The Role of Bone Marrow Derived Cells (BMDCs) in Burn wound Healing

Suzanne Marie Rea

MB BCH BAO MRCSI FRCSI (Plast)

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School of Surgery
ABSTRACT

Introduction

The reparative process that results in scar formation after tissue injury results in permanent functional and aesthetic deficits. Re-epithelialisation of wounds and dermal cell re-population has been thought to be driven by cells peripheral to the wound site. However, recent research has demonstrated that cells originating from the bone marrow can contribute to healing wounds in other tissues and also after incisional injury. The transition from embryonic complete tissue regeneration to adult scar formation and repair after injury may in part be due to these cells originating in the bone marrow, either hematopoietic stem cell derived (HSC), or mesenchymal stem cell derived (MSC), or potentially both. Currently, many studies are focused on the clinical potential of bone marrow stem cells, in particular MSCs. However, our current understanding of their role in repair and clinical potential remains limited, particularly with respect to burn injury. This project aims to clarify the role of bone marrow derived cells in burn injury repair, and further explore both HSC and MSC populations in wound repair and scar formation.

Hypothesis

Bone marrow derived cells (BMDCs) are involved in the dermal and epidermal repair processes, and not just inflammation, after thermal injury

Materials and Methods

A murine model of a minor (3% TBSA) and moderate (8% TBSA) full-thickness burn was developed. Using a chimeric bone marrow model, generated by lethal irradiation and reconstitution of bone marrow with EGFP+ cells from a transgenic mouse, the fate of BMDCs for 120 days post-injury was tracked. Using a mouse model of burn injury, we have also investigated the activity of exogenously applied mesenchymal stem cells when applied both at
the time of injury and at time-points post-injury to assess whether MSC injection may be a useful clinical tool.

Finally, to determine whether the HSC population within the bone marrow is important in scar formation and repair after burn injury, a transgenic mouse model with all hematopoietic stem cells labelled with a reporter gene was used.

**Results**

Our study shows that BMDCs contribute to tissue repair and not just inflammation after both minor and moderate burn injury. In contrast to the minor injury, BMDCs were also present in the epidermal compartment after burn injury suggesting greater involvement of BMDCs in repair of more severe injuries.

After exogenous application, MSCs were detected in the wound tissue, but the evidence suggests there is little directed migration when applied either at time of injury or 24 hours post-injury, with MSCs also detected in many other tissues and surviving for the duration of the experiment. Timing of administration does impact on cell migration to multiple tissues and to presence in the skin over the long-term, although whether this is due to the wound status rather than discrete signalling is unclear.

Finally, whilst hematopoietic derived cells were detected in the healing wound post the acute inflammatory phase, no long-term contribution of HSCs was detected.

**Conclusion**

The results show that BMDCs are involved in the response to burn injury. The data regarding exogenous application suggests clinical application may not be ideal and the presence of applied cells in multiple tissues in the long-term could be problematic. Finally, it is clear that HSCs do not contribute significantly to the healed wound, and therefore future work should
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<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>BM</td>
<td>Bone Marrow</td>
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<td>BMDC</td>
<td>Bone Marrow Derived Cells</td>
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<tr>
<td>BMSC</td>
<td>Bone Marrow Stem Cell</td>
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<tr>
<td>BMMSC</td>
<td>Bone Marrow Mesenchymal Cells</td>
</tr>
<tr>
<td>BMHSC</td>
<td>Bone Marrow Hematopoietic Stem Cells</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
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<tr>
<td>DMEM</td>
<td>Dubelcos Modified Eagles Medium</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth Factor</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>GFP</td>
<td>Green Flourescent Protein</td>
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<td>GVHD</td>
<td>Graft Vs Host Disease</td>
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<td>GTP</td>
<td>Guanosinetriphosphatases</td>
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<td>HSC</td>
<td>Hematopietic Stem Cell</td>
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<tr>
<td>HC</td>
<td>Hematopoietic Cell</td>
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<tr>
<td>KGF</td>
<td>keratinocyte growth factor</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MC</td>
<td>Mesenchymal Cell</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>MSP</td>
<td>Macrophage Stimulating Protein</td>
</tr>
<tr>
<td>NHMRC</td>
<td>National Health and Medical Research Council</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>SMA</td>
<td>Smooth Muscle Actin</td>
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<tr>
<td>TBSA</td>
<td>Total Body Surface Area</td>
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<tr>
<td>TICHR</td>
<td>Telethon Institute for Child Health Research</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor β</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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CHAPTER 1 INTRODUCTION

1.1 Incidence, prevalence and clinical significance of wounds and scars

A wound can be defined as a break in the skin (epidermis or dermis) that can be related to trauma, including surgical intervention, or to pathological changes[1].

Wounds are a major burden worldwide; whether accidental, surgical or non-surgical, the management of patients with wounds places enormous demands on health resources and budgets[2]. Pressure ulcers treatment alone accounts for 4% of the health budget in the United Kingdom[3]. There is no doubt that the presence of a wound such as burn injury, pressure sore, leg ulcer, or malignant wound (Fig. 1.1) places significant strain on individuals and society in terms of quality of life of affected individuals and their carers[4-5].

The prevalence of wounds in Western Australia has recently been assessed by the Wound West Survey, the first such study in Australia. This survey examined 2,777 patients across 85 hospitals over a 4 week period in May 2007. The results are startling 49% of all patients examined had 1 or more wounds at some point during their hospital stay and 26% of patients had 3 or more wounds. Across the state 2,687 wounds were identified in 1,363 patients. Wound prevalence tended to increase with age with patients 60 years and older accounting for 59% of all wounds identified[6].

Scarring

One of the major long term consequences of wounds is the scar. Each year in the developed world, 100 million patients acquire scars, some of which cause considerable problems, as a result of 55 million elective operations and 25 million operations after trauma (Fig. 1.2A, B)[7].
Common skin injury types include partial thickness burn injury (A), pressure sore (B), malignancy (C), and full thickness burn injury (D). These wounds cause a significant burden for both the individual and their families.

There are an estimated 4 million burn scars (Fig. 1.2C) and 11 million keloid scars (Fig. 1.2D) in the developed world, 70% of which occur in children[7]. Global figures are unknown but almost certainly much higher. Scars arise after almost every injury. Rare exceptions include tattoos and superficial scratches. Scars are often considered trivial, but they can be disfiguring” aesthetically unpleasant and cause severe itching, tenderness, pain, sleep disturbance, anxiety, depression, and disruption of daily activities[8]. Other psychosocial sequelae include development of post-traumatic stress reactions[9], loss of self esteem[10] and stigmatisation[11] leading to diminished quality of life. Physical deformity as a result of skin scar contractures(Fig 1.3) can be disabling[12].
Figure 1.2 Scars are the result of repair after significant skin injury. They can result from elective surgery (A), surgery after trauma (B), burn injury (C) and in some instances form excessive scarring, such as keloid scar formation (D).

Figure 1.3 Scar contracture occurs as a result of delayed wound healing and inadequate postoperative management. This can be disfiguring and even more important disabling, restricting movement and use of the affected limb or joint.
1.2 Clinical and economic importance of burns and burn scar

Burns are a major public health problem. The World Health Organisation has reported that 322,000 deaths in the world in 2002 were as a direct result of fire related burns, the vast majority of these occurred in the developing world (Table 1.1). In the United States more than 1.25 million people sustain burns each year[13] and the corresponding figure in the United Kingdom is 250,000[14]. Burn injury is one of the most devastating conditions encountered in medicine. It affects all aspects of patient’s lives from the physical to the psychological. A burn can be major or minor. In Western Australia severe injuries account for approximately nine deaths and 500 hospitalisations annually, in addition a further 500 people per year attend a specialized burn clinic for outpatient management of their injury[15]. From 1983 to 2008, there were 23,450 hospitalizations for an index burn injury. In Western Australia, this reflects the number of patients who required hospital admission for burn injury, but not those treated as outpatients [16]. The effect of a burn injury can lead to chronic disability which maybe physical or psychological or a combination[17].

<table>
<thead>
<tr>
<th>REGION:</th>
<th>AFR</th>
<th>AMR</th>
<th>EMR</th>
<th>EUR</th>
<th>SEAR</th>
<th>WPR</th>
<th>WORLD</th>
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<tr>
<td>INCOME GROUP</td>
<td>LOW</td>
<td>MIDDLE</td>
<td>HIGH</td>
<td>LOW</td>
<td>MIDDLE</td>
<td>HIGH</td>
<td>LOW</td>
</tr>
<tr>
<td>Burn deaths x 1000</td>
<td>39.2</td>
<td>3.9</td>
<td>4.5</td>
<td>0.0</td>
<td>31.9</td>
<td>2.8</td>
<td>35.4</td>
</tr>
<tr>
<td>Death rate per 100,000</td>
<td>5.8</td>
<td>1.2</td>
<td>0.8</td>
<td>0.6</td>
<td>6.4</td>
<td>0.7</td>
<td>7.4</td>
</tr>
<tr>
<td>% of global mortality due to fires</td>
<td>12.2%</td>
<td>1.2%</td>
<td>1.4%</td>
<td>0.0%</td>
<td>9.9%</td>
<td>0.9%</td>
<td>11.0%</td>
</tr>
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Source: WHO Global Burden of Disease Database, 2002
* WHO Regions do not correspond exactly with geographic regions. They are made up of countries and areas under the six WHO regional administration.

Table 1.1 Global distribution of Burn mortality

Mortality after burns in the regions-Africa (AFR), Americas (AMR), Eastern Mediterranean (EMR), Europe (EUR), South East Asia (SEAR), and Western Pacific (WPR).
Burns affect all age ranges with the paediatric and elderly populations most vulnerable. Morbidity and mortality rates are higher in these groups. Figures from the Australian Bureau of Statistics 1995-2000 for burns show that the risk of hospitalisation was 2.1 times higher for males than females, 5.7 times higher for Indigenous people and 2.3 times for those from rural regions[18-19]. Burns rank fourth as a cause of unintentional child injury related death in the USA.

The last twenty years has resulted in enormous changes in burn care practise, while the patterns of injury have remained static. Fires and scalds remain the most common burning agent. An epidemiological study of all burn hospitalizations in children less than 5 years from 1983-2008 showed that in Western Australia most burns occurred in the home and resulted from exposure to a household hazard and as such are preventable injuries[16, 20]. There have been major changes and development in all aspects of clinical practice. Referral patterns have changed with more people being referred for specialist treatment. Good resuscitation, intensive care support, early and extensive surgical excision of the burn wound have all contributed to improved patient outcomes. Adult survival rates of 70.2 % of all patients with burns greater than 50 % have been reported[15]. In paediatrics it has been shown in some units that 50 % of children will survive a 90- 95% burn injury[21]. The need to achieve rapid wound closure in patients with massive burns and limited skin donor sites has led to new developments in replacement skin technologies such as cultured epithelial auto graft[22].

However with the increasing numbers of survivors from major burn injuries there have been other problems identified. Delayed wound healing results in infection, hypermetabolism, sepsis, immunosuppression and multi organ, including bone marrow, failure. If wound healing is delayed excessively and the race to achieve wound closure is not achieved these systemic effects of the injury may become fatal. This has been reported as occurring typically at approximately 3 months post injury[15].
The initial admission may involve many months in hospital with average length of stays of approximately 1.5 days per percentage burn[15]. In addition ongoing rehabilitation and re-integration into the community can take years. Burn survivors are often in close contact with the burns treatment team for many years, scar management is protracted and may require further surgical interventions over years to allow for growth. This is especially true in the growing child. In long term outcome studies a gender specific increased incidence of cancer has been identified[23]. A fatal hepatocellular carcinoma has also been reported in a boy who survived a massive paediatric burn[24-25].

This acute treatment phase is resource intensive, however there is little known about the cumulative cost of burn injury to the Australian community with regard to the wide resource utilization that spans many years. In the USA the cost of caring for a patient with a burn injury is crudely estimated at US$ 3000 to US$5000 per day. But these expenses may only represent 25 % of the actual costs. It is important to emphasise that the economic impact of a burn injury also extends to include indirect costs including loss of wages and the costs relating to deformities and scarring, including loss of skills, further surgery and emotional trauma[26].

1.3 Classification of Burn

A burn is the partial or complete destruction of skin caused by some form of energy, usually thermal energy. Burns can be classified in a number of ways; mechanism, extent and depth of injury. The most common mechanisms of injury are thermal, chemical, electrical and radiation. A thermal burn occurs when the cells of the skin are exposed to temperatures above 45°C. At this temperature, enzyme systems begin to malfunction, oxygen free radicals are produced and denaturation of protein occurs to the extent that the cell’s inherent mechanisms of repair are inadequate[27]. The level of damage is proportional to the energy (heat) applied and the duration of application. Necrosis occurs in cells exposed to 45°C for 1 hour and in 5-10
In seconds for cells exposed to 60°C[27]. As temperatures increase, the damage is predominantly due to coagulative necrosis, where proteins, including those associated with all cells and nerves, universally denature. This leads to total loss of the functions of the skin. A model of the damage to the cells at a burn wound has previously been postulated, based on the studies described above[28]. This considers three regions of a burn injury (Fig. 1.4). The region exposed directly to the heat will be most damaged, and consists primarily of unsalvageable necrotic material. This is surrounded by a zone of stasis, which is damaged tissue but may be salvaged through the body’s own mechanisms and optimal treatment. This in turn is surrounded by a zone of mild damage (hyperaemia), which will recover through the normal physiological processes. The extent of injury is a description of the total body surface area (TBSA) that has been burned[29]. The depth of injury (See Section 1.4) refers to the layers of the skin that are involved in the injury and remains the most clinically useful description of the injury given that the epidemiological data is available based upon this classification.

Figure 1.4 Jackson’s model of burn injury
Three zones are described in this model of injury – necrotic (unsalvageable), stasis (potentially salvageable) and hyperaemia (recovers without intervention)
1.4 Burn Wound Depth

Burns can be described as superficial, partial or full thickness, which equates to the still commonly used terminology of first, second and third degree burns[30-31].

1.4.1 Superficial Burns

Superficial burns involve damage to the epidermis only and are rarely clinically significant other than being painful. The involved area is initially erythematous due to vasodilatation. Eventually, desquamation ensues but is followed by complete scarless healing within 7 days(Fig 1.5).

Figure 1.5 Superficial burn showing epithelial loss and surrounding erythema. This injury was caused by sunburn.
1.4.2 Partial thickness burns

Partial thickness burns are further categorized into superficial or deep. In superficial injuries, all of the epidermis is destroyed as well as varying superficial portions of the dermis. These lesions are usually very painful due to the survival of nerve endings in the superficial and mid dermis. Blistering is often present. Healing will generally occur rapidly and completely through migration to the surface by epithelial cells which survive in deeper portions of the hair follicles as well as sweat and sebaceous glands. Relatively little scarring occurs in a superficial injury, owing to the contracted inflammatory phase, which is cut short by wound closure (re-epithelialisation) occurring within 2 weeks.

Deep partial thickness injuries involve destruction of the dermis and the few remaining epithelial cells are located in the deepest recesses of the epidermal appendages (Fig. 1.6). Heat kills the nerve endings rendering the wound relatively insensate. Pressure sensation may remain, owing to survival of deeply situated pressure receptors. Blistering is usually absent due to the thicker adherent overlying eschar, which prevents the lifting effect of oedema. Due to the depth from which migrating epithelial cells must travel, re-epithelialisation is greatly retarded in these wounds. When healing does occur, often weeks post injury, the epidermis is very thin, tenuous and sometimes non functional. Due to the prolonged period before wound closure, the inflammatory phase is protracted, allowing extensive deposition of collagen, which manifests as extensive scarring.
1.4.3 Full thickness burn injury

Full thickness burn injuries involve necrosis of the entire thickness of the skin, leaving no chance for healing except for very small wounds which may heal by contraction and epithelialisation from the wound edges, but will still likely incur profound scarring (Fig 1.7). Full thickness wounds are routinely treated with excision and skin grafting or the use of dermal substitutes such as Integra, which may be applied in combination with negative pressure [32-37].
1.5 Biology of wound healing

A major review of the processes involved in wound healing was published by Singer et al in 1999[38]. This review describes the dynamic interactive processes involved and the three main phases in cutaneous wound healing, which are inflammation (early and late phase), tissue formation and tissue remodelling (Fig. 1.8).

1.5.1 Inflammation

Briefly, following tissue injury the inflammatory phase is activated following disruption of blood vessels and formation of blood clot. Release of multiple vasoactive mediators recruit inflammatory leukocytes to the wound. These act to remove necrotic and non viable tissue, reclaim salvageable tissue and offer some protection against further wound colonisation. This

Figure 1.7 Full thickness flame burn, covering the anterior torso with no surrounding transition zone
phase is augmented by many pro inflammatory cytokines and growth factors including but not limited to; transforming growth factor β (TGFβ), monocyte chemo-attractant factor, tumour necrosis factor α (TNFα), interleukin-1, insulin like growth factor (IGF) and macrophage derived growth factors. The formation of the fibrin clot not only stimulates the inflammatory response, but the same cytokines that are released by platelets in the provisional matrix also recruit cells required for tissue formation (Fig 1.9).

Figure 1.8 This diagram shows the overlapping phases of wound healing and the timescale post-injury that they occur
1.5.2 Tissue formation

Reepithelialisation Phase

This important phase commences within hours of injury as epidermal cells lining skin appendages act to remove clotted blood and damaged stroma from the wounded area. In addition they change their phenotype, disrupting cell to cell connections and allowing lateral cell movement to occur. The initial phase of epidermal movement is to allow for separation of the eschar from viable tissue. This leading edge is then followed by a proliferating wound margin. This process continues in an ordered sequence until establishment of re-epithelialisation and basement membrane formation. Once completed the epidermal cells cease to be hyperproliferative and appear to function as normal epidermal cells.

Figure 1.9 Contractile fibroblasts secrete and organize extracellular matrix fibres (blue) that are loaded with growth factor complexes (green), resulting in a turquoise overlay colour. The contractile fibres inside the cells are visualized by detecting a smooth muscle protein (red). The cells' nuclei are visualized in yellow. Taken from Reference : [39]
1.5.3 Formation of granulation tissue and neovascularisation

At the same time as epidermal cells proliferate and migrate to re-cover the wound surface, macrophages, fibroblasts and blood vessels move into the wound space from about day 4 post-injury (Fig 9B). In the presence of multiple growth factors especially platelet derived growth factor (PDGF) and transforming growth factor β (TGFβ), macrophages and fibroblasts are stimulated for the formation of fibrous tissue, angiogenesis and extracellular matrix formation. The establishment of new blood vessels is essential to maintain this newly formed granulation tissue. And there are many complex interactions among endothelial cells and between endothelium and the ECM and fibroblasts to coordinate this process (summarised in the review by Singer et al)[38].

1.5.4 Remodelling phase

Briefly, macrophages, fibroblasts and blood vessels move into the wound space concurrently[40]. The macrophages provide a continuing source of growth factors necessary to stimulate fibroplasia and angiogenesis; the fibroblasts produce the new extracellular matrix necessary to support cell growth (Fig. 1.9) and blood vessels carry oxygen and nutrients necessary to sustain cell metabolism. Growth factors, especially PDGF and TGFβ[41] in concert with the extracellular matrix molecules [42-43] stimulate fibroblasts of the tissue around the wound to proliferate, express appropriate integrin receptors, and migrate into the wound space.

The structural molecules of newly formed extracellular matrix, termed the provisional matrix [44] contribute to the formation of granulation tissue by providing a scaffold or conduit for cell migration. These molecules include fibrin, fibronectin, and hyaluronic acid[45]. In fact, the appearance of fibronectin and the appropriate integrin receptors that bind fibronectin, fibrin or both on fibroblasts appears to be the rate limiting step in the formation of granulation tissue[43, 46]. The fibroblasts are responsible for the synthesis, deposition and remodelling of
the extracellular matrix. Conversely, the extracellular matrix can have a positive or negative effect on the ability of fibroblasts to synthesize, deposit, remodel and generally interact with the extracellular matrix[43, 47]. Cell movement into a blood clot of cross-linked fibrin or into a tightly woven extracellular matrix may require an active proteolytic system that can cleave a path for cell migration. A variety of fibroblast derived enzymes in addition to serum derived plasmin are potential candidates for this task, including plasminogen activator, collagenases, gelatinase A, and stromelysin[48].

After migrating into wounds, fibroblasts commence the synthesis of extracellular matrix[47, 49]. The provisional extracellular matrix is gradually replaced with a collagenous matrix [47, 49], stimulated by secretion of TGFβ1[47]. Once an abundant collagen matrix has been deposited in the wound, the fibroblasts stop producing collagen, and appear to undergo apoptosis, triggered by as yet unknown signals. Eventually, the fibroblast rich granulation tissue is replaced by a relatively acellular scar. Disruption of these finely balanced processes results in fibrotic disorders such as keloid formation, morphea and scleroderma[38].

During the second week of healing, fibroblasts assume a myofibroblast phenotype characterized by large bundles of actin containing microfilaments disposed along the cytoplasmic face of the plasma membrane of the cells and by cell-cell and cell-matrix linkages[49-50]. The appearance of myofibroblasts corresponds to the commencement of connective tissue compaction and the contraction of the wound.
1.6. **Major Cell types involved in wound healing process**

1.6.1 **Keratinocytes**

Reepithelisation is the resurfacing of a wound with new epithelium and is one of the early stages in tissue formation occurring simultaneously with granulation tissue formation. It consists of both migration and proliferation of keratinocytes, which in the case of severe injury, occurs from the periphery of the wound. For this to happen the keratinocytes undergo marked phenotypic alteration, resulting in changed morphology and ability to migrate (*Fig 10*), including; disassembly of hemidesmosomal links between epidermis and basement membrane, retraction of intracellular tonofilaments and keratin filaments, dissolution of most desmosomes, formation of peripheral cytoplasmic actin filaments (lamellipodia) and focal contacts[38]. As epidermal migration progresses, keratinocytes at the wound margin then proliferate behind these actively migrating cells. Although all the important stimuli for migration and proliferation have not been determined, there are a number of different factors which are known to play a role. There is a physical effect called the free edge effect which refers to the lack of adjacent or neighbor cells at the margin of the wound thus promoting migration. A number of growth factors have been implicated including PDGF, Epidermal growth Factor (EGF), TGFβ, keratinocyte growth factor (KGF) and Macrophage Stimulating Protein (MSP). It is well established that EGF, TGFα and KGF are central to the proliferation process. The complex interactions between these growth factors, cellular and molecular mechanisms such as the integrins, extracellular matrix molecules and mettaloproteinases are explored in detail in a review by Santoro and Gaudino[51].
1.6.2 Hair follicles and epithelial stem cells

Hair follicles and epithelial stem cells from the hair follicle bulge have been of interest to researchers of wound healing as it is known that re-epithelialisation of partial thickness wounds occurs, at least in part, from adnexal structures in the wound bed. However, the mechanism by which stem cell populations in the hair follicle are stimulated remain unclear and are still being investigated.

In normal circumstances, the prime function of a hair follicle is to produce a hair fiber, but these follicles also contribute to wound healing, in particular superficial and partial thickness wounds. Hair follicles maintain a number of the skin’s stem cell populations, including keratinocyte and melanocyte populations[52-53]. These are found in the bulge region of the hair follicle. These stem cells contribute to both cyclical homeostasis of the epidermis and the re-epithelialisation required for wound healing. Using a number of labeling systems such as cre-recombinase – based lineage tracing, it has been shown that following injury between 25 and 50 % of cells in the repaired interfollicular epidermis are of hair follicle origin[54-55]. Langton et al have shown recently that absence of hair follicles in a cutaneous wound results in an acute delay in wound healing. This is then followed by expansion of the region of activated
epidermis, which is greater than that normally seen. However, it is important to note that appropriate wound closure can and does occur, even in the absence of hair follicles[56]. Whether this is by a normal wound repair mechanism, or an alternate pathway, is not yet known.

1.6.3 Fibroblasts

Fibroblasts are the cells responsible for connective tissue. They are however poorly characterised and there is a large degree of variability in their phenotypes. Fibroblasts are derived from many different organs and tissue types including mesenchymal cells such as adipocytes.

Differences in fibroblast differentiation can be seen depending on anatomical location. In the dermal fibroblasts the differences can be seen even in layers of the dermis. Deeper layer fibroblasts showed reduced collagen expression compared with fibroblasts from more superficial layers with differences in collagen type I and III and mRNA expression[57]. This also proves true from different body sites [58-59] The HOX gene expression pattern in fibroblasts appears to be closely associated with which body site the fibroblast has been derived from[58].

