The effect of Hoxa3 overexpression on macrophage differentiation and polarisation

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

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Abstract

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The effect of Hoxa3 overexpression on macrophage differentiation and polarization.

The regulated differentiation and polarisation of macrophages are essential for successful wound healing process. During wound repair, macrophages are involved in the early inflammatory process of healing, as well in later regenerative phases by producing cytokines and growth factors relevant for each stage. Their plasticity made macrophages able to change their phenotype from M1 inflammatory during the inflammatory phase of healing to M2 reparative during regenerative phases of healing. Diabetes affects the ability of macrophages to mature from the bone marrow and on their ability to polarise to different phenotypic subsets. Whereas the non-diabetic macrophages can mature normally to M2 macrophages during mid-stages of healing, diabetic wound continues to display immature proinflammatory macrophages resulting in mixed M1/M2 macrophages in the wound that remain until late stages of healing. We previously showed that sustained expression of Hoxa3 reduced the excessive number of leukocytes recruited to the wound, suggesting an anti-inflammatory effect of Hoxa3 upon all leukocytes population. Hoxa3 protein transduction also promoted the differentiation of HSC/P into pro-angiogenic Gr1+CD11b+ myeloid cells. Here we showed that Hoxa3 promoted the differentiation of macrophages and upregulated the transcriptional machinery controlling macrophage differentiation, in THP-1 monocytes and primary macrophages from non-diabetic and diabetic mice. Using qRT-PCR and protein analysis of bone marrow derived macrophages from diabetic mice, we showed that Hoxa3 upregulated the master regulator of macrophages differentiation, Pu.1 transcriptionally and post-transcriptionally and that Hoxa3 protein interacted with Pu.1 protein in vitro and in vivo within macrophages proposing a mechanism of their regulation. Hoxa3 also inhibited proinflammatory markers in classically activated macrophages and augmented pro-healing markers in alternatively activated macrophages. Investigating the IL-4/Stat6 pathway of M2 macrophage activation revealed that Hoxa3 upregulated Stat6 and increased Stat6 phosphorylation, a novel effect of Hoxa3 on the signaling pathway of alternative macrophage activation. In vivo analysis of Hoxa3’s effect on wound derived macrophages in diabetic mice, confirmed that Hoxa3 promoted the generation of pro-healing macrophages and showed reduced Nos2+ (M1) cells and increased Arg1+ (M2) cells suggesting that Hoxa3 can rescue the phenotype of diabetic macrophages in the wound. Altogether, this work has delineated the specific role of Hoxa3 in rescuing maturation and phenotype of diabetic macrophages thereby providing a better understanding of the therapeutic role of this transcription factor for myeloid cells dysregulation in diabetes.
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# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>Ant-c</td>
<td>Antennapedia complex</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BX-c</td>
<td>Bithorax complex</td>
</tr>
<tr>
<td>Ccl2</td>
<td>Chemokine C-C motif ligand</td>
</tr>
<tr>
<td>Cebpα</td>
<td>CCAAT-enhancer-binding protein alpha</td>
</tr>
<tr>
<td>Chi3</td>
<td>Chitinase-like secretory lectin</td>
</tr>
<tr>
<td>CM</td>
<td>condition media</td>
</tr>
<tr>
<td>c-MYB</td>
<td>c- terminal myeloblastosis</td>
</tr>
<tr>
<td>CoIP</td>
<td>co Immunoprecipitation</td>
</tr>
<tr>
<td>Db</td>
<td>Diabetic</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>Elf</td>
<td>E 74-like fator1</td>
</tr>
<tr>
<td>Erg</td>
<td>Ets-related gene</td>
</tr>
<tr>
<td>Exd</td>
<td>Extradenticle</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Fizz</td>
<td>Found in inflammatory zone 1</td>
</tr>
<tr>
<td>GATA-2</td>
<td>Globin transcription factor 2</td>
</tr>
<tr>
<td>GEMM</td>
<td>Granulocyte-erythocyte-megakaryocyte-macrophage</td>
</tr>
<tr>
<td>GM-CFU</td>
<td>Granulocyte-monocyte progenitor colony forming unit</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S transferase</td>
</tr>
<tr>
<td>HIF1</td>
<td>Hypoxia inducible factor 1</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human dermal microvasculer endothelial cells</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cells</td>
</tr>
<tr>
<td>HSC/P</td>
<td>Hematopoietic stem cell/progenitor</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INFγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>iNos</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>mφs</td>
<td>macrophages</td>
</tr>
<tr>
<td>M-CFU</td>
<td>Macrophage colony forming unit</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescent intensity</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MMP14</td>
<td>Matrix metalloproteinase 14</td>
</tr>
<tr>
<td>ndb</td>
<td>non diabetic</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>Relmα</td>
<td>Resistin-like molecule</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Runt-related transcription factor1</td>
</tr>
<tr>
<td>SCL</td>
<td>Stem cell leukemia</td>
</tr>
<tr>
<td>Sec</td>
<td>Secretin sequence</td>
</tr>
<tr>
<td>SP</td>
<td>Signal peptide</td>
</tr>
<tr>
<td>TALE</td>
<td>Three amino acid loop extension</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>uPAR</td>
<td>Uroplasminogen activator receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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</tbody>
</table>
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This thesis is dedicated to my son Hamoody...
Chapter 1: General Introduction

1.1 Introduction

Wound healing is a normal physiological process that occurs in response to injury to the skin and forms part of a larger complicated process of tissue repair and regeneration. Tissue regeneration, a prolonged form of wound healing, takes place in organs to regenerate tissue, particularly after surgery. Throughout these processes, macrophages play a diverse role and, because of their plasticity, can change their phenotype from inflammatory to reparative depending on the stage of healing in a highly regulated fashion. However, evidence from diabetic patients and animal models show that intrinsic factors within macrophages together with the diabetic environment bring about dysregulation of this process. This affects how macrophages mature from the bone marrow and how they respond to signals and polarise into different phenotypes creating a state of chronic inflammation and impaired healing. (Bannon et al., 2013; Mirza and Koh, 2011).

Hoxa3 is a homeobox transcription factor that is expressed endogenously in a normal wound but not in diabetic wounds. The potential therapeutic role of Hoxa3 was identified in our laboratory using gene transfer and protein transduction methods (Mace et al., 2009; Mahdipour et al., 2011). However, though administration of Hoxa3 has been shown to accelerate healing, it is not clear whether it plays a role in macrophage maturation and polarization, particularly in diabetic wounds. My aim in this project is to identify the role of Hoxa3 in the maturation process of monocytes to macrophages and bone marrow (BM)-
derived macrophages in both normal and diabetic mouse models. This is followed by investigating whether Hoxa3 can rescue the phenotype of diabetic macrophages from persistent inflammation into normal functioning cells that can polarise into a reparative fate during the latter stages of healing. It is imperative to understand these aspects to evaluate strategies that would employ Hoxa3 to improve diabetic wound healing.

In the following section, the phases of wound healing in a coetaneous wound are elucidated with a particular focus on macrophages. This summary will be followed by a description of alterations in macrophage as a result of diabetes. Available treatment options and challenges for managing of chronic wounds, as well the potential of Hoxa3 in reprogramming myeloid cells, are then discussed. Finally, I investigated the mechanisms controlling the maturation and polarisation of macrophages focusing on genes that could be a potential downstream target for Hoxa3, including the Ets transcription factor Pu.1.

**1.2 Wound healing**

When skin barrier is breached, acute wound follows a well regulated process involving recruited immune cells, endothelial cells, keratinocytes, fibroblasts and regulated by cytokines, growth factors, signalling proteins, and transcription factors. The normal process of wound healing involves orderly progression from inflammatory response to proliferation, migration and tissue regeneration, then to remodelling phase (reviewed in (Eming et al., 2014)). These phases are not discrete and each phase can overlap with the next and the time span needed for the resolution of each phase and the total period needed to complete wound repair varies depending on multiple factors, like the site and the severity of the wound, health status, and
genetic susceptibility (Guo and DiPietro, 2010). Figure 1.1 provides a brief overview of the phases involved in the cutaneous wound healing process.

Figure 1.1: Summary of the wound healing phases. a. Haemostasis and inflammation. The wound area contains blood clots and infiltrating neutrophils, monocytes, and M1 macrophages. b. Proliferation. Keratinocytes migrate to and proliferate to close the wound surface by re-epithelisation. The new tissue formed is granulation tissue that replaces the damaged dermis. Neutrophils are removed from the wound by macrophages. Fibroblasts together with macrophages (particularly the M2 type) are the main cellular components of granulation tissue and repair of damaged vessels take place by Neovascularisation. c. Tissue remodelling phase. After closer of wound epidermis, the collagen produced within dermis is remodelled in a process that balances collagen formed with collagen breakdown. Myofibroblasts differentiated from fibroblasts involved in contraction of the wound and collagen production.
1.2.1 Homeostasis and inflammation

Injury leads to immediate activation of coagulation cascade, which through the formation of a fibrin clot, sustain haemostasis and isolate the wound from the external environment. The clot occurs within minutes to hours after injury and triggered by released blood component from injured vessels into the extravascular tissue. The first cellular response in this process occurs in platelets. Their activation and aggregation lead to initiation of the coagulation cascade (Nurden et al., 2008). The end product of the coagulation cascade is the formation of a platelet plug that is stabilised by fibrin (Figure 1.1a). In addition to maintaining haemostasis, the fibrin clot serves as a provisional matrix to which growth factors can bind. (Nurden et al., 2008). Activated platelets trapped within the clot are the earliest provider of growth factors such as platelet-derived growth factor (PDGF), transforming growth factor β (TGF)-β, and TGF-α that set the wound for inflammatory phase of healing (Su et al., 2009). The relevance of platelets and their product in wound resolution is reflected in the clinic by the use of platelet rich plasma as mean to accelerate healing ((Martinez-Zapata et al., 2012).

This stage is followed by an inflammatory response which begins with passive leakage of leukocytes from damaged blood vessels into the wound as well by an activation of wound resident immune cells such as epidermal dendritic cells ‘langerhans’ cells.’ (Cumberbatch et al., 2000), γδ Tcells (Jameson et al., 2004) and mast cells (Noli and Miolo, 2001). Activated cells release a rapid pulse of proinflammatory cytokines and chemokines. These pulses together with factors released from platelet degranulation (Deppermann et al., 2013) and from foreign epitopes of invading micro-organisms such as lipopolysaccharides (LPS) and damage associated molecular pattern (DAMP) and pathogen-associated molecular pattern (PAMP)
released from damaged cells (Bianchi and Manfredi, 2009), triggers inflammatory cell recruitment. Inflammation begins with an influx of polymorphonuclear (PMN) cells followed by monocyte and macrophage recruitment (Eming et al., 2007a; Shaw and Martin, 2009). This recruitment process is facilitated by activation of endothelial cells that express P-selectin and E-selectin molecules, which in turn allows immune cells to roll along the vessel wall and extravasate from the circulation into the wound tissue (Yukami et al., 2007).

This process is also associated with enhanced vascular permeability that is promoted by nitric oxides (NO) produced by PMN and macrophages together with plasminogen activators and other factors (Eming et al., 2007a; Shaw and Martin, 2009). Recruited neutrophils become activated by pro-inflammatory cytokines, such as interleukin-6 (IL-6), IL-1α, IL-1β and tumour necrosis factor (TNF) (Eming et al., 2007a). Monocytes come later in inflammatory phase and play role in phagocytosis and their number peak around 24h following injury (Shaw and Martin, 2009) (Figure 1.1a).

Studies that aimed to test the function of each immune cell lineage throughout the healing response provided us with clues about the role of leukocytes subpopulation within the wound. The role of PMN is mainly bactericidal, associated with killing micro-organisms and releasing oxidative bursts like reactive oxygen species (ROS), proteases (elastase, cathepsin G, and urokinase plasminogen activator) that involved in clearing cellular debris and antimicrobial activities (reviewed in (Eming et al., 2007a)). However, their depletion from the wound accelerates healing process (Dovi et al., 2003). On the other hand, macrophages can play more diverse role and take over the phagocytic role of PMN and monocytes and due to their plasticity, they can transit from being inflammatory ‘M1’ macrophages during early stages of
healing to anti-inflammatory/reparative ‘M2’ macrophages during proliferative stage (Daley et al., 2010; Koh and DiPietro, 2011). Analysis of macrophage polarisation gene expression profile showed that macrophages from both M1 and M2 profile exist in early inflammatory stages whereas in intermediate phase of healing, their phenotype is predominated with M2 macrophages (Figure 1.2a,b) (Novak and Koh, 2013). The origin of macrophages within the wound either comes from tissue resident macrophages (CCR2<sup>lo</sup>, Cx3CR1<sup>hi</sup>) which form a small proportion of macrophages within the wound, or more abundantly from the monocyte-derived macrophages (CCR2<sup>hi</sup>, CX3CR1<sup>lo</sup>) that differentiate from recruited monocytes and can expand in number in response to inflammatory stimuli (reviewed in (Wicks et al., 2014). The presence of macrophages, particularly of inflammatory phenotype is controlled mainly by pro-inflammatory mediators and chemokines, including RANTES and MCP-1 that are released from platelet degranulation, fibrin clots, keratinocytes, fibroblasts and leukocytes themselves (Eming et al., 2007). At the end of the inflammatory phase, macrophages are the dominant cell type in the wound that maintain antimicrobial activity through production of IL-1, IL-6, TNF and inducible nitric oxide synthase (iNos) (Daley et al., 2010; Koh and DiPietro, 2011).

1.2.2 Proliferation and tissue regeneration phase of healing

This phase demonstrates the cellular proliferation, migration and active repair of damaged blood vessels and dermis. Morphologically, the damaged dermis is replaced by granulation tissue which composed of invading capillaries that give it a granular appearance (Baum and Arpey, 2005). Within this tissue, repairing the damaged blood vessels can take place by two
methods: angiogenesis or vasculogenesis (Bauer et al., 2005). In angiogenesis, blood vessels sprout from intact capillaries to replace the damaged vessels. While in vasculogenesis, the new vessels are formed from aggregates of endothelial cells independent of the existing capillaries (Bauer et al., 2005; Eming et al., 2007b). The most important regulator of angiogenesis and vasculogenesis is VEGF, which is produced by M2 macrophages but not from M1/pro-inflammatory population (Jetten et al., 2014a). Keratinocytes also play a role in the synthesis and production of VEGF and this growth factor together with TGF-β and Fibroblast growth factor (FGF) can contribute synergistically in neovascularisation (Bae et al., 2015; Ferrari et al., 2009; Werner and Grose, 2003; Xue and Greisler, 2002). Reparative macrophages, which also referred to as wound-healing macrophages in this phase can release a plethora of growth factors and molecular targets that not only associate with angiogenesis but also in collagen production and ECM remodelling. These factors including platelet-derived growth factor (PDGF), transforming growth factor (TGF)β, FGF and Relmα, the latter mediate the production of lysyl hydroxylase for the formation of the collagenous scar (Knipper et al., 2015; Lucas et al., 2010a). Bannon and Colleagues demonstrated that the presence of these reparative macrophages in human ulcer can be a prognostic factor that defines the healing from non-healing ulcers (Bannon et al., 2013). In parallel to repairing the damaged blood vessels, this phase of healing is characterised by migration of cells mainly Fibroblasts and Keratinocytes. Fibroblasts activation and migration are triggered by factors associated with the tissue being damaged such as H₂O₂ and Calcium, serum exposure and loss of mechanical tension and changes in electrical gradient (Achterberg et al., 2014; Chang et al., 2004; Wang et al., 2011). These stimulators induced the recruitment of fibroblasts from the edge of the wounds as well
from the BM, particularly from Mesenchymal stem cells (MSC) (Martin and Nunan, 2015; Sasaki et al., 2008). Activated fibroblasts involved in a mechanical role in that is to differentiate into myofibroblasts, a specialised cell with a contractile ability that helps to re-approximate the wound edges promoting wound closure (Shaw and Martin, 2016) (Figure 1.1b,c). Importantly, fibroblasts contribute to repairing the damaged dermis mainly by producing collagen, component of scar tissue that replaces the fibrin-based ECM formed in haemostasis with collagen-based extracellular matrix (ECM) (McDougall et al., 2006). Migration of Keratinocytes in this phase is also evident (Gurtner et al., 2008) and as with fibroblasts, keratinocytes are being triggered by stress signals released from damaged tissue involving serum exposure, peroxides, and changes in mechanical tension and electrical gradient (Freedberg et al., 2001; Lisse et al., 2016; Riding and Pullar, 2016; Zhou et al., 2015). Activated Keratinocytes lay across the granulation tissue to fulfil re-epithelisation (Figure 1.1b). Re-epithelisation would not be possible without the signals from encountering growth factors like FGF and Hepatocyte growth factor (Eming et al., 2014). TGF-β on the other hand, negatively regulates the process of re-epithelisation and their low level is associated with epidermal hyperproliferation (Martinez-Ferrer et al., 2010; Pastar et al., 2010). Even though the outer epidermal layer of the skin is typically closed after the proliferative phase, the inside of the wound is still infiltrated by collagen and cellular infiltrates of granulation tissue that did not regain the integrity and plasticity of healthy skin.
1.2.3 Tissue remodelling phase of healing

In this phase, the collagen-based ECM is remodelled in a process that balances the collagen formed from fibroblasts with collagen breakdown (Baum and Arpey, 2005). The end product of healing, which is either tissue regeneration or replacement of damaged tissue by scar are largely determined by inflammatory cells recruited early in healing process (Mori et al., 2008). Macrophages particularly, the reparative macrophages are a master regulator of fibrosis and contribute to collagen production from fibroblasts by producing TGF-β, fibroblast growth factor (FGF)-2 and insulin-like growth factor (IGF)-1 (reviewed in (Wynn and Barron, 2010)). Importantly, Resistin-like molecule alpha (Relmα), a reparative macrophage marker, was found to direct the fibrotic collagen formation by producing lysyl hydroxylase 2 (LH2) enzyme (Knipper et al., 2015) thereby further supporting the previously identified mechanisms that link inflammation to fibrosis and tissue scarring (Mori et al., 2008).

In normal remodelling, capillaries infiltrating the granulation tissue begin to degrade or regress and macrophages together with any cellular components are removed from the wound by the end of this stage either through apoptosis or migration and wound become contracted and closed (Figure 1.1c; Gurtner et al., 2008). However, abnormal remodelling can result in pathological scarring such as hypertrophic scars or keloids (reviewed in (Martin and Nunan, 2015)).

1.3 Role of macrophages in wound healing and tissue regeneration

Following to skin injury, an inflammatory influx commences that begin with Neutrophils and tissue-resident dendritic cells. Macrophages then take over the inflammatory role and remain
the dominant immune cell type until mid-to-late stages of repair (Figure 1.2). Due to their plasticity, macrophages can change their phenotype from inflammatory to anti-inflammatory fate as well have a factory role by producing growth factors essential for repair and regeneration and scar tissue formation (Figure 1.2).

**Figure 1.2: Role of macrophages during skin wound healing.** Following to a skin injury, activation of innate immune response initiated by neutrophils and dendritic cell. Macrophages then take over the phagocytic role and dominate the wound environment during mid-stages of repair and remain in the wound until late stages of healing. The plasticity of macrophage permits them to be involved in inflammatory function, synthesis of growth factors as well in anti-inflammatory function. Adopted from (Willenborg and Eming, 2014).
Even though macrophages within the wound is an extensively studied topic in the literature, their role within different types of wound remain controversial. Lesions in neonatal mice recruit very few, if any macrophages but healed efficiently and effectively even in the absence of macrophages (Hopkinson-Woolley et al., 1994). Similarly, neonatal Pu.1 null mice that lack macrophages (but also neutrophils and B-cells) resulted in efficient scarless healing (Martin et al., 2003). On the other hand, macrophage depletion studies showed that deficiency of macrophages from the wound is detrimental to healing. The first depletion study took place with a guinea pig model in the 1970s using macrophage anti-sera and glucocorticoids (Leibovich and Ross, 1975). This study yielded the first evidence for impaired healing in the absence of macrophages, including delay in the clearance of erythrocytes, neutrophils, and debris from the wound. The appearance of fibroblasts, the main component of ECM remodelling, was also delayed, and their rate of proliferation was slower. (Leibovich and Ross, 1975). This study was limited by the fact that glucocorticoids can impair the wound healing process and may have been involved in the negative repair outcome observed in these experiments.

Recent studies involving the selective ablation of macrophages from the wound overcame these previous limitations but also confirmed the essential role of macrophages in healing. (Goren et al., 2009; Lucas et al., 2010; Mirza et al., 2009). In their studies, Goren et al., (2009) and Lucas et al., (2010) used transgenic mice (LysM-Cre/DTR) containing human diphtheria toxin-sensitive macrophage receptors under the expression of lysosome M promoter, modulated by Cre-recombinase to deplete macrophages before wounding. Similarly, Mirza et al., (2009) made use of the same concept to deplete macrophages using diphtheria toxin but
driven by the CD11b promoter. All these groups discovered that depletion of macrophages resulted in reduced re-epithelisation, delayed wound contraction, and decreased levels of certain growth factors, including VEGF and TGF-β. As a result, these wounds demonstrated impaired neovascularization, granulation tissue formation and reduced numbers of myofibroblasts, thereby delaying the overall wound repair process (Goren et al., 2009; Mirza et al., 2009).

In their study of macrophage depletion in a time-restricted pattern, Lucas et al. (2010) made a large contribution to clarify the specific role of macrophages during each phase of healing. Their investigation revealed that depletion occurred during the inflammatory phase not only delayed the initial repair process but also affected the late repair response. The author also demonstrates that macrophages recruited during the early stages of healing are the same one that can transit to the reparative phenotype and can produce VEGF and TGF-β that contribute to wound angiogenesis and myofibroblast differentiation (Lucas et al., 2010). Macrophage depletion during the proliferative phase of healing, altered the active repair process and increased apoptosis of endothelial cells. Macrophages during this stage also functioned in dermal and epidermal interactions as evidenced by the detachment of the wound epidermis from underlying granulation tissue in the macrophage-depleted wound (Lucas et al., 2010). All the above studies demonstrate that adult wound healing would not be successful without the presence of macrophages and that these particular cells can have multiple functions during the various stages of healing.

Macrophages in the dermis, requires careful identification as they share some overlapping surface markers and morphology with other mononuclear phagocytic cells such as dendritic
cells and monocytes. Immunological Genome Project provided a comparative analysis to discriminate between macrophages, dendritic cells and monocytes in mice (Gautier et al., 2012; Miller et al., 2012). Based on gating F4/80+ population that were co expressing CD64 as well MerTK, a protein-tyrosine kinase receptor that recognises apoptotic cells identified them separately from CD64+MerTKlo to − monocytes (Gautier et al., 2012). However, this analysis could not differentiate macrophages from other CD11b expressing leukocytes in the dermis and identified CD11b+ population as a broad heterogeneous group of leukocytes including dendritic cells, macrophages and monocytes (Gautier et al., 2012; Miller et al., 2012).

Tamoutounour et al., (2013) and colleagues used a multicolour flow cytometry panel to identify CD11b+ macrophages from other CD11b+ cells. Based on CD64 (FCγ receptor 1), MerTK, CCR2, major histocompatibility complex (MHCII) and CD24, they identified five subpopulations of dermal CD11b+ cells (Tamoutounour et al., 2013). These populations identified as CD11b+ dendritic cells, P1 (monocytes), P2 and the more differentiated P3, referred to as monocytes derived dendritic cells and P4, and the more differentiated P5 were identified as macrophages (see Table 1.1).
Table 1.1: identification of CD11b+ dermal leukocytes in the skin.
Adapted from (Tamoutounour et al., 2013).

<table>
<thead>
<tr>
<th>CD11b+ populations in the skin</th>
<th>Cell surface markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b+ DC</td>
<td>CD11b+, CD64-, MerTK-, CCR2+, Ly-6C-, MHCII-, CD11c^lo^to^+^, CD24^lo^</td>
</tr>
<tr>
<td>P1 dermal monocytes</td>
<td>CD11b+, CD64^lo^, MerTK^-to^lo^, CCR2^+, Ly-6C^hi^, MHCII^lo^, CD11c^-, CD24^-</td>
</tr>
<tr>
<td>P2 monocytes derived DC</td>
<td>CD11b^+, CD64^lo^, MerTK^-to^lo^, CCR2^+, Ly-6C^hi^, MHCII^lo^, CD11c^-to^lo^, CD24^-</td>
</tr>
<tr>
<td>P3 monocytes derived DC</td>
<td>CD11b^+, CD64^+, MerTK^-to^lo^, CCR2^+, Ly-6C^hi^, MHCII^lo^, CD11c^-to^lo^, CD24^-</td>
</tr>
<tr>
<td>P4 dermal macrophages</td>
<td>CD11b^+, CD64^hi^, MerTK^+, CCR2^lo^, Ly-6C^lo^, MHCII^-, CD11c^-to^lo^, CD24^-</td>
</tr>
<tr>
<td>P5 dermal macrophages</td>
<td>CD11b^+, CD64^hi^, MerTK^+, CCR2^lo^, Ly-6C^lo^, MHCII^-, CD11c^-to^lo^, CD24^-</td>
</tr>
</tbody>
</table>

The author argues that the common marker used to identify macrophages in the skin such as F4/80 and Cx3CR1, and CD68 can not distinguish CD11b^+ macrophages from dermal DC or monocytes (Tamoutounour et al., 2013). Therefore, in a skin wound models, it would be essential to consider identification of macrophage carefully by more than one marker to separate them from a population of monocytes and dendritic that share several similarities morphologically and in term of their surface marker expression.

The ability of macrophages to eliminate apoptotic cells is important phenotypically to characterise macrophages from other population ‘as demonstrated above’ but also have substantial functional impact. A process known as efferocytosis, the removal of apoptotic neutrophils from the wound by macrophages (Cvetanovic and Ucker, 2004), not only prevents
the persistence of neutrophils that may damage the tissues but is also proven to be a mechanism promoting the transition of macrophage phenotype from pro-inflammatory to pro-healing (Khanna et al., 2010). Thus, macrophage function spans the bridge between the inflammatory and proliferative healing phases as they facilitate the successful transition to the proliferative phase (Khanna et al., 2010).

In diabetes, the function of macrophages is impaired, affecting the resolution of inflammation, the removal of neutrophils and the transition from inflammatory to reparative mode (Khanna et al., 2010; Lecube et al., 2011). These defects involved at least partially in creating a chronic inflammatory environment of the wound. Recent evidence shows that the removal of apoptotic cells by macrophages can even impact the polarisation to inflammatory phenotype (Shaw and Martin, 2016). Analysis using translucent Drosophila embryo provided evidence that naive macrophages that lack the genes needed for 'programmed cell death' fail to be recruited to sites of tissue damage and fail to recognise any bacterial infection (Shaw and Martin, 2016). On the other hand, macrophages that can engulf the apoptotic cells can develop an immunological memory that facilitates their response to tissue damage and bacterial infections and functioned as inflammatory macrophages (Shaw and Martin, 2016). All the above studies emphasise on the role of phagocytosis in the ability of macrophage to polarise into both inflammatory and reparative phenotypes. These findings also support the potential of macrophages in being re-programmed and being a desired therapeutic target to treat conditions like chronic infections and non-healed wounds.
1.4 The phenotype of macrophages

1.4.1 The phenotype of macrophages in the wound

To understand macrophage phenotype within the wound and how it can be affected in a diabetic wound, it is important first to elucidate the concept of macrophage polarisation and the differences between each phenotype. The concept of classical activation of macrophages identified since the 1960s when macrophage in mice showed the first sign of anti-microbial activity in response to infections with Mycobacterium Bovis bacillus (BCG) or Listeria monocytogenes (Mackaness, 1964). This concept was extended further and developed by Dalton and colleagues, who identified the product that activates these macrophages as interferon gamma (INF-γ) coming from T helper 1 cells or natural killer cell (NK) (Dalton et al., 1993). Indeed, direct activation of macrophages by the bacterial cell wall product, lipopolysaccharides (LPS), alone or together with INF-γ was identified as the main inducer of classical activation of macrophages. Stein et al., (1992), increased the complexity of macrophage phenotype by identifying the macrophages produced from IL-4 cytokines as alternatively activated macrophages. By 2003, the concept of alternative activation of macrophages was fully appreciated to be identified as macrophages derived from IL-4 or IL-13 exposure that were distinct from macrophages derived from IL-10 ‘immune suppressor macrophages’ but share some overlapping characteristics such as inhibiting the inflammatory cytokines and upregulating mannose receptor CD206 (Gordon, 2003). The term M1 and M2 were initially proposed to describe the two antagonistic pathways of nitrogen metabolism in LPS-induced or INF-γ-induced macrophages from Th1 mouse models C57BL/6 or Th2 models
BALB/c mice (Mills et al., 2000). Studies since then identified other stimulators for the M1 or M2 phenotype where M-CSF-differentiated macrophages showed a phenotype similar to the alternatively activated: upregulating IL-10 but not IL-12, and GM-CSF-derived macrophages showed a phenotype of increased TNF and IL-1 closely resembling the M1-macrophages (Fleetwood et al., 2007). More understanding of the M2-like phenotype took place after that, suggesting that these macrophages share some characteristics of IL-4 and IL-13 induced macrophages such as increased IL-10, high expression of mannose and angiogenic factor. M2-like phenotype can be induced by TGF-β, IL-10 or immune complexes (Biswas and Mantovani, 2010; Mantovani et al., 2004). A simplified history of the phenotypic classifications of macrophages is illustrated in (Figure 1.3).