Fibroblasts have been shown to have a prominent role in abnormal wound healing in particular hypertrophic and keloid scar formation. In this type of wound healing fibroblasts form within the abnormal scar area, lesional fibroblasts, have been shown to differ from surrounding fibroblasts[60-61]. There is increased mRNA for activin and a strong expression of αSMA. These changes are also demonstrated in culture with high baseline expression of activin A, follistatin, αSMA, collagen 1, thrombospondin-1 and the ectodermal dysplasia-A fibronectin present in keloid fibroblasts when compared to normal fibroblasts in culture[62-64]. It has also been demonstrated that increased fibrin gel [65] and collagen-gel contraction are seen in fibroblasts from hypertrophic scars. It is unknown currently how the keratinocyte – fibroblast
interactions differ in keloid and hypertrophic scars compared to normal interactions or indeed whether it is the keratinocyte or fibroblast which is the driver of this interaction

1.6.4 **Myofibroblasts**

Myofibroblasts may be defined morphologically and immunologically through identification of expressed cytoskeletal proteins[66-67]. The simplest definition is that they are smooth muscle like fibroblasts. Some investigators chose to call them smooth muscle like cells or activated smooth muscle cells [68-70] whilst others refer to them as lipocytes because of their propensity to store retinoids, or as stellate cells because of a shape change of transiently differentiated myofibroblasts. Myofibroblasts may well represent an intermediate state between fibroblasts and smooth muscle cells (**Fig. 1.11**). This is best demonstrated in the prostate [71-73] in the pericytes surrounding the foetal vessels of the placental stem villus [74] and in the stromal myofibroblasts of the breast[75].

Both *in vivo* and *in vitro* they possess several distinguishing morphological characteristics, some of which are present in fibroblasts or smooth muscle cells. They display prominent smooth muscle cytoplasmic actin microfilaments (stress fibres) and they are connected to each other by adherens and gap junctions[76-77]. These cells are also in contact with the ECM by focal contacts once known as the fibronexus, a transmembrane complex made up of intracellular contractile microfilaments and the ECM protein fibronectin[78]. Both fibronexus formation and stress fibre assembly are regulated by Rho, a newly described member of the RAS superfamily of small guanosine triphosphatases (GTPases) specifically in mammalian cells by Rho A[79]. These small monomeric GTP binding proteins also regulate myofibroblast morphology[80]. Often an incomplete basal lamina surrounds the myofibroblasts. Gap junctions couple some myofibroblasts to the tissue smooth muscle, and the cells are commonly in close apposition to varicosities of nerve fibres[74, 81].
Activated fibroblasts or myofibroblasts are derived from several different sources including resident stromal fibroblasts, epithelial cells (epithelial-mesenchymal transition (EMT)), endothelial cells (endothelial-mesenchymal transition (EndMT)), and bone marrow-derived fibrocytes. Myofibroblasts synthesize and deposit ECM components, which mainly include collagen type I and smaller amounts of collagen type III, fibronectin, elastin, laminin, proteoglycan and glycosaminoglycan, and release various cytokines and mediators, which stimulate myofibroblasts in a paracrine manner. Infiltrating inflammatory cells, parenchymal cells and other cells also release cytokines and mediators. TGFβ, transforming growth factor-β; IL-6, interleukin-6; Ang II, angiotensin II.

Immunohistochemical characterization of myofibroblasts is based on antibody reactions to two of the three filament systems of eukaryotic cells these three systems are composed of 1) actin, a component of the microfilaments 2) vimentin, desmin, lamin or glial fibrillary acidic protein (GFAP), members of the intermediate filament system; and 3) the tubulins of the microtubules[82]. Vimentin, desmin and alpha smooth muscle actin are the three filaments most often used to classify myofibroblasts[83]. Expression of these proteins may vary with the tissue studied within species and is subject to environmental factors e.g. whether the cells are studied in situ or in culture and even within a given tissue whether the cells are activated by hormonal or cytokine treatment or by disease[84].
**1.6.5 The origin of fibroblasts and the role of circulating fibrocytes**

A distinct population of blood borne fibroblast like cells that rapidly enter areas of tissue injury was described in 1994 by Bucala et al[85]. Termed fibrocytes, these cells comprise 0.1- 0.5 % of non erythrocytic cells in peripheral blood and display an adherent, spindle shaped morphology when cultured *in vivo*. Cultured cells express the fibroblast products collagen types I and III and fibronectin, as well as the leucocyte common antigen (Ag) (CD 45RO) the pan myeloid Ag (CD13), and the haemopoeitic stem cell Ag (CD 34). In addition fibrocytes express MHC Class II and co stimulatory molecules (CD 80 and CD 86) and have the capacity to present Ag *in vitro* and *in vivo*[86-87]. Fibrocytes differ from monocytes / macrophages, dendritic cells and B cells by their morphology, growth properties, cell surface markers and cytokine profile[66]. In culture they do not express CD14, 16 or 19 – which are typical monocyte/ macrophage and B cell markers, nor do they express typical surface proteins of dendritic cells or their precursors (CD1a, CD 10, CD 25 and CD 38).

Although these cells make up only 0.5 % of peripheral blood leucocytes they constitute 10 % of cells infiltrating subcutaneously implanted wound chambers in mice[85]. An important role for fibrocytes in cutaneous wound healing *in vivo* has been postulated based on immunolocalisation of CD34* fibroblast- like cells in dermal scar tissue[85-86]. The expression of the chemokine receptor CCR7 on fibrocytes and interaction with lymphoid chemokines is involved in the migration of these cells to the wound[88]. More recently it has been reported that fibrocytes induce angiogenesis both *in vitro* and *in vivo*[89-90]. More recent studies have demonstrated that an interaction between CCR7, expressed on fibrocytes and the SLC chemokine (secondary lymphoid chemokine) is necessary for the trafficking of fibrocytes to the wound bed[88].

Recently it has also been shown that the development of fibrocytes is upregulated in burns patients. The extent of upregulation also appears to correlate with increased percentage TBSA[91]. However, it despite the evidence for the role of fibrocytes, it is important to note
that the co-expression of the hematopoietic stem cell Ag CD34 and Collagen I has only been detected in circulating fibrocytes to date[85-86, 88, 92], and not in cells within the wound. It is not yet clear why this has been the case.

In vitro, fibrocytes differentiated from an adherent population of CD141-enriched peripheral blood cells when cultured in DMEM and FBS (with no additional growth factors). This differentiation process appears to require T cell interaction. Further studies will be necessary to identify the molecules involved in functionally significant interactions between T cells and fibrocytes that are required for fibrocyte maturation[88]. The T cell requirement observed for fibrocyte differentiation is reminiscent of the maturation of dendritic cells, also known for their ability to process and present Ag[88].

It has been postulated that circulating fibrocyte precursor cells interact with activated T cells, which permits their early differentiation (toward the fibrocyte phenotype), and they then migrate to the wound site. Within the wound site, these early differentiated fibrocytes might further interact with recruited T cells and fully differentiate and mature following exposure to TGF-β. These fully differentiated, mature fibrocytes express increased levels of αSMA and produce collagen and other extracellular matrix proteins that promote wound healing and contracture[88]. However, it is important to note that the mechanism of activation and role for fibrocytes in wound healing remains unclear, and will require future research to clarify the role of fibrocytes in wound repair and scar formation.
1.7 Biology of burn scar

Burn wounds differ from incisional wounds in both the direction and cellular mechanism of injury. Burn wounds are characterized by heat–induced tissue coagulation at the time of injury [93] and in contrast to incisional wounds, the predominant direction of tissue injury is horizontal not vertical[94]. These differences result in distinct healing mechanisms of incisional vs. thermal wounds. Vertical injuries such as those seen with surgical incisions heal rapidly by blood clot formation, re-epithelialization and fibroblast proliferation[95], whereas cutaneous burn wounds heal more slowly, in part due to the oedema, extensive necrosis and relative hypoxia of the burn wound [94](Fig 1.12).

![Figure 1.12 In large open wounds the phases of wound healing overlap, with more extensive and prolonged inflammatory and proliferative phases (Modified from Total Burn Care by D.N Herndon)](image)

1.7.1 Factors influencing burn wound healing and subsequent burn scar formation

The factors that influence all types of wound healing also impact the burn wound including patient factors, wound factors and treatment factors. There are also a number of important factors specific to the burn wound including depth of injury, extent of injury, time to healing and treatment received. Burn wound depth is discussed in more detail above. It is sufficient to
state that the deeper the burn the less potential for regeneration and the more aggressive surgical interventions are required, thus the greater potential for significant scar formation.

The TBSA impacts on scar formation as in the absence of suitable donor sites the task of wound healing may be overwhelming and require many months of surgery and dressings to achieve closure. It is this time to healing that has been most robustly shown to impact negatively on scar formation. Deitch et al noted that wounds that healed within 2-3 weeks had a much lesser tendency to scar formation. In contrast those that remained unhealed for longer periods were much more likely to develop hypertrophic scar formation[96].

1.8 Burn Scar Formation

The major source of morbidity after burns is excessive scarring. In the absence of injury there is a balance between the synthesis and degradation of collagen in human dermal tissues[97]. However, extensive thermal injuries result in alterations in collagen metabolism that lead to
alterations in extracellular matrix components of the dermis, and in particular to excessive collagen deposition which is responsible for scar formation [57, 98-101].

1.9 Abnormal scar formation

Extensive thermal injuries disrupt the skin structure and the repair process, whilst requiring extensive collagen deposition, can become excessive, a characteristic feature of several forms of fibrotic conditions[98-99]. Hypertrophic scars and the related, but more exuberant condition known as keloid are among the dermal fibrotic diseases which are characterized by increased collagen deposition[100-101]. Hypertrophic scarring represents the dermal equivalent of fibro-proliferative disorders and is a common clinical problem for patients who survive extensive thermal injury[57].

It remains a source of frustration to all who treat burns patient that as yet, while we can attribute risk as a result of certain factors which predict scar outcome including patient factors, wound factors and treatment factors, there is still no way of predicting accurately scar outcome. This is indicative of the lack of knowledge of all the important genetic and environmental factors involved in the wound healing process.
Figure 1.14 The unpredictable nature of scarring. These photographs illustrate that at 6 months the scar is reasonable (A). Although it is red, it is smooth and flat. Two years later however (B), it is now a mature scar and has raised and uneven surface. In addition it has contracted significantly pulling on the developing breast and the axilla affecting arm movement.

It is important to mention that it is routine practise to continue with scar management techniques in the year following a burn injury, including such techniques as pressure therapy and topical silicone. Although this is routine treatment there is only limited evidence exploring it’s mechanism of action and to support its use. Costa et al describe the structural changes in the dermis of pressure treated post burn hypertrophic scars and although only descriptive provide direct evidence for close relationships in vivo between mechanical modifications (i.e. pressure treatment) cell phenotype, and matrix remodelling[102]. This work showed that modification of collagen fibre organization can be induced by pressure, in that the disorganized orientation of collagen originally observed was replaced under pressure by a parallel arrangement similar to the pattern observed in normal healing. This pressure induced reorganization has also been observed previously by Kischer et al [103] and Baur et al[104].
1.10 Biology of fibrosis and Scar in other Sites

Fibroproliferative diseases can affect all tissues and organ systems. Scarring post burn is just one such condition, but it shares many similarities with other pathologies such as pulmonary fibrosis, systemic scleroses, liver cirrhosis, cardiovascular disease, progressive kidney disease and macular degeneration. In all these conditions fibrosis occurs as a result of “out of control” repair. These other conditions have been investigated much more extensively than burn injury due to their prevalence. Most fibrotic conditions have in common a persistent irritant that results in persistent chronic inflammation and sustains the production of growth factors, proteolytic enzymes, angiogenic factors and fibrogenic cytokines that progressively remodel and destroy normal tissue architecture[105-106]. This is similar to the slow healing burn wound in terms of chronicity or indeed the recurrent minor skin breakdowns that burn patients routinely experience. It is this persistent irritation that has been suggested as a possible aetiology with many conditions having a background of infection, with bacteria, viruses, fungi and multicellular parasites driving the chronic inflammation and development of fibrosis. It has been argued that this constant exposure maintains cells at a heightened state of activation and that inhibiting pathogen mediated fibroblast activation may offer a potential therapeutic intervention[107-110].

The cell types implicated in the pathogenesis of fibrosis include fibroblasts, myofibroblasts and fibrocytes, and research is continuing to determine the underlying changes that cause fibrotic conditions.
1.11 The origin of cells involved in wound healing

The wound healing cascade activates a number of cell types from the periphery of the wound, the systemic circulation and the bone marrow. The normal tissue adjacent to the wound is the area thought to supply the majority of the cells involved in wound healing, particularly for dermal and epidermal repair, when the defect is small. However, as the wound increases in size this wound margin may no longer be sufficient as a source of reparative cells, and there may be a more involved systemic response with cell recruitment from the circulation. Whilst it is clear that the inflammatory phase of the wound healing response involves the recruitment of circulating cells, the origin of cells involved in tissue remodelling, scar formation and re-epithelialisation is not as clear. There is increasing evidence that at least some of these cells are not recruited from the region local to the wound, but involve recruitment from the circulation, and potentially other organs. Of particular recent interest is the potential role of the bone marrow as a source of dermal and epidermal cells in the repair of skin injury.
1.12 Stem Cells

Stem cells are defined as cells capable of both self renewal and differentiation into at least one mature cell type. They can be further sub classified based on their species of origin, tissue of origin, and potential to differentiate into one or more specific types of mature cells[111]. Classification by source is important, as this is the division into embryonic, foetal or adult type stem cells. Embryonic stem cells, for example the zygote and its immediate daughter cells are thought to be totipotent. That is the cells are capable of differentiating into any cell type, including all the cells of the embryo, developing foetus, adult organism and all the extra embryonic tissues and structures such as the placenta. Embryonic stem cells are the only truly totipotent stem cell population. Adult stem cells, while not totipotent, can be pluripotent (form many cell types). The extent of the pluripotency is dependent on the tissue source and other factors. It was originally thought that adult stem cells, although pluripotent, had a more limited differentiation capacity, which was most likely organ specific[111]. However it is now recognized that these adult stem cells can give rise to tissues not normally present in the organ in which the stem cells are located. All the stem cells used in this research proposal, and described in this work, are adult in nature, unless otherwise stated.

1.13 The Bone marrow

The bone marrow stroma contains precursor cells that are capable of differentiating along hematopoietic cell (HC) and mesenchymal cell (MC) lineages. The hematopoietic stem cells in the bone marrow continually replenish the immune and erythrocyte cell populations, whilst they are also known to be capable of contributing to liver, skeletal muscle and endothelial cells under certain conditions[112][Fig. 1.15]. In contrast, the mesenchymal cells have been shown to differentiate into epithelial, neuronal, muscle (cardiac and skeletal) and bone lineages [113-117][Fig. 11.15]. These two cell populations are described in more detail below.
1.13.1  Mesenchymal Stem Cells (MSC)

Mesenchymal stem cells, or stromal cells, reside in the stromal portion of the bone marrow. It was assumed that their function was to provide the cellular microenvironment for hematopoiesis. However, it has been shown that these cells can also differentiate into a number of cell types. Bone marrow is the most useful source of MSC’s there has been a number of other sources identified in the body with adipose tissue an area of great interest due to the relative ease of harvest[118-119]. Periosteum, tendon, muscle, synovial membrane have also been used[120]. It is of particular interest that there appears to be no difference in the source of the MSC the properties remain the same[121-123]. Mesenchymal stem cells can differentiate to form osteocytes, chondrocytes, adipocytes, and bone marrow stromal fibroblasts. Recent reports illustrate that bone marrow contains stem and/or progenitor cells capable of differentiating into non haematopoietic tissue [124-126]. After transplantation of bone marrow haematopoietic stem cells or non hematopoietic mesenchymal stem cells, muscle, [113] heart [116], liver [117], and lung cells [114-115] of donor origin have been detected (Fig 1.15).
Bonemarrow plasticity
This demonstrates how the pluripotent stem cells of the bone marrow of HSC and MSC type can form different lineages in a number of organs including skin, heart, liver, muscle.

1.13.1.1 Identification & Isolation

MSC are typically isolated by flushing the stromal fraction of bone marrow in small animal studies or by BM aspirates in humans. In fresh BM, MSCs account for 0.01-0.0001% of nucleated marrow cells[121, 127]. The minimal criteria by the International Society of Cellular Therapy to define MSCs are 1) plastic adherent in culture; 2) expression of CD105, CD73, and CD 90; 3) lack of expression of hematopoietic makers such as CD45, CD34,CD14, Cd11b, Cd19, CD79a, and HLA-DR; and 4) ability to differentiate into osteoblasts, adipocytes, and chondrocytes[128]. The ratio of MSCs to marrow mononuclear cells is estimated to be 10
MSCs per million marrow cells[129]. Despite relatively low numbers a 2mL aspirate of bone marrow can be expanded 500-fold ex vivo to 12 billion to 35 billion MSCs within 3 weeks[130].

Three main approaches have been described for isolating MSCs and can be used independently or in combination. The traditional method relies on the fact that MSCs selectively adhere to plastic in contrast to HSC. Hence the HSCs can be removed with medium changes[127]. Percoll gradient separation techniques based on cell density have also been described[129, 131-134]. Sorting of bone marrow populations using flow cytometry (FACS) is becoming increasingly popular, and necessary for pure population isolations. This is based on MSC reactivity, or non-reactivity, to a number of antibodies. This can be performed either by positively selecting for expressed antigens or by a process of immunodepletion of cells expressing hematopoietic and/or other lineage antigens. Antibodies against CD34, a hematopoietic surface marker, can be used to identify and remove non mesenchymal cells from a marrow culture[135]. Markers used to identify MSCs include but are not limited to, CD13, CD29, CD31, CD44, CD54, CD63, CD73, CD105, CD106, CD140b, CD166 and Stro 1[136-137]. There is no consensus as to the optimal marker combination for identification of MSCs but the majority of subsets include both CD29 and CD105 as positive markers, and CD34 as a negative marker[127]. MSCs can be grown in culture and have been demonstrated in vitro and in vivo to be capable of differentiating into osteoblasts, chondrocytes, and adipocytes, which is also a property that can be used to characterise the MSC population[138].

1.13.1.2 Therapeutic Potential

The therapeutic potential of these cells is promising. MSCs are readily accessible from patients and healthy donors, offering a source of cells for clinical applications in regenerative medicine. In addition to providing a scaffold for HSCs, MSCs have been demonstrated to play a role in hematopoiesis themselves[139]. This has been demonstrated in patients undergoing chemotherapy, through co-transplantation of MSCs and HSCs vs. HSCs alone. The addition of the MSCs was shown to significantly improve hematopoiesis [140].
1.13.1.3 **Graft Versus Host Disease**

MSCs are suitable for allogeneic transplantation as evidence suggests that they are immune privileged with low MHCI and no MHCI expression [141]. MSCs have also been observed to play an immunoregulatory role. MSCs can exert profound immunosuppression by inhibiting T cell responses to polyclonal stimuli [140] and to their cognate peptide[127]. This inhibition is not antigen specific [142-144] and targets both primary and secondary T-cell responses[145]. The characterization of the phenotype of MSC induced T cells showed that the inhibitory effect of MSC is directed mainly at the level of cell proliferation[142]. The immunosuppressive effect of MSCs has led to the use of MSCs in graft versus host disease GVHD. Co-infusion of donor derived MSCs together with HSCs has been shown to reduce the incidence and severity of GVHD[142].

1.13.1.4 **Skeletal Pathology**

MSCs are in clinical use for a number of skeletal conditions including defective fracture healing and cartilage repair. MSCs have been used with and without scaffolds to repair large bone defects[145-146]. Cartilage repair has been performed in patients with osteoarthritis, a collagen gel has been embedded with MSCs and re-implanted with formation of hyaline cartilage like tissue compared to controls[147]. The majority of the skeletal research to date has focussed on topical administration of cells, but recently systemic administration of allogenic MSCs has been given to children with osteogenesis imperfecta. Transplanted MSCs were shown to migrate to bone and produce collagen, thus providing a new and efficient way to treat this debilitating condition[148].

1.13.1.5 **Myocardial Infarction**

Current clinical trials are underway examining the role of MSCs in the treatment of myocardial infarction[146, 149]. These involve injecting either whole bone marrow or undifferentiated MSCs directly into the heart. Although the mechanism of action has yet to be established,
significant improvement in terms of extent of damage and recovery of function has been detected[150]. It has been suggested that the improvement in left ventricular ejection fraction post treatment is as a result of the formation of new cardiomyocytes and angiogenesis triggered by the infusion[151]. Theories abound as to how the MSCs effect this recovery including direct action by *in situ* differentiation or alternatively by fusion with resident myocytes[112]. It is also postulated that the effects of applied MSCs is simply through secretion of pro-myogenic factors promoting endogenous myocardial repair after MSC cell death[116-117, 152].

Whilst the mechanism of action of MSCs remains unclear, in many cases of clinical use there have been marked improvements in outcome or other measurements. It will be important to validate these results, as well as identify the mechanisms of action, for the full clinical potential of MSCs to be realised in the many situations that they can be applied. Of particular interest for this research proposal is the potential of MSCs in the amelioration of scar formation after burn injury, and the current findings in this area are discussed further below (Sections 1.15-1.19).
1.13.2 Hematopoietic Stem Cells (HSC)

Hematopoietic stem cells potentially can be sourced from bone marrow, peripheral blood and umbilical cord blood. However most of these cells, particularly in the circulation, are not true stem cells but rather immature hematopoietic and immune cells rather than true HSCs. The true HSC population resides in the bone marrow of adults, and even when isolated from the bone marrow extensive purification of these source cells needs to occur (using cell surface markers) to isolate the HSC population. A single hematopoietic stem cell can reconstitute the entire hematopoietic cell population, as demonstrated in animal models [153], and there may be a potential of these cells to be harnessed in promoting wound healing.

1.13.2.1 Animal model for investigating role of HSC

The model selected for ease of identification of HSC where all hematopoietic and endothelial cells are labelled. To determine whether the HSC population within the bone marrow is important in scar formation, transgenic mouse model is required. The Vav-Cre C57BL/6 mouse has Cre recombinase under the control of the Vav-promoter element, and has been demonstrated to be active in haematopoietic stem cells and consequently all cells derived from HSCs [154]. Whilst endothelial cells are also labelled in this model, we will selectively identify this cell type using additional markers. In addition, mosaic animals (when Cre recombinase is activated in cells other than HSCs) will be identified with pre-injury biopsies [155]. We will cross this mouse with the ROSA26R LacZ reporter mouse (also on C57BL/6 background), to generate mice with LacZ reporter expression restricted to HSCs and progeny (Fig1.16).
1.13.2.2 Identification & Isolation

The technique used to isolate HSCs predominantly involves surface antigen detection by flow cytometry. There have been a number of markers identified and it has been demonstrated that single-cell transplantation of cells with the strongest Hoechst 33342 dye efflux activity (Tip-SP) and the CD34+ Ckit + Sca-1− Lin-(CD34+ KSL) phenotype resulted in more than 90% engraftment activity and long term multilineage hematopoietic reconstitution[116].

Figure 1.16 The model selected for ease of identification of haemopoietic stem cells (HSCs) where all hematopoietic and endothelial cells are labelled [See text for description]
1.13.2.3 Therapeutic Potential

The clinical use of adult HSC’s is expanding, far beyond the traditional therapies for malignancy. A comprehensive review [112] identified all studies from January 1997 to December 2007 on the use of HSC’s in autoimmune, cardiac or vascular disease. In summary this report has highlighted the exponential increase in the use of HSCs clinically. There were 926 reports identified, over 600 were excluded as these were reviews, editorials, commentaries or ethical discussions. Three hundred and twenty three were reviewed, with 69 included in analysis of HSC therapy. This review concluded that in studies of autoimmune disease transplantation of HSCs may have a potent disease remitting effect, in the active inflammatory stage, but the duration of remission is uncertain. In cardiovascular disease there has been some modest improvement in cardiac function in patients with acute myocardial infarctions and chronic coronary artery disease. However the conclusion is that currently larger clinical trials are required to answer several questions including; most appropriate cell type, dose, method, timing of delivery, and adverse effects of adult HSC’s. The role of HSC in relation to wound healing and burns is discussed in detail below.
1.14 Bone Marrow Derived Cells, Fibrosis and Scar formation in other organs

There is evidence that in several forms of pathological fibrosis, such as pulmonary fibrosis induced by bleomycin and scarring after myocardial infarction, that the deposition of collagen largely occurs from fibroblasts and/or myofibroblasts that are derived from the bone-marrow and have migrated to the site of fibrosis from the circulation[114, 116-117, 156].

Hashimoto et al have demonstrated that substantial bone marrow-derived fibroblast like cells migrated to the lung in a murine model of bleomycin – induced pulmonary fibrosis[156]. This data was obtained using a transgenic bone marrow from a donor mouse that had been genetically modified to express the gene that encodes the Green Fluorescent Protein (GFP) into otherwise isogenic recipient mice. Thus, all bone-marrow derived cells in the recipient mouse expressed GFP, allowing these cells to be detected by ultraviolet microscopy or flow-cytometry. Most GFP+ cells in fibrotic lung tissue were macrophages or neutrophils, but 27 % of the GFP+ cells synthesised type I collagen. Furthermore, these cells constituted more than 80% of all Col I expressing cells in the fibrotic lung, indicating that the predominant collagen producing cell in fibrosis was derived from the bone marrow and had migrated into the lung in response to signals released in reaction to lung injury.