**Figure 1.3**: Key discoveries in classical and alternative macrophage activation. Simplified history of the study of macrophage polarisation, focusing on their functional aspects and exogenous stimuli.
From a functional point of view, classically activated-M1 macrophages govern resistance against microbes, parasites, and tumours by producing high levels of TNF, IL-12, IL-6, ROS, and reactive nitrogen intermediates (RNI) and reactive oxygen intermediate (ROI) (Martinez and Gordon, 2014). On the other hand, M2 macrophages promote tissue remodelling, angiogenesis, allergic reaction and parasite clearance. The well-characterised M2 macrophages stimulated by IL-4 and IL-13 are now identified as the M2a macrophages. Other M2 subtypes including the M2b and M2c, differentiated in response to immune complex or IL-10/TGF-β respectively. Although the mode of activation of M2 subtypes can vary, these M2 subsets share some overlapping function and gene expression profile. M2 macrophages are often characterised by their increased arginase activity, induced mannose receptors, scavenger receptors, and TGF-β and down-regulated pro-inflammatory markers including IL-12, TNF and nitric oxide synthase (Nos2) (Dinarello, 2005; Martinez et al., 2009a; Munder et al., 1998). Markers that were also used to identify alternatively-activated macrophages have been employed in studies with the phenotype of wound macrophages are: Found in inflammatory zone 1 (Fizz1), also known as Resistin-like molecule α (Relmα), as well as the chitinase-like secretory lectin (Chil3), also known as Ym1 (Raes et al., 2002) (Figure 1.4).
Figure 1.4: Schematic representation of macrophage polarization. M1 macrophages are induced by interferon gamma, Lipopolysaccharides, or by a tumor necrosis factor and function as inflammatory macrophages. M2 macrophages are further subdivided into M2a, M2b, and M2c, all sharing various characteristics but differing with respect to their stimulus, mediating immune suppression and Th2 responses. In the wound, transition from M1 to M2 profile of macrophages is mediated the following stimulus: IL-4, IL-13, adenosine, miR21 or anti-microbial effector molecule LL-37. TLR: toll-like receptor, IC: immune complex, Gluc: glucocorticoids. miR21; microRNA-21 Adapted from (Ferrante and Leibovich, 2012; MacLeod and Mansbridge, 2016).

In vivo classification of macrophages is not as absolute as is the case with in vitro studies.

Within the wound, macrophage phenotype is best described as a continuous spectrum of various phenotypic states that can be changed depending on the wound microenvironment and the cell’s own intrinsic factors (Bannon et al., 2013; Mirza and Koh, 2011). This heterogeneous population within the wound is dominated by M1-like proinflammatory macrophages during the early inflammatory phase and then switches to M2-like pro-healing macrophages during the proliferative and tissue remodelling phases. However, it is also
common to find a population that presents with a mixed phenotypic state that can, for example, express markers of alternative activation, such as CD206, as well as expressing TNF (Bannon et al., 2013; Daley et al., 2010; Koh and DiPietro, 2011). The M1-M2 switch in vivo is certainly associated with normal wound resolution, collagen production and angiogenesis. This is due to the fact that the M2 macrophages, which are often referred to as ‘wound-healing macrophages’, are a rich source of factors such VEGF and TGF-β (Mantovani et al., 2013; Pollard, 2009). Indeed, the in vivo switch of macrophage phenotype is associated with changes in myeloid cells such as acquiring markers, including scavenger and mannose receptors, increase in arginase activity and reduced iNOS activity (Mantovani et al., 2013).

Although all previous evidence supports the transition from M1 to M2 macrophage during normal healing, it remains unclear to what extent a cell that has an M1 phenotype at the start of healing, acquires the M2 phenotype later in healing. It could be that additional cells also recruited from the BM or that the resident non-activated macrophages become activated to the desired phenotype.

Studies using a sterile wound model or polyvinyl alcohol sponge derived macrophages provided some answers for the precursors of macrophages within the wound and their relation to the phenotypic changes (Crane et al., 2014; Daley et al., 2010). During early stages of healing, F4/80⁺Ly6C⁺ Inflammatory monocytes population can either remain without any further differentiation or rapidly became proinflammatory monocytes by acquiring markers such as CD14 and TNFα (Crane et al., 2014). As healing proceeds to mid stages, macrophages derived from F4/80⁺Ly6C⁻ “more mature population” begin to acquire other surface markers like co-expression of CD64, MerTK as well as CD206 (Crane et al., 2014). These
Ly6C<sup>lo</sup>MertK<sup>+</sup>CD64<sup>+</sup> cells were releasing pro-repair mediators such as VEGF and TGFβ, suggesting that acquiring the M2/reparative phenotype in wound was accompanied by an increase in macrophages' maturation potential. On the other hand, pro inflammatory phenotype was associated with less mature population expressing Ly6C<sup>hi</sup> (Crane et al., 2014). Similarly, macrophages in gut, showed that alternative activation in response to nematode infection that assessed by the expression of Relmα, was determined in a population identified as resident macrophages expressing CX<sub>3</sub>CR1<sup>hi</sup>F4/80<sup>+</sup>Ly6C<sup>−</sup> and mature inflammatory macrophages expressing CX<sub>3</sub>CR1<sup>int</sup>F4/80<sup>+</sup>CD11c<sup>−</sup> but not in immature population of Ly6C<sup>+</sup> monocytes (Little et al., 2014). Thus, supporting the hypothesis that macrophage phenotype, notably M2 integrates with the more mature developmental stage in skin wound model and intestinal infection model. With that connection in mind, diabetic wound macrophages that fail to mature normally as stated in many studies (Bannon et al., 2013; Miao et al., 2012; Mirza and Koh, 2011), likely to be the same cells that fail to polarise pro- healing fate associated with increased inflammatory cellular infiltrate and chronic wound pathologies (this will be discussed in details in the next sections).

IL-4 and IL-13, the central mediators of alternative activation of macrophages in vitro, (Gordon, 2003; Martinez et al., 2009b; Mosser and Edwards, 2008) are proposed to give rise to the reparative phenotype of macrophages in the wound (Salmon-Ehr et al., 2000). However, in a model of a sterile wound, there was no evidence of the presence of IL-4 or IL-13 cytokines, their receptor IL-4Rα or their downstream target, pStat6, in wound cells (Daley et al., 2010). This could be because of the absence of invading microbes that may alter the inflammatory
stage and subsequently disturb the pathway that facilitates the switching to M2 reparative macrophages. The IL-4/Stat6 signalling pathway in macrophages is discussed in more detail in Section 5.1.

Knipper and colleagues provided important clues about the regulation of M2 macrophages \textit{in vivo} and demonstrated the existence of functional IL-4Rα in wound macrophages. Conditional depletion of IL-4Rα from whole wounds or from leukocytes attenuated the M2 signature in the wound as evidenced by reduced CD163, CD206, and IL-10 and enhanced NOS (Knipper et al., 2015). Furthermore, the functionality of the macrophages was impaired as a result of IL-4Rα\textsuperscript{-/-}, leading to haemorrhage of granulation tissue, impaired epithelisation, and reduced proliferation of macrophages (Knipper et al., 2015). These reports suggest that the mechanism influencing M2 macrophages within the wound could be partially through the involvement the IL-4Rα. In the above section, several functions of macrophages involved in each phase of healing were discussed. The ability to adopt different phenotypic states makes macrophages an attractive target that can be modified in pathological states such as chronic wounds with delayed healing, and fibrosis. The following sections will discuss the changes that occur in macrophages in the diabetic environment, and different therapeutic approaches that taken for chronic wounds.

\textbf{1.4.2 Dysregulation of macrophage phenotypes is associated with impaired wound healing}

Several observations from our laboratory, as well as others, demonstrate that diabetes impairs the function of myeloid cells, including macrophages, and that the changes in wound leukocytes contribute to the phenotype of chronic wounds (reviewed in (Wicks et al., 2014)).
Dysregulation of myeloid cells in diabetes affects their transition from an ‘M1-like activation profile’ to an ‘M2-like activation profile’. Several reports on diabetic macrophage phenotype described them as macrophages with a prolonged M1-like inflammatory profile that ineffectively switches to the reparative profile needed for intermediate and later stages of healing (Bannon et al., 2013; Koh and DiPietro, 2011; Miao et al., 2012) (Figure 1.4). Investigating markers of M1 and M2 macrophages from (db/db) mice, a model of type 2 diabetes, demonstrates that while the non-diabetic-derived macrophages can effectively express the M2 profile later in healing, the diabetic cells continue to display M1 markers even during late stages of repair (Bannon et al., 2013; Mirza and Koh, 2011). The presence of M1-like inflammatory cells in the wound until late stages of healing can negatively impact the healing outcome, causing diabetic wounds to remain in inflammatory phase and inhibit their progression to proliferative and tissue regeneration phases of healing.

Type 1 diabetic rat models displayed a different pattern of impaired phenotype that also correlates with impaired wound repair. Wound macrophages from these models showed insufficient M1 markers early in healing and excessive level of prohealing markers present in late stages of repair (Miao et al., 2012). These observations encourage the theory that any impairment in macrophages’ phenotype plat important role for delayed healing in different models of diabetes. Moreover, using adoptive transfer experiments, in which GFP+ diabetic-derived macrophages were transplanted into non-diabetic and diabetic wounds, showed that db-derived macrophages over-respond to ‘normal’ inflammatory stimuli in a non-diabetic wound as assessed by M1 marker expression, but not to ‘normal’ anti-inflammatory stimuli.
This indicates that intrinsic defects within diabetic-derived macrophages are a major causative factor for their chronic inflammatory phenotype (Bannon et al., 2013). Diabetic macrophages continued to display impaired phenotypes even when cultured outside their diabetic environment in the same conditions as their non-diabetic counterparts (Bannon et al., 2013).

Gr1⁺CD11b⁺ cells, a population of immature myeloid progenitors and mature myeloid cells known to act as immunosuppressor as well as promoting neovascularisation (Yang et al., 2004, Shojaei et al., 2007), showed impaired gene expression and behaviour in Leptin receptor deficient (db/db) diabetic mouse models. This suggests a problem in their development and differentiation or a problem in the signals they receive from the environment (Mahdipour et al., 2011). Extrinsic factors in the wound microenvironment are likely to play important role in this dysregulation of myeloid cells, such as increased levels of inflammatory cytokines like TNF and IL-1 (Wetzler et al., 2000) and reduced expression of growth factors, including PDGF, TGF-β, VEGF, and FGF2 (Beer et al., 1997; Frank et al., 1995; Greenhalgh et al., 1990). However, our lab has provided evidence that intrinsic factors from the cells themselves play an equally important role (Bannon et al., 2013; Wicks et al., 2015).

A population of CCR2⁺ macrophages that recruited early in the healing process exhibited dual marker from both M1 and M2, were VEGF expressing cells that promoted neovascularisation (Willenborg et al., 2012). However, these mixed-polarised macrophages expressing markers from both M1 and M2 that retained in diabetic wounds, failed to stimulate angiogenesis (Bannon et al., 2013). In an attempt to explain these defects, it was hypothesised that diabetic macrophages and myeloid cells altogether fail to mature properly and, therefore, cannot
perform their functions such as angiogenesis. This is supported by reduction in F4/80 and CD11b maturation markers in BM-derived mouse macrophages isolated from diabetic mice (Bannon et al., 2013). This demonstrates that problems associated with macrophage maturation in diabetes originate in the bone marrow rather than being affected by the wound environment alone.

Aberrant expression of endogenous transcription factors might be one mechanism explaining the diabetic phenotype of persistent immaturity. For example, the CCAAT enhancer binding protein alpha (Cebpα) gene that controls macrophage and granulocyte development was significantly reduced in myeloid cells from diabetic mice (Wicks et al., 2015). Moreover, Hoxa3 and Hif1 transcription factors also show aberrant endogenous gene expression in diabetes that can negatively impact the healing outcomes (Mace et al., 2005, 2007). Importantly, enforced expression of these transcription factors can rescue the diabetic phenotype and impact different phases of healing. Further details of transcription factor overexpression will be discussed in details in the subsequent sections.

To summarise, the diabetic phenotype of macrophages represent cells with prolonged M1 signature that continues until the late stages of repair in addition to ineffective switching to the M2-like prohealing fate that promotes angiogenesis, tissue regeneration, and wound closure. By looking at the phenotype of macrophages in diabetic wounds and the phenotype of macrophage-depleted wounds presented earlier (Goren et al., 2009b; Lucas et al., 2010b; Mirza et al., 2009a), one can say that the phenotype of chronic wounds resembles that of
macrophage-depleted wounds. Both involved delayed repair process, reduced activity of M2-prohealing growth factors such as VEGF, TGF-β and arginase that promote angiogenesis, collagen production and wound closure. Altogether this shows that macrophages are critical to multiple phases of healing.

Figure 1.5: The phenotype of macrophages in normal and diabetic healing. Pro-inflammatory macrophages (left) derived from CCR2^{hi}Cx3CR1^{lo} population together with resident macrophages (CCR2^{lo}Cx3CR1^{hi}) population are present in large numbers during the initial phase of inflammation to perform the antimicrobial activity. As the healing progresses into the regeneration and remodelling phase, macrophages within the wound can acquire reparative markers associated with switching their fate to reparative macrophages (right). Reparative macrophage marker Rlemα involved in the mechanism that leading to collagen production from fibroblast resulting in the formation of scar tissue. Diabetes affects macrophage phenotype in the wound, including CCR2^{hi}Cx3CR1^{lo} and CCR2^{lo}CX3CR1^{hi}, leading to persistent upregulation of pro-inflammatory macrophages and ineffective switching to the reparative phenotype. This dysregulation of macrophage phenotype in diabetic wounds is marked by (x) on the arrow. Overview from (Knipper et al., 2015; Wicks et al., 2014).
1.5 Current treatment and challenges in wound healing

Despite being diverse clinically and molecularly, all chronic wounds are generally defined as full thickness coetaneous wound that fail to heal within 3 months, which occur in one of three major clinical complications: leg ulcers, diabetic foot ulcers or pressure ulcers (Table 1.2; reviewed in (Nunan et al., 2014)).