Fibrocytes are a population of circulating bone-marrow derived cells that represent the most likely candidate as the cell type that migrates into the lung and is responsible for the observed collagen deposition[88, 157]. Within the site of fibrosis it is likely that fibrocytes differentiate into fibroblasts or myofibroblasts, or potentially both[105]. The mechanisms responsible for the homing of bone marrow derived cells that produce collagen have not been completely elucidated. There is evidence that fibrocytes can express several chemokine receptors, especially CXCR4 and CCR7, and migrate in response to their respective ligands[88]. There is
also evidence that T-cells and transforming growth factor (TGF-) may play a role in the migration and differentiation of fibrocytes during wound healing[88, 158-161].

In both humans and in mice models of renal transplantation, there is data to suggest that bone marrow derived cells may become renal epithelial cells. Further studies have demonstrated a major role of the bone marrow in renal epithelial cell repair as tested by a lethal irradiation, bone marrow transplantation followed by ischemia reperfusion injury in a β- gal transgenic Rosa mice model. These studies demonstrated that infusion of large numbers of lineage negative, bone marrow cells, essentially bone marrow stem cells, have the capacity to participate in renal tubule repair, whereas the HSC population mediates a lesser protective effect[162]. However a number of more recent studies have questioned this and suggested that this original mouse model over represented the contribution from the bone marrow[163-164]. Both of these studies have been reviewed by Krause et al and the current understanding is that MSC’s when cultured and administered in large numbers do have a protective anti-apoptotic effect, suppress inflammation and promote proliferation or can augment the influx and differentiation of endogenous renal stem cells. However, there are a number of areas that remain uncertain including whether this protective effect of the MSC’s requires them to leave the bone marrow and transit through the renal circulation or whether these cells can exert protective effects from distant sites[162].

The heart has traditionally been described as a “post mitotic” organ, that is that cardiomyocytes are terminally differentiated and that any subsequent injury results in irreversible scarring without regeneration[152]. Orlic et al have explored the role of bone marrow derived cells in treatment of myocardial infarction[117]. In this study male lin`c-kit[POS] bone marrow cells from transgenic GFP+ve mice, were injected in the area adjacent to an experimentally induced myocardial infarction from a previously ligated coronary artery in female mice. This study showed repair in 40 % of the treated mice. The developing tissue comprised proliferating myocytes and vascular structures. Functionally a 36% reduction in left
ventricular end diastolic pressure and an improvement in systolic pressure development and relaxation were observed in the treated group. In a further study administration of stem cell factor (SCF) and granulocyte colony stimulating factor (G-CSF) for 5 days pre injury and 3 days post injury resulted in a decreased mortality, infarct size and increased left ventricular performance[165]. The percentage of new myocytes, endothelial cells and smooth muscle cells expressing EGFP was 53+/−9 %, 44+/−6% and 49+/−7% respectively. The values were consistent with the fraction of transplanted lin−c-kitpos bone marrow cells that expressed EGFP, 44+/−10%[117]. Based on this promising animal research a number of clinical trials are currently underway[152].

The liver is another organ where there is significant interest in the potential of stem cells as a therapeutic tool. The shortage of organs available for transplantation and the associated problem of life- long immunosuppression have all added to the search for alternative treatment options. There are a number of animal models of liver disease including toxin induced model such as carbon tetrachloride (CCL4) or chronic hepatocellular injury such as the hepatitis B surface antigen transgenic mouse. Studies exploring the role of BMDC’s in these and other models are reviewed by Lorenzini. This shows a wide variation in results with varying figures of engraftment from 0.16% to 50 %[166].
1.15 Bone Marrow Derived Cells in Skin and Wound Healing

Normal skin contains dendritic cells, which are bone marrow–derived cells involved in host defense and inflammatory processes (predominantly antigen presentation), including after injury[167]. After tissue injury, hematopoietic and multipotent progenitor inflammatory cells are mobilized both from the circulation, where monocytes differentiate to macrophages and migrate to the injury site, and also from the bone marrow, where many cell types, including neutrophils and monocytes are increasingly produced and enter the circulation. These cells migrate to the site of injury, where they regulate the proliferation and migration of epithelial and dermal cells during the early inflammatory phase, as well as being involved in initiating matrix formation[115]. As the inflammatory response subsides, the wound is remodelled to form a stratified epithelial layer over a collagen-rich matrix containing several mesenchymal cell types, including fibroblasts, capillaries and peripheral nerve fibres. Although the bone marrow contribution of inflammatory cells in the acute response to injury is well established, the long-term fate and role of non-inflammatory bone marrow–derived cells in a healed cutaneous wound is uncertain[168].

Previous studies have concentrated primarily on examining the BM response to small punch biopsy excisional wounds, linear incisional wounds and tape stripping to generate epidermal loss. The wounds were typically 2-3mm in diameter full thickness circular excisions or linear incisional wounds, size not stated, similar to our minor burn injury model in terms of severity (discussed in Results 3.1.1). All of these studies showed a small or negligible keratinocyte population which appeared to originate from the bone marrow[115, 169-170]. It has been postulated that in these smaller wounds there is no exhaustion of the epidermal keratinocyte stem cell niche and that therefore BMDCs are not required to replenish keratinocyte stem cells and stimulate keratinocyte repopulation after injury[170-171].

The contribution of BMDC to the epidermis has been explored in a number of studies examining both steady state epidermis and the healing wound. In contrast to the results
described above, in the landmark paper from Krause et al in 2001, examining multi-organ, multilineage engraftement by a single BMD stem cell, it was observed that up to 2% of all cytokeratin positive cells (keratinocytes) in the skin were BM derived.

A number of studies have followed with quite conflicting results. Fan et al suggest that the contribution of the bone marrow is negligible at approximately < 0.0001% in a healed wound and only marginally more in a healing wound[170]. In contrast, a contribution of 8% in the early days after wounding falling to 1% at a later time point of 56 days has also been reported[171]. At even earlier time points of 4 days post injury the BMDC contribution to keratinocytes in a healing wound was said to be up to 11.5%[172].

Harris, in a multi-organ study examining the presence of fusion requirements in the development of BM derived epithelia also assessed the engraftment of BMDCs as keratinocytes[169]. The skin injury was a full thickness excision model, size not stated. These wounds were examined at 10 and 21 days, which showed an engraftment rate of 0.1% and lack of fusion.

These studies were broadly similar in terms of their design and methodology with the major differences relating to both time of injury post transplant and time points selected for examination of the wounds. These factors alone do not explain the inconsistencies found, in particular with regard to the contribution of BMDC to uninjured but transplanted skin. The differences may be attributable to the models utilised, in particular the use of transgenic EGFP expressing reporter strains. There can be considerable biological variability of GFP expression between strains and among similar cell types in the same strain[173]. Even mice that appear to glow bright green under appropriate fluorescent light may not be uniformly green at a cellular level[174], whilst other studies have suggested reporter gene expression may deplete with time in specific strains, further complicating analysis of data that relies on reporter gene detection[175]. A further difference has been attributed by some to detection methods utilising a rabbit polyclonal antibody which may have exhibited non specific and background
staining. A recent paper by Krause et al discussing the role of expression of EGFP in lineage studies suggests that the site of transgene integration may account for some of the variability of expression[176].
1.16 Contribution of Bone Marrow Derived Cells to fibroblast populations

There is now recent evidence that bone-marrow derived cells make a substantial contribution to cutaneous fibrosis after injury[177]. This work has shown that the bone marrow contribution to the healing of an incisional cutaneous wound is substantial. Again, by the use of a technique that utilised GFP+ bone marrow transplantation, the mean percentage of EGFP +ve cells in the healed dermis was 37% at day 28 and 19.2% at day 42. Furthermore, the bone marrow-derived cells were able to contract a collagen matrix and transcribe both collagen types I and III, whereas the fibroblasts that were derived from the peripheral tissue surrounding the wound transcribed only collagen type I. The study concluded that wound healing involved local cutaneous cells for reconstituting the epidermis but distant bone marrow-derived cells and the adjacent uninjured dermal mesenchymal cells. Collagen type I is the predominant collagen in normal human skin and exceeds type III by a ratio of 4:1. During wound healing, this ratio decreases to 2:1 because of an early increase in the deposition of collagen type III[177-178].

In addition, Ishi et al, in an excisional skin wounding study, reported that there was an increase in the number of GFP +ve fibroblasts recruited into the wounded area compared to non-injured skin, with 32.3% of all fibroblasts GFP+ve[179]. This study however only investigated a single timepoint, at day 7 post injury. The GFP+ve fibroblasts were found adjacent to the striated muscle of the dermis. This study also reported that almost all of the BM derived fibroblasts produced type I collagen. The study concluded that the BM can potentially contribute to the turnover of fibroblasts and may have a direct role in the process of pathological fibrosis[179].
These studies support the hypothesis that bone marrow derived cells play a role in skin wound repair, but to date there is no data regarding the response to burn injury. This will be the focus of this research proposal.

1.17 The Therapeutic potential of Hematopoietic & Mesenchymal stem cells in wound healing

All previously described studies have explored the role of the bone marrow using either transgenically labelled or sex mismatched chimeric bone marrow models. However, as previously described, the bone marrow contains two distinct cell populations, mesenchymal and hematopoietic, and there is growing interest in the role these distinct cell populations play, particularly for therapeutic interventions. Currently, while there is interest in both populations, it is likely the MSCs may show more promise, given their relative lack of immunogenicity, potential for expansion ex vivo, storage properties and consequent potential for allogeneic administration. Some have even referred to MSCs as the “potential universal healing cell” [180]. As a direct result it has been proposed that administration of such cells to critically ill patients would be beneficial, especially patients with major trauma. This administration could be autologous or allograft from living or dead donors. It could be administered whole or in a cultured form, allowing adequate expansion of the desired populations. This has been advocated as a potential and real treatment option[181-182].
1.18 Hematopoietic Stem Cells and Wound Healing

A study by Orgill et al, examined the role of a selected side portion of hematopoietic stem cells[183]. This subset were identified based on their ability to efflux the vital dye Hoechst 33342, and were Sca-1+, C-kit+, Lin^neg/low and CD34+. In this study BM cells were collected, prepared and sorted based on these markers, with the remaining cell population of the marrow used as a control. These two bone marrow populations were then applied topically to treat an excisional 1.0 cm² full thickness wound on the dorsum of a diabetic mouse. There was a statistically significant improvement in wound healing, assessed histologically by H&E staining, in the group treated with Side Population Cells. One of the hypotheses put forward included the suggestion that in these diabetic mice the application of stem cells has led to a rescue therapy. The authors also stated that while the improvement was dramatic the knowledge is still rudimentary. The proposed mechanisms included cytokine activation and elaboration or alternatively they suggest that engraftment and incorporation is a possibility. However, cell fate tracking was not conducted in these studies, and therefore the mechanism currently is not clear.
1.19 Mesenchymal Stem Cells and Wound healing

Similar to the study described above by Orgill et al, the benefit of MSCs in a wound excision model in diabetic and non-diabetic mice has also been investigated[180]. In this study MSCs were isolated from GFP+ve donors and injected intradermally around the wound at 4 injection sites. BM-MSCs significantly enhanced wound healing in normal and diabetic mice compared with the effects of neonatal dermal fibroblasts. The study showed engraftment of 27% at 7 days, 7.6% at 14 days, and 2.5% at 28 days post-injury of total BM-MSCs administered. BM-MSC-treated wounds exhibited significantly accelerated wound closure, with increased re-epithelialisation, cellularity, and angiogenesis. BM-MSCs, but not CD34+ bone marrow cells in the wound, expressed the keratinocyte-specific protein keratin and formed glandular structures, suggesting a direct contribution of BM-MSCs to cutaneous regeneration. In addition, high levels of vascular endothelial growth factor (VEGF) and angiopoietin-1 (ANG1) were detected in BM-MSCs and significantly greater amounts of the proteins were detected in BM-MSC-treated wounds. This suggests that BM-MSCs promote wound healing through differentiation and release of proangiogenic factors.

Human mesenchymal stem cells in combination with bFGF have also been used in combination with a porcine derived dermal template in a rat model and the initial results are promising for reconstruction of large skin defects including those created following debridement of major burns[181]. In this study, full thickness skin and soft tissue defects of 1.5x1.5 cm in size, were excised and covered with hMSCs and bFGF soaked skin substitute. The wound size was assessed as significantly smaller in the hMSC group (p<0.01) and any dose of bFGF (1,10,100μg) enhanced the healing. In addition the reepithelialisation markers integrin α3 and skin derived antileucoproteins were remarkably increased with the presence of bFGF in a dose dependent manner. The role of MSC is further supported by a recent study by Yamaguchi[184].
In a deep partial thickness contact burn model they found that BM-MSCs injected 5 days prior to injury homed to the bone marrow initially. They were not detected at day 7 in the wound, but were detected at days 10 and 14, mainly in the upper dermis and at the edge of the regenerating dermis. They concluded that the presence of BM-derived myofibroblasts in the upper dermis just beneath the regenerating epidermis mainly during the granulation phase of deep dermal burn wound healing suggested a role for MSCs not only in promoting granulation tissue but also for the enhancement of dermal-epidermal interactions.

Lastly, an experiment on the delivery of autologous bone marrow-derived cultured mesenchymal stem cells after skin injury, using a fibrin spray, showed significant improvements in wound repair [185]. This was true in both human and animal studies.
1.20 Human studies – Chronic wounds & Burn Injuries

Treatment of chronic wounds with bone marrow derived cells has been performed in a small number of patients in a study by Badiavas[186]. In this study patients with recalcitrant chronic wounds that had not responded to conventional therapy, including bioengineered skin application and grafting with autologous skin, received autologous bone marrow derived cells. It was reported that this application resulted in complete wound closure and that there was evidence of dermal rebuilding. This study was described as proof of principle that bone marrow contains progenitor cells and can engraft into wounds and contribute to wound healing and regeneration of dermis.

Although a small study of only 3 patients there are a number of important aspects to this study including the interesting histological analysis. Some of the new cells resembled immature, committed hematopoietic progenitor cells. In addition many spindle shaped cells and elongated cells were noted after treatment. These spindle shaped cells were most prominent following topical application of cultured cells. These changes are different from those seen with other wound dressings or bioengineered skin[186].

In the case of thermal injury, Rasulov et al have reported the use of BM-MSCs in a patient with 40% TBSA injury, treated with a transplantation of allogenic fibroblast- like bone marrow cell suspension. There is no real detail in the case report, other than the clinical findings of a systemic improvement following topical application of cryopreserved allogenic fibroblast –like bone marrow mesenchymal stem cells[187]. The lack of clinical experience in this area is highlighted by the fact that a case report stating intention to treat was published despite the fact that the patient died prior to attempts to culture and administer autologous MSC[188].

A further study examining the circulating response to acute burn injury examined blood samples from burn patients and healthy donors. They employed a large monoclonal antibody panel including CD44, CD45, DR, DC34, CD19, CD13, CD29, CD105, CD1a, CD90, CD38 and
CD25. MSC phenotype was considered positive for CD44, CD13, CD 29, CD90 and CD105. This study found cells phenotypically suggestive of MSC’s present in large amounts during acute burn wounds. The samples were all taken at day 3 post injury and the TBSA ranged from 20 to 55%[189]. However, no longer term data was obtained.

These small scale clinical applications and investigations provide potentially interesting observations, but without a better understanding of MSC and HSC populations in the burn injury response, it is unlikely that clinical applications will become more widespread, or benefit patients. However, the potential of BMDCs to improve patient outcomes appears very real, and there is a need for further extensive investigation to increase understanding and allow the design of improved clinical intervention trials.
1.21 Summary

It is widely accepted that the previous dogma of the dermal and epidermal cells involved in wound repair always being derived from the wound periphery is unlikely to be the case. This is particularly true in the case of severe injuries. Currently, the most convincing data strongly suggests that cells either from the circulation or the bone marrow, or potentially both, are involved not only in the inflammatory response but in the repair, remodelling and eventual scar formation that occurs after injury. There is also currently strong evidence that Bone marrow derived cells, either of hematopoietic of more likely mesenchymal origin, have significant therapeutic potential in multiple conditions.

However, much of the evidence for the role of BMDCs in wound healing is currently conflicting. In addition, there is little data on how BMDCs respond in the case of thermal injury. Lastly, the mechanism of action, and whether cells of bone marrow origin play a positive or negative role in scar formation remain unknown.

This project will therefore build on the current knowledge and potential of BMDCs in wound repair. Specifically, the role and extent of involvement of BMDCs in the process of burn injury repair and scar formation will be investigated. The role of the two predominant populations, Mesenchymal and Hematopoietic, will also be studied in greater detail to attempt to delineate the cell functions within the wound and subsequent scar.
1.22 Hypotheses

1. Bone marrow derived cells (BMDCs) are involved in the dermal and epidermal repair processes, and not just inflammation, after thermal injury

2. The response of the BMDCs changes qualitatively or quantitatively as the extent of injury increases

3. Mesenchymal stem cell derived and not hematopoietic stem cell derived cells are predominantly involved in cutaneous repair after thermal injury

4. BMDCs can promote wound healing and ameliorate scar formation

1.23 Aims

1. To identify whether BMDCs migrate to the site of thermal injury in a mouse model

2. To characterise the longitudinal response of BMDCs to thermal injury of differing severity (minor and moderate) in a mouse model

3. To identify whether exogenous BMDCs contribute to burn wound healing

4. To characterise the roles of MSC and HSC populations in the response to thermal injury in a mouse model
CHAPTER 2 MATERIALS AND METHODS

2.1 Ethics Approval

All animal experiments were carried out with approval by the relevant institutional animal ethics committees and all experiments were conducted in accordance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Experiments were carried out at the Telethon Institute for Child Health Research, Murdoch University (ethics approval # R2080/07 and R2081/07) and Royal Perth Hospital, Perth, Western Australia. Bone marrow cell donor mice (C57BL/6-Tg(ACTB-EGFP)1Osb/J) were obtained from Jackson Laboratories (JAX mice and services, Maine, USA) and the colony maintained at the Animal Resource Centre, Murdoch, Perth, WA.
2.2 Determining the role of bone marrow derived cells in burn wound repair using an EGFP chimeric murine bone marrow model

2.2.1 Preparation of transgenic donor bone marrow cells for transplant

Bone marrow cells for transplantation were isolated from the femurs of C57BL/6-Tg(ACTB-EGFP)1Os/J mice. Adult animals (20-30 weeks old) were euthanased by cervical dislocation and femora dissected. The isolated femora were flushed with a 25 gauge needle and 500 l GKN buffer. This solution was then diluted in GKN buffer (Table 2 A) to a total volume of 5ml, centrifuged @ 300 × g for 7min at room temperature and cells resuspended in 5mls of fresh GKN buffer. A viable cell count was performed by adding an equal volume of 2× trypan blue to an aliquot of the cell suspension with cells counted using a haemocytometer. Cells were then re-centrifuged and resuspended to 1 x 10⁷/ml BM cells in sterile GKN buffer.

2.2.2 Generation of Transgenic Bone Marrow Chimeric Mice

Eight week old wild-type C57BL/6 mice were lethally irradiated using a Gammacell 3000 Elan irradiator (MDS Nordian, Ottawa, Canada) with two doses of 550 cGy and 2hr between each dose. After the second irradiation, 2 × 10⁶ donor bone marrow cells (EGFP positive, prepared as described in 2.1) were injected in a total volume of 200μl of sterile GKN buffer via the tail vein. Mice were maintained on antibiotic water (neomycin/polymyxin) for one week prior to and one month post-irradiation. Ten weeks following lethal irradiation and tail vein injection, mice were
bled via the tail vein and the degree of chimerism assessed by FACS analysis of white blood cells for EGFP expression (Section 2.2.3).

2.2.3 Validation of chimeric bone marrow mice

To assess the degree of chimerism after bone marrow transplant, and to ensure the radiation dose was sufficient to prevent reconstitution of wildtype (non-EGFP expressing) bone marrow derived cells, mice were bled via the tail vein and the percentage of EGFP positive white blood cells determined. 100μl of blood was diluted in 1ml of red blood cell (RBC) lysis buffer (Table 2 A) to remove red blood cells. The solution was incubated for 10min at room temperature in the dark. The sample was centrifuged at 300 × g for 5min at room temperature. The supernatant was removed and sample washed once in 1ml staining buffer (Table 2 A). The sample was then centrifuged at 300 × g for 5min at room temperature and finally resuspended in 1ml of staining buffer. Cells were kept on ice and analysed for EGFP expression using a FACS Calibur flow cytometer (Becton, Dickinson, Franklin Lakes, NJ) using excitation at 488nm and fluorescence detection at 530nm. Flow cytometry analysis of data was carried out using FlowJo flow cytometry analysis software Version 7.2.2 (Tree Star Inc.).
Table 2 A: Solution compositions for validation of chimeric model

**2.2.4 Murine Burn Injury Model**

The murine burn wound model used is based on a model described previously by Bruen *et al* and modified to create a full thickness injury [190]. Following induction of general anaesthesia with isoflurane inhalation, mice are shaved on the dorsum and swabbed with povidone iodine antiseptic solution. Mice receive a full thickness flank/contact burn via a 10sec application of a 65g brass rod heated to 95°C. Two different injury severities were induced; a moderate injury, using a 2cm diameter brass rod (3.14 cm² which translates to 8% TBSA injury) and a minor injury, using a 1cm diameter brass rod (0.785 cm² which translates to2% TBSA). Immediately post induction of anesthesia, animals were given a single dose of buprenorphine, 0.05-0.1mg/kg by subcutaneous injection. Post-injury, mice were returned to their cages and given feed and water *ad libitum*. Mice received paracetamol in drinking water (1mg/ml) for five days, to ensure adequate analgesia.
At days 1, 3, 7, 10, 14 and 21 for both minor and moderate injury model, and in addition at days 28, 56 and 120 post-burn for the moderate injury only, mice were euthanized (n=5 at each time point) and the wound area and/or scar assessed. In addition, five Day 0 no-injury controls were also analyzed. At each time point, the entire wound, including the adjacent 3mm skin margins were excised. The wound was bisected along the cranial caudal axis. One half of the sample was fixed in 4% paraformaldehyde solution, processed, paraffin embedded and sectioned at 5μm for histological and immunohistochemical analysis. The second half of the sample was prepared for FACS analysis as detailed below. In addition, samples of uninjured skin were also isolated and prepared for histology and immunohistochemistry.

2.2.5 Flow cytometric analysis of skin, wound and scar tissue samples

For flow cytometric analysis it is important to obtain a clear single cell suspension with little or no debris. It is also important to consider whether the protocol used to obtain the single cell suspension may remove important cell surface marker proteins that will be analysed by flow cytometry. The protocol below is modified from a previously described protocol demonstrated to maintain skin cell surface markers intact [191].

To prepare excised scar, skin or wound samples for FACS, the following protocol was used. The tissue sample was cut finely by hand using a surgical scalpel, on ice, until it was minced thoroughly. Digestion solution (5ml/g tissue or 1ml/cm² tissue) (Table 2 B) was added, and the solution incubated at 37°C for 2-3hrs, with periodic agitation until tissue was completely digested.

Following digestion, the cell suspension was briefly vortexed and then passed through a 100μm cell strainer (BD Biosciences, Franklin Lakes, NJ) and centrifuged at 300 × g for 10min at 4°C. The supernatant was removed and the cell pellet washed in 5 ml ice-cold PBS, centrifuged at 300 × g
for 10 min at 4°C and resuspended two more times. The final cell pellet was then resuspended in 1ml PBS or 100μl Staining Buffer (Table 2 B) for FACS analysis.

**COMPOSITION OF SOLUTIONS**

<table>
<thead>
<tr>
<th>Digestion Solution</th>
<th>Staining Buffer</th>
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<tr>
<td>2mg/ml Collagenase D</td>
<td>2% FBS</td>
</tr>
<tr>
<td>1mg/ml Hyaluronidase I</td>
<td>0.1% sodium azide</td>
</tr>
<tr>
<td>20μg/ml DNAse I</td>
<td>In Dulbecco’s PBS (pH 7.4)</td>
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<td>In DMEM</td>
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</table>

*Table 2 B: Solution compositions for processing of skin, wound and scar tissue for FACS analysis*

Using the cell suspension prepared as described above, non-specific antibody binding to Fc receptors was blocked using 0.5μg of rat anti-mouse CD16/32 antibody (BioLegend, San Diego, CA) to minimize false positive staining, briefly vortexed and incubated on ice for 10 minutes. Cell suspension was then centrifuged at 300 x g for 5min at 4°C, pellet washed twice in ice cold staining buffer, centrifuged and finally resuspended in 100μl staining buffer. The cell population was then analyzed using multiple primary antibodies (Table 2 C) to determine the percentage of bone marrow derived cells within the wound (EGFP positive) and the cell types that were bone
marrow derived (EGFP/Cell specific marker double positives). Controls included samples of cells with no antibody added as well as negative control samples with each antibody added separately to accurately determine the point at which cells are positive.

<table>
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<th>Primary Antibody marker used</th>
<th>Antibody marker used</th>
<th>Antibody source</th>
<th>Primary Antibody Concentration</th>
<th>Cell permeabilization required</th>
<th>Cell type identified</th>
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<td>EGFP – Alexa Fluor 488</td>
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</tbody>
</table>

Table 2 C: Summary of Primary antibodies, conditions used and cell types identified

Antibodies were supplied by Pharmingen, San Diego, CA for inflammatory markers, Millipore, Billerica, MA for col1, Lab Vision, Fremont, CA for α-SMA, Molecular Probes, Eugene, OR for secondary antibodies.
1 μl of conjugated primary antibody was added to each 100 μl cell suspension after non-specific blocking with CD16/32 (2.2.5.2). Cell suspensions were incubated with the antibodies for 30 min on ice, in the dark. After incubation, cells were washed, centrifuged (300 × g for 5 min at 4°C) and resuspended in staining buffer (Table 2 B), repeating twice. Final cell pellet was resuspended in 500 μl staining buffer, transferred to a FACS tube and kept on ice in the dark until FACS analysis.

For cytoplasmic cell markers, cells were first permeabilised and fixed to allow antibodies to penetrate the cell membrane whilst retaining the intracellular marker proteins within the cell. For fixing and permeabilisation, the cells were centrifuged at 300 × g for 5 min at 4°C and then resuspended in 250 μl of Cytofix/Cytoperm (BD Diagnostics) solution at 4°C for 20 min. Cells were then washed twice in 1 ml of Perm/Wash solution (BD Diagnostics). The prepared cells were finally resuspended in 100 μl Perm/Wash solution.

1 μl of primary conjugated antibody was then added to each prepared cell suspension, briefly vortexed and incubated for 30 min on ice in the dark. Cells were then washed twice using 1 ml of the Perm/Wash solution. Finally, the cells were resuspended in 500 μl Perm/Wash solution, transferred to a FACS tube and kept on ice in the dark until FACS analysis.

2.2.5.1 Flow Cytometry Procedure

Labelled cells were analysed with a FACS Calibur flow cytometer (Becton, Dickinson, Franklin Lakes, NJ) using excitation at 488 nm and fluorescence detection at 530 nm (Alexa 488), 585 nm (PE, R-PE), 661 nm (APC, Alexa Fluor 647) and 670 nm (PI, PE-Cy5). Ten thousand cells were counted and analysed using FlowJo flow cytometry analysis software Version 7.2.2 (Tree Star Inc.).
2.2.6 Immunohistochemical and histological analysis of skin, wound and scar tissue

Immunohistochemistry was carried out on 4% paraformaldehyde fixed and paraffin embedded sections of tissue (prepared from 2.2.4). Paraffin sections were used in preference to frozen tissue sections for the superior histology. Sections were sliced to a 7μm thickness using a Leica RM2255 automated microtome (Leica Microsystems, North Ryde, NSW) and mounted on Lomb Superfrost Plus slides. Prior to immunohistochemistry or Haematoxylin and Eosin staining, paraffin was removed and the sections rehydrated by sequentially incubating the prepared slides as follows;

**A: Dewax and Rehydration (all at room temperature)**

1. Toluene 2 min x 2
2. 100% Ethanol 2 min x 2
3. 70 % Ethanol 2 min x2
4. Distilled Water (DW) 2min x2
5. Tris Buffered Saline (TBS) 2 min

**B: Antigen Retrieval**

Rehydrated sections were placed in a bath of 10mM sodium citrate (pH 6.0) at room temperature. Bath was then placed in a microwave (1000W) and heated on high for 4min then on medium for 4min. Sections were then allowed to cool for 20min at room temperature. Finally, sections were washed three times for 2 min in TBS at room temperature **(Table 2 D)**.
**C: Blocking Endogenous Peroxidase Activity**

After antigen retrieval, endogenous peroxidase activity was blocked by incubation of the sections for 10 min at room temperature with 3% H$_2$O$_2$ in TBS. Sections were then washed three times for 5 min in TBS at room temperature.

**D: Antibody Incubations**

The sections were then outlined using a wax pen and the primary antibody applied. The primary antibody, rabbit anti-EGFP (Molecular Probes, Eugene, OR), was diluted 1:1000 in 10% normal goat serum/TBS-T (Table 2 E), applied on sections and incubated overnight in a humidified chamber at 4°C. Sections were then rinsed three times for 5 min in TBS-T (Table 2 D). The secondary antibody, biotinylated goat anti-rabbit IgG (Pharmingen, San Diego, CA) was diluted 1:450 in 10% normal goat serum / TBS-T, applied to each section and incubated in a humidified chamber for 1 hr at room temperature. Sections were then washed twice in TBS-T for 5 min, followed by two further washes in TBS for 5 minutes.

**E: Colour Reaction**

Detection of the antibody was conducted using a Vectastain ABC kit (Vector Labs, Burlingame, CA), as per manufacturer’s instructions. The ABC reagent contains avidin-biotinylated peroxidase complexes, which bind to the biotinylated antibody. Detection is through the use of the peroxidase substrate Diaminobenzidine (DAB) which turns brown.
The ABC reagent was applied to the sections and incubated in a humidified chamber for 1 hour at room temperature. Sections were then washed in TBS three times for 5 minutes at room temperature. Liquid DAB was then applied for 5-10min (with colour development being observed to determine end point), and three further 5min washes with TBS performed. Sections were then counterstained with haematoxylin for 30sec, and finally washed three times in water. Stained sections were mounted using Aquamount (Sigma-Aldrich, St Louis, MO).

*F: Analysis*

Sections were then analysed using a light microscope and Stereo Investigator 7 software package (MBF Bioscience, Williston, VT) at the Centre for Microscopy, Characterisation and Analysis (CMCA), University of Western Australia.

**COMPOSITION OF SOLUTIONS**

<table>
<thead>
<tr>
<th>Tris buffered saline (TBS)</th>
<th>TBS-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mM Tris</td>
<td>0.05% Tween20 in TBS (pH 7.4)</td>
</tr>
<tr>
<td>150mM NaCl</td>
<td></td>
</tr>
<tr>
<td>pH to 7.4</td>
<td></td>
</tr>
</tbody>
</table>

*Table 2 D: Solution compositions for Immunohistochemical and histological analysis of skin*
2.3 Determining the effect of administration of whole bone marrow systemically and topically after burn wound injury on healing and scar formation

2.3.1 Preparation of whole Bone Marrow for systemic and topical administration

Adult female C57BL/6-Tg(ACTB-EGFP)1Osb/J mice were euthanased by cervical dislocation and femora dissected. The isolated femora were flushed with a 25 gauge needle using 200 μl GKN buffer per femur. The resulting cell suspensions were combined and diluted to 10 ml in GKN buffer. The cell suspension was then centrifuged @ 300 × g for 7 min at 4°C. Supernatant was removed and cell pellet resuspended in 4 ml of GKN buffer. The mononuclear fraction of the bone marrow was isolated using Lympholyte™-Mammal density gradient (Cedarlane®, Ontario, Canada). The 4 ml of cell suspension was layered onto 3 ml of Lympholyte-Mammal in a 15 ml centrifuge tube. Resulting mix was centrifuged at 800 × g for 20 min at room temperature. The monocytes and lymphocytes reside in the interface, with a pellet of dead cells, matter and red blood cells and neutrophils. The interface layer was pipetted carefully and transferred to a clean centrifuge tube. Cells were diluted in GKN buffer, centrifuged at 800 × g for 10 min at room temperature and a final cell suspension in GKN buffer was prepared. Cells were counted on a haemocytometer using an equal volume of 2x trypan blue added to an aliquot of the cell suspension. Final cell suspension was then prepared to a concentration of 1x10^7 cells/ml.
2.3.2 Burn injury and application of systemic and topical cells

The burn injury was conducted as described previously (2.2.4). 72 mice received a 2cm diameter full thickness burn. At time of injury, the cell suspension (2.3.1) was applied either topically to the wound site or systemically by tail vein injection. For systemic administration, 120μl of the cell suspension (1.2 × 10^6 cells total) were injected via the tail vein whilst the animal was anesthetized and after the burn injury had been conducted (n=18). For topical application, 120μl of the cell suspension (1.2 × 10^6 cells) was injected underneath a 3M™ Tegapore™ Wound Contact Material (3M Australia, Pymble, NSW) and allowed to bathe the burn wound (n=18). Control animals included those injected with GKN buffer only (n=18) and topically applied GKN buffer only (n=18).

2.3.3 Cell fate monitoring and wound healing assessment

At days 7, 14, 21 28 and 55 post-burn mice were euthanased (3 from each group at each time point) according to standard procedures. The entire wound including the adjacent 3mm skin margins were excised. The wound was bisected along the cranial caudal axis. One half of the sample was fixed in 5% paraformaldehyde, processed, paraffin embedded and sectioned at 5μm for histological and immunohistochemical analysis using procedures described in 2.2.6. The second half of the sample was prepared for FACS analysis using anti-EGFP-Alexa Fluor 488 antibody as described (2.2.5). Gross re-epithelialization was quantified at day 12 and daily until >95% of the wound was re-epithelialized. To assess healing, digital planimetry of collected digital photographs of the wounds were analysed using Advanced Wound Assessment and Measurement System software (Savant Imaging, 2009). Areas of re-epithelialization were traced and the results were expressed as a percentage of re-epithelialization by comparison with the original burn area.[192]
2.4 Determining the effect of Bone Marrow Mesenchymal Stem Cells (MSCs) on burn wound repair

2.4.1 Isolation and Culture of bone marrow mesenchymal stem cells

Bone marrow was collected by flushing the femurs of three C57BL/6-Tg(ACTB-EGFP)1Osb/J mice (JAX® Mice and Services, Bar Harbor, ME) with a 25 gauge needle using 200 μl GKN buffer per femur. The mononuclear fraction of the bone marrow was isolated using Lympholyte®-Mammal density gradient (Cedarlane®, Ontario, Canada) as described (2.3.1) and resulting cells were plated in 75cm² tissue culture flasks in minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 0.5μg/ml Fungizone® antimycotic liquid, 100μg/ml kanamycin, 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen, Carlsbad, CA) and incubated for 1 week at 37°C in 5% CO₂. After 48hrs the non-adherent cells were removed and fresh medium was added to the cells. Medium was then changed every 2 days. The adherent cells were further propagated for 3 passages.

2.4.2 Characterization of cultured BMSCs

Cultured MSCs were resuspended in phosphate buffered solution (pH 7.4) supplemented with 2% FBS (Invitrogen, Carlsbad, CA) at 10⁶ cells/ml. Cell aliquots (100μl) were incubated in CD16/32 antibody (BioLegend, San Diego, CA) to block non-specific immunoglobulin binding to the Fc receptors before being incubated with phycoerythrin (PE) conjugated monoclonal antibody specific for CD105 (endoglin) and Alexa Fluor 647® conjugated monoclonal antibodies specific for
CD29 (integrin β1), CD44, CD90.2, CD45, CD34, CD19, (BioLegend, San Diego, CA) on ice for 30min. Ten thousand events were analysed with a FACS Calibur flow cytometer (Becton, Dickinson, Franklin Lakes, NJ) using excitation at 488nm and fluorescence detection at 585nm (PE), 661nm (Alexa Fluor 647). Flow cytometry analysis of data was carried out using FlowJo flow cytometry analysis software Version 7.2.2 (Tree Star Inc.).

2.4.3 Burn injury and application of BMSCs

C57BL/6 wildtype mice received a tail vein injection of $2 \times 10^6$ cultured MSCs in 200μl of GKN buffer via a 24-gauge needle administered; 5 days prior to burn injury (T-5d), at time of injury (T 0) or 24hrs post burn injury (T 24hr). The burn injury was carried out as previously described (2.2.4).

Mice were divided into the following groups;

1. No MSCs + 1cm burn (n=25)
2. MSCs + no burn (n=25)
3. MSCs T-5d + 1cm burn (n=35)
4. MSCs T-5d +2cm burn (n=35)
5. MSCs T 0 + 1cm burn (n=25)
6. MSCs T 24hr + 1cm burn (n=25)

Total number of mice is 170

At days 7, 14, 21 28 and 56 post-burn mice were euthanized (5 from each group 1, 2, 5, 6 and 7 from groups 3 and 4 at each time point) according to standard procedures.
2.4.4 Cell fate monitoring and wound healing analysis

Wound and skin sample preparation and analysis is previously described (2.2.5; 2.2.6). In addition, the potential for administered cells migrating to tissues other than the wound/skin was investigated. Liver, lung, heart, spleen, leg muscle and brain tissue samples were all prepared and analysed for the presence of transgenic donor derived cells (EGFP positive).

Organs or a portion of the organ was dissected from the mouse and placed in staining buffer. These were then minced finely and passed through a cell strainer. All tissues were substantially softer tissues than skin and cells could be isolated by physical methods rather than enzymatic digestion. The filtered cells were centrifuged @ 300 x g for 5min @ 4°C, supernatant removed and sample was resuspended in 1ml red blood cell lysis buffer. This was incubated at room temperature for 10min. The sample was centrifuged @ 300 x g for 5min @ 4°C. The supernatant was removed and cells resuspended in 1ml staining buffer. The sample was centrifuged @ 300 x g for 5min @ 4°C. Then supernatant is removed and cells resuspended 100μl staining buffer. Liver, spleen, bone marrow and brain required a further 1:20 dilution in staining buffer as cells were too concentrated for accurate analysis by flow cytometry. A rabbit anti-EGFP Alexa Fluor 488® conjugated antibody (Molecular Probes, Eugene, OR) was used for identification of EGFP positive cells. Labelled cells were then analysed with a FACS Calibur flow cytometer (Becton, Dickinson, Franklin Lakes, NJ) using excitation at 488nm and fluorescence detection at 530nm. Flow cytometry analysis of data was carried out using FlowJo flow cytometry analysis software Version 7.2.2 (Tree Star Inc.).
2.5 Assessing the contribution of hematopoietic lineage derived cells using the Vav-Cre Transgenic mouse

The cre-lox transgenic mouse model has been widely used to monitor the importance of specific cell types in development and disease. [193-194] Briefly, the system uses a mouse strain containing a reporter gene that is silenced by the presence of LoxP sites within the reporter. The strain most commonly used is the Rosa26LacZ strain, which has the LacZ gene (which codes for beta-galactosidase) inserted into the genome with LoxP sites [195]. This reporter strain contains the LacZ/LoxP construct in all the cell lineages. [195] The reporter strain is then crossed with a transgenic mouse containing the Cre-recombinase protein under the control of a specific promoter. This restricts Cre-recombinase expression to the cells of interest. When the two strains are crossed, the progeny express Cre-recombinase in the specific cell lineage of interest, and in these cells the LoxP sites are removed and the LacZ reporter gene is expressed. In all other cell types, the LacZ gene remains switched off by the presence of the LoxP sites.

In these experiments, we used the Vav-Cre mouse, which has the Cre-recombinase under the control of specific Vav elements and has been shown to restrict expression to hematopoietic stem cells and endothelial cells (and therefore all hematopoietic lineages contain an active reporter gene) [154]. This mouse was crossed with the Rosa26LacZ reporter mouse, and the offspring genotyped and characterized to confirm specific LacZ expression to the hematopoietic cells. Mice were then used for the standard burn injury model (Methods 2.2.4) and the fate of hematopoietic cells monitored longitudinally.
2.5.1 Mouse Genotyping

Mouse genotyping used tail tissue DNA and blood samples to identify the recombined fragments in hematopoietic cells. The protocols used was slightly modified from a previous report [196].

Briefly, tail tissue was digested overnight at 55ºC in TNES (Table 2 E) buffer containing Proteinase K. Blood samples did not undergo this step. Proteins were then precipitated using saturated NaCl solution, incubating at 4ºC for 30 min and then centrifuging. Supernatant was removed and genomic DNA precipitated, washed in 80% ethanol and air dried. The DNA was resuspended in TE buffer (Table 2 E) at 55ºC for 10 min and DNA quantified using a nanodrop 1000 spectrophotometer (Thermoscientific).

2.5.2 PCR reactions

3 PCR reactions were used to assess genotype. One reaction to determine the presence of the Cre recombinase gene, and two reactions to determine whether the LacZ product had undergone recombination (in blood cells of transgenic mice) or was still disrupted by the presence of the LoxP sites. Reactions used the primer combinations and conditions as previously reported [154-155]. R26R primers were used to differentiate intact and recombined R26R (1 and 2 = intact, 500 bp product, 1 and 3 = recombined 250 bp product) as below;

Primer 1 - R26R1 5’-AAAGTCGCTCTGAGTTGTTAT

Primer 2 - R26R2 5’-GCGAAGAGTTTGTCCTCAACC

Primer 3 - R26R3 5’-GGAGCGGGAGAAATGGATATG

Cre PCR also used previously reported primers and conditions generating a 262 bp fragment from the correct genotype; [154-155]
CRE1 5’-CGCAAGAACCTGATGGACAT

CRE2 5’-TGCTGTCACTTGGTCGTGG

COMPOSITION OF SOLUTIONS

<table>
<thead>
<tr>
<th>TNES Buffer</th>
<th>Saturated NaCl Solution</th>
<th>TE Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris pH 7.55</td>
<td>Approx. 6M NaCl solution</td>
<td>10 mM Tris pH 8</td>
</tr>
<tr>
<td>400 mM NaCl (MW: 58.44)</td>
<td></td>
<td>1 mM EDTA (MW: 292.25)</td>
</tr>
<tr>
<td>100 mM EDTA (MW: 292.25)</td>
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<td></td>
</tr>
<tr>
<td>250 mM SDS (MW: 288.38)</td>
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<td></td>
</tr>
<tr>
<td>0.2mg/ml Proteinase K</td>
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</tr>
</tbody>
</table>

Table 2 E: Solution compositions for Immunohistochemical and histological analysis of skin

2.5.3 Mouse Burn Injury

The murine burn wound model is based on a contact thermal full-thickness injury model [197] and has been previously described (Methods 2.2.4). Mice were divided into the following groups;

1. Vav-cre x rosa26lacZ transgenic mice with no injury (n=3)

2. Vav-cre mice + 2cm diameter burn injury (n=5, (no lacz gene control))

3. rosa26lacZ mice + 2cm diameter burn injury (n=5, (non-recombined control))

4. Vav-cre x rosa26lacZ mice + 2cm diameter burn injury (n=20, test animals)
At day 7, control animal groups (Vav Cre only and Rosa26LacZ only) were euthanased and skin and wound tissue analysed for lacZ expression. This was to ensure no lacZ detection occurred in negative controls. At day 7, non-injured Vav-creRosa26LacZ reporter mice were also euthanased to determine whether there was any lacZ expressing cells present in the skin without a burn injury. At days 7, 14, 21 and 28 post-burn test mice (n=5 at each timepoint) were euthanased. The entire wound including the adjacent 3mm skin margins were excised. An area of unburnt skin was also taken from each mouse. The wound was bisected along the cranial caudal axis. One half of the sample was processed for lacZ staining and the second half of the sample was prepared digestion and culturing of cells.
2.6 Analysis of LacZ expression in mice after burn injury

2.6.1 LacZ Whole Mount Staining and histology

The wound tissue was removed from euthanased animals at day 7, 14, 21 and 28 post-burn injury. The wound was then bisected and half the wound used for cell culture (see below) and the remaining half of the wound was used for whole mount staining. For whole mount staining, tissue was placed into wash buffer at room temperature for 20 minutes, repeated three times. The wound was then placed in LacZ staining buffer overnight (Table 2 F) in the dark at 37°C with rotation. Tissue sample was then washed in 3%DMSO/PBS three times for 10 minutes, and tissue sample then fixed in 4% paraformaldehyde and processed for tissue sectioning. 5 μm skin sections were deparaffinised, stained in Eosin stain for 45 sec, covering with cover slips and viewing under a light microscope. Types of cells that were stained blue (LacZ positive) were determined viewing multiple fields to ensure random sampling and accurate statistical analysis.
2.6.2 **Cell isolation and characterization**

One half of the bisected wound and surrounding skin was dispersed into a single cell suspension using a tissue dispersion protocol as previously described.[197] The single cell suspension obtained by this process was then washed thoroughly before being plated in T25cm² tissue culture flasks in Dulbecco’s minimum essential medium/F12 Glutamax supplemented with 10% fetal bovine serum (FBS), 0.5μg/ml Fungizone® antimycotic liquid, 100μg/ml kanamycin, 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen, Carlsbad, CA) and incubated at 37°C in 5% CO₂. After 24hrs the cells were washed with PBS (pH7.4) and fresh media added. Following another 24hrs, the media was removed and fresh media minus Fungizone® and kanamycin was added to the cells. Once the flask was confluent, the cells were seeded onto round coverslips fitted into 6-well plates before being fixed in glutaraldehyde (diluted 1 in 100 PBS pH7.4) and stained using a LacZ staining solution (2mM MgCl₂, 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆, X-gal 1mg/ml in PBS) for 18hr at 37°C in a dark chamber.

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**Table 2 F: Solution compositions for the analysis of LacZ expression**

<table>
<thead>
<tr>
<th>Wash Buffer</th>
<th>Staining Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>5mM EGTA</td>
<td>5mM K₃Fe(CN)₆</td>
</tr>
<tr>
<td>0.01% deoxycholate</td>
<td>5mM K₄Fe(CN)₆</td>
</tr>
<tr>
<td>0.02% NP-40</td>
<td>5mM EGTA</td>
</tr>
<tr>
<td>2mM MgCl₂</td>
<td>0.01% deoxycholate</td>
</tr>
<tr>
<td>20mM Tris (pH 7.3)</td>
<td>0.03% NP-40</td>
</tr>
<tr>
<td>In PBS (pH 7.4)</td>
<td>2mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>20mM Tris (pH 7.3)</td>
</tr>
<tr>
<td></td>
<td>1mg/ml X-Gal</td>
</tr>
<tr>
<td></td>
<td>In PBS (pH 7.4)</td>
</tr>
</tbody>
</table>
2.6.3 Characterisation of LacZ positive cultured cells

Cells that exhibited blue LacZ staining underwent further characterization by double staining. Immunofluorescence was conducted using a rabbit anti-collagen type 1 to stain for collagen type 1 producing fibroblasts and rabbit anti-α-smooth muscle actin antibody for myofibroblasts. Secondary antibody goat anti-rabbit IgG Alexa Fluor 488 (Molecular Probes, Eugene, OR) was used. Slides were viewed using a fluorescence microscope at the Centre for Microscopy, Characterization and Analysis (CMCA), University of Western Australia.
3.1. Optimization of a mouse contact burn injury model

3.1.1 Determination of burn injury depth and reproducibility

The animal burn wound was based on a model described previously [190]. This protocol was then modified to create a uniform, reproducible full thickness burn injury. To determine burn depth and reproducibility, 5 test mice received 2 separate burn injuries whilst under general anaesthesia. The injuries were generated using a brass rod weighing 65g and 1 cm in diameter, which equated to approximately 2% total body surface area (TBSA) injury on an 8 week old C57BL/6 mouse.

The brass rod was heated to 95°C using boiling water and then applied to the dorsal skin of the mouse for 10 seconds, and all animals were euthanased immediately post procedure without recovery. Burn wounds healed well, with no signs of infection, and wounds were >95% healed by day 11 post injury and completely healed by day 14 post-injury (using the 1cm diameter injury, (Fig. 3.1, Fig. 3.2C)

Burn injured tissue was excised, bisected and processed for histology prior to examination by two independent and experienced dermatopathologists, Dr Peter Heenan and Dr Trevor Beer, (Cutaneous Pathology, Perth, WA). After serial section analysis, both dermatopathologists confirmed that each wound was consistently full-thickness in depth and reproducible in the extent of damage to the tissue (Fig 3.2A). All 10 burn tissue samples were assessed as full-thickness injuries. The extent of damage was assessed by demarcation of the boundaries of the injury site. For the 1cm diameter brass rod induced injuries, burn injury diameter was 10.2mm ± 0.23.
Figure 3.1 Burn Wound Model

Macroscopic appearance of burn wound as seen on an 11 week old female C57BL/6 mouse; (A), at time of burn injury, (B) day 3, and (C) day 11 post injury. Wounds were healed by Day 11 in this model of burn injury.

Figure 3.2 Validation of burn wound model

A 1 cm (A) diameter brass rod was in contact with dorsal mouse skin for 10 seconds after heating to 95 C. This results in a Full thickness burn with no transition zone and is an equal thickness throughout (B&C) healed burn wound at day 14.

To generate a more severe injury model using the same technique, a 2 cm diameter brass rod, weighing 260g, was used with the same method. This was also assessed by the same dermatopathologists and all 10 samples were determined to be full thickness (Fig. 3.2B), with
wound diameter assessed as 20.5 ± 0.15 mm. The 2cm diameter brass rod equated to an injury size of approximately 8% TBSA. This data validated the extent of injury caused by use of the 1cm diameter and 2cm diameter brass rods at 95°C and in contact with the mouse dorsal skin for 10 seconds. This method was then used for all subsequent experiments.
3.2 Generation of a stable chimeric (EGFP+) Bone Marrow (BM) in wildtype mice

EGFP+ cells were isolated from the bone marrows of C57BL/6-Tg (ACTB-EGFP)1Osb/J (Jackson Labs) transgenic donor mice (Methods 2.2.2) and 2x10⁶ EGFP+ isolated cells injected in the tail vein of each lethally irradiated recipient wildtype 8 week old C57BL/6 mouse. The mice were given antibiotics during recovery and after 8 weeks had reconstituted a transgenic EGFP+ bone marrow cell population in a wildtype background.