Table 1.2: Forms of chronic wounds in human
Adapted from (Nunan et al., 2014)

<table>
<thead>
<tr>
<th>Chronic wound</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leg ulcers</td>
<td>Common chronic wound caused by reduced venous or arterial blood flow to lower limbs</td>
</tr>
<tr>
<td>Diabetic foot ulcers</td>
<td>The most common type of diabetic complication caused by neural or visceral damage and associated with 58% of diabetic foot amputations (Pecoraro et al., 1990)</td>
</tr>
<tr>
<td>Pressure ulcers</td>
<td>Also known as bed sores caused by sustained pressure in elderly or immobile patients that blocks blood flow to tissue</td>
</tr>
</tbody>
</table>

The problem in chronic wounds, whether in mouse or human, as discussed earlier, is that they fail to close within the expected time frame, remaining in the inflammatory phase. The best way to lead this healing process towards the reparative phase is by successive treatment that removes the excess inflammatory cells and then induces re-epithelisation and angiogenesis. Therefore, many therapeutic strategies for chronic wounds have been directed toward controlling the inflammatory phase. One of the potential treatments that have been tested to limit persistent inflammation is the use of anti-TNF antibodies (infliximab) that was topically applied on patients with chronic ulcers. Neutralizing TNF levels improved the wound surface
area by 50% in 4 weeks and by 75% in 8 weeks of treatment (Streit et al., 2006). Aschroft et al., (2012) and colleagues provided mechanistic insights into the positive contribution of anti-TNF antibodies to wound of secretory leukocytes protease inhibitor null mouse (SLPI) models that present with excessive inflammation and severely impaired healing (Ashcroft et al., 2012). They showed that neutralising TNF levels downregulate NF-κB that is known to be responsible for induction of several pro-inflammatory targets, including TNF itself (Ashcroft et al., 2012). In addition anti-TNF treated mice demonstrated enhanced matrix synthesis, reduced leukocyte recruitment and diminished inflammatory markers and may be applicable as treatment or prophylaxis for chronic wounds in humans (Ashcroft et al., 2012). Although anti-TNF drugs are available for treating other inflammatory conditions, like rheumatoid arthritis, the accompanied risk of malignancy and infections need to be considered (Antoni and Braun, 2002). Therefore, even if blocking TNF can significantly benefit the healing outcome, concerns about increased risk of inflammation and cancer could profoundly influence their use.

Another pathway of investigation to therapeutically accelerate and repair impaired skin wounds is the use of growth factor-based therapy. The critical role of growth factors in healing includes directing cell function to angiogenesis and tissue repair, renders them a potential therapeutic factor for treating chronic wounds. Administration of human recombinant VEGF growth factor improved neovascularisation, enhanced the levels of TGF-β and PDGF and affected the recruitment of bone marrow (BM) derived cells to diabetic mouse wounds (Galiano et al., 2004). Several types of growth factors including VEGF-A, keratinocyte growth factor (KGF), FGF, placental derived growth factor (PIGF) and platelet derived growth factor
(PDGF) have multiple functions in wound healing and were clinically tested on different forms of chronic wounds. However, only PDGF-BB (becaplermin gel) was FDA approved while other factors had limited success in clinical trials (Eming et al., 2014). The hostile environment of the wound, particularly an infected wound, inhibits the effects of therapeutic molecules and can biologically degrade these factors (Eming et al., 2007; Neely et al., 2000). Therefore, for multifactorial diseases, such as diabetes, therapeutic approaches targeting one pathway or one gene may have limited success.

An alternative and promising approach to treat chronic wounds is by manipulating or reprogramming immune cells within the wound. Because cell intrinsic factors play an important role in the dysregulation of the macrophage phenotype causing their persistent M1/inflammatory state within diabetic wounds (discussed in section 1.4.2). Overexpression of reduced transcription factors can reverse the diabetic phenotype. This make-the case for macrophages being an attractive target for cell reprogramming to improve healing. One opportunity might be to utilize endogenous anti-inflammatory signals within macrophages such Chemerin15, or endogenous anti-inflammatory transcription factors that promote a normally healing wound (Cash et al., 2014; Mace et al., 2005, 2009). Hoxa3 and hypoxia-inducible factor 1 (Hif1) are examples of endogenous transcription factors present in normal wounds but reduced in diabetic wounds that show impaired healing (Mace et al., 2005, 2007). Gene transfer of Hoxa3 into wounds of diabetic mice improved healing and enhanced endothelial progenitor cell recruitment while limiting the excessive inflammatory response (Mace et al., 2005, 2009). Importantly, ex vivo protein transfer of Hoxa3 into haematopoietic
stem cells (HSCs) can positively reprogram their differentiation and proliferation, enhance their migration toward MIP-1 and MCP-1 chemoattractants and reduce their adhesion to TNF-activated endothelial cells in a safe and efficient way (Mahdipour et al., 2011). In addition, Hox genes are important regulators of hematopoietic development. Transcriptome analysis of CD34+ human HSCs revealed that they express a wide array of Hox genes, particularly from A, B and C clusters (Sauvageau et al., 1994). Lessons from retroviral overexpression knock in and knock out mouse models have provided a rich body of data on the potential of Hox gene in Hematopoietic cells differentiation and maturation. For example, overexpression of Hoxa10 in cord blood progenitors inhibit the differentiation of HSC to lymphoid lineages, whilst promoting the differentiation of monocyctic lineages thereby affecting lineage commitment and differentiation (Taghon et al., 2002). Hoxa9 knockout in mice (Hoxa9−/−) on the other hand resulted in severe defects in lymphoid, myeloid and erythroid maturation as well as affecting HSC repopulating ability suggesting the important endogenous role of Hoxa9 in controlling the development of several lineages of Hematopoietic cells (Izon et al., 1998; Lawrence et al., 1997, 2005). The next section will briefly introduce the Hox genes and their role in development followed by discussion of the important contributions of Hoxa3 to wound healing.

1.6 Homeobox transcription factors and reprogramming of myeloid cells

1.6.1 The discovery of Hox transcription factors

Homeotic genes were originally identified as genes that regulate body parts or segments in mammals, insects and plants (Hirth et al., 1998). The main families comprising the Homeotic
genes are the Hox family and the ParaHox (reviewed in (Garcia-Fernàndez, 2005)). Hox genes were first identified in Drosophila, as genes involved in specifying segment identity (Krumlauf, 1994). Mutations in Homeotic genes can lead to transformation of a segment or part of their structure into another. For example, mutations in the Antennapedia (Antp) gene, the first identified mutation in the Hox complex of Drosophila, elicit a transformation of the adult antennae into legs (Schneuwly et al., 1987).

Hox genes, such as Antp, are one subset of Homeotic genes that were established in the fruit fly, Drosophila melanogaster, where they were grouped into two unique gene complexes. The Antennapedia complex (Ant-C) resembles the structure of the mammalian anterior Hox gene 1-8 paralogue groups, and the bithorax complex (BX-C) closely resemble to the Hox posterior paralogue groups 9-13 (Tupler et al., 2001) (see Figure 1.6). Mammalian Hox genes are arranged into four clusters - HOXA, HOXB, HOXC, and HOXD - and are located on four different chromosomes. Each cluster may contain between 9-11 members of paralogue groups. A striking feature of each cluster is that Hox genes are expressed, in order, along the chromosome corresponding to how they are expressed along the embryonic anterior-posterior axis (Tupler et al., 2001). Interestingly, transcriptional analysis of human CD34+ cells uncovered that most genes in the 3’ position of the cluster (HOXB4, HOXA3) are found in early progenitors, whereas Hox genes located near the 5’ position of the cluster (e.g., HOXB7, HOXA10) are expressed in later developmental stages of more differentiated population of CD34+ cells (Kim et al., 2009). Of note is the fact that members of the same Hox paralogue group (e.g., HOXA4, HOXB4, HOXC4, HOXD4) have an exceptional ability to compensate for one another in many circumstances (Figure 1.6).
Figure 1.6: Cluster of organization of Hox genes in Drosophila and mammals. The four Hox clusters each contain 9-11 members where 3’ genes (anterior Hox) are expressed in anterior tissues and 5’ genes (posterior Hox) are expressed in posterior tissues. Individual Hox genes fall within the same paralogue group (denoted by the same colour), share the same sequence in the homeobox region and have common sequence homology with the drosophila HOM-C homeotic gene. Paralogue groups of Hox genes also share functional similarities. Blank squares show missing genes. Adapted from (Alharbi et al., 2013).

The term homeobox was first assigned to the common sequence element of 180 base pairs that all Hox genes share, which encode the Homeodomain, a DNA binding domain that is composed of 60 amino acids (Gehring et al., 1994) (Figure 1.7). The Homeodomain contains three alpha helices that form a helix-turn-helix motif. This highly conserved structure in helix 3 is particularly involved in DNA-protein binding. The preferred DNA recognition site of many Hox proteins is TAAT, important for selecting an in vivo binding site (Seeman et al., 1976). Although Hox proteins like many other transcription factors are often localise in the nucleus, cytoplasmic localisation has been observed for many Hox proteins such as Hoxb13 expression.
in the epidermis of developing skin (Kömüves et al., 2003). Homeoproteins have an interesting ability to transfer between cells; this is mediated by two separate steps of secretion and internalization (Dupont et al., 2007). Secretion of homeoproteins from cells is mediated by 11 amino acids between the second and third alpha helix in the Homeodomain, referred to as the secretin sequence (Sec). However, internalization of homeoproteins inside the cells is an endocytosis-independent mechanism and occurs without the need for vesicle compartment. The penetratin sequence, located in the third alpha helix, contains all of the necessary information for the homeoprotein to be taken up by the cells (Derossi et al., 1994, 1996). This feature of homeoproteins is of great importance to the research presented in this thesis, as it allows internalisation of the protein by THP-1 monocytes or BM-derived macrophages, two models that used in this research. Another important aspect of Hox structure is the hexapeptide motif that contains the IFPWMK consensus sequence. This motif forms the binding site for the anterior Hox gene, like Hoxa3, with members from three amino acid loop extension (TALE) proteins, known as Hox cofactors such as myeloid ectopic viral insertion site, Meis, and pre-B cell leukaemia homeobox (PBX) proteins (Moens and Selleri, 2006).
Figure 1.7: Structure of homeoproteins. Homeoproteins contain 180 base pairs that encode a DNA binding domain, the Homeodomain that is conserved among all HOX proteins. Homeodomain contain 3 alpha helices. This Figure shows the sites involved in protein internalization and secretion. The secretion (Sec) sequence is located between the second and the third α helix. It contains 11 amino acids and facilitates secretion of the protein through the vesicles; whilst the penetratin sequence is contained within the third α helix and mediates HOX protein internalization and can pass through tight epithelial junctions. All together, the secretion sequence and penetratin (Sec Pen) peptide form 27 amino acids. The Figure also shows where the HOX cofactors, PBX and MEIS, bind to the HOX protein in an area just before the homeodomain structure (hexapeptide). aa: amino acids.

1.6.2 Hoxa3 as potential therapy to treat diabetic wounds

1.6.2.1 Hoxa3 and angiogenesis

Early studies by Chisaka and Capeechi (1991) showed the first evidence of Hoxa3 regulating vertebrate neovascularisation using Hoxa3 knockout mice (previously known as Hoxa5.1). These mice did not survive and died shortly after birth because of an abnormal heart size and the absence of a carotid artery (Chisaka and Capecechi, 1991). However, they also saw that this phenotype was reversed once Hoxd3 was placed in the right place within the Hoxa3 locus, indicating that the function of Hoxa3 and Hoxd3 are interchangeable (Greer et al., 2000). Several years later, this was reflected in the role of exogenously expressed Hoxa3 and Hoxd3,
as both were found to accelerate wound closure and promote angiogenesis in genetically diabetic mice (Hansen et al., 2003; Mace et al., 2005). The ability of Hoxa3 to enhance endothelial cell migration in human immortalized endothelial cells (HMEC) was similar to applying VEGF growth factor to HMEC cells. However, these studies also noted that Hoxa3 can further improve the healing process by increasing keratinocyte migration and re-epithelisation, a feature that was not observed with Hoxd3 (Mace et al., 2005).

Hoxa3 is expressed endogenously in the wound and in unwounded skin; Hoxa3 is expressed within hair follicles but not in vascular tissue. However, following injury, Hoxa3 expression becomes apparent in wound microvessels and endothelial cells from day 1 after wounding reaching the highest level in vessels composing granulation tissue by day 4 of healing (Mace et al., 2005). Given that angiogenesis normally commences around day 4 of healing, this emphasizes the endogenous role of Hoxa3 in angiogenesis in a normal wound. Diabetic wounds that have impaired angiogenesis do not express high levels of endogenous Hoxa3 and Hoxd3 levels are downregulated, even in wounded skin (Hansen et al., 2003; Mace et al., 2005).

1.6.2.2 Hoxa3 can reprogram myeloid cells to become anti-inflammatory

BM derived cells, particularly inflammatory and progenitor cells can re-populate any part of the body in response to injury. They can be involved directly in tissue regeneration by differentiating to particular cell types, such as keratinocytes, sebaceous gland cells, fibroblasts, and endothelial cells, or indirectly by producing cytokines and growth factors important for neovascularisation and tissue regeneration (Badiavas et al., 2003). However, disease
situations, like diabetes, can alter the behavior and function of BM derived cells and affects their migration, adhesion, and angiogenic potential.

Diabetic BM derived cells showed increase in the inflammatory cells component within diabetic wounds, that failed to recruit, migrate, and differentiate normally, contributing to excessive inflammation and impaired wound healing. (Mace et al., 2009; Tepper et al., 2002). Gene transfer of Hoxa3 significantly reduced the inflammatory subset of BM derived cells recruited to the Hoxa3-treated wound by day 7 of healing (Mace et al., 2009). Interestingly, at day 4, which denotes period of progression of the healing process from inflammation to the proliferative phase, there was significant alteration in gene expression profile but not in cellular recruitment, inhibiting members of NF-κB gene pathways and promoting several genes involved in neovascularisation and endothelial cell migration (Mace et al., 2009). These findings, together with data on role of Hoxa3 in angiogenesis (section 1.6.2.1), supports the same hypothesis: Hoxa3 can reprogram cells in diabetic wounds to become proangiogenic and less inflammatory at cellular and molecular level. These findings form the basis of our hypothesis, which is Hoxa3 can reprogram dysregulated macrophages in diabetes and rescue them from the persistent inflammatory phenotype, most likely by promoting the M2 reparative fate.

The effects of Hoxa3 on myeloid populations were investigated in more specialized population of BM derived cells, the Gr1⁺CD11b⁺ subset that were dysfunctional and dysregulated in diabetics and demonstrate attenuated M2/prohealing markers. Hoxa3 protein transfer mediate the differentiation of HSC/P into normal functioning Gr1⁺CD11b⁺ and restored their
normal angiogenic function and promoted an increase in their TGF-β levels (Mahdipour et al., 2011). All of the above findings support the potential for Hoxa3 to modify the phenotype of myeloid cells thereby promoting wound healing and angiogenesis in diabetes. Essentially, these data also support the role of Hoxa3 in correcting the intrinsic defects of total BM derived cells and Gr1+CD11b+ subpopulations of myeloid cells at the molecular level by modifying their gene expression to less inflammatory and more angiogenic profile, and at the cellular level by improving their function through restoring their balance and altering their migration/recruitment potential.

## 1.7 Differentiation of macrophages and regulatory mechanisms

Tissue macrophages are terminally differentiated cells derived from monocyte precursors that originate from the bone marrow, but a subset also originate from the yolk sac before birth and can maintain their population independent of monocytes (Ginhoux et al., 2010; Wynn et al., 2013).

In the bone marrow, the differentiation of macrophages from haematopoietic stem cells (HSC) depends on gradual expression of lineage-specific genes and on the action of critical transcription factors that determine cell-type specific gene expression (Figure 1.8).

During haematopoiesis, HSC choose to either undergo self-renewal or become multipotent progenitor cells, also referred to as a granulocyte-erythocyte-megakaryocyte-macrophage progenitor colony forming unit (GEMM-CFU) (Hamilton, 1993; Lopez et al., 1992). Multipotent progenitor cells can give rise to granulocyte/monocyte GM-CFUs under the influence of IL-1 and IL-3 (Lopez et al., 1992). GM-CFU precursors can become a committed precursor,
macrophage colony-forming unit (M-CFU), which can in turn differentiate into monoblasts, promonocytes, and mature monocytes (Figure 1.8).

While granulocyte-macrophage colony stimulating factors (GM-CSF) controls the early stage of differentiation before the cell commits to the monocytic lineage, macrophage colony stimulating factor (M-CSF) is critical in regulating the differentiation of latter stages, from a monoblast to fully differentiated macrophages (Chitu and Stanley, 2006; Hamilton and Achuthan, 2013).

As part of the haematopoiesis, macrophage maturation undergoes several cell-fate-decision branch points along the differentiation pathway, which is controlled by lineage-specific transcription factors and interleukins. The transcription factors, growth factors and cytokines involved in the process of macrophages maturation are illustrated in (Figure 1.8).

Several transcription factors are present throughout the stages of macrophage differentiation, indicated by genetic manipulation of cell models and knockout mouse models. Two main groups have been determined based on these analyses. First, there are those transcription factors that are essential for macrophage development, either by directly controlling myeloid cell differentiation, like SPI1, or because they control the survival of Haematopoietic stem cells and myeloid progenitor populations. These factors include GATA binding protein 2 (GATA-2), stem cell leukaemia (SCL) gene and c-terminal myeloblastosis (c-MYB). The second group involves transcription factors that control macrophages development indirectly, by activating or repressing the expression of target genes for macrophage development, such as CEBPα and acute myeloid leukaemia/runt-related transcription factor 1 (AML1-RUNX1) (Hoogenkamp et al., 2009; Huang et al., 2008; Yeamans et al., 2007; Zhang et al., 1996a).
Figure 1.8: Differentiation of Hematopoietic stem cell to macrophages. Schematic shows a number of transcription factors, cytokines, and growth factors involved in the differentiation of HSC along monocytic lineage to macrophages. Curved arrows denote successive branch points to different lineages and transcription factors involved in the differentiation are illustrated in black. Interleukins and growth factors are in red. HOXB3 and HOXB4 are involved with the earliest progenitor committed to myeloid, erythroid, and megakaryocytic lineage (GEMM-CFU). HOXB7 is involved in the differentiation of monoblast to monocyte. PU.1 transcription factor is involved in almost every stage through the differentiation of HSC to fully mature macrophages. HOXB3: Homeobox transcription factor 3, HOXB4: Homeobox transcription factor 4, PU.1: PU box transcription factor, HOXB7: Homeobox transcription factor 7. Adapted from (Valledor et al., 1998).

1.7.1 The master regulator of macrophages, Pu.1

Genome-wide DNA binding analysis as well as gain-of-function and loss-of-function mutations identified the ETS family transcription factor, Pu.1 (also known as Spi1) as the master regulator for macrophage development and identity. It is the product of spleen focus-forming virus proviral integration SFFV-1 protoncogen and binds to the consensus sequence, GAGGAA, also referred to as the PU box (Klemsz et al., 1990). The central core of this GGAA sequence can be
recognised by all members of the ETS family. The flanking nucleotide from this core region is specific to PU.1, thus preventing other ETS proteins from competing for the same promoter region (Nye et al., 1992).

In haematopoietic cells, Spi1 is expressed in mature monocytes, macrophages, neutrophils, B-lymphocytes and mast cells (Chen et al., 1995; Hromas et al., 1993). Even though deletion of Spi1 affects other myeloid cell development, particularly lymphocytes, Spi1−/− knockout mice showed clear evidence of an absolute requirement of Spi1 for macrophage development (McKercher et al., 1996; Scott et al., 1994). Spi1 is essential for the development of monocyte-derived macrophages in adults (Dakic et al., 2005) as well for the yolk sac-derived tissue macrophages (Schulz et al., 2012).

Given the vital role of Spi1 in macrophages, the regulation of Spi1 itself is of significant interest. Early in the differentiation process, Csf1 can directly induce Spi1 production and increase the number of SPI1-positive cells from HSC (Mossadegh-Keller et al., 2013). This likely take place through activating myeloid transcription factors that can activate the Spi1 gene, such as Runx1 (Huang et al., 2008; Zhang et al., 1996a) and Cepbα (Yeamans et al., 2007). Initial induction of Spi1 requires Runx1, but Spi1 can then maintain its own level by an autoregulatory feedback loop (Hoogenkamp et al., 2007; Leddin et al., 2011; Lichtinger et al., 2012). The Spi1 downstream target, Csf1R/M-CsfR, is regulated by cooperation between Spi1, Runx1 and Cepbα (Zhang et al., 1994a, 1996a). At the promoter region and enhancer element of Csf1R, Runx1 can complex with the Spi1 protein (Hoogenkamp et al., 2009) and the close proximity of the binding sites between Runx1 and Cepbα suggest protein–protein interactions (Figure 1.9). Mounting evidence from recent observations suggest that regulation of Csf1R gene
occurs via the enhancer region, also known as the c-fms intron regulatory element, or FIRE element (Sauter et al., 2013) (Figure 1.8). As a result of Spi1, Runx1, and Cebpα functional and physical interaction, CSf1R is activated and responds to signals from its ligand, Csf1/M-CSF, to promote macrophage proliferation and differentiation (reviewed in Molawi and Sieweke, 2013). All these observations suggest that SPI1 is essential for macrophage differentiation, proliferation, and survival as it controls the expression of Csf1R.

Figure 1.9: The regulation of the CSf1r gene involving protein-protein interactions. Promoter region of CSf1R showing binding sites for Spi1 (left). Enhancer region located in intron 2 where C/EBPα is bound as a heterodimer to the C/EBP binding site, and the close proximity of the binding site to Runx1 suggests a protein-protein interaction. Runx1 requires its cofactor core binding factor (CBF) to enable its binding to the Runx1 binding site (Runx1 BS). Runx1 also physically interacts with the Spi1 protein and their synergistic DNA binding activates the Csf1r gene. This activation is essential for the macrophages to respond to M-CSF in order to be terminally differentiated. C/EPB BS: CAAT enhancer binding protein binding site, RUNX1 BS: RUNX1 binding site, Spi1 BS: Spi1 binding site, CBF: Core binding factor.
1.8 Summary

For multifactorial conditions, such as the diabetic wound, in which several pathways are involved in disease pathophysiology, the chances of treating the condition by targeting one pathway, such as angiogenesis or inflammation, are poor. The physiological involvement of macrophages in healing and the plasticity of these cells make them an attractive target to treat chronic wounds. The pathological consequences of diabetes on the maturation and activation states of macrophages imply that targeting macrophages, particularly by limiting their inflammatory phenotype or by promoting their reparative fate, might be a desired approach to mediate successful repair. Previous interventions of applying several growth factors that are known to stimulate macrophage activations may have a number of drawbacks, not the least of which the proteolytic activity of unlimited inflammatory cells in diabetic wound that could degrade these factors.

Hoxa3 is an excellent candidate to therapeutically target these macrophages as it has been shown previously to reprogram BM derived cell populations of myeloid cells in diabetes to be more angiogenic. In addition, the unique ability of Hox proteins to translocate between biological membranes without the need for cell-specific receptors increase their chance of being uptake by macrophages and their use as successful topical agents to treat non-healing wounds.
Understanding the transcriptional program that Hoxa3 can influence to drive macrophage maturity, as well signaling molecules that induce distinct activation states for macrophages, is necessary to unravel any therapeutic contributions of Hoxa3 to reprogram macrophage activity in the desired direction. Previous approaches with gene therapy or protein transfer of Hoxa3 into the wound identified Hoxa3 as the key transcription factor that itself targeted several wound healing pathways, including reprogramming BM derived cells, limiting inflammation, and promoting angiogenesis. Thus, key questions about whether Hoxa3 can reprogram macrophages to promote their maturation and limit their inflammatory M1 phenotype as one mechanism by which Hoxa3 drive successful healing remains to be answered.

1.9 Aims and hypothesis:

The main hypothesis of this thesis is that Hoxa3’s accelerated wound healing in diabetes could be by modifying macrophage differentiation to become more prohealing/M2 and less inflammatory/M1 macrophages which may rescue the persistent inflammatory phenotype of macrophages.

Aims

- To test if Hoxa3 protein transduction can influence the differentiation of THP-1 monocytic cell line and change their gene expression focusing on macrophage transcription factors that regulate their differentiation. To thereby obtain an idea on the potential of Hoxa3 in
differentiating macrophages and to obtain a presumed idea on which target genes that can be manipulated by Hoxa3 overexpression.

- To investigate the potential of Hoxa3 overexpression in rescuing impaired maturation of diabetic macrophages using BM-derived macrophages ex vivo cultured from ndb and db mice. Also to investigate the possible downstream target/transcription factors that can be regulated by Hoxa3 to mediate macrophage maturation.

- To assay the changes in the phenotype of BM derived macrophages and wound derived macrophages following Hoxa3 protein transduction and Hoxa3 gene transfer. To thereby discover the potential of Hoxa3 in re-programming the phenotype of macrophages to potentially treat the chronic inflammatory macrophage phenotype in diabetes.

- To identify molecular mechanisms that Hoxa3 undertake to reprogram macrophage to particular phenotypic state focusing on the IL-4/Stat6 pathway of alternative activation to discover novel pathways mediated by Hoxa3 overexpression.

In summary, the work presented in this thesis demonstrates an important therapeutic role for Hoxa3 in treating macrophage deregulation in diabetes. Understanding the mechanisms associated with Hoxa3’s effect on macrophage maturation and phenotype is crucial for the future of using Hoxa3 in gene- or protein-based therapy for the treatment of chronic wounds.
Chapter 2: Materials and methods

2.1 Animals

All animals were housed at the University of Manchester animal care facility, and all procedures were approved by the local ethics review committee and the Home Office. Procedures were performed under the strict legal requirements of the Animal (Scientific Procedures) Act 1986 (as amended). Diabetic Lepr\textsuperscript{db/db} and heterozygous Lepr\textsuperscript{db/+} mice were purchased from Harlan (Oxfordshire, UK). All animals used were 8–16 weeks old and were age- and sex-matched to controls.

2.2 Cell culture

2.2.1 Culture of bone marrow-derived macrophages

Freshly euthanized diabetic or non-diabetic mice were sprayed with 70% alcohol, and the skin from the lower half of the mice was cut and removed. The femurs and tibiae were dissected and cleaned from the surrounding muscles and tendons. Both ends of the femurs and tibia were cut to expose bone marrow (BM), which was flushed with Dulbecco’s Modified Eagle’s Medium (DMEM) using a 27-gauge needle. Aggregates from the bone marrow were passed multiple times through a 19-gauge needle and a 70-µm cell strainer (BD Falcon) to remove tissue aggregates. In one well of a 6-well plate or in a 10-cm dish, cells were plated at $1 \times 10^6$ cells/ml or $5 \times 10^6$ cells/ml, respectively. Macrophage medium was prepared from Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma), which was supplemented with 10% heat-inactivated foetal bovine serum (FBS; Sigma) and 1× penicillin/streptomycin (P/S; Sigma).
induce macrophage differentiation, the medium was supplemented with 10% L929 (ATCC) conditioned medium, containing macrophage colony-stimulating factor (M-CSF). Cells were allowed to differentiate for 7 days at 37 °C in 5% CO₂. At day 3, cells were fed with half of the initial volume added to the culture medium on day 0, and the medium was replaced with new macrophage growth medium on day 5.

2.2.2 Culture of THP-1 cells and induction of macrophage-derived THP-1 cells

The human promonocytic leukaemia THP-1 cell line, provided by the American Type Culture Collection (ATCC, #TIB-202), was maintained in Roswell Park Memorial Institute (RPMI)-1640 (Sigma) growth medium supplemented with 2 mM L-glutamine, 10% FBS (Sigma), and 1% P/S (Sigma).

For maintenance of the THP-1 cell line, the medium was replaced every 2 to 3 days via centrifugation and subsequent resuspension of the cell pellet in fresh, warmed growth medium. Alternatively, the cells were resuspended at 2–4 × 10⁴ cells/ml when the concentration reached 8 × 10⁵ to 1 × 10⁶ cells/ml by centrifuging at 300 × g for 5 minutes.

For the differentiation of THP-1 cells into macrophage-like cells, cells were seeded at 1 × 10⁶ cells/ml and cultured in standard culture medium supplemented with 5 ng/ml phorbol-12-myristate B-acetate (PMA; Sigma).

2.2.3 Culture of the 293T cell line

Human 293T cell cultures, derived from the human embryonic kidney 293T (HEK 293T) cell line (ATCC), were maintained in DMEM (Sigma) supplemented with 10% heat-inactivated FBS (Sigma) and 1× P/S (Sigma). Cells were cultured in a T75 flask (Corning) at 37 °C in a 5% CO₂
incubator until they were 90% confluent and then split. To split the cells, the medium was removed, and the adherent cells were detached using 5 ml of phosphate-buffered saline without calcium (PBS; Sigma). The cell suspension was pipetted thoroughly to generate a single cell suspension, and the cells were split at a 1:10 ratio into new complete medium.

2.3 Transfection and protein transduction

2.3.1 Overexpression of Hoxa3 through calcium phosphate transfection

Cells were seeded in a 10-cm dish at a 1:6 ratio. When the cells were 20% confluent, the SP-Hoxa3-mCherry and SP-mCherry expression plasmids (10 µg each) were transfected into 293T cells using the calcium phosphate method. In brief, plasmids were diluted with 61 µl of 2 M calcium chloride, and the total reaction volume was increased to 500 µl with the addition of sterile H$_2$O. The mixture was then added slowly to 500 µl of 2× HEPES-buffered saline (HBS; 50 mM HEPES, 1.5 mM Na$_2$HPO$_4$, 280 mM NaCl) and subsequently added drop-wise across a 10-cm dish. The cells were incubated under standard culture conditions. Cells were incubated for 12–24 hours, and the medium was replaced with fresh 293T growth medium. Cells were then viewed under a microscope, and the transfection efficiency was determined. Conditioned medium was collected from cells that showed a transfection efficiency of 70% or more at 24, 48, and 72 hours post-transfection. The conditioned medium was filtered through a 0.45-µm filter and stored at -20 °C for later use.