The extent of bone marrow chimerism and effectiveness of the irradiation and transplant procedure was assessed using FACS analysis of a 100μl tail vein blood sample and in cells isolated from the bone marrow of euthanased mice. FACS analysis was used to identify EGFP+ cells. Assessment of the chimeric status of the mice was conducted at time of injury and at each time point post-injury that animals were euthanased for analysis (days 7, 14, 21, 56 and 120). In peripheral blood at time of injury, 98.52% ± 2.8% of nucleated blood cells were EGFP+ve (Fig. 3.3B). Wildtype blood samples gave a background positive stain of 1.72% ± 0.46 (Fig. 3.3A).

![Figure 3.3](image)

**Figure 3.3** FACS analysis of peripheral blood

FACS analysis of EGFP+ve cells in peripheral blood in wild-type controls (A), and in chimeric mice (B) at time of injury. Wild-type mice had a low background level of positive cells in this analysis (1.72 +/- 0.46%), whilst chimeric mice were strongly positive (98.52 +/- 0.46%).
Directly isolated bone marrow cells were 32.38% ± 4.4 positive at time of injury and wildtype bone marrow cells were 0.05% ± 0.1 (data not shown). By Day 120, the percentage of positive nucleated cells in peripheral blood was maintained at 97.07% ± 1.6, whilst the bone marrow was 33.02% ± 7.21, suggesting the chimeric status was stable for the duration of the experiments (Fig. 3.4).

**Figure 3.4**

Percentage EGFP+ve cells from FACS analysis of peripheral blood over time in chimeric mice. The Chimera appears to be stable for the duration of the experiments with greater than 97% nucleated peripheral blood cells EGFP+ve at day 120 post injury.
3.3 **Determination of the contribution of bone marrow derived cells (BMDCs) to normal skin in the absence of injury**

To determine the contribution of bone marrow derived cells to normal epidermal and dermal homeostasis, EGFP+ bone marrow recipient animals were monitored at the same time-points as injured mice for EGFP+ cells in the skin (days 7, 14, 21, 28, 56 and 120 post-injury, which equated in non-injured animals to 19, 20, 21, 22, 26 and 38 weeks old). There was no detection of EGFP+ cells in the skin samples from non-injured animals at any time-point tested above background levels (compared to wildtype mice, no EGFP+ BM transplant). Therefore analysis focused solely on the presence of bone marrow derived cells in the wound and scar after burn injury.

3.4 **Determination of the contribution of BMDCs to dermal and epidermal cell populations after a minor burn injury (2% TBSA)**

At day 1 post-injury 36.5% ± 11.9 of cells isolated from the wound site were EGFP+ (Fig. 3.5). BMDC EGFP+ cell number at the wound site decreased significantly (p<0.05) to 8.4% ± 1.4 at day 3 and 7.1% ± 2.5 at day 7 (Fig.4). At day 14, 11.03% ± 5.95 were EGFP+ and finally at day 21 post-injury only 4.6% ± 1.3 of the total cell population within the wound area were EGFP+ (Fig. 3.5).
3.4.1 Determination of BMDC derived cell types within the wound post minor burn injury

To determine the phenotype of cells identified as EGFP+ (and therefore postulated to be derived from the bone marrow cell population) immunohistochemistry using anti-EGFP antibodies in combination with histological assessment and other cell type specific markers was conducted.

Figure 3.5

Percentage of total cells in the healing 1cm diameter full-thickness burn wound that were EGFP+ve by FACS. At Day 1, greater than 35% of cells are EGFP+ve in the wound. This is significantly reduced by Day 3, and at all subsequent timepoints. A small percentage of EGFP+ve cells persist (<0.5%) in the scar after healing at Day 21.
3.4.1.1 Optimization of Immunohistochemistry protocols to identify BMDCs in wound and scar tissue

Since the BMDCs in this experimental model were EGFP+, it was postulated that BMDCs within the wound could be identified from fixed and processed paraffin embedded or frozen tissue sections using the fluorescence of the EGFP+ cells. However, initial investigations showed significant autofluorescence, in particular of the stratum corneum (Fig. 3.6A) and collagen matrix (Fig. 3.6B). In addition, keratinized structures such as adnexal structures were also autofluorescent at a similar wavelength to EGFP (Fig. 3.6C). Therefore the protocol was modified and we subsequently used non-flourescent 3.3’-Diaminobenzidine (DAB) colorimetric staining of EGFP+ cells using an EGFP specific biotinylated antibody and streptavidin-Horse radish peroxidase (HRP) conjugated secondary antibodies together with the DAB substrate to detect the presence of EGFP+ cells (Methods 2.2.6). This staining protocol allowed for clear histology to identify cell type and structures (Fig. 3.7A), and there was negligible non-specific staining (Fig. 3.7B, C) allowing for accurate quantitation of BMDCs within the wound.

Figure 3.6

Autofluorescence of (A) the stratum corneum, (B) collagen matrix, and (C) adnexal structures when imaged using the same settings as for GFP. The presence of significant autofluorescence determined the use of alternative staining protocols (No scale bars).
3.4.1.2 *Cell types of bone marrow origin identified in the burn wound and scar*

Immunohistochemical analysis of early timepoints was not conducted, due to the significant inflammatory infiltrate and cell debris within the wound site (Fig. 3.8A). It is widely accepted that there is substantial inflammatory infiltrate in the acute phase post-injury, and it was not part of this investigation. The inflammatory infiltrate and debris masked the proportion of EGFP+ cells that would be considered part of the dermal and epidermal cell populations and limited the possibility quantitation and identification of the BM derived cells in the dermal and epidermal compartments. However, qualitative analysis of longitudinal samples showed that, as expected, at early time-points there is a substantial inflammatory infiltrate within the wound that is EGFP+ (Fig. 3.8A). This appears to subside and is replaced by day 14 with a predominantly dermal cell population that is EGFP+ (Fig. 3.8B). By day 21, there are fewer EGFP+ cells present in the healed wound, but endothelial, fibroblast and epidermal cells that are EGFP+ can still be detected (Fig. 3.8C).
Figure 3.8

Immunohistochemistry of EGFP +ve cells in burn-injured tissue at (A) Day 1, (B) Day 7, and (C) Day 21 post injury. Initial inflammatory infiltrate subsides but dermal and epidermal cells that are EGFP +ve persist.

Figure 3.9

Percentage of cells originating from the bone marrow (EGFP+ve) identified using histology in the scar at Day 21 post burn injury following a 1 cm diameter full thickness burn injury.
Quantitation and phenotype of the EGFP+ cell types within the dermal and epidermal layers during the remodeling and established scar phases of wound repair was conducted using histology at day 21. At day 21 post-injury, 53.6% ± 7.8 of the BMDCs in the wound area were fibroblasts, 30.7% ± 9.2 were inflammatory cells and 4.5% ± 3.9 were epithelial cells (Fig. 3.9).
3.5 Determination of the contribution of BMDCs to dermal and epidermal cell populations after a moderate burn injury (8% TBSA)

In the moderate wound model (1.9cm diameter full-thickness burn injury) we identified 17% ± 0.7 of the total wound cell population as BMDCs at day 3 post injury using FACS analysis (Figure 3.10). By day 7, the number of BMDCs increased significantly to 38.1% ± 5.2. Due to technical difficulties, FACS analysis results for day 28 post-injury were not obtained. At day 56, the percentage of BMDCs found in the wound area was 5.1% ± 4.2. By day 120, no BMDCs were detected by FACS analysis (Figure 3.10).

![Figure 3.10](image)

Figure 3.10

Percentage of total cells in the healing 2 cm diameter full-thickness burn wound that were BMDCs (EGFP+) over time. There is a substantial percentage of cells in the acute phase, which decreases over time. However, even at Day 56, with a fully healed scar, there continues to be a small percentage of EGFP+ cells in the tissues.
Immunohistochemical analysis for BMDCs within the healed wound identified both endothelial and fibroblast cell types (Fig. 3.11). Using histology and cell counting we identified the proportion of BMDCs (EGFP+ cells) that were fibroblasts, endothelial and epidermal cells (Fig. 3.12). From this analysis, at day 28 post-injury, 66.5% ± 6.6 of the total BMDCs were fibroblasts, 28.9% ± 9 were inflammatory cells and 4.1% ± 2.5 were keratinocytes (Fig. 3.11). At day 56 post-burn, a similar profile of BM-derived fibroblasts (64.4% ± 7.5) and inflammatory cells (30.1% ± 2.3) was found, however, the contribution of BMDCs to the epidermal layer decreases (1% ± 0.8) and BM-derived endothelial cells (3.3% ± 3.3) appear in some sections. By day 120 post-injury, no BMDCs were detected using histology.

At day 28 post-burn, over half 66.5% ± 6.6 of all BMDCs are fibroblasts, almost one third of BMDCs 28.9% ± 9 are inflammatory cells whilst less than 5%, 4.1% ± 2.5 of BMDCs are found in the epidermis (A). At day 56 post-burn, a similar profile of BM-derived fibroblasts 64.4% ± 7.5 and inflammatory cells 30.1% ± 2.3 was found, however, the contribution of BMDCs to the epidermal layer decreases 1% ± 0.8 and BM-derived endothelial cells 3.3% ± 3.3 appear (B).
Figure 3.11

Cell types originating from the bone marrow that were found in a healing wound following a 2 cm diameter full-thickness burn injury. A and B: Day 15 post injury. EGFP+ cells appear to be predominantly epithelial and inflammatory cells. Epithelial BMDCs are found in all layers of the hyperproliferative wound edges (A) and inflammatory BMDCs are found in the dermis of the healing wound (B). C and D: Day 28 post injury. Epithelial BMDCs are found only in the differentiated layers of the epidermis (C) and EGFP+ fibroblasts appear within the dermis(D). E and F: Day 56 post injury. At this time point the wound is fully healed. Very few BMDCs (EGFP+ cells) are found in the epidermia (E) and few fibroblasts are found in the dermis (F). By Day 120 post injury, no EGFP+ cells were identified (not shown).
Summary

In summary BMDCs contribute to healing in both a minor and moderate burn wound. They do not contribute to normal skin. The contribution diminishes over time but was still present after burn wound healing at day 11. Immunohistochemistry indicates that the predominant cell type in the later stages of wound healing are fibroblasts. This suggests that BMDCs may contribute to scar formation.
3.6 Topical and intravenous administration of exogenous Bone marrow derived cells and their effect on burn wound healing

The results of the previous experiments provide clear evidence for the migration and long-term persistence of bone marrow derived cells within both minor and moderate burn wounds. However, the cells do not appear to remain in the scar tissue permanently but rather are involved in mediating wound healing. In addition, recent evidence has emerged that specific bone marrow cell populations may promote wound healing in vivo [183]. We therefore in light of this and the previous chapter’s results decided to determine whether exogenous BMDCs could promote healing. We administered isolated BMDCs both topically and intravenously in the mouse model of burn injury. These experiments aimed to clarify whether exogenous BMDCs could;

1. Provide a source of viable cells that were involved in the wound healing process
2. Promote or slow the wound healing process

This is critical to understanding whether there is therapeutic potential for the use of these cells in promoting repair after burn injury.

3.6.1 Topical administration of BMDCs at the time of injury

At the time of injury, 1 x 10^6 transgenic EGFP+ BMDCs were applied to the burn injury wound surface on wildtype animals (C57BL6/J mice). The burn injury model used for the moderate injury (8% TBSA, 2cm diameter full-thickness injury). This was used for both topical and IV administration, as it takes longer to heal than the 1cm minor injury and we expected any difference in time to heal to be more easily observed in this more severe model of injury. Cell fate was then monitored over time using FACS and histology of tissue biopsies. FACS analysis showed that at day 7, 0.13% ± 0.03 of live cells were EGFP+ (Fig. 3.13A). At day 14 this was 0.78% ±0.30 (Fig. 3.13B), at day 21 there were 1.06% ±0.31 EGFP+ live cells (Fig. 3.13C), at day 28 0.89% ±0.16 were EGFP+, and at day 56, 1.06% ±0.11 cells were EGFP+ (Fig. 3.13 E).
Background positive staining in animals with no EGFP+ cells administered was 0.22% ± 0.13. There was no significant difference between these values at any timepoint when compared to the negative background control. This strongly suggests there were no viable EGFP+ cells persisting at the site of the wound from day 7 post-injury when cells were topically administered.

Figure 3.13
FACS analysis of burn injury tissue biopsy at Day 7 (A), Day 14 (B), Day 21 (C), Day 28 (D) and Day 56 (E) post-injury. No tissue samples showed elevated live EGFP+ cell numbers when compared to the negative control (no EGFP+ cell application). Columns show cells detected in IV control, IV burn, Topical Administration control and Topical Administration burn injury from left to right.

For immunohistochemical analysis, no cell counts were performed prior to day 14 as the aim was to assess potential long-term contribution of exogenous cells rather than short-term survival. In addition, in the acute phase tissue immunohistochemistry is of limited use given the extensive cell damage and presence of high levels of inflammatory cells. However,
immunohistochemical detection was used to assess cells that were EGFP+ at day 14 post-injury and all later timepoints (day 21, day 28 and day 56) despite the lack of EGFP positive cells identified using FACS.

Cells that were identified as EGFP+ were further characterized by histology to determine cell type. At day 14, 71% ± 4.24 of the total EGFP+ cells in the wound area were fibroblasts, 3.30% ± 1.4 were endothelial cells and 10.73% ± 8.2 were hair follicle cells. In addition a further 6.38 % ± 1.40 of cells were not sufficiently identified using histology (Fig 3.14A).

At day 21, 48.41% ± 7.97 of the total EGFP+ cells in the wound area were fibroblasts, 0.6% ± 0.59 were endothelial cells and 0.16% ± 0.14 were hair follicle cells. In addition a further 50.81% ± 7.35 of cells were epithelial (Fig. 3.14B).

At day 28, 39.95% ± 36.75 of the total EGFP+ cells in the wound area were fibroblasts, and 1.17% ± 0.7 were hair follicle cells, in addition a further 57.05% ± 40.59 of cells were epithelial, no endothelial cells were identified (Fig. 3.14C).

By day 56, 18.89% ± 6.25 of the total EGFP+ cells in the wound area were fibroblasts, in addition a further 80.34% ± 6.22 of cells were epithelial and 0.77% ± 0.02 were hair follicle cells, no endothelial cells were identified (Fig. 3.14D).

For immunohistochemical analysis, no cell counts were performed prior to day 14 as the aim was to assess potential long-term contribution of exogenous cells rather than short-term survival. In addition, in the acute phase tissue immunohistochemistry is of limited use given the extensive cell damage and presence of high levels of inflammatory cells. However, immunohistochemical detection was used to assess cells that were EGFP+ at day 14 post-injury and all later timepoints (day 21, day 28 and day 56) despite the lack of EGFP positive cells identified using FACS.
Fig 3.14 Cell Phenotypes of cells originating from the bone marrow (EGFP+) that were identified using immunohistochemistry of wound or scar tissue at Day 14 (A), Day 21 (B), Day 28 (C) and Day 56 (D), following a topical administration of $1 \times 10^6$ cells from a freshly isolated whole BMDC preparation at the time of injury. Note that detection of any EGFP+ cells was very low, therefore these charts represent very small numbers.

Cells that were identified as EGFP+ were further characterized by histology to determine cell type. At day 14, 71% ± 4.24 of the total EGFP+ cells in the wound area were fibroblasts, 3.30% ± 1.4 were endothelial cells and 10.73% ± 8.2 were hair follicle cells. In addition, a further 6.38% ± 1.40 of cells were not sufficiently identified using histology (Fig 3.14A).

At day 21, 48.41% ± 7.97 of the total EGFP+ cells in the wound area were fibroblasts, 0.6% ± 0.59 were endothelial cells and 0.16% ± 0.14 were hair follicle cells. In addition, a further 50.81% ± 7.35 of cells were epithelial (Fig. 3.14B).

At day 28, 39.95% ± 36.75 of the total EGFP+ cells in the wound area were fibroblasts, and 1.17% ± 0.7 were hair follicle cells, in addition, a further 57.05% ± 40.59 of cells were epithelial, no endothelial cells were identified (Fig. 3.14C).
By day 56, 18.89% ± 6.25 of the total EGFP+ cells in the wound area were fibroblasts, in addition a further 80.34% ± 6.22 of cells were epithelial and 0.77% ± 0.02 were hair follicle cells, no endothelial cells were identified (Fig. 3.14D).

3.6.2 Intravenous administration of BMDCs at the time of injury

A second group of animals were administered 1x10^6 freshly isolated bone marrow derived cells by tail vein injection at the time of injury. FACS analysis showed that, as with the topical administration, at no time-point was there any significant elevation in numbers of EGFP+ cells above the level of the negative control (mice with no EGFP+ cells administered after burn injury, (Fig. 3.13)). Using histology to identify cell types, at day 14, 86.06% ± 4.4 of the total EGFP+ cells in the wound area were fibroblasts, 3.3% ± 1.4 were endothelial cells and 4.24% ±
5.4 were hair follicle cells, in addition a further 5.3 % ± 4.11 of cells were not determined. (Fig 3.15A)

At day 21, 21.13% ± 12.85 of the total EGFP+ cells in the wound area were fibroblasts, 78.24% ±11.79 of the cells were epithelial, and 0.63% ±1.09 were hair follicle cells, no endothelial cells were identified (Fig 3.15B).

At day 28, 74.95% ±29.6 of the total EGFP+ cells in the wound area were fibroblasts, in addition a further 3.897% ± 4.65 of cells were epithelial and 17.69% ± 28.16 were hair follicle cells, 0.0676% ± 28.16 were endothelial cells.(Fig 3.15C).

At day 56, 34.21% ± 14.68 of the total EGFP+ cells in the wound area were fibroblasts, 40.06% ± 36.47 of cells were epithelial, and 25.69% ± 42.25 were hair follicle and 0.03% ± 0.05 endothelial cells were identified (Fig 3.15D).

3.6.3 Impact of exogenous BMDC administration on time to healing

We monitored the wounds from each group for time taken to heal. All wounds healed by day 19, with most wounds healed at day 14 post-injury. No significant differences were observed between groups, although there was a slight trend to slower healing in the treatment groups compared to control (Fig. 3.16). No further data on wound healing rate was collected (wound closure using planimetry) on these samples.
Summary

The administration of freshly isolated bone marrow cells (whole population) either topically or intravenously did not appear to provide a source of viable cells at the site of injury nor have any observed positive impact on wound repair. However, this may be in part due to the use of whole isolates rather than specific populations of cells. Recently, interest in the potential efficacy of the mesenchymal stem cell subset of bone marrow cells has been increasing, with reports of positive effects in some tissue injury models [198]. There is also some interest in the potential role of hematopoietic derived cells (such as fibrocytes) in wound repair and scarring.
[199]. We therefore determined to assess the role and therapeutic potential of mesenchymal and hematopoietic cells in burn wound repair.
3.7 Identifying the role of Mesenchymal Stem Cells in burn wound healing

From our previous studies we have reported evidence that BMDCs migrate to the site of burn injury and persist during healing and scar formation[197]. However, two distinct cell populations exist within the bone marrow cell compartment, mesenchymal and hematopoietic, and to date the relative contributions of these distinct cell populations has not been identified. In addition, there is some evidence that lethal irradiation, whilst effective in replacing all hematopoietic lineages, does not remove all the endogenous MSCs within the bone marrow [200-201] and is therefore not as effective a model for identifying the role of MSCs. Finally, there is increasing interest in the possible clinical potential for MSCs in a number of different trauma types, and even small scale clinical applications of MSCs after burn injury have been reported [[202-204]. Therefore a series of experiments using isolated EGFP+ bone marrow derived MSCs was conducted to determine;

1. Whether bone marrow derived MSCs are involved in the wound healing response after burn injury

2. Whether isolated and exogenously administered MSCs migrate specifically to and persist in the wound site or whether they migrate and persist in other tissues after administration in vivo

3. To identify possible interactions and/or effects of MSCs on other cell types involved in the wound healing process to determine whether MSCs may be a positive or negative influence on scar formation and wound healing outcomes

For Aims 1-3 above, $2 \times 10^6$ EGFP+ donor MSCs were administered through intravenous tail vein injection into wildtype mice at three different time points;

A. five days prior to injury

B. at time of injury (T0)

C. twenty four hours post injury (T24)
These time-points were chosen to assess the fate of MSCs both in a context similar to the endogenous situation (injected 5 days prior to injury, therefore the donor cells will not be subject to initial wound healing signalling) and also in a therapeutic context (administered at time of injury to determine the response of BMDCs in the immediate time frame post-injury and 24 hours post-injury to determine the longer term role of BMDCs and potential for clinical application). Cell fate and contribution to wound healing of the administered MSCs was examined.

Fig 3.17  FACS analysis of isolated and cultured mesenchymal stem cells prior to injection into the mice. Greater than 89% of cells were CD44+, CD29+, CD90.2+, CD105+, CD34-, and CD19-.
3.7.1 Selection of MSC population for tail-vein injection

In this study we used the selective adherence of MSC’s in culture to isolate MSCs from whole bone marrow isolates. Cells that were administered by tail vein injection were assessed prior to injection using validated markers of MSC’s [111]. The antibody panel used consisted of, CD105, CD90.2, CD44, CD 34 and CD29 positive markers and CD19, CD45 and CD34 negative markers. Prior to injection, FACS analysis showed that 99.55% of cells were CD34+, 99.32% CD44+, 89.31% CD105+, 98.4% CD90.2+ and 96.36% CD19− (Fig 3.17).

3.7.2 The contribution of MSCs to burn wound healing after intravenous administration 5 days prior to injury in a minor burn wound model

After administration of isolated EGFP+ MSCs by tail vein injection 5 days prior to injury, EGFP+ cells can be detected in wound tissue samples at day 7 with 1.46% ± 1.1 of cells EGFP+. This is significantly elevated when compared to both control groups (no MSC injection and burn injury (0.49% ± 0.32, p<0.05) and MSC injection with no injury (0.61% ± 0.46, p<0.05)). At days 14, 21, 28 and 49, a small percentage of EGFP+ cells were still detected, but this was not significantly different from the background controls (Fig. 3.18).
As in previous studies, the wounds and subsequent scars were sectioned and examined histologically and using anti-EGFP antibodies to detect MSC derived cells and assess phenotype. This was done at all time-points even though FACS suggests there are no significant EGFP+ cell populations post day 7. At day 7 in the minor wound (1cm diameter), 34.13% ± 41.73 of the total BMDCs in the wound area were inflammatory, 8.51% ± 4.99 were immunological cells and 26.43% ± 22.92 were fibroblasts, in addition a further 30.11% ± 26.68 of cells were epithelial (Fig. 3.19A).

At day 14, 61.18% ±14.26 of the total BMDCs in the wound area were epithelial, 20.89% ±10.07 were fibroblasts and 16.11% ± 13.99 were hair follicle cells, in addition a further 0.80% ±1.24 of cells were undifferentiated, endothelial and immunological respectively (Fig. 3.19B).

By day 21, 70.91% ± 37.47 of the total BMDCs in the wound area were fibroblasts, 27.1% ± 39.31 were inflammatory 0.73% ± 1.04 were immunological (Fig. 3.19C).
By day 28, 60.37% ± 27.07% of the total BMDCs in the wound area were fibroblasts, in addition a further 23.32% ± 31.9% of cells were epithelial, 3.22% ± 5.58% were immunological, 2.15% ± 3.72% were undifferentiated and 2.78% ± 2.80% were hair follicle cells (Fig. 3.19D). By day 49, 99.03% ± 1.09% of all the BMDC’s seen were fibroblasts (Fig. 3.19E). It is important to note that there were very few EGFP+ cells identified even by immunohistochemistry, supporting the FACS data suggesting very limited survival or migration to the wound of the injected cells.

**Fig 3.19** Cell Phenotypes of cells originating from the EGFP+MSCs that were identified using immunohistochemistry of wound or scar tissue at Day 7 (A), Day 14 (B), Day 21 (C) and Day 28 (D) and Day 49 (E) following an intravenous (tail vein) administration of 2x10^6 cells from a freshly isolated MSC preparation 5 days prior to injury. Note that detection of any EGFP+cells post day 7 was very low, therefore these charts represent very small numbers.
3.7.3 The contribution of MSCs to burn wound healing after intravenous administration 5 days prior to injury in a moderate burn wound model

In the 2cm diameter moderate burn injury model the results mimic closely those found with the minor injury model. However, at no time point was there a significant elevation in the percentage of EGFP+ cells compared to controls. There was a trend that suggests a small population of EGFP+ cells may be present preferentially in the wound site of treated animals, and this was assessed using immunohistochemistry.

![Graph](image)

**Fig 3.20** Percentage EGFP+ cells in wound tissue after 2cm burn injury and administration of $2 \times 10^6$ cells isolated EGFP+MSCs 5 days prior to injury by tail vein injection. No significant difference was observed between treated and control groups at any timepoint.

From this analysis at day 7 in the 2cm wound 74.55% ± 38.45 of the total BMDCs in the wound area were inflammatory, 13.39% ± 22.77 were immunological cells and 10.05% ± 17.41 were fibroblasts, in addition a further 1.39% ± 1.21 of cells were epithelial (Fig 3.21A).

At day 14, 81.39% ± 6.89 of the total BMDCs in the wound area were inflammatory, 14.54% ± 5.88 were undifferentiated and 4.06% ± 1.67 were immunological (Fig 3.21B).
Fig 3.21 Cell Phenotypes of cells originating from the EGFP+MSCs that were identified using immunohistochemistry of wound or scar tissue at Day 7 (A), Day 14 (B), Day 21 (C) and Day 28 (D) and Day 49 (E) following an intravenous (tail vein) administration of $2 \times 10^6$ cells from a freshly isolated MSC preparation 5 days prior to a 2cm burn injury. Note that detection of any EGFP+cells post day 7 was very low, therefore these charts represent very small numbers.

At day 21, 97.35% ± 2.41 of the total BMDCs in the wound area were fibroblasts, 1.23% ± 2.14 of cells were undifferentiated, and 1.06 % ± 1.56 were epithelial and 0.35% ± 0.34 were immunological (Fig 3.21C). Due to technical problems there were no day 28 samples available for histology. This was due to some mice needing to be euthanased as they had injured themselves from fighting in the cages.

At Day 55, 98.77% ± 1.07 of the BMDC in the wound were fibroblasts, 0.44% ± 0.40 were epithelial, 0.19% ± 0.32 were undifferentiated, 0.44 % ± 0.50 were hair follicles (Fig 3.21D).

**Summary**

The administration of MSCs 5 days prior to injury did not result in many MSCs being appearing in the wound. MSCs were detected at Day 7 with minimal amounts after this. In the moderate
wound there were no significant MSCs detected. It is most likely that the cells are not surviving as they are delivered to early. As a direct result of these findings a further study was performed administering the MSCs at the time of injury.
3.7.4  The distribution and contribution of MSCs administered intravenously at time of injury to burn wound healing in a moderate burn injury

Given that the contribution of exogenous MSCs to both the minor and moderate burn injury appeared similar in the previous experiments, the analysis of the distribution of these cells and contribution to healing when administered at time of injury and 24 hours post-injury was analysed only using the moderate burn injury model.

FACS analysis shows that for the 2cm burn with MSC injected at time of injury there are 3.75% ± 1.13 EGFP +ve cells at day 7, 3.1% ± 1.68 at day 14, 4.78% ± 1.17 at day 21, 1.95% ± 0.77 at day 28 and 1.35% ± 0.44 at day 56 (Fig. 3.22). Cells also appear elevated in normal tissue taken at day 21 only.

Fig 3.22 Percentage EGFP+ cells in skin samples after a 2cm burn injury and the administration of 2x10^6 isolated EGFP+MSCs at time of injury by tail vein injection. Administration of cells results in significantly elevated levels in both normal and injured tissue at days 7, 14, 21, 28, and 56 compared to no MSC controls. The injury shows elevated levels of EGFP+ cells compared to no injury control at day 21 only.
from injured animals at all time-points compared to no MSC injection controls. At day 7, 1.75% ± 1.04 of the cells were EGFP+, at day 14, 2.025% ± 2.37 of the cells were EGFP+, at day 21 4.34% ± 4.63 were EGFP+, at day 28, 2.27% ± 0.92 of the cells were EGFP+ whilst at day 56 0.026% ± 0.019 were EGFP+ (Fig. 3.22). These are all significantly elevated compared to the No MSC injection control group (p<0.05), demonstrating the detection of EGFP+ cells is effective and that MSCs appear to migrate to normal skin and injured tissue after application at time of injury. However, compared to the no burn injury group injected with MSCs, the burn injury appears to significantly increase the EGFP+ cells in the skin at day 21 post injury (Fig. 3.22).

Immunohistochemistry shows that at day 7 post-injury, inflammatory cells comprised 75.94% ± 34.89 of all the EGFP+ cells identified (Fig. 3.23A). At day 14, inflammatory cells comprised 52.78% ± 3.92 of all the EGFP+ cells identified, while 18.56% ± 23.85 were hair follicle cells and 26.45% ± 24.51 were fibroblasts (Fig. 3.23B). At day 21, 70.61% ± 25.7 of all EGFP+ cells identified were fibroblasts while 14.54% ± 14.9 were hair follicle cells (Fig. 3.23C). At day 28, fibroblasts accounted for 76.64% ± 6.96 (Fig. 3.23D) EGFP+ cells identified while at day 56, total cell number was reduced and there appeared to be small numbers of fibroblasts 38.58% ± 20.43 epithelial cells 10.69% ± 14.75 and hair follicle cells 26% ± 4.33 (Fig. 3.23E).
Fig 3.23 Cell phenotypes of cells originating from the administered EGFP+MSCs (administered at the time of injury-T0) that were identified using immunohistochemistry of wound or scar tissue at Day 7 (A), Day 14 (B), Day 21 (C) and Day 28 (D) and Day 56 (E). The pattern of cell types seen was similar over time to that observed in our previous studies, but more EGFP+ cells were detected when MSCs were administered at time of injury.

3.7.4.2 Analysis of MSC engraftment to multiple other organs after burn injury and exogenous MSC intravenous administration

To examine therapeutic potential in more detail, we also examined tissues other than the site of injury, to determine whether exogenous MSCs can engraft in the long-term and the specificity of the response to the burn injury. We examined non-injured skin and the liver, spleen, brain, muscle, heart and lung for the presence of injected MSC’s.

At all time-points tested (days 7, 14, 21, 28 and 56 post-injury), EGFP+ cells were detected in all tissues tested, both in uninjured animals and in animals receiving a burn injury. The detection of EGFP+ cells was significantly elevated compared to background detection using FACS on non-injected animals. However, there was no significant difference at any time-point between injured and uninjured animals when animals were injected at time of injury (Fig.
Less than 1% of all cells were EGFP+ in any tissue tested, with the exception of the spleen, liver and bone marrow. These tissues had the greatest number of EGFP+ cells detected post-injury (Fig. 3.24).

**Fig 3.24** Detection of EGFP+ cells in Liver (A), Spleen (B), Bone Marrow (C), Skeletal Muscle (D), Lung (E), Heart (F) and Brain (G) isolated from uninjured (blue) and burn-injured (red) mice after tail-vein injection of 2x10⁶ isolated MSCs (injection at time of injury-T0). The injury had no significant effect on the levels of EGFP+ cells detected in any tissue, but the administration of cells led to significantly elevated EGFP+ cells in all tissue tested at all time-points in both injured and uninjured animals when compared to No MSC injection controls.
3.8 The distribution and contribution of MSCs administered intravenously 24 hours post-injury to burn wound healing in a moderate burn injury

3.8.1 Analysis of the Burn Wound/Scar site and non-injured skin

In the 2cm moderate full-thickness burn injury group who received the MSC’s at 24 hours post injury the FACS analysis showed that at day 7, 2.75% ± 2.40 cells were EGFP +ve, at day 14, 0.868% ± 0.157 were EGFP +ve, at day 21, 4.00% ± 2.08 cells were EGFP+, by day 28 4.387% ± 0.70 cells were positive and at day 56 0.086% ± 0.89 cells were EGFP+ from burn injured animals. Fig 3.25) This was not significantly elevated over the number of cells detected in normal skin from these burn injured animals at any timepoint (Fig. 3.25), but was significantly elevated over negative controls at all timepoints (both uninjured and injured skin compared to no MSC injection + burn injury controls, (Fig. 3.25).

From histological and immunohistochemical analysis, in this group at day 7, hair follicles accounted for 92.99% ± 7.78 of all EGFP +ve cells, 2.05% ± 3.56 were endothelial and2.56 % ± 4.44 were not identified (Fig. 3.26A). In the day 14 sample, inflammatory cells accounted for 80.11% ± 10.48, fibroblasts were12.73% ± 17.99 and indeterminate 7.18% ± 7.55 (Fig. 3.26B). At day 21, 71.98 % ± 14.34 of all EGFP+ cells were fibroblasts, the remainder were undifferentiated 6.71% ±5.39, langerhans 0.24% ± 0.34, immunological 1.32% ± 1.86, hair follicles 4.10 % ± 5.81 and inflammatory 15.64% ± 13.24 (Fig. 3.26C). By day 28 the EGFP positive cells were fibroblasts 76.81 ± 14.35, indeterminate 15.95% ±4.76 and inflammatory 6.36% ± 11.01 (Fig. 3.26D). At day 56 fibroblasts accounted for 17.52% ± 7.35 of EGFP+ cells, hair follicles 61.41% ± 5.55 and there remained a small inflammatory population of cells 9.28 % ± 10.08 (Fig. 3.26E).
Fig 3.25 Percentage EGFP+ cells in tissue samples after 2cm burn injury after tail-vein injection of 2x10^6 isolated MSCs 24 hours post injury. Administration of cells results in significantly elevated levels in both normal and injured tissue at days 7, 14, 21, 28, and 56 compared to no MSC controls. There is no significant difference between injury and no injury animals in EGFP+ cell detection at any timepoint.

Fig 3.26 Cell phenotypes of cells identified using immunohistochemistry of wound / scar tissue at Days 7, 14, 21, 28 and 56 (A-E sequentially) after tail vein injection of 2x10^6 isolated MSCs 24 hours post injury. The pattern of cell types seen was similar over time to that observed in our previous studies.
3.8.2 Analysis of migration of exogenously administered MSCs to organs after burn injury

At all time-points tested (days 7, 14, 21, 28 and 56 post-injury), EGFP+ cells were detected in all tissues tested, both in uninjured animals and in animals receiving a burn injury. The detection of EGFP+ cells was significantly elevated compared to background detection using FACS on non-injected animals. However, there was no significant difference at any time-point between injured and uninjured animals when animals were injected at 24 hours post-injury at (Fig. 3.27). The highest number of cells were detected in the liver, spleen, bone marrow and lung tissue, but significantly elevated albeit small populations of EGFP+ cells were still detected in the brain at day 56 post-injury (Fig. 3.27).
Fig. 3.27 Detection of EGFP+ cells in Liver (A), Spleen (B), Bone Marrow (C), Skeletal Muscle (D), Lung (E), Heart (F), and brain (G) isolated from uninjured (BLUE) and burn-injured (RED) mice after tail-vein injection of 2x10^6 isolated MSCs (24 hours post injury). The injury had no significant effect on the levels of EGFP+cells detected in any tissue, but the administration of cells led to significantly elevated EGFP+ cells in all tissue tested at all time-points in both injured and uninjured animals compared to No MSC injection controls.
3.9 Investigation of the impact of burn injury and the timing of administration of MSC on cell fate.

The next aim was to use statistical modelling to understand how cell survival over time in each tissue is affected by time of administration. This is important to understand with respect to potential therapeutic use, and the opportunities for administering cell based therapies after a burn injury. For future potential therapeutic trials, an understanding of the impact of timing of administration may be critical. To do this, a mixed model method of statistical analysis was used. This methodology was chosen as this data requires longitudinal and multilevel analysis.

There are multiple levels of clustering within this data so that observations at time-points are nested within organs that are nested within individuals. This means that the normal statistical assumption of independent sampling is not valid without using a multi-level model. In addition, the counts of cells obtained do not follow a normal distribution, restricting the appropriate statistical models that can be used. In this case, the mixed model is not dependent on the assumption of normality and is resistant to bias from missing observations, making it the most suitable method for comparisons between the groups. For this analysis of the data, statistical advice and support was provided by Dr Michael Philips, Biostatistician, Western Australian Institute for Medical Research, University of Western Australia.

3.9.1 Analysis of the impact of timing of cell delivery (at time of injury or 24 hours post-injury) on cell distribution and survival

Prior to conducting the modelling analysis of the impact of cell administration timing, a number of statistical modelling controls were conducted to ensure any assessments were valid. Initial modelling was done to test whether there was increased EGFP cell detection after EGFP cell administration compared to no administration controls.
A mixed model analysis with time as a random effect and group membership as fixed effects indicates that ALL MSC injected groups have a significantly higher intensity measure in wounded skin when compared to No MSC injection and burn injury (Table 3.1). This result suggests that the EGFP measurements of wounded skin are not simply an artifact caused by injured tissue or protocol issues, but is dependent on the injection of EGFP+ cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>Coefficient</th>
<th>95% Conf interval</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>2.96</td>
<td>2.32 to 3.61</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T24</td>
<td>3.41</td>
<td>2.75 to 4.07</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Burn-No MSC</td>
<td>0.02</td>
<td>-0.81 to 0.85</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 3.1 EGFP+ cell detection in T0 and T24 MSC administered groups. EGFP+ cell detection is significantly elevated in both T0 and T24 MSC groups compared to No MSC+burn injury control. This demonstrates detection is not an artifact associated with the burn injury but is detecting transplanted MSCs.

Background levels of EGFP+ cell detection was also assessed in all tissues in animals not injected with EGFP+ cells (Table 3.2). Mean intensity of detection was not significantly different to 0 in 6 tissues tested. However, there was a detection of EGFP+ signal in both the liver and lung that was significantly above a 0 value (Table 3.2). This indicates a low level of background fluorescence in cells from these tissues that was important to note for further experimental analysis.
Table 3.2: EGFP+VE cells detected in animals not administered MSCs. In all tissues there is no significant detection of EGFP+ cells (as evidenced by confidence intervals overlapping 0), except in lung and heart where there is a small positive detection of cells.

Modelling of the cell populations after administration at time of injury or 24 hours post-injury showed no significant difference in detection of EGFP+ (MSC) cells in 6 of the 8 tissues tested (Fig. 3.28) between the two modes of administration. However, there was a significant difference between the EGFP+ cells identified at both the wound site and in the liver over time, when administered at different times with respect to the injury (Fig. 3.28). In particular, at both day 7 and day 28, a larger population of EGFP+ cells were detected at the wound site when cells were administered at 24 hours post-injury (Fig. 3.28). This suggests greater migration or survival of cells at the site of the wound when administered at 24 hours after the injury, when compared to administration at the time of injury. The liver also showed
differences in EGFP+ cell detection, with more cells detected at day 7 post-injury when cells were administered at the time of injury (Fig. 3.28). In skeletal muscle, heart, spleen, bone marrow, lung and brain, there were no differences observed between the two groups.
Figure 3.38 A-D
Details in legend on following page
Fig 3.28 E-H

EGFP+ cell detection in each tissue when cells were delivered at time of injury (T0) and 24 hours post injury (T24) compared to non-injured controls with cells administered. (A) Burn wound scar, (B) Liver, (C) Bone Marrow, (D) Lung, (E) Brain, (F) Heart, (G) Skeletal Muscle and (H) Spleen. No difference was seen in EGFP+ cell survival over time between delivery at 24 hours following, or at the time of injury except in the wound and the liver (A&B).
Summary

Administration of MSC intravenously at the time of injury and at 24 hours post injury has shown the following.

Administration of MSC prior to injury on FACS analysis showed very low levels of MSCs in the wound at all time point. There was no significant difference between the groups. Administration of MSC at time of injury and at 24 hours post injury shows an increased number of EGFP +ve cells in the wounds over time in those animals injected 24 hours post injury (P=0.038). Systemic detection of MSCs was greatest in bone marrow, wounded skin, normal skin and spleen. Long term persistence of MSCs in other tissues was identified up to 8 weeks post injury in all the organs examined except the spleen.
3.10 The Role of hematopoietic derived cells in the post-inflammatory phase of wound repair

Over the past few years, there has been increasing interest in the role of fibrocytes in wound repair. Fibrocytes are thought to be derived from the hematopoietic cell lineage, and have been shown to be important in wound repair [205-206]. Since we previously identified that BMDCs are present during normal wound repair, and have subsequently investigated the potential therapeutic use of the mesenchymal cell population in healing, we also determined to investigate the contribution of the hematopoietic cell lineage to the post-inflammatory phase of burn injury repair.

We obtained two transgenic strains of mice, a VAV-Cre mouse, which has the Cre recombinase protein under the control of the VAV promoter, and thus restricts Cre recombinase expression to hematopoietic stem cells. [195] These animals were crossed with ROSA26 LacZ animals, which have the LacZ reporter gene inserted into the genome in a form which can only be activated by Cre recombinase expression. Hence in the offspring of the VAV-Cre x ROSA26LacZ mice, the LacZ reporter gene is expressed in the hematopoietic stem cells and in all the cells derived from them. Therefore the entire hematopoietic lineage is LacZ positive, while other cells do not express the LacZ reporter gene.

3.10.1 Animal genotyping and experimental design

All animals were genotyped using PCR prior to use in the burn injury experiments (Methods 2.5.1). Those animals that had recombined and were positive for LacZ expression were given a 2cm diameter burn injury and the presence of LacZ positive cells in tissue biopsies assessed at day 7, 14, 21 and 28 post-injury using whole mount staining (Methods 2.6.1). Tissue samples were also used for cell isolation and culture, to assess the phenotype and presence of LacZ positive cells (Methods 2.6.3).
3.10.2 Whole mount staining of wound tissue biopsies after burn injury

Pre-injury skin biopsies taken from transgenic animals confirmed no expression of LacZ positive cells in the skin samples isolated prior to injury (Fig. 3.29). This was done to insure there were no mosaic animals being used that already have aberrant LacZ expression in the skin. Previous reports have demonstrated that the Cre recombinase can be activated aberrantly during embryogenesis and thereby creating mosaic animals [154]. Pre-injury biopsies confirmed no mosaic expression in animals used (Fig. 3.29 A, B). Some mosaic animals were detected (Fig. 3.29 C), but these were not used. Subsequently, whole mount staining identified LacZ positive cells in the healing wound post burn injury (Fig. 3.30). At day 7 post-injury, the presence of immune infiltrate, as expected, was positive for LacZ expression (Fig. 3.30 A). By day 14, there were still lacZ positive cells detected in the healed wound tissue (Fig 3.30 B), which appeared to reside in the dermis and have a fibroblast like morphology. At day 21 and 28, no positive cells were detected in any of the healed skin samples obtained (Fig. 3.30 C, D). This suggests any hematopoietic derived cells are present in the healing wound only transiently in this model. Control samples without the recombined gene expression did not

Fig 3.29 Representative samples of pre-injury biopsies confirm that no lacZ positive cells are present in the skin prior to injury. This confirms that the transgenic animals were not mosaic and the there were no lacZ positive cells in the skin prior to injury (A and B). A sample of a mosaic transgenic animal sample that was detected but not used is also shown (C).
show any positive LacZ expression, confirming that LacZ expression was only detected in transgenic animals (Fig. 3.30 E,F).
Fig 3.30 LacZ positive cell detection using whole mount staining technique after a burn injury. LacZ positive cells are derived from the hematopoietic lineage. Cells are present in the wound at Days 8, 14, 21, and 28 (A-D respectively). However, cells within the dermal or epidermal compartments are no longer observed post Day-14 following injury. Representative controls animals at Day 7 (VavCre only, E), and Rosa26LacZ only (F) are shown. No positive LacZ staining was detected at any time-point in control animals. The solid triangles indicate LacZ positive blue cells. At Day 7, these are largely inflammatory cells (A), but by Day 14 there are cells within the dermal layer similar to fibroblasts (B). These are no longer present at Day 21 (C), but blood vessels stain positive confirming that the methodology and animals are correct.
3.10.3 Isolated cell LacZ expression and phenotypic analysis

Cells from the wound tissue at each time point were isolated and grown in culture. At day 7 (Fig. 3.31A) and 14 (Fig. 3.31B), LacZ positive cells were detected in the cell culture. However, consistent with the whole mount staining, no LacZ positive cells were detected in cell samples isolated at day 21 (Fig. 3.31C) or day 28 (Fig. 3.31D) post-injury.

**Fig 3.31** Isolated LacZ positive cells were detected from the tissue samples at Day 14 (A,B), but were no longer detectable at Day 21 post injury(C,D). Cells adhere to the flask and appear to be similar in morphology to fibroblasts. Many of the cells without LacZ staining also appear to be fibroblast type cells.
To determine whether these cells were fibroblast type cells, an anti-collagen I antibody was used to double stain lacZ positive cells. LacZ positive cells do not appear to express collagen I (Fig. 3.32), whilst other isolated cells growing in culture are positive for collagen I expression (Fig. 3.32). This suggests some isolated cells are expressing fibroblast specific proteins, but that the lacZ positive cells are not collagen I expressing cells.

Fig 3.32 Isolated LacZ positive cells do not detect collagen I. LacZ positive cells isolated ad Day 14 were stained for collagen I. Positive cells identified for LacZ expression (A,C) do not stain for Collagen I expression (B,D). Other isolated cells are Collagen I positive (B,D), suggesting that these are fibroblasts.
Further analysis of these cells was also conducted using anti alpha-smooth muscle actin antibodies, to determine if the cells were expressing this marker of myofibroblast type cells. Similar to the collagen I analysis, no lacZ positive cells appeared to express a-smooth muscle actin (Fig. 3.33). However, other isolated cells in culture were positive for a-smooth muscle actin, demonstrating that myofibroblast type cells had also been isolated but that these are not the same as the lacZ positive cell population.

**Fig 3.33** Isolated LacZ positive cells do not detect α-smooth muscle actin. LacZ positive cells isolated at Day 14 were stained for smooth muscle actin. Positive cells identified for LacZ expression (A,C) do not stain for smooth muscle actin expression(B,D). Other isolated cells are α-SMA positive (B,D), suggesting that there are isolated myofibroblasts present from the isolated wound tissue.
Summary

In summary we have demonstrated that the HSCs provide a transient population of cells in the wound. They appear to be more than just an immune infiltrate and to persist up to day 14. However our findings suggest that as they do not double stain with α smooth muscle actin or with Collagen I, they are not fibroblast type cells.
Chapter 4 DISCUSSION

4.1 Animal models of burn injury

The first burned mouse model was described in 1975 [207]. There have been many other models utilised since then, specifically to examine the effect of thermal injury on cutaneous wound healing and the systemic effects associated with burn injury, including immunosuppression, vasodilatation, shock, sepsis and multiple organ failure [208-212]. Other mammals have also been used for partial thickness burn models, including pigs, dogs, rabbits and guinea pigs [213-218]. The injury sizes, depth and use of contact method for this study was selected for a number of reasons; detailed below.

4.1.1 Animal Welfare

The National Health and Medical Research Council’s Australian Code of Practice provide the guidelines strictly adhered to by all facilities within Australia that perform biomedical research on animals. These guidelines specify that each researcher must reduce the numbers of animals used and refine techniques used to reduce the adverse impact on animals [219]. In accordance with these guidelines and to comply with local animal ethics committee concerns we have performed a full thickness burn. As this is a full thickness injury all nerve endings will have been destroyed at the time of injury and the wound will be insensate. This reduces the amount of pain experienced by the animal lessening the impact on the animal during recovery.

4.1.2 Reproducibility

It is important to emphasise that this model offers us many advantages. As we have shown it is reliable and reproducible (Results Fig. 3.2). The contact burn wound results in a circumscribed injury which does not have a transition zone adjacent to normal skin. This results in uniform wound healing throughout the injury (Results Fig 3.2). It is well known that rodents especially mice will heal greater than 90% of their wounds by contraction [220-222]. This contractile
healing can cause concerns in studies exploring potential therapeutics and their impact on
time to heal. However our line of investigation pursues qualitative information with regard to
cell type and the biology underlying burn wound repair, rather than quantitative comparisons
of wound healing rates. We have only examined wound healing rates in a single study
examining topical application of whole BMDCs in this work (Results Fig 3.16). The mechanisms
underlying murine wound repair, including the inflammatory response, deposition of
granulation tissue and hyperproliferation of the epidermal cells, all closely mimic the
mechanisms underlying human skin repair. Therefore the use of the mouse model, with its
advantages of reproducibility, cost and ability to monitor cell activity (using transgenic models)
was the optimal choice for the studies presented in this thesis. The examination of burn
wound healing and scar quality is probably best assessed in porcine models [218]. However,
the use of a large animal model would not have been well suited to the mechanistic
experiments that have been carried out, whilst the potential of porcine experiments to
examine the potential therapeutic effects of the cells tested here provides the most promising
method for assessing the impact of exogenous cells on time to heal in the future.

4.1.3 Use of Transgenics

Mice are also advantageous for this type of investigation since it is relatively easy to study
strain related mutants and transgenic animals that allow for the study and monitoring of
specific cell types. Green fluorescent protein (GFP) transgenic mice have been used in many
chimeric mice models of other disease states. These have been used to effectively track the
fate of bone marrow cells in the pathological process in nearly every organ in the body from
solid organ tumours to Alzheimer disease [156, 171, 223-226].

4.1.4 Good model of human for cellular processes

Since the process of wound healing has been shown to be similar throughout mammalian
species, more attention has turned to rodent models of thermal injury, both for ease of
handling and for the ability to use large numbers of animals for ease in achieving statistical significance.

There are however important differences in rodent skin and human skin. In normal epidermis, proliferation is confined to the basal layers that contain both stem cells and more numerous transit amplifying cells. In thin rodent epidermis the suprabasal cells are arranged in columns (stacks) that interdigitate with neighbouring stacks; each stack is associated with a seemingly defined group of basal cells, and a more slowly dividing cell underneath the centre of each stack has been proposed to be the stem cell for the so-called epidermal proliferative unit [227-228]. Human epidermis is much thicker and is generally not stacked, and the identity of stem cells is more controversial[227, 229].