2.3.2 Overexpression of Hoxa3 in mouse macrophages via protein transduction

Conditioned medium was collected from 293T cells transfected with SP-Hoxa3-mCherry or SP-mCherry that contained the secreted Hoxa3-mCherry or mCherry proteins because of the
presence of a signal peptide (SP), as explained in (Dupont et al., 2007). BM-derived macrophages (BMDMs) or THP-1 cells were treated with conditioned medium (0.5× total volume) containing Hoxa3 or mCherry and the Hoxa3-mCherry in the medium was internalised by mouse macrophages via passive translocation, as previously described in (Amsellem et al., 2003; Derossi et al., 1996; Mahdipour et al., 2011).

2.3.3 Amaxa nucleofection

An Amaxa human monocytes nucleofection kit (Lonza) was used to transflect THP-1 cells, and an Amaxa mouse macrophages nucleofection kit (Lonza) was used to transflect mouse BMDMs. For human monocyte nucleofection, a modified version of the protocol described in (Schnoor et al., 2009) was followed. To prepare the Amaxa Nucleofector solution, 82 µl of the appropriate Nucleofector solution (human monocytes or mouse macrophages) was mixed with 18 µl of Nucleofector supplement. One hundred microlitres of this solution was mixed with either 5.8 × 10^5 THP-1 cells or 1 × 10^6 mouse macrophages. Four micrograms of the expression plasmid was added to each reaction, and the whole mixture was transferred to the supplied cuvette, which was inserted into the Amaxa Nucleofector 2b device. Cells were nucleofected using the program setting Y-01 for mouse macrophages and human monocytes. Immediately after electroporation shock, the cells were equilibrated by adding 1.5 ml of recovery medium (Iscove's Modified Dulbecco's Media [IMDM] supplemented with 20% human serum [Sigma], 1% P/S [Sigma], 1× non-essential amino acid solution [NEAA; Sigma], and 1% sodium pyruvate). Transfected cells were maintained in a 12-well plate under
standard culture conditions, and each transfection was performed in triplicate. Fresh growth medium, specific for each cell type, was added 6 hours after nucleofection.

2.4 Sample preparation for quantitative RT-PCR

2.4.1 RNA extraction

RNA was isolated from BMDMs at specified time points via cell homogenization in TRIzol (Life Technologies). Briefly, cells were incubated with TRIzol for 5 minutes and then scraped and mixed with one-fifth volume of chloroform. The mixture was incubated for 3 minutes at room temperature and centrifuged for 20 minutes. The aqueous solution containing the RNA was transferred to a new tube and precipitated with 0.5 volumes of isopropanol. The RNA pellet was washed with 75% ethanol and re-dissolved in nuclease-free water (Qiagen). The RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific).

2.4.2 DNase I treatment

DNase I treatment was performed using an RNase-free DNase set (Qiagen) with 45 µl of RNA. The isolated RNA was mixed with 5 µl of 10× RDD buffer (Qiagen). DNase I (1.25 µl of 2500 Kunitz U/mL) was added to the 50-µl reaction and incubated for 10 minutes at room temperature. The reaction was stopped by denaturing the enzyme at 75 °C for 10 minutes. A second RNA precipitation was then performed using isopropanol and 75% ethanol, as described in (section 2.4.1).
2.4.3 Complementary DNA (cDNA) synthesis

Extracted RNA was reverse transcribed to cDNA using BioScript (Bioline) according to the manufacturer’s instructions. The first reaction mix was prepared by combining RNA, oligo (dT) (Bioline), random hexamers (Life Technologies), and 10 mM dNTPs, as shown in Table 2.1. The mixture was denatured at 65 °C for 15 minutes.

Table 2.1: First reaction mixture for cDNA synthesis

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>Volume of 1 µg</td>
</tr>
<tr>
<td>Random hexamer</td>
<td>1 µl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1 µl</td>
</tr>
<tr>
<td>Oligo (dT)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>to 15 µl</td>
</tr>
</tbody>
</table>

A second reaction mix comprising 5× buffer (Life Technologies), RNaseOUT (Life Technologies), and BioScript reverse transcriptase (Bioline) was prepared as described in Table 2.2. Reverse transcriptase-negative (no RT) controls were included to ensure the absence of genomic contamination. The cDNA or no RT master mix (5 µL) was added to the first reaction mix and incubated for 10 minutes at 25 °C and for 30 minutes at 42 °C. The reaction was terminated by incubating the samples at 85 °C for 5 minutes followed by a hold temperature at 4 °C. The cDNA was stored at -20 °C for use in qRT-PCR.
### Table 2.2: cDNA and Reverse Transcriptase negative control master mixes (volume per reaction)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>cDNA (volume)</th>
<th>No RT control (volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5× Reaction buffer</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>RNaseOUT</td>
<td>0.5 µl</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>BioScript enzyme</td>
<td>0.5 µl</td>
<td>0.5 µl of nuclease-free water</td>
</tr>
</tbody>
</table>

### 2.5 Quantitative real-time PCR (qRT-PCR)

#### 2.5.1 TaqMan assay

qRT-PCR analysis of mouse macrophages was performed using histone 2A (H2A) and heat shock protein 90 (Hsp90) as the internal standard reference genes, with no RT and no template reactions included as negative controls. PCR reactions containing 1× Fast Universal Master Mix (Life Technologies), 1X TaqMan probe for target or reference gene (Life Technologies; Table 2.3) and 1:15 diluted cDNA in total 10µl volume into each well of a 96-well optical plate (Life Technologies). The plate was centrifuged at 300 × g for 5 minutes to remove air bubbles. Quantitative RT-PCR was performed using a StepOnePlus system (Applied Biosystems) adjusted for the TaqMan fast assay. For qRT-PCR analysis of human THP-1 cells, the same protocol was followed, using 18S or RPL37A as the internal standard reference gene (Table 2.4).
### Table 2.3: TaqMan assay probes used for qRT-PCR analysis of mouse macrophages

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoxa3</td>
<td>Mm01326402_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Hoxc13</td>
<td>Mm00802798_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Itgam</td>
<td>Mm00434455_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Emr1</td>
<td>Mm00802529_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Cd14</td>
<td>Mm00438094_g1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Csf1r</td>
<td>Mm01266652_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Ym1 (Chi313)</td>
<td>Mm00675889_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Arg1</td>
<td>Mm00475988_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Tgfβ</td>
<td>Mm01178820_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Mrc1</td>
<td>Mm01329362_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>IL-10</td>
<td>Mm00439614_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Nos2</td>
<td>Mm01309897_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Tnf</td>
<td>Mm00443258_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Cd86</td>
<td>Mm00444543_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>IL-12</td>
<td>Mm00434165_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Ccl2</td>
<td>Mm00441242_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Spi1</td>
<td>Mm00488142_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Cepba</td>
<td>Mm01265914_s1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Stat6</td>
<td>Mm01160477_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>Hsp90ab1</td>
<td>Mm00833431-g1</td>
<td>Reference gene</td>
</tr>
<tr>
<td>Hist2h2aa1</td>
<td>Mm00501974_s1</td>
<td>Reference gene</td>
</tr>
</tbody>
</table>
Table 2.4: TaqMan assay probes used for qRT-PCR analysis of human THP-1 cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOXA3</td>
<td>Hs00601076.m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>HOXC13</td>
<td>Hs00600868_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>HOXB7</td>
<td>Hs04187556_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>SPI-1/PU.1</td>
<td>Hs02786711_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>SPIB</td>
<td>Hs00162150_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Hs00257856.s1</td>
<td>Target gene</td>
</tr>
<tr>
<td>CSF1/M-CSF</td>
<td>Hs99999084_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>MCSFR</td>
<td>Hs00911250_m1</td>
<td>Target gene</td>
</tr>
<tr>
<td>CEBPα</td>
<td>Hs00269972.s1</td>
<td>Target gene</td>
</tr>
<tr>
<td>CD68</td>
<td>Hs02836816_g1</td>
<td>Target gene</td>
</tr>
<tr>
<td>18S</td>
<td>Hs99999901_s1</td>
<td>Reference gene</td>
</tr>
<tr>
<td>RPL37A</td>
<td>Hs01102345_m1</td>
<td>Reference gene</td>
</tr>
</tbody>
</table>

2.5.2 SYBR Green qRT-PCR

To prepare qRT-PCR SYBR green reaction, 1x SYBR master mix (Life Technologies) was added to 10 µM forward and reverse primer each, and the reaction volume was made up to a total volume of 6 µl. Equal amounts of 1:7 diluted cDNA were mixed with the primer mix in a 10 µl reaction. The plate was analysed using StepOnePlus system adjusted for fast SYBR Green amplification.
Table 2.5: List of oligonucleotides used for SYBR Green real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Target</th>
<th>5’ forward primer</th>
<th>3’ reverse primer</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2A</td>
<td>mouse</td>
<td>GTCGTGGCAAGCAAGGAG</td>
<td>GATCTCGGCCGTAGGTACT</td>
<td>Primers verified in our lab</td>
</tr>
<tr>
<td>Csf1</td>
<td>mouse</td>
<td>AGTATTGCCAGGAGGTGCTCA</td>
<td>ATCTGGCATGAAGTCTCCATT</td>
<td>(Wei et al., 2010)</td>
</tr>
<tr>
<td>Runx1</td>
<td>mouse</td>
<td>CACCGACAGCCCAACT</td>
<td>CCCAGTGCACCACACT</td>
<td>(Himes et al., 2005)</td>
</tr>
<tr>
<td>RPL37A</td>
<td>human</td>
<td>ATTGAATACGCAGCACGC</td>
<td>AGGAACACAGTGCCAGATCC</td>
<td>(Maeß et al., 2010)</td>
</tr>
<tr>
<td>CD14</td>
<td>human</td>
<td>CGCTCCGAGATGATGTG</td>
<td>ACGACAGATTGAGGAGTT</td>
<td>(Song et al., 2001)</td>
</tr>
<tr>
<td>CD11b</td>
<td>human</td>
<td>CAACAAGCAGGTCTAGATGGT</td>
<td>TGAGCCACACAGAGCTCTG</td>
<td>(Moeenrezakhanlou et al., 2008)</td>
</tr>
<tr>
<td>CD11c</td>
<td>human</td>
<td>CCGATTGTCCATGCCTCAT</td>
<td>AACCCCAATTGCATAGCG</td>
<td>(Caprodossi et al., 2005)</td>
</tr>
</tbody>
</table>

2.6 Flow cytometry

2.6.1 Flow cytometry using BMDMs

For flow cytometry labelling, BMDMs were cultured in 6-well plates and differentiated as described in section 2.2.1. First, the medium was removed, and the cells were washed twice with PBS. Each well was then treated with 0.05% trypsin/0.02% EDTA (Sigma) for 30 minutes at room temperature. After the incubation, an equal amount of macrophage medium was added. The cells were detached by gentle scraping and centrifuged for 5 minutes at 400 × g.
The supernatant was then discarded, and the pellet was resuspended in 4 ml of macrophage medium. The cells were counted using a haemocytometer. For each staining reaction, \(1 \times 10^6\) cells were resuspended in 100 µl of FACS buffer (2% FBS in PBS without Ca\(^{++}\) or Mg\(^{++}\)). An appropriate amount of Fc block was then added (Table 2.6) followed by the addition of a conjugated monoclonal antibody or isotype control. A list of antibodies used to detect mouse macrophage cell surface markers is presented in Table 2.6. Unlabelled cells were also used as a negative control in order to assess background autofluorescence. All antibodies were incubated for 30 minutes in the dark. After the labelling reaction, the cells were washed 3 times with FACS buffer, resuspended in 400 µl of FACS buffer, and analysed on a Beckman Coulter CyAn ADP Analyser or Fortessa LSR II (BD Bioscience) flow cytometer. Images of the labelled population were generated using FlowJo software from Tree Star.
Table 2.6: Monoclonal antibodies used for mouse flow cytometry

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Manufacturer</th>
<th>Working concentration</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16/32 (FC block)</td>
<td>BD Pharmingen</td>
<td>0.5 µg/µl</td>
<td>Rat anti-mouse</td>
</tr>
<tr>
<td>CD14 (APC)</td>
<td>eBioscience</td>
<td>1 µg/µl</td>
<td>Rat IgG2a kappa (R&amp;D Systems)</td>
</tr>
<tr>
<td>CD11b (eF450)</td>
<td>eBioscience</td>
<td>0.06 µg/µl</td>
<td>Rat IgG2b kappa</td>
</tr>
<tr>
<td>F4/80 (eF450)</td>
<td>eBioscience</td>
<td>0.25 µg/µl</td>
<td>Rat IgG2a kappa</td>
</tr>
<tr>
<td>CD115 (PE Cy7)</td>
<td>eBioscience</td>
<td>0.125 µg/µl</td>
<td>Rat IgG2a kappa</td>
</tr>
<tr>
<td>CD124 (Fluorescein)</td>
<td>R &amp; D systems</td>
<td>10µl/100 ul</td>
<td>Goat IgG kappa</td>
</tr>
</tbody>
</table>

APC, allophycocyanin; eF450; eFlour 450, PE Cy7; phycoerythrin cyanine.

2.6.2 Flow cytometry using the THP-1 cell line

For flow cytometry labelling, THP-1 cells were cultured as described in section 2.2.2. THP-1 cells in suspension were washed twice with PBS, and the adherent cells were detached using trypsin/EDTA as described in section 2.6.1. THP-1 cells (1 x 10^6) in FACS buffer were first blocked for 20 minutes on ice with human FC block. The appropriate antibody, as listed in Table 2.7, was then added and incubated for 45 minutes at 4°C on a nutator. For intracellular CD68 staining, cells were washed twice and resuspended in intracellular staining FACS buffer (3% FBS and 1% bovine serum albumin [BSA] in PBS). While vortexing, 100 µl of IC fixation buffer
(eBioscience) was added to 100 µl of cells in FACS buffer and incubated for 20 minutes at room temperature. Without a subsequent wash, 2 ml of permeabilization buffer (0.25% saponin in 3% FBS and 1% BSA in PBS) was added, and the cells were centrifuged at 300x g at room temperature for 5 minutes. The supernatant was removed, and the pellet was vigorously vortexed with 100 µl of permeabilization buffer and centrifuged as described above. Cells were blocked with human FC block (Table 2.7), and the appropriate amount of CD68 or isotype control diluted in FACS buffer was added and incubated for 45 minutes at 4°C. Cells were then washed 2 times with 2 ml of permeabilization buffer, Finally, the cells were resuspended in 300 µL of FACS buffer at the final wash and analysed using a LSR II Fortessa flow cytometer.