The structural differences in epidermis may impact on this study. However we are examining the maintenance of the epidermis which is similar in the murine model. In addition it is the healing burn wound that is of greatest interest to us and the cellular composition of scar but rather the impact of injury. The cellular responses to injury in mice do closely mimic those in humans, albeit with some studies suggesting there are differences [230]. In addition whilst scars seen in mice are often very small due to the significant wound contraction, the cellular and matrix composition of scar remains similar to human.

A full thickness burn as mentioned above is less likely to cause pain and as a result should mean less if any analgesia will be required post injury. This results in a good model of actual injury so that the normal responses to injury can occur without being subdued by excessive analgesia. Hence the circulating catecholamines and inflammatory mediators can circulate as would normally occur in a clinical scenario and are less likely to be affected [231-232]. However, the use of the full-thickness model does represent a type of injury that is less
common in the clinical setting which does restrict the interpretation of the results presented [16]. Overall, the advantages of using the full thickness model, including reproducibility and reduced need for analgesia seem significantly greater than the disadvantages, and therefore this was the preferred model.

4.1.5 Limitations of the mouse model

The principal disadvantages are that all rodent models heal by contraction to a greater extent than human skin [220]. Rodents also have a very high density of hair follicles which facilitate re-epithelialisation. This is more marked in partial thickness wound healing and less important in the full-thickness burn animal model used here, but still relevant to the quality of the scar, which is overall better in rodents than in human skin. This can be seen by the difficulty in creating a murine model of hypertrophic scarring [218, 233].

A lesser limitation with this model relates to the small size of the animal. Animal welfare must remain a priority. This in turn limits the size of injury which we can perform within the NHMRC guidelines in Australia. As we aim to recover our animals and follow them up over time we are restricted in the TBSA we can perform, currently limited to a 2cm burn wound which equates to 8% TBSA in our animals. Our main focus of interest is in the biology of the burn wound itself and not on the associated effect of major trauma such as the immunosupression and septic responses which have been well described and are less related to the burn specifically but rather to the extent of trauma [209, 231, 234]. Non-severe burn wounds, those that are less than 10% TBSA as in this model are the most commonly sustained injuries in adults and children. In a twenty six year population based study of hospital admissions for burns in Western Australia 84.5% of males and 90% of males had a burn of less than 10% TBSA [16]. This supports the use of the limited TBSA model, although it does still restrict the interpretation of the findings, as it is possible the role of bone marrow cells changes significantly with increasing injury size.
A major focus of our research is the investigation of the impact of the non-severe burn wound and the associated systemic effects. We are confident from examining weight loss post injury and other activity records in mice using this model of burn injury that these injuries are non-severe in extent (personal communication E. O’Halloran). Thus they are a very good model to represent the vast majority of burns seen in the developed world.

In summary the model has the advantage of using animals which are inexpensive to purchase, maintain and have the benefit of transgenic strains.

The burn procedure is quick, simple to perform, requires no specialized equipment and yields a highly accurate and reproducible result.

4.2 Generation of a stable chimeric (EGFP+) Bone Marrow (BM) in wildtype mice

4.2.1 Transgenic Chimeric Mice

The majority of studies exploring the role of bone marrow derived cells employ a form of chimeric mouse model. The original model involved transplantation of male BM into female recipients and identification of the donor mice using the presence of the Y chromosome [235]. However the introduction of transgenic technology has enabled more sophisticated methods to be used.

4.2.2 Methods of generation and evaluation

Transgenic mice are created by direct injection of a gene of interest into a recently fertilized one-cell embryo of mice [236-237]. In subsequent embryonic divisions, the extra foreign DNA is distributed to every cell in the mouse. Microinjected embryos are implanted into foster mothers by oviduct transfer. Each transgenic pup represents a unique chromosomal integration site; as such each is a unique transgenic “founder”. Transgenic founder mice are
then bred into a desired genetic background to establish permanent transgenic lines of mice. Arbeit et al explain this process in a comprehensive review of the process [235].

4.2.3 Green Fluorescent Protein (GFP)

GFP is responsible for the bioluminescence in the jellyfish Aequorea Victoria. The primary amino acid sequence was determined in 1993 and this protein is now used as a simple marker both in vitro and in vivo [174, 238-241]. A further modification of GFP led to an enhanced and more stable fluorescence in 1995. This is referred to as enhanced green fluorescent protein or EGFP [242]. Expression of EGFP seems to be non toxic, and the immunological function of the mouse is sustained at normal levels [243]. The introduction of EGFP as a reporter had a significant advantage over other commonly used reporters as it emits fluorescence without any substrates or cofactors, allowing researchers the ability to monitor the presence of GFP by illuminating living cells [244-246].

A stable chimeric (EGFP+) bone marrow (BM) in wildtype mice was created which persisted throughout the duration of the study. (Results Fig 3.3 & 3.4) The extent to which durable engraftment had been established is similar to that shown in animal models of skin wounding and other injury states such as pulmonary fibrosis showing levels of 85-95% up to one year from injury in circulating cells [156, 177]. The lower levels of engraftment seen in the bone marrow are to be expected as it has been shown previously that in EGFP mice bone marrow donors only approximately 50% of all cells analysed by FACS were EGFP positive [177]. The lower levels of engraftment may also be in part due to the difficulty of getting lethal irradiation correct without actually causing fatalities. Hence a balance has to be achieved between generating a stable 100% chimeric animal and achieving a suitable level of survival of irradiated animals. In addition, there is some evidence to suggest that the MSC component of the bone marrow is more radiation resistant than the HSC populations leading to the bone
marrow containing a consistently lower percentage of transgenic cells than can be detected circulating in the blood [200-201].

Since this study has been completed a number of papers have discussed the limitations of green fluorescent protein as a cell lineage marker [173-174, 176]. These relate primarily to variable levels of expression both between and within species. It has been shown that even mice that glow bright green may not have uniform expression of GFP at a cellular level. The transgenic strain selected in this work, C57BL/6-Tg(ACTB-EGFP)1Osb/J (Jackson Labs) have been shown to be a good reliable source of transgenic GFP mice with stable and consistent expression [176]. It has also been suggested is that over time high levels of EGFP may have potentially toxic effects so that over time EGFP expression is downregulated in cells in longitudinal studies and ultimately not detectable in transgenic animals. This has the potential to influence the interpretation of the results using this model. However, at all time points we monitored wound healing and tissue samples, we also analysed blood samples to confirm maintenance of EGFP+ expression in the cells. (Results 3.2) Therefore in the studies presented here, we can be confident that there was not a significant deterioration in EGFP+ expression over time. Rather, the lack of EGFP+ cells detected in the skin at later timepoints is likely to truly reflect a change in the origin of the cells in the tissue being tested.
4.3 Bone marrow derived cells (BMDCs) in minor and moderate burn injury

The origin of both keratinocytes and fibroblasts in any healing wound has been widely assumed to be the periphery and normal skin adjacent to the site of injury [38]. However, recent studies of tissue repair in other organs, including lung, liver and heart have demonstrated the existence of a population of cells involved in scar formation that originate not from the peripheral tissue but from the bone marrow [113-114, 116-117, 156, 171]. In addition, more recent studies also demonstrated a bone marrow response that may contribute to scar in models of excisional and incisional skin injury [169-171, 247].

Burn wounds differ from incisional wounds in both the topography and cellular mechanism of injury. Burn wounds are characterized by heat induced tissue coagulation at the time of injury [93] and the predominant direction of tissue injury is horizontal not vertical [248], resulting in a unique and slower mechanism of wound healing for burns. In addition the extent of damage and denatured material remaining at the site of a burn wound rather than at an incisional wound is considerable and has an impact on the acute inflammatory response. Therefore, in light of these differences and the findings described, it was important to determine whether BMDCs are involved in burn wound healing and the extent and nature of BMDCs in the burn wound scar.

The key finding in this study is that both dermal and epidermal cells involved in burn wound healing originate not only from the peripheral tissue but also from the bone marrow. We have also demonstrated that this bone marrow response occurs in both minor and moderate severity wounds. As expected, in both models there is a substantial inflammatory infiltrate during the acute early phase. However, as the inflammation subsides, there is a discrete population of cells from the bone marrow that persist until the wound is healed and scar established (day 21 in the minor wound and day 56 in the moderate wound model). From our
data, it is clear that in both minor and moderate injury models there is a substantial proportion of fibroblast cells in both the healing wound and scar that are originating from the bone marrow. Up to seventy percent of all fibroblasts identified within the scar in sections by immunohistochemistry at day 28 in the moderate burn group were BM-derived. This response was similar at day 56 as the scar matures and further remodelling occurs.

While considering the above results a minor limitation of the study needs to be considered. Although histological identification is a reliable technique and was performed following training with senior histopathologists (Results 3.1.1) there is a possibility that subgroups of cell types such as fibroblasts and myofibroblasts may have been amalgamated in cell counts. Ideally we had planned to perform double staining to clearly identify these subgroups. However a number of the antibodies tested did not perform sufficiently well to assist with this identification and so histology and cell counting was used for this study. As discussed in Results 3.4.1.1 autofluorescence of the stratum corneum, collagen matrix and adnexal structures was a major issue when imaged with the settings required for EGFP (Results Fig 3.6). As a result alternative staining protocols were developed (Results Fig 3.7) which allowed visualisation of EGFP+ cells in the sections without interference of autofluorescent structures. At the time of established scar in the minor and moderate wound, we found no statistically significant difference in the total percentage of BMDCs within the healed tissue, suggesting that the extent of injury had no significant impact on the migration of BMDC to the site over the long-term. We also found no statistically significant difference when we compared the percentage of BM derived fibroblasts, epithelial cells, immune cells and endothelial cells in the minor and moderate wounds at day 21 and 28 respectively. This suggests that the bone marrow response to a wound is not simply a stochastic response to the level of tissue damage but rather that the BM response is providing a specific cell population even in minor wounds that does not appear to differ with increasing injury severity.
As there is no previous research examining the BM response to burn wounds we can only extrapolate from other studies involving cutaneous injuries. These studies have concentrated primarily on examining the BM response to small punch biopsy excisional wounds, linear incisional wounds and tape stripping to mimic epidermal loss. The excisional wounds were typically 2-3mm in diameter full thickness circular excisions similar to our minor injury model in terms of severity. Similar to our findings in the minor injury model, all of these studies also show a small or negligible keratinocyte population from the bone marrow in response to injury [169-171]. It has previously been postulated that in smaller wounds there is no exhaustion of the epidermal keratinocyte stem cell niche and that therefore BMDCs are not required to replenish keratinocyte stem cells [170]. In our model of moderate injury which demonstrates a significant BM derived keratinocyte population, these cells do not appear to persist once scar is mature at day 120, suggesting the cells originating from the bone marrow are not true epidermal stem cells, and that therefore epidermal stem cell replenishment is provided by cells either from the peripheral tissue or other source rather than BM derived. This correlates with the absence of BM derived epidermal cells observed in non-injured skin controls and the data of others which demonstrates the absence of BMDCs required for normal skin homeostasis [170]. Therefore, epidermal stem cell populations appear to be self-maintained in both normal skin and in wound healing.

The dermal component of burn wound healing is vital for scar formation. Extensive thermal injuries disrupt the balance between collagen synthesis and degradation and lead to abnormalities in collagen metabolism, a characteristic of several forms of fibrotic conditions, hypertrophic scars and keloid scars [98-99]. Hypertrophic scars and the related but more exuberant condition known as keloid are among the dermal fibrotic diseases which are characterized by increased collagen deposition [100-101]. Alteration in extracellular matrix (ECM) components is thought to be responsible for the undesirable physical properties of fibrotic scar tissue. Hypertrophic scarring represents the dermal equivalent of fibro-
proliferative disorders and is a common clinical problem for patients who survive extensive thermal injury [57]. Our study has shown that the BM contributes up to 30% of fibroblasts present in the wound at day 28 in the moderate wound injury during the intense tissue formation and remodelling phase. The BM contribution to the fibroblasts during wound healing is important, particularly clinically, as it presents a potential opportunity for intervention to alter scar formation. It has been clearly demonstrated that these BM derived fibroblasts in other fibroproliferative diseases synthesise collagen type I (col I) [179], the most important collagen type with respect to scar formation [249-252]. Furthermore, these cells constituted more than 80% of all col I expressing cells in the fibrotic lung, indicating that the predominant col I producing cell in fibrosis was derived from the bone marrow and had migrated into the lung in response to signals released in reaction to the lung injury and fibrosis [156].

The signal that activates or promotes migration of BM cells to the wound site remains unknown. It is most likely mediated by either a single or combined chemokine signal at the wound site, and there is some evidence to suggest that T-cells play a role [253-256], but this has yet to be fully explored. Inokuma et al [257] speculate that chemokine/chemokine receptor interactions mediate the migration of the BM keratinocyte precursor cells via a cutaneous T-cell attracting chemokine, CTACK/CCL27. CTACK is the major regulator involved in the migration of keratinocytes [257]. In addition, intradermal injection with CTACK/CCL27 into the periphery of skin wounds significantly enhanced BM-derived keratinocyte migration [257]. However in this study there was no substantial long-term contribution of BM derived cells to the epidermis. Therefore whilst this signaling may attract BM cells to the wound which then differentiate to keratinocytes to provide faster coverage of the injured tissue, there is no contribution of the BM to this cell population in the scar. Therefore the importance of these migratory signals remain unclear.
It is also unclear from this data, whether the BMDCs observed at the wound site in this study are circulating prior to injury or whether the cells are actively migrating from the BM niche after injury in response to a signal. It is possible that the bone marrow itself is activated by circulating cytokines or some other signal to secrete the cells that then migrate to the wound. There is some evidence that circulating cells known as fibrocytes are the most likely source for the BM derived dermal cells observed in the wound [258-259]. This correlates with our observations that BM derived dermal cells populate the wound at early time-points during healing, suggesting the cells may be circulating rather than recruited indirectly through bone marrow stimulation and then migration to the wound. However, this has yet to be definitively demonstrated.

Finally, it is important to note the transient nature of the response observed with the moderate injury model. Our minor burn wound was healed at day 10 approximately and a stable scar was present at the last time point of 21 days. Our results demonstrated that a small number of BMDCs were still present at this stage. On histological examination of the healed wound almost half the total number of fibroblasts was bone marrow derived. Our study examining the moderate burn wound was continued over a much longer time frame, up to 120 days. The results demonstrated that the BM response continued to 56 days and was similar in profile to the minor wound. However by 3 months post injury there were no detectable BMDC present in the mature scar. This was observed both by FACS and immunohistochemistry, despite the presence of a persistently strong chimeric model confirmed by FACS analysis of nucleated blood cells of animals at this timepoint. At 3 months post injury in the mouse burn model the wound is fully healed and the scar mature, it is pale and shiny and almost invisible. From this data, we believe that the minor injury model was not completely remodeled by day 21 and that continued observation of the minor wound would result in the loss of all BMDCs as
observed with the moderate injury model. The long-term but transient nature of the BMDCs in the healing wound suggest a role for the BM cell in wound healing but not in long-term homeostasis of skin. Critically, it suggests that the cells originating from the BM are not stem cells, but are differentiated either prior to or once they reach the wound site. If it was truly a population of stem cells then they would be detected at this 3 month timepoint if they continued to produce progeny cells contributing to the dermal or epidermal layers. In conjunction with the observation that the percentage of cells in the wound originating in the bone marrow is similar in both moderate and minor injury, we believe this indicates that the bone marrow is the source of a specific cell type that is required for normal wound healing and scar formation but not in normal skin. Indeed, it is possible that the BMDCs augment and direct the wound response until a steady state has occurred and that in a model of unfavorable or hypertrophic scarring maybe a persistence of the BM response by potentially related to ongoing inflammation that contributes to this.

The bone marrow contains precursor cells capable of differentiating along hematopoietic [153] and mesenchymal (MC) lineages [129]. Hematopoietic stem cells can reconstitute the entire circulating population of HCs [153], while mesenchymal stem cells can differentiate to form osteocytes, chondrocytes, adipocytes, and bone marrow stromal fibroblasts [129]. The most likely source of BMDCs in the healing wound is the mesenchymal population. This has been suggested by a recent transplant study in which MSCs were transplanted and observed in the dermal layer during early timepoints of burn wound healing [187]. However, whilst the MSCs are likely to be the source of the cells observed, it cannot be ruled out that it is in fact trans-differentiation of hematopoietic progenitors that results in the BMDC population in the wound. Answering this will further our understanding of the bone marrow role in wound healing, and clarify the nature of plasticity of hematopoietic cells in burn repair.

In summary, this study provides evidence that the bone marrow is an important source of cells involved in burn wound healing.
A key point that needs to be addressed for clinical intervention is whether this BM response promotes scarring or reduces scarring. If it can be shown that the quality of wound healing may be altered by enhanced, reduced or ablated BMDC response either in terms of speed of wound healing or scar quality, then intervening in this response will be key to promoting better outcomes for burn patients.

4.4 Does the topical or IV administration of whole bone marrow contribute to wound healing and scar formation?

Administration of whole EGFP+ ve bone marrow was performed in this study. Application of whole BM has been shown to be advantageous in other animal models but not in burn models. The previous models have shown improved healing rates in diabetic mice wounds [183].

It has also been reported in two case reports of adults humans with major burns as a salvage treatment when all else appeared to be failing. These patients both died as a result of their injuries, however it is indicative of the interest in this area that both of these reports were published [187-188].

In light of these studies the possibility of utilising autologous BM without the need for a BM transplant and the associated morbidity and mortality was investigated. This study has yielded a number of aspects worthy of further discussion. There was a very poor survival rate of transplanted BMDC in both groups of animals who received topical and intravenous administration as seen by FACS analysis. However, histological analysis was carried out as in the previous experiment, even with the very low cell yields to verify the cell counts were correct and also to confirm the presence of small numbers of EGFP cells within the wound. The cell breakdown by type which persisted are predominantly fibroblasts and epithelial cells. But these results have to be interpreted with caution in view of the very low cell yield. This
may be partly explained as a result of the well recognised immunogenicity of whole BMDC [142]. In addition, the topical application of cells could be administering the cells in a very hostile environment, with the wound containing large numbers of phagocytes, proteases and other mediators after acute injury. The intravenous administration, whilst not directing the cells into a hostile environment immediately, most likely leads to significant losses of cells through breakdown and may also enhance the immune response to the presence of the cells. Alternatively, cells administered intravenously may also migrate to other tissues and not contribute to the wound healing process at all.

In this study the cells did not survive, or were not detectable beyond one week post administration. However modulation of chronic wounds by the application of BMDCs has been reported although again no evidence for cell engraftment or survival in this case is presented, and the current thinking is that rather than direct cell survival and contribution to the wound the administration of cells in some way changes the nature of the wound to a more acute type thereby leading to healing [186, 260]. This improvement in wound healing is particularly marked in the acute phase after topical application [260]. It is most likely that these effects are seen as a result of local growth factors and the paracrine effects of the BM to recruit macrophages and endothelial cells [261-262]. The application of BM cells may also involve the secretion of VEGF or FGF which can help prevent apoptosis, promote angiogenesis assist in matrix reorganisation and increase in the recruitment of circulating MSC’s [262-263]. It cannot be ruled out that subsequent cell death of the applied cells also acts as a stimulus for growth factor release and subsequent wound healing effects [264].

The potential therapeutic role of topical cell application may relate primarily to the timing of the application and the conversion effect of the wound from chronic back to more acute in nature, thus rendering the usefulness of the application questionable in the acute burn wound setting. At the time of the acute burn wound the wound itself is very active in terms of cytokines, chemokines and growth factors and it maybe that the application of the cells just
gets incorporated within the inflammatory cascade and the therapeutic potential is lost, whereas in the chronic wound the application of cells maybe the trigger for reactivation of the wound healing response [203]. Indeed, one current understanding of chronic wounds proposes that these wounds are ‘stuck’ in the inflammatory phase of healing and unable to progress (due to currently unclear factors), and it is possible the administration of cells in this context stimulates the immune response and triggers the healing process. If this is the case, as stated above, the potential benefits of these cells for acute wound healing would be limited, although the cells in the acute wound situation may alternatively interact in a different and still positive manner to promote repair.

One of the aims of this study was to clarify whether exogenous BMDCs would accelerate or slow the burn wound healing process. The administration of the BMDCs has resulted in no significant difference between the groups but identified a trend toward delayed wound healing, (Results Fig 3.16) This observed trend, while small, may simply be a result of physical properties related to the application of the cells in the topical group. In this group an adhesive dressing was applied and the cells injected underneath and were allowed to bathe the wound (Methods 2.3.2). This occlusive wound dressing may have had a role to play in the delayed healing but this technique has been used by our group in other wound healing studies with no delay [265] and the control animals received a similar dressing. This dressing does take some time to apply and is cumbersome and therefore this may have had some impact on the timing of cell delivery to the wound. This dressing was not used in the mice who received an intravenous injection of BMDCs. Again it is important to note that the very small numbers of EGFP+ cells that survived support the theory that exogenously applied cells, at least in this context, are likely to impact on healing either through release of soluble factors prior to cell death or by the cell death itself stimulating the intrinsic response to promote repair. However, in this study, no impact on healing was observed. Identifying whether cell death is a prerequisite for improved healing, as reported in other studies, will be important to further understand the interaction between applied cells and tissue repair.
4.5 The role of Mesenchymal derived BM cells in burn wound healing

Mesenchymal stem cells (MSCs) or stromal cells reside in the stromal portion of the bone marrow. It was assumed that their function was to provide the cellular microenvironment for hematopoiesis [266]. However, it has been subsequently shown that these cells can also differentiate into a number of cell types and may be important in wound repair. MSCs are defined by 3 criteria; 1) adherence to plastic in standard culture conditions, 2) Markers CD105, CD73 and CD90 ≥95% positive, CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR ≤2% negative and 3) demonstrated differentiation into osteoblasts, adipocytes and chondroblasts in vitro [128].

The therapeutic potential of MSCs is very promising. MSCs are readily accessible from both patients and healthy donors, offering a potential source of cells for clinical applications in regenerative medicine. In addition to providing a scaffold for HSC proliferation, MSCs have been demonstrated to play a role in hematopoiesis themselves [127]. This has been demonstrated in patients undergoing chemotherapy, where MSCs have been shown to significantly improve hematopoiesis compared to autologous blood stem cell transplantation alone [138]. MSCs are suitable for allogeneic transplantation as evidence suggests that they are immune privileged with low MHCI and no MHCII expression [135]. MSCs have also been observed to play an immunoregulatory role. MSCs can exert profound immunosuppression by inhibiting T-cell responses to polyclonal stimuli and to their cognate peptide [139]. The characterization of the phenotype of MSC induced T-cells showed that the inhibitory effect of MSCs is directed mainly at the level of cell proliferation[139]. This immunosuppressive and immune privileged role has led to the use of autologous and allogeneic MSCs in graft versus host disease (GVHD)[142, 144]. Co-infusion of donor derived MSCs together with HSCs have been shown to reduce the incidence and severity of GVHD. MSCs are also in clinical use for a number of skeletal conditions including defective fracture healing and cartilage repair. MSCs
have been used with and without scaffolds to repair large bone defects whilst cartilage repair has been performed in patients with osteoarthritis [267]. The majority of the skeletal research to date has focused on topical administration of cells, but recently systemic administration of allogeneic MSCs has been given to children with osteogenesis imperfecta. Transplanted MSCs were shown to migrate to bone and produce collagen, thus providing a new and efficient way to treat this debilitating condition [148]. Therapeutic effect of MSCs has been shown in many more human diseases including muscular dystrophy, stroke, diabetes [204, 268-269]. With the significant interest in MSCs and the potential for tissue regeneration in multiple diseases, some studies have been conducted on the potential for these cells in skin repair. Transplanted BMDCs were shown to be integrated and differentiated into non-haemotopoietic skin structures, which accelerated wound closure, with increased re-epithelialisation, cellularity and angiogenesis [270]. In addition, MSCs but not CD34+ BMDCs in the wound expressed keratin and formed glandular structures, suggesting a direct contribution of MSCs to cutaneous regeneration [180] [271]. Other studies have ranged from clinical applications in humans including a case reporting a failed attempt to salvage a major burns patient with application of MSCs, and initial treatment of radiation burns with MSCs [187, 272-273]. Systemic administration of MSCs in a rat model of wounding, given either as a single dose or a repeated dose for 4 days post injury, has shown the presence of MSCs in the wound. This study also reported an increase in the time to wound healing and an associated increase in tensile strength [274]. MSCs have been delivered to wounds in diabetic mice via a fibrin spray and this study also suggested that the MSCs may persist and act to stimulate wound healing [185]. MSCs administered to mice with full thickness skin defects either by IV or topical injection have also been shown to improve wound healing when compared to controls [182]. Intravenously administered MSCs from GFP transgenic mice, have also been shown in a murine model to transdifferentiate into keratinocytes, endothelial cells and pericytes [275].
In order to examine the role of MSCs in the healing burn wound and subsequent scar formation injection of MSC by tail vein was performed at three different time points, 5 days prior to injury, at time of injury and 24 hours post burn injury.