Table 2.7: Monoclonal antibodies used for human flow cytometry

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Manufacturer</th>
<th>Working concentration</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD46/32/16</td>
<td>eBioscience</td>
<td>20 µl/100 µl</td>
<td>N/A</td>
</tr>
<tr>
<td>(Human FcR binding inhibitor)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11b</td>
<td>eBioscience</td>
<td>0.06 µg</td>
<td>Rat IgG2b kappa</td>
</tr>
<tr>
<td>(AF 660)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11c</td>
<td>eBioscience</td>
<td>0.5 µg</td>
<td>Mouse IgG1 kappa</td>
</tr>
<tr>
<td>(eF450)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD68</td>
<td>BD Pharmingen</td>
<td>5 µl/100 µl</td>
<td>Mouse IgG2b kappa</td>
</tr>
<tr>
<td>CD14</td>
<td>eBioscience</td>
<td>5 µl/100 µl</td>
<td>Mouse IgG2a kappa</td>
</tr>
</tbody>
</table>

ef, eFluor; BV, Brilliant Violet; FITC, fluorescein isothiocyanate; AF, Alexa Fluor.
2.6.3 Cell proliferation assay

The proliferation of THP-1 human monocytic cells was investigated using Cell Proliferation Dye eFluor 450 (eBioscience) according to the manufacturer’s instructions. In brief, a single cell suspension was prepared from THP-1 cells treated with Hoxa3 or mCherry control conditioned medium. Cells were then washed twice with PBS to remove any serum, and cells were suspended in PBS at 2× the desired concentration for labelling. Cell proliferation dye eF450 solution (20 µM) was prepared in PBS. Equal volumes of cell solution and dye solution were mixed and incubated in the dark for 20 minutes at room temperature. To stop the labelling reaction, 5 volumes of complete growth medium (RPMI 1640 supplemented with 2 mM L-glutamine, 10% FBS, and 1% P/S) was added and incubated for 5 minutes on ice. Cells were washed with complete medium and cultured in standard culture conditions or resuspended in FACS buffer (PBS supplemented with 2% FBS) when ready for analysis. To assess cell division, the fluorescence intensity of eF450 was measured on day 0 and 48 hours after labelling. Cell proliferation was determined as the reduction in fluorescence intensity, and the dye dilution rate was calculated from the day 0 and day 2 measurements. All fluorescence measurements were obtained using a Beckman Coulter Cyan ADP with 450/50 band-pass filters.

2.6.4 Apoptosis assay

Apoptosis assays were performed using the Vybrant FAM Poly Caspase Assay kit (Molecular Probes) according to the manufacturer’s protocol. In brief, a single cell suspension was prepared from THP-1 cells treated with Hoxa3 or mCherry control conditioned medium. A
minimum of $0.5 \times 10^6$ cells/ml was used per reaction and resuspended in 300 µl of THP-1 complete medium. The fluorescent labelled inhibitor of caspases (FLICA) regent, provided as a lyophilized solution, was dissolved completely in 50 µl of DMSO (provided by supplier) to generate a 150× FLICA reagent solution. This solution was diluted to a 30× FLICA reagent solution using PBS, pH 7.4, and 10 µl of 30× FLICA was added to the cell suspension. The cell suspension was incubated for 1 hour at 37 °C under standard culture conditions and mixed twice during the incubation. In the meantime, 1× apoptosis wash buffer was prepared from 10× buffer (Molecular Probes), and 2 ml of the buffer was added to the labelling reaction. The cells were spun down, and the pellet was re-suspended in 400 µl of 1× wash buffer. FAM poly caspase-labelled cells were detected using a 530/40 band-pass filter, and the FLICA fluorescent signal was used to determine the number of apoptotic cells. Unlabelled control cells were used to assess the background fluorescence in the medium.

2.7 Macrophage activation assay

Macrophages from diabetic or non-diabetic mice differentiated for 7 days were serum starved for 6 hours and activated as follows: (1) non-activated controls received serum-free DMEM medium; (2) macrophages were classically activated using serum-free macrophage medium supplemented with 100 ng/ml of lipopolysaccharide (LPS; Sigma) and 100 of ng/ml interferon-γ (INF-γ; Sigma); and (3) macrophages were alternatively activated using serum-free medium supplemented with 20 ng/ml of interleukin (IL)-4 (Peprotech) and 50 µg/ml of anti-INF-γ (Bio X Cell). Cells were incubated at 37 °C in 5% CO₂. Twenty-four hours after activation, RNA was isolated from each group independently for gene expression analysis. Forty-eight hours after
activation, supernatants were collected for enzyme-linked immunosorbent assays (ELISA) and Griess assays, and the cells were scraped and lysed for arginase assays.

2.8 Enzyme-linked immunosorbent assay

2.8.1 Human M-CSF detection by ELISA

ELISAs for human M-CSF were performed according to the kit manufacturer’s instructions (R&D Systems). In brief, 96-well plates (Nunc MaxiSorp flat-bottom) were coated with 2 µg/ml of capture antibody diluted in PBS and incubated overnight at room temperature. The wells were then washed 3 times with washing buffer (0.05% Tween 20 in PBS). At the final wash, the remaining wash buffer was removed by blotting the plate on absorbent tissue. The plate was then blocked with reagent diluents (1% BSA in PBS) for 1 hour at room temperature and washed. Samples or standards were added to each well in triplicate and incubated at room temperature for 2 hours. The standard curve for human M-CSF ELISA was generated using 2-fold serial dilutions over eight points from 1000 pg/ml–8 pg/ml. The wells were then washed, and 200 ng/ml of detection antibody was added for 2 hours at room temperature. The wells were again washed, and streptavidin-conjugated horseradish peroxidase (HRP) was added for 20 minutes in the dark at room temperature. The wells were washed again, and substrate solution (1:1 mixture of colour reagent A [H₂O₂] and colour reagent B [tetramethylbenzidine]) was then added to each well and incubated for 15 minutes at room temperature. Finally, stop solution (2 N H₂SO₄) was added to each well to stop colour development. The plate was then read on a plate reader at 450 nm and again at 570 nm to correct for optical imperfections.
2.8.2 Mouse TNF, IL-12, and TGF-β ELISAs

Mouse tumour necrosis factor (TNF)-α and interlukin-12 p70 (IL-12) were detected using Ready-Set-Go ELISA Kits (eBioscience). TGF-β release was assessed using the Ready-Set-Go (2nd generation) ELISA Kit (eBioscience). Experiments were performed according to the manufacturer’s instructions. First, 100 µl of capture antibody diluted in 1× coating buffer (10× provided by eBioscience) was added to each well of the ELISA plate and incubated overnight at 4 °C. The plate was washed 3 times and blocked by the addition of 200 µl of 1× ELISA/ELISPOT diluent for 1 hour at room temperature. During the blocking step, standards were prepared from 1 µg/ml mouse TNF alpha or IL-12 p70 recombinant proteins, using 2-fold serial dilutions. The concentration range for each protein standard is indicated in Table 2.8. At the end of the blocking step, the plate was washed once with washing buffer. Standards or blank reagent (1× assay diluent) were then added in triplicate.

Table 2.8: Standard concentration ranges for mouse ELISA assays

<table>
<thead>
<tr>
<th>Kit</th>
<th>Standard concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>1000–8 pg/ml</td>
</tr>
<tr>
<td>IL-12</td>
<td>2000–23 pg/ml</td>
</tr>
<tr>
<td>TGF-β</td>
<td>1000–8 pg/ml</td>
</tr>
</tbody>
</table>

Undiluted samples from cell culture supernatants were added in triplicate for IL-12 and TNF-α, ELISAs. For human/mouse TGF-β ELISAs, the cell supernatants were pretreated with acid activation and neutralization to separate the latency associated peptide (LAP) from mature
TGF-β before adding samples to the wells. Samples were acidified by the addition of 20 µl of 1 N HCL to 100 µl samples for 10 minutes at room temperature. Samples were neutralised by the addition of 20 µl of 1 N NaOH. All final concentrations of TGF-β were corrected for the 1.4× dilution factor. Samples or standards were incubated for 2 hours at room temperature for IL-12 and TNF-α and overnight for TGF-β to increase the sensitivity of the assay. Plates were washed 5 times, and 100 µl of biotin-labelled detection antibody was added and incubated for 1 hour at room temperature. Plates were washed 5 times, and 100 µl of avidin-HRP-conjugated antibody was added and incubated for 30 minutes. Plates were washed 5 times, and the plate was soaked in washing buffer for 1 minute during each wash. To each well, 100 µl of 1× tetramethylbenzidine (TMB) substrate solution was added and incubated for 15 minutes. To stop colour development, 50 µl of 2 N H₂SO₄ stop solution was added. The plate was analysed as described in section 2.8.1.

2.9 Nitric oxide release (Griess assay)

Nitric oxide release was determined using supernatants from CA or NA macrophages treated with Hoxa3-mCherry or mCherry control conditioned medium. Griess reagent, comprising Griess 1 (1% sulphanilamide in 5% phosphoric acid) and Griess 2 (0.1% N-(1-naphthyl ethylenediamine in dH₂O) was added to the supernatant from macrophages for 10 minutes. Standards ranging from 100 µM to 1.5 µM were prepared using sodium nitrite diluted in DMEM. The Griess mixtures containing standard or samples were added in triplicate to a 96-well plate. Nitric oxide release was determined using a colorimetric plate reader that measured the absorbance at 570 nm and the background absorbance at 630 nm.
2.10 Arginase assay

Arginase activity was measured in activated macrophages or non-activated controls at 48 h post-activation. Macrophage cell lysates were prepared by trypsinization and cell scrapping as described in section 2.6.1. Cells (1 × 10^6) were vortexed in 100 µl of permeabilization buffer (0.1% Triton-X in PBS) and incubated for 30 minutes at room temperature. Assay buffer (100 µL; 10 mmol MnCl₂ in 50 mmol Tris, pH 7.5) was added, and the mixture was heated to 56°C for 10 minutes to activate the enzyme. From each sample, the reaction was performed in triplicate, and 1 volume of L-arginine (0.5 M arginine, pH 9.7) was added and incubated for 1 hour at 37 °C. Urea standards (200 µg/ml to 10 µg/ml) were prepared in assay buffer, and assay buffer alone was used as a blank. The reaction was stopped by the addition of an acid solution (H₂SO₄:H₃PO₄:dH₂O at 1:3:7, v/v/v), and samples and standards were transferred to a 96-well plate. The formation of urea by active arginase from within the macrophages was analysed by adding one volume of 9% isonitrosopropiophenone to all samples and standards and incubating the reactions in the dark at 100 °C for 45 minutes. The coloured product was measured at 570 nm, and the background absorbance was measured at 450 nm.

2.11 Western blot

2.11.1 Protein extraction

For protein extraction, RIPA lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate [SDS] in 1× PBS) was supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma), 2 mM sodium orthovanadate, and 1x protease inhibitor cocktail solution (100x PIC; Sigma). To extract protein from adherent cells,
the medium was removed, and the cells were washed twice with cold PBS. Samples were kept on ice throughout the process. Cold lysis buffer (200 µL) was applied, and the adherent cell sheet was disrupted and homogenized with the use of a cell scraper. The cell lysate was thoroughly mixed by pipetting to ensure complete homogenization. The homogenate was then gently transferred to a pre-cooled microfuge tube and centrifuged in 14,000 × g for 15 minutes at 4°C to separate the cell debris from the protein. The supernatant was then transferred to a new tube and stored at -20°C for further analysis.

2.11.2 Bradford assay

The protein concentration was measured using the Bradford assay, with BSA (Sigma) as the protein standard. A 10× solution of BSA was prepared in RIPA lysis buffer, and 2-fold serial dilutions were used to prepare standards ranging from 4 mg/ml to 0.5 mg/ml. Standards and samples were diluted 1:10 in dH2O, added in triplicate to a 96-well plate (COSTAR 96-well flat bottom), and mixed with 200 µl of 1:5 diluted Bradford dye (Bio-Rad). The protein concentration was determined using a colorimetric plate reader, which read the absorbance at 595 nm.

2.11.3 Gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein samples. The protein samples were separated using a 10% separating gel and a 4.2% stacking gel. The separating gel was prepared using 4 mL ddH2O, 5 mL Protogel (30% acrylamide/1% N,N-methylenebisacrylamide, (Bis) (Sigma), 5.6 ml 1 M Tris-HCl, pH 8.8, 150 µl 10% SDS, 24 µl N,N,N′,N′-tetramethylethlenediamine (TEMED; Sigma-Aldrich), and
100 µl of 10% ammonium persulphate (APS). The latter was added last to induce polymerization. The separating gel solution was poured between two glass plates separated by 1.5-mm spacers. The stacking gel was then prepared by adding 700 µl protagel, 650 µl 1 M Tris-HCl, pH 6.8, 38.8 µl 10% SDS, and 12 µl TEMED to 3.61 ml ddH₂O, with 50 µl APS added to the mixture at the end. The stacking gel solution was poured between the plates, and a 1.5-mm-thick 10-well comb was inserted. After polymerization, the gel was submerged in 1× running buffer (192 mM glycine, 25mM Tris base, and 0.1 % SDS).

2.11.4 Sample preparation for SDS-PAGE

Laemmli sample buffer (5×; 2% SDS, 5% β-mercaptoethanol (Sigma), 50% glycerol (Sigma), 1% bromophenol blue (Sigma), and 0.06 M Tris-HCl, pH 6.8) was used for sample loading and protein denaturation. Protein (10 µg) was mixed with 5× Laemmli sample buffer (LSB) in a total reaction volume of 25 µl, and the mixture was heated at 95 °C for 5 minutes. To determine the molecular weight of the target proteins, a protein ladder of 10–250 kDa (NEB) was used.

2.11.5 Protein transfer

Protein transfer was performed using the Trans-Blot Turbo Transfer Pack (mini format, 0.2 µm PVDF). The bottom pack containing the PVDF blot was placed on the bottom cassette, and the protein gel was placed on the top of the pack. Air bubbles were removed by rolling. The labelled top half of the pack was added on the gel, and air bubbles were removed by rolling on the top of the layers. The cassette was inserted into the Turbo transfer device, and the program was set for a 1.5-mm gel, with a transfer time of 10 minutes.
2.11.6 Western blotting

To verify the success of the transfer, the blot was stained with 0.1% w/v Ponceau for 15 min and then washed with Tris-buffered saline (TBS). Next, the blot was blocked with blocking buffer (5% skimmed milk diluted with 0.1% Tween TBS) for 1 h at 4 °C. The blot was then washed with Tween TBS and incubated with diluted primary antibodies overnight at 4°C (Table 2.9). The blot was incubated 3 times in Tween TBS and then incubated with HRP-conjugated secondary antibodies (Table 2.9) for 1 hour at room temperature.