4.5.1 The Contribution of MSCs to burn wound healing after intravenous administration 5 days prior to injury in a minor and moderate burn wound model

The selection of a five day prior injection while not clinically relevant in patients with an acute burn injury is useful as this study was performed principally to mimic the endogenous state and demonstrate the normal migration of cells in animals with labelled MSC’s. The first MSC experiment showed that in animals “primed “ with an intravenous injection of MSC prior to injury results in a small population of EGFP+ve cells being identified in the wound at all time points.

The cell types identified were similar to those seen in the bone marrow transplant model as identified on histology. However in the moderate wound there seems to be a longer inflammatory phase up to day 14 compared to the smaller injury that by day 21 has progressed to the remodelling phase and consequently the majority of EGFP +ve cells identified appear to be fibroblasts.

It is important to place these results in context. The true number of cells as demonstrated by FACS analysis in both the minor and moderate group was very small. Less than 1.5% of all cells detected at all time points examined (Results Fig 3.18). Hence the further data from the immunohistochemistry interpretation is of limited importance.

The findings in the larger burn wound model were very similar to the minor wound model. There was a trend suggesting that the EGFP+ cells were preferentially present in the wound, but no statistically significant differences when compared to controls.
Histological analysis again showed similar findings with a strong inflammatory response and more fibroblast cells seen later. Again the numbers are very low and suggest that the injected cells probably have not contributed significantly to the burn wound.

The fate of the MSC’s injected prior to injury is of interest. It is most likely that the majority have not survived the intervening time period between administration and injury. If they have survived they did not migrate to the wound. Unfortunately in this experiment we did not examine other tissues and organs and attempt to detect the presence of MSC’s distributed in the different animal organs. Therefore we cannot determine whether the cells have all been cleared by the animal or are simply migrating to other tissues with no injury to promote skin migration at the time of injection. In view of this result our subsequent MSC experiments were improved by analysis of additional tissues and organs for MSC populations to better understand the fate of these cells.

4.5.2 The distribution and contribution of MSCs administered intravenously at time of injury to burn wound healing in a moderate burn injury.

A further important aspect in study design for this experiment was to assess if there was a systemic effect from the administration of the MSCs and in order to investigate any such effect, non-injured skin, bone marrow, muscle, brain, liver, spleen and muscle were all harvested at the same time as wound tissue and analysed for MSC presence.

Initially the stimulus for MSC therapy was the thought that the plasticity of the multipotent stem cells were the key to providing an additional source of cells to engraft and contribute to repair. It is becoming increasingly clear that there are more complicated mechanisms at play rather than simple engraftment of the cells.
We have demonstrated in this study that the timing of administration of MSCs does not seem to alter cell fate but does appear to have an impact on relative cell numbers migrating to the wound and scar site. MSCs administered at time of injury were detected in burn injury and subsequent scar at all time-points examined, in contrast to the application of cells prior to the injury (Results Fig 3.25).

The quantity of cells detected was low across all time points with the greatest yield at the early time points. This level has been shown in other studies both in cutaneous and other injury models[198]. There is now significant evidence that MSC can have a beneficial effect on tissue repair even if the numbers of MSC present are small and time in the damaged tissue is transient [202].

4.5.3 The distribution and contribution of MSCs administered intravenously 24 hours post-injury to burn wound healing in a moderate burn injury.

MSCs administered 24 hours post injury, were detected in burn injury and subsequent scar at all time-points examined. There was greater migration of MSCs to the wound when administered 24 hours post injury, and there was also greater migration to the uninjured skin when administered at this later time-point compared to at the time of injury (Results Fig 3.25).

Once again, the quantities of cells detected was low across all time points with the greatest yield at the early time points, reflecting most likely limited survival of cells over the duration of the healing process.

The immunohistochemistry shows the expected pattern of early inflammatory type cells present in large numbers initially, at least in part because the MSC population is unlikely to be 100% free of HSC type cells. The populations changes over time such that as seen in the other models, there is an increasing number of fibroblasts present in the wound. However, in this
group there are also a variety of other cell types present, including langerhans cells, epithelial cells and hair follicles (Results Fig 3.26). Again, how much of these cell types truly represent a result of MSC differentiation rather than contamination by other cell types in the original administered cell population is not clear.

4.5.4 Analysis of migration of exogenously administered MSCs to organs after burn injury

The distribution of MSCs to organs following tail vein injection shows that MSCs do appear in other organs. In particular the BM, spleen and liver and uninjured skin at all time-points analysed appear to have MSC derived cells above that detected in the relevant controls (Results Fig 3.28). This is an area of controversy with some studies suggesting that MSC’s specifically migrate to areas of injury after application. Other studies have identified different findings with a major area of “trapping” of cells said to be the lung [198]. The key difference in our study may relate to the timing of sampling as many of the previous studies sample within the first 12-24 hours. In a human study five out of six children who received two administrations of intravenous gene marked donor marrow derived MSC for the treatment of osteogenesis imperfecta had MSC’s detected in bone, skin and marrow stroma [276]. Here, we have clearly demonstrated multi-organ involvement. This may in part be due to the fact that although this is a relatively small injury there is still a significant systemic response driven by inflammation. Work from our group has previously identified, using the same animal model, systemic neurological changes even with such a moderate size injury [277-278]. This is despite there being no evidence for hypermetabolic or other similar systemic issues as is often seen in more severe injuries. It is also possible that the resulting cell populations in these other organs simply reflect a non-specific engraftment of the cells after administering intravenously. It will be important to determine whether these cells continue to persist in the even longer term or potentially continue to produce progeny. At all time-points the populations detected were very small, but this is to be expected given the numbers administered. What is important to
determine in the future is what happens to these cells after the duration of these experiments, particularly as this could have substantial implications for clinical treatment of burn injuries rather than life-threatening late stage diseases.

### 4.5.6 Investigation of the impact of burn injury and the timing of administration of MSC on cell fate

The timing of administration of MSCs is another controversial area, but critical to understand for therapeutic applications. Using a mixed model method of statistical analysis (Dr Michael Phillips, WAIMR) there is a significant difference between the EGFP+ cells at both the wound site and the liver when administered at different times (Results Fig 3.28, (time of injury and 24 hours post-injury)). In particular at day 7 and day 28 there was an increase in the EGFP +ve cells following the later administration of MSCs. This may occur because of the requirement for specific signalling from the wound to cause migration of the MSCs into the environment, as previously indicated by experiments suggesting the importance of CTACK signalling for bone marrow mobilisation [73]. Alternatively as changes occur in the wound it is possible the MSCs monitor the changing microenvironment through other means and this significantly influences cell fate and survival in the wound [121].

As the wound is healing there are substantial changes over time in the cellular makeup and it may be that the later administration has resulted in a more profound inflammatory response developing, which may have been attenuated by the earlier injection. Alternatively, it maybe that the pro-inflammatory response has encouraged the honing of cells to the wound, facilitating their survival in contrast to the immediate administration.

Since this work commenced many of the original theories around the role of adult stem cells MSCs have evolved. This is summarised by Prockop [202]. Initially it was thought that their primary role was to support hematopoiesis, and the main clinical role was to support the role of the hematopoietic system following bone marrow transplantation [138]. It was later
thought that MSCs when administered either topically or intravenously could facilitate tissue repair by engraftment and differentiation. This has been discussed in detail in the introduction and this was widely embraced as a concept, in all fields of tissue repair. However in more recent times there has been a further shift in thinking that suggest there is a complex series of interactions between the MSCs and the injured tissues. This may result in changes to the production of TNF α and other inflammatory mediators, thus limiting the tissue injury. Alternatively, and one of the most common suggestions currently, is that any cell type applied for skin therapies acts only as a source of growth factors, cytokines or chemokines and that survival of the cells is not important for their role in promoting healing. This may be true of the application of skin cells, which has been used extensively, as well as the more recent applications of bone marrow derived MSCs or even adipose derived stem cells [279-280]. These changes to our understanding of the potential role of these cells will have significant implications to future therapies and the choice of optimal cell type. In particular, it can be envisioned that if the main role of applied cells is to provide a source of stimulatory factors, this may be best achieved using specific cell types or artificially enhanced cells to deliver the factors, rather than heterogeneous stem cell populations. However, there is substantial further work required before the optimal cell type can be determined.

4.6 The role of hematopoietic derived cells in the post-inflammatory phase of wound repair.

Hematopoietic stem cell (HSC) derived cells are well-known to be important in the acute aspects of wound healing through the coagulation cascade and the inflammatory pathways.[38] It is however uncertain if there is a role for these cells in the post inflammatory phase. The HSC derived cell which is said to have the most influence in wound healing and
subsequent scar is the fibrocyte. Fibrocytes were first identified in the mid 1990’s and described as fibroblast like cells within the peripheral blood which move into areas of damaged tissue [85]. They have now been implicated in all types of healing in many other organs, including liver, lung and hypertrophic scars, Bucala has a comprehensive review [205-206, 281-282]. Fibrocytes are a unique cell population, although whilst they are mostly assumed to be from the hematopoietic lineage their origin is not completely clear, in part because they express both hematopoietic and mesenchymal cell markers [199]. One possibility is that fibrocytes are mesenchymal progenitor cells from hematopoietic origin [283]. Fibrocytes secrete a range of cytokines, growth factors and chemokines, all of which contribute to facilitate wound repair. It has been shown that CD34, the hematopoietic marker, is initially expressed by fibrocytes but decreases over time in cutaneous wounds. This occurs at approximately the same time as Prolyl-4 –hydroxylase, an enzyme associated with the stabilisation of collagen, also decreases [284]. This is said to occur as the inflammatory stage decreases and the fibrocytes mature into more mature connective tissue cells. The CD45 or leucocyte common antigen is said to display a similar decrease in expression potentially due to differentiation of the fibrocyte type cells [285].

It has been shown in burn injuries that the proliferation of fibrocytes can reduce transforming growth factor-beta (TGF-beta)-mediated alpha-smooth muscle actin (alpha-SMA) expression. This may indirectly regulate dermal fibroblasts though mechanisms are uncertain and haven’t been explained further at this stage [258, 286-287].

The studies to date have been small and the methodologies used make the interpretation of the origin of the fibrocyte unclear. However, it is clear that interest in the fibrocyte and it’s specific role in wound healing and scar formation has increased significantly over the last 5 years. Coupled to the increasing interest in the role of fibrocytes, the role of HSCs has also been examined increasingly in burn wound healing and scar formation over the last few years [258, 286, 288]. However, the data continues to remain inconclusive as to whether
hematopoietic cells have a role in wound repair other than driving the early inflammatory response.

The purpose of this study was to use a transgenic mouse model to determine whether hematopoietic cells are involved in wound repair post-inflammation after burn injury. In order to do this, we utilised the VAV-Cre transgenic mouse model (Methods 2.5). The VAV-Cre mouse, which has the Cre recombinase protein under the control of the VAV promoter, and thus restricts Cre recombinase expression to HSC’s, was crossed with ROSA 26Lac Z animals which have the LacZ reporter gene inserted into the genome in a form which can only be activated by Cre recombinase expression. The offspring of this cross have the LacZ reporter gene expression restricted to the entire hematopoietic lineage.[195] Hence all HSCs and their derivatives will be clearly marked.

This is a highly reliable model provided appropriate animals are selected (Results 3.10.2). The reliability and accuracy of the model was validated by using and examining pre-injury biopsies of normal skin (Results Fig 3.29). This is because previous studies have demonstrated the creation of mosaics in this line with expression in the skin. Mosaic expression is a common problem with the use of Cre recombinase transgenic mice [154]. Mosaic animals, with expression of the reporter gene not restricted to the designated cell type, arise during embryonic development. Ectopic expression of the Cre recombinase during development, even at low levels, can lead to reporter gene recombination and subsequent expression in the incorrect cell types as well as those being targeted. The types of mosaic generated are promoter dependent, in that the use of specific promoters driving Cre recombinase expression often have ‘leaky’ expression in the same ‘incorrect’ cell types during development. In the case of the Vav-Cre mice, there is evidence of mosaic expression in the skin in some animals [154]. This would impact significantly on our experiments. Therefore we have used pre-injury biopsies of the skin to confirm there is not mosaic expression in any animals used in these experiments. The use of these biopsies was necessary to confirm no LacZ expression in normal
skin cells in addition to the genotyping to confirm the LacZ gene was correctly recombined and expressed in the animals used in the hematopoietic cells. All animals used were positive for recombination and expression in HSCs, and negative for mosaic expression in skin (Results Fig. 3.29).

The results clearly show the expected hematopoietic component to early burn wound healing hemostasis and inflammation (Results Fig 3.29A). This inflammatory infiltrate is clearly seen at day 7 and as expected is substantially and by day 14 few inflammatory cells are observed but cells within the dermis become apparent, whilst no cells in either the interfollicular dermis or epidermis were detected at day 21 or day 28. Positive expression at days 21 and 28 consisted solely of endothelial cells and hematopoietic cells within blood vessels which is consistent with the expression of the Vav-Cre recombinant mice [155].

At day 14 as well as a small number of inflammatory cells there were cells observed that were similar in morphology to fibroblasts and resided in the dermal compartment in the wound tissue (Fig. 3.29). To characterise these cells further tissue samples were used for cell isolation and culture. Interestingly, whilst these cells morphologically appear to be fibroblast-like, they do not appear to express either Col I or α-SMA. This strongly suggests they are not normal fibroblasts, but potentially a sub-population of resident macrophages that persist in the wound longer than other immune cells during the healing process, potentially contributing to healing. It is important that the cell type is accurately identified, and ongoing experiments to further characterise these isolated cells are underway.

In summary, the data clearly shows a transient population of hematopoietic cells within the healing wound. This appears to be predominantly inflammatory, but there is a small number of cells persisting longer than the acute phase response in the healing dermis. These cells do not appear to be fibroblasts, and therefore this experiment shows no evidence for hematopoietic cells contributing to long-term healing through fibrocyte differentiation [258-259, 285]. This is in stark contrast to a number of other reports investigating the role of fibrocytes. Although the
use of the transgenic model provides very reliable data, one of the limitations of this model is that it is murine and may not be a true mimic of the human situation. This has been shown to be the case in some other recent work looking at the inflammatory response to burn injury, [230] but many other studies suggest the mouse is a useful model for wound healing mechanisms and accurately, or at least largely mimics the human response [289]. In addition, other mouse studies have shown contrasting results to those we have shown using the transgenic model, suggesting that the absence of any HSC derived cells in the wound is not solely due to the model we used. A further point that needs to be considered is the size of the injury. The burn injury in this case equates to a moderate burn TBSA of less than 8%. This is the largest injury that we are permitted to perform under the local animal ethics review panel and is in keeping with our strategy to investigate the most common types of burn injury which are predominantly non-severe. In many other reported burn studies investigating the role of fibrocytes or hematopoietic cells, the burn wound is much larger in size, typically 40 % TBSA [210, 290-291]. This much more significant trauma will have a more profound effect on the body and on its response to the injury. In particular, these more severe injuries lead to a much greater inflammatory response and potentially overwhelm the normal response mechanisms [231]. This could lead to the differentiation of cells, including hematopoietic ones, into other lineages as part of the more response, and would in part explain some of the differences observed. Other studies have also focused on chronic wounds, which by their nature are very different in pathology to acute wounds and therefore quite likely to involve different responses. These studies also generally involve the use of diabetic mice to generate a chronic wound-like state, which again alters the underlying pathology away from that being studied here [292-293].

A previous study exploring hematopoietic response to wound healing used a parabiotic mouse model [294]. This parabiotic model is an alternative method to explore the role of mature blood borne circulating cells in cutaneous wound healing. These blood borne circulating (BBC)
cells are both hematopoietic and mobile mesenchymal stem cells. In this model two genetically identical and physiologically normal mice are surgically attached [295]. This provides a conjoined circulation. In this study one of the two conjoined animals was a GFP transgenic mouse.[294] The results from this cutaneous wound healing study show that BBC cells predominate over resident skin cells in the early stages of wound healing. The first time point for this study was at day 3. When neutrophils are discounted the predominant cell type is undifferentiated leucocytes at this timepoint. Interestingly the GFP+ve cells expressed mesenchymal markers Coll I and αSMA in the early wound [294]. This is in contrast to our double staining of cultured cells. (Results Fig 3.31 & Fig.3.32). This may be more likely as a result of the mobile mesenchymal elements in the BBC differentiating within the wound, rather than reflecting hematopoietic cell contribution.

Since this study has been performed there has been further interest and debate in the literature with regard to the role of the fibrocyte and it’s origin [199, 259, 296]. Since the original description there has been a steady increase in our knowledge about this cell type and it’s mechanism of action [85, 206]. It has, for example, been shown that in cutaneous wounds the CD34 hematopoietic marker initially expressed by fibrocytes decreases over time. This occurs as the inflammatory state of the wound is down regulated and fibrocytes are said to differentiate into more mature connective tissue cell types [284]. This has been suggested to explain why hematopoietic markers are not found in the long-term cell populations, despite being derived from fibrocytes [199]. However, importantly in this study, once the LacZ gene has been activated in the hematopoietic stem cells it is unlikely that even with differentiation of the cells into mesenchymal type cells that the LacZ expression will be diminished. This is because the LacZ gene is not under the control of a hematopoietic specific promoter, rather it is under a ubiquitous promoter that is only activated specifically by the Cre recombinase expression. Therefore LacZ expression should be consistent irrespective of the differentiation status of any cell derived from the HSCs. This means we can be confident in this study that it is
not because of trans-differentiation that we no longer detect LacZ positive cells in the late stages of the healed wound, but rather represents the lack of contribution of HSC derived cells to the long-term scar population in a mouse model of moderate burn injury.

One very important consideration that was revealed by this study is the limitation of histological sections as an indicator of cell function and contribution to the wound. In this study, cells within the whole tissue sections were identified at Day14 post-injury. These cells resided in the dermis and their morphology was such that they were identified as fibroblasts. However, after culturing these cells and identifying them using the LacZ expression, no expression of the expected Col I or a-SMA markers was detected. This was despite the fact that other cultured cells that were LacZ- did show positive Col I and/or a-SMA expression in the same cultures, demonstrating the staining protocol was effective but that the LacZ positive cells did not express the marker. This has implications for much of the previous research which used histology as an indicator of cell phenotype.

In light of these findings the interpretation of our previous histology may have been significantly improved by the addition of further double staining to truly assess cell phenotype, rather than rely on morphology and histology alone as a guide. There is the possibility that cells which were previously identified as fibroblasts may indeed not be so, or at least be expressing different markers to other cells in the wound. Whilst this would only have a limited impact on the key findings of the previous studies, it would certainly improve future research to use additional staining techniques. In this study we hypothesise that the resident cells in the dermis at day 14 were most likely longer-lived macrophage type cells. There is evidence for macrophages or monocyte precursors differentiating into fibrocytes and fibroblast cells particularly in the context of wound healing. There is also evidence that the monocyte-macrophage-fibroblasts lineage is closer to a continuum rather than distinct lineages of cells [297-298]. However, these cells do not express fibroblast markers, and so whilst the morphology suggests they are similar to fibroblasts they are unlikely to be functioning in the
wound in a similar manner. Further characterisation of these cells will be important to better understand any potential role in healing.

This study clearly shows that there is no long-term hematopoietic cell derived contribution to scar tissue after moderate burn injury. This is in contrast to much of the literature regarding fibrocytes and their role in wound repair. The use of this well-characterised transgenic model provides strong data and circumvents many of the limitations of other studies, with respect to the influence of cell differentiation, cell characterisation and phenotype assessment on the interpretation of results. It will be important to use this model to assess other injury extents (e.g., severe burn injury) and modalities to further determine the role, if any, that hematopoietic derived cells play in wound repair (other than inflammation). Understanding the source of the conflicting data and the true role of these cells is critical to enable new cell therapies to be developed and to be effective in the treatment of burn injury.

4.7 Future directions

These studies have furthered our understanding of the role that is played in wound healing and scar formation after burn injury by cells originating from the bone marrow. It is clear from these studies that the bone marrow is one of the sources of cells that contribute to the healing process long after acute inflammation has subsided. It is also clear that there is no long-term contribution from cells that are hematopoietic in origin, and therefore the contribution we have observed must be due to cell originating from the mesenchymal population within the bone marrow. What is not clear from the studies to date is how these findings might change in a more severe injury model. This would be an interesting next step, especially given the contrasting findings that have been reported in other studies with respect to a role for hematopoietic cells.
From a therapeutic perspective, the data suggests that mesenchymal cells can migrate to the wound when delivered systemically. However, migration and survival of these cells was also observed in other tissues, and this would suggest the use of the cells should proceed with caution until the fate of cells not migrating to the wound is better understood. It may be that after a further period of time all these cells would have apoptosed, and it would be useful to extend the timeframe of these studies to determine whether the cells really do persist or even proliferate in the long-term. What was not shown by these studies was whether the exogenous mesenchymal cells impacted positively on the wound healing process, as the mouse model is not best suited to measuring changes in the healing process. Further studies using exogenous application of mesenchymal stem cells in a well-designed study using a large animal model (porcine) are warranted, to ascertain whether this source of cells has the potential to improve outcomes.

Wound healing and scar formation continue to be a major challenge in modern medicine. Survival rates from severe injuries have significantly increased, but scarring, loss of function, chronic pain and psychological effects drastically reduce quality of life. Adults are affected, losing their ability to return to work, and children have lifelong functional deficits.

As discussed above there are still a number of questions related to how the MSCs contribute to tissue repair, the so called “evolving paradigms”[202]. It is critical that further work clarifies our understanding of this role for MSCs to be realised as a therapeutic option. However, despite a clear need for a better understanding of the role, function and potential of MSCs, a number of clinical studies have already been commenced, often in more serious conditions with few available treatment options but also recently in burn injury. The difficulty with these studies is that given the lack of basic knowledge around the cells and no well-designed large animal trials to base therapeutic trials on, the studies themselves do not generally satisfactorily answer whether MSCs truly have therapeutic potential.
Examples of some of the many small trials of treatment that have been reported include spinal cord lesions, pulmonary hypertension [204, 299-300]. Each of these papers reports different methods of administration and varying methods of cell isolation and preparation, as well as varying dosage regimes. It is also not clear from the studies yet whether the use of autologous or allogeneic cells is preferable. In the case of burns, a small number of studies have been reported. These consist of a case report of allogenic, MSCs being administered topically in a 40% TBSA burn with little clinical detail reported other than an improvement [187]. Currently there is an Argentinian Phase I/II study exploring the application of cadaveric MSCs in a fibrin based spray over an acellular dermal matrix. This will be performed in 10 major burn patients [301]. This study is similar to others where stem cell capturing collagen scaffolds are being used, impregnated with stem cell specific antibodies and which in cardiac studies have been shown that used as a patch there is observed regeneration of cardiomyocytes [302]. However, whilst the use of MSCs in these contexts may be promising, there are other alternatives that may provide a better therapeutic option in the future. Certainly, since the demonstration of the ability to reprogram cells to become induced pluripotent stem cells (iPS cells, there has been increasing interest in reprogramming cells in situ [303-304]. This has recently been achieved with the reprogramming in vivo of cardiac fibroblasts to create functional myocytes, which highlights the potential for using a reprogramming approach of cells at the site of the wound rather than necessarily using exogenous cell applications [305]. Alternatively, it may be that combining the reprogramming approach with a source of cells amenable to programming such as MSCs will also be an avenue to explore for future cell based therapies.

The potential clinical application of MSCs in the treatment of burns patients is multi faceted. Their role in the acute major burn patient may be more akin to the haematological conditions, that is with the extensive inflammatory response, there is evidence that the bone marrow becomes overwhelmed[188] and it may be that culturing autologous BM on admission and augmenting the BM with Erythropoietin and Granulocyte macrophage Colony Stimulating
factor may be useful as a source of replenishing depleted bone marrow cells at later stages in the healing process when some of the later deaths occur [306]. In future clinical practise the potential for topical administration is most likely to involve the use of collagen type scaffolds or other smart type dressings. There is increasing interest in the use of scaffolds in particular in combination with current cell therapies to further direct the healing process and it is reasonable to expect these studies to be piloted clinically in the near future.

An alternative application for MSCs may be in the treatment of chronic type wounds in the burn patient. These are common in the major burn patients who may go on and require dressings for a considerable length of time. It is possible that good wound bed preparation and administration of MSCs may convert the chronic type wound back to an acute healing wound state and allow wound healing to occur [203].

Regardless of the potential clinical role of the MSCs, there continues to be questions regarding safety. This not only relates to the use of autologous or allogeneic cells, but also the potential risks associated with their administration. There have been concerns raised with regard to teratomas and malignancies [307], which coupled to the data presented here showing long-term discrete populations of applied cells in multiple tissues, provides cause for concern, in particular if these cells are to be applied to improve cosmetic or functional outcomes rather than in lifesaving situations.
4.8 CONCLUSION

This study has substantially progressed our understanding of how cells mobilised from the bone marrow contribute to non-severe burn wound healing. Based on the findings here, continued work on the potential therapeutic benefits of exogenous MSCs is warranted, whilst re-examination of the role or origin of fibrocytes in healing is also necessary. Cell therapies have made an important contribution to burn treatment to date, and it is hoped that further work will help to improve current and identify better cell therapies into the future.
REFERENCES


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