Bands were detected using a Pierce ECL substrate detection kit (Thermo Scientific) and visualized using Kodak BioMax XAR film (Sigma) or a ChemiDoc XRS+ system connected to Image Lab software.

Table 2.9: Primary and secondary antibodies used for western blot

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species isotype</th>
<th>Dilution</th>
<th>Manufacturer</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mCherry</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>BioVision</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-Pu.1</td>
<td>Goat</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology</td>
<td>D-19</td>
</tr>
<tr>
<td>Anti-Flag</td>
<td>Mouse monoclonal</td>
<td>1:2000</td>
<td>Sigma</td>
<td>M2</td>
</tr>
<tr>
<td>Anti-Stat6</td>
<td>Rabbit monoclonal</td>
<td>1:2000</td>
<td>Abcam</td>
<td>YE361</td>
</tr>
<tr>
<td>Anti-Stat6</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
<td>Abcam</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>(phospho Tyr641)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Tubulin</td>
<td>Mouse monoclonal</td>
<td>1:5000</td>
<td>Sigma</td>
<td>DM1A</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------</td>
<td>-------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Anti-Rabbit</td>
<td>Goat</td>
<td>1:25000</td>
<td>Abcam</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-Goat</td>
<td>Donkey</td>
<td>1:2000</td>
<td>Abcam</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-Mouse</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>Abcam</td>
<td>N/A</td>
</tr>
</tbody>
</table>

### 2.12 Co-Immunoprecipitation assay

The co-immunoprecipitation (CoIP) protocol was modified from (Yamada et al., 2008).

#### 2.12.1 Transfection of 293T cells

To overexpress proteins of interest, 293T cells were transfected in 10-cm dishes using the calcium phosphate transfection method, according to the protocol described in section 2.3.1. 5 µg Pu.1 expression plasmids, Hoxa3-mCherry or mCherry were co-transfected into 293T cells for use in CoIP with cells maintained under standard culture conditions.

#### 2.12.2 Protein extraction and CoIP

Protein was extracted from cells at 72 hours post-transfection by adding 200 µl of CoIP lysis buffer (1× PBS with 1% Triton X-100, 1× PIC, and 0.01% IGEPAL CA-630) to the 10-cm dish and following the procedure described in 2.11.1. For endogenous CoIP, protein was extracted from mouse macrophages, which endogenously express Pu.1, 48 hours after treatment with Hoxa3-mCherry or mCherry control. The extracted protein was pre-cleared to remove non-specific proteins that can bind to the beads. Pre-clearing was performed with 2µg of an irrelevant antibody with the same isotype as antibody used in the immunoprecipitation
reaction. The mixture was incubated for 1 hour on ice. Thereafter, 100 µl of pre-washed Dynabeads (FlowComp Flexi System; Invitrogen) was added to the mixture and incubated for 20 minutes at 4 °C with occasional tapping. Non-specific protein bounded to the beads was discarded, and the cell supernatant was removed using a magnet (EasySep™; Stemcell Technologies) for use in subsequent immunoprecipitation reactions. Rabbit anti-mCherry (BioVision), goat anti-Pu.1 (D-19; Santa Cruz Biotechnology), goat anti-CD34 (C-18; Santa Cruz Biotechnology) IgG antibody control, and mouse anti-Flag (Sigma) were labelled with biotin (DsB-x; Molecular Probes) according to the manufacturer’s instructions. Pre-cleared extracted protein lysate (300 µg) was incubated with 2 µg of biotin-labelled antibody (anti-Pu.1, anti-FLAG, or anti-mCherry) together with 0.4 mg of pre-washed Dynabeads in extraction buffer. The volume of the protein-antibody-beads mixture was brought to 300 µl using CoIP lysis buffer and incubated overnight at 4 °C with gentle rotation. At the end of the incubation, the mixture was washed 3 times with wash buffer (0.1% SDS, 150 mM NaCl, 10 mM HEPES, pH 7.5, 0.2% IGEPAL CA-630). The cell supernatant and unbound protein were removed using a magnet. The unwashed CoIP reaction that bound to the beads was resuspended in 20 µl of extraction buffer and 7 µl of 4x LSB. The mixture was heated at 95 °C for 5 minutes and the eluate was western blotted as described in section 2.11.

2.13 Immunofluorescence staining

2.13.1 Immunofluorescence staining of THP-1 cells

For immunofluorescence staining, THP-1 cells were grown on sterile cover slips (GG-12; 12 mm in diameter; Biokeystone) in 24-well plates (Costar; Sigma). Cells were maintained under
standard culture conditions for 24 h. At the time of analysis, the medium was removed, and the cells were fixed with 4% paraformaldehyde in 1× PBS for 10 minutes on ice. The cells were washed with PBS and treated with the permeabilizing agent 0.25% Triton X-100 for 3 minutes at room temperature. The cells were then blocked for 15 minutes with 5% western blocking reagent (Roche) diluted in 1× PBS. Cells were then incubated with primary antibody (mouse anti-Flag, 1:1000) for 2 hours at 37 °C and washed 3 times with PBS. The donkey anti-mouse Alexa Fluor 488 secondary antibody was then applied at a 1:1000 dilution for 45 minutes at 37 °C.

The cells were washed with PBS, and the cover slips were mounted with VECTASHIELD® Mounting Medium with DAPI (Vector Labs). The samples were stored at -20°C. An Olympus BX51 upright microscope with a 10× objective was used to view the cells. Images were captured using a CoolSNAP ES camera (Photometrics) and processed using MetaView Imaging Software (Molecular Devices). Specific band-pass filter sets for DAPI and FITC were used.

### 2.13.2 Immunohistochemistry (IHC) staining of wound sections

Sections taken from wounds of diabetic mice that were treated with Hoxa3 or control plasmid that were previously prepared in our lab. The protocol used for the processing of wound sections is described in (Bannon et al., 2013; Mahdipour et al., 2011). Briefly, 5-µm sections were generated using a microtome (Leica RM2255) from a formalin-fixed wound embedded in paraffin. Before IHC staining, the sections were first stained with haematoxylin and eosin (H and E) using Shandon Varistain 24-4 (Thermo Scientific) to locate the granulation tissue and peri-wound dermis that will be used for IHC imaging. Matching sections were selected for
IHC and heated at 55 °C for 20 minutes to melt the paraffin. Subsequently, to de-wax and rehydrate the sections, the slides were placed in a slide holder and subjected to 3 washes in xylene, 1 wash in 50% xylene/50% ethanol, 3 washes in 100% ethanol, and 2 washes in 100% methanol. Each wash was performed for 3 minutes under a fume hood. Heat-mediated antigen retrieval solution (2.94g tri-sodium citrate, pH=6.0 in 1L of distilled H₂O) was prepared while the sections were rinsed carefully under slow running cold tap water to avoid scratching the sections. The slides within the slide holder were immersed in antigen retrieval solution and heated in a microwave until bubbling was observed and then heated on high for 3 minutes. The sections were cooled at room temperature for at least 20 minutes to allow the denatured antigen to re-nature before antibody staining. Then sections were washed 3 times in PBS/TBS as appropriate and twice in PBST using 0.1% Tween or 0.3% Triton-X as the permeabilizing agent (see Table 2.10). Tissue sections were blocked in donkey serum (DS) in PBS (Table 2.10) and subsequently incubated for 14 hours at 4 °C with the appropriate primary antibody diluted in blocking buffer, as listed in Table 2.11. After several washes in the appropriate buffer, the following secondary antibodies were applied for 1 hour at room temperature: donkey anti-rat Alexa Fluor 488 (1:500; Invitrogen/Life Technologies, A-21208), donkey anti-goat Cy5 (1:500; Abcam, ab6566) and donkey anti-rabbit Alexa Fluor 555 (1:500; Invitrogen/Life Technologies, A-31572). At the end of the incubations, sections were washed 3 times in TBST or PBST and 1 time in TBS/PBS without Tween. ProLong Antifade Reagent with DAPI (Invitrogen) was added to each section. Cover slips were applied, and the mounted sections were dried at room temperature, and then stored at -20.
2.14 Microscopy and data analysis

Coloured Images from H and E staining were obtained using Nikon Eclipse 80i microscope attached to SD-Fi1 coloured camera and Images generated on NIS-Element software.

Fluorescent Images were obtained using an Olympus IX81 inverted microscope (Olympus, Japan) and processed using FluoView 1000 software. Four fields from each wound were used for the statistical analysis of Mac3⁺ Nos2⁺ Arg1⁺, Mac3⁺ Nos2⁺ Arg1⁺, or Mac3⁺ Nos2⁻ Arg1⁺ cells or Mac3⁺ VEGF⁺, Mac3⁺ VEGF⁻, Mac3⁺ TGF-β⁺, or Mac3⁺ TGF-β⁻ cells in each sample using the Imaris software module or using Image J cell counter option.

Table 2.10: Specific conditions used for antibody staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Washing buffer</th>
<th>Permeabilization buffer</th>
<th>Blocking buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Mac3</td>
<td>TBS or PBS</td>
<td>0.1% Tween in TBS or 0.3% Triton-X in PBS</td>
<td>5% Donkey serum (DS) in TBS</td>
</tr>
<tr>
<td>Anti-Nos2</td>
<td>TBS</td>
<td>0.1% Tween in TBS</td>
<td>5% DS in TBS</td>
</tr>
<tr>
<td>Anti-Arg1</td>
<td>TBS</td>
<td>0.1% Tween in TBS</td>
<td>5% DS in TBS</td>
</tr>
<tr>
<td>Anti-VEGF</td>
<td>PBS</td>
<td>0.3% Triton-X in PBS</td>
<td>5% DS in PBS</td>
</tr>
<tr>
<td>Anti-TGF</td>
<td>PBS</td>
<td>0.3% Triton-X in PBS</td>
<td>10% DS in PBS</td>
</tr>
</tbody>
</table>
Table 2.11: Primary antibodies used in immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Concentration</th>
<th>Dilution</th>
<th>Buffer</th>
<th>Manufacturer</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Mac3</td>
<td>Rat IgG</td>
<td>0.5 mg/ml</td>
<td>1:50</td>
<td>5% DS in TBS or PBS</td>
<td>BD Pharmingen</td>
<td>M3/84 (RUO)</td>
</tr>
<tr>
<td>Anti-Nos2</td>
<td>Rabbit IgG</td>
<td>0.2 mg/ml</td>
<td>1:100</td>
<td>5% DS in TBS</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-651</td>
</tr>
<tr>
<td>Anti-Arg1</td>
<td>Goat IgG</td>
<td>0.2 mg/ml</td>
<td>1:200</td>
<td>5% DS in TBS</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-18354</td>
</tr>
<tr>
<td>Anti-VEGF</td>
<td>Rabbit IgG</td>
<td>0.294 mg/ml</td>
<td>1:400</td>
<td>5% DS + 4% BSA in PBS</td>
<td>Abcam</td>
<td>EP1176Y</td>
</tr>
<tr>
<td>Anti-TGF</td>
<td>Rabbit IgG</td>
<td>0.59 mg/ml</td>
<td>1:150</td>
<td>10% DS in PBS</td>
<td>Abcam</td>
<td>EPR12678(B)</td>
</tr>
</tbody>
</table>

2.15 Plasmids

The pcDNA3.1 Hoxa3-mCherry, pcDNA3.1 mCherry, SPmCherry pSecTag2A and SPHoxa3mCherry pSecTag2A plasmids were previously designed in the Mace lab. The pcMV-sport6-Pu.1, pcDNA3.1 Flag-Hoxc13, and pFLAG-CMV2-Pu.1 plasmids were generously donated by Dr Kathryn Hentges. The lentivirus vector PLVTHM, packaging plasmids psPAX and pMD2G were obtained from the laboratory of Professor Charles Streuli. All plasmid maps are provided in (Supplementary figure 1-10).
2.16 Generation of GFP-Pu.1shRNA-PLVTHM

2.16.1 Design and amplification of mouse Pu.1shRNA

The Pu.1shRNA sequence and pLVTHM primers used to amplify Pu.1shRNA were obtained from Eurogentec. The Pu.1 shRNA sequence is as follows:

```
Pu.1 shRNA sense
5' TGCTGGACAGTGACGGCTGTGGATG 3'
Loop
TAGTGAAGCCACAGATG3' TGCCTACTGCCTCGGA3'
Pu.1 shRNA anti sense
5' catccagctgagctccagc 3'
```

The Pu.1 shRNA sense sequence 5’ gctggagctcagtggatg 3’ and antisense sequence 5’ catccagctgagctccagc 3’ were copied from (Lin et al., 2012). The structure of the 5’ TAGTGAAGCCACAGATG3’ sequence creates a loop that connects the sense and antisense sequences, promoting the formation of a short hairpin structure. The forward and reverse pLVTHM primers used for the amplification of the Pu.1shRNA insert sequence before cloning into the vector are as follows:

```
Forward primer
5' GAC ACGCGT TGCTGGACAGTGACGGCG 3'

Reverse primer
5' GAC ATCGAT TTTTT TCCAGGGCAGTGGCA 3'
```

The pLVTHM primers contain Mlu1 and Cla1 restriction enzyme sites used in the digestion reaction. They also contain an extra flanking region to allow for attachment of the restriction enzyme. The reverse primer contains a poly-T sequence that terminates the polymerase reaction. The Pu.1shRNA sequence (10 µM) was amplified using 10 µM of the forward and
reverse primers with 1x ready-made PCR master mix in a 25-µl reaction. The reaction was performed under the conditions described in Table 2.12.

**Table 2.12: PCR reaction conditions for the amplification of the Pu.1shRNA sequence**

<table>
<thead>
<tr>
<th>Step</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94 °C, 5 minutes</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C, 30 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>56 °C, 30 seconds</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C, 30 seconds</td>
</tr>
<tr>
<td>Final extension</td>
<td>72 °C, 7 minutes</td>
</tr>
<tr>
<td>Final hold</td>
<td>4 °C</td>
</tr>
</tbody>
</table>

The PCR product was detected in a 2% agarose gel, and a product of 200 bp was extracted for subsequent purification using the GE Healthcare GFX PCR and Gel Band Purification Kit (Fisher Scientific) according to manufacturer’s instructions. At the end of the purification process, 20 µl of nuclease-free water was used to elute the Pu.1shRNA product.

The purified Pu.1shRNA sequence and 10 µg of the pLVTHM vector were digested with 1 U of MluI (Roche) and ClaI (New England Biolabs) enzymes in compatible buffer to generate sticky ends. Purified Pu.1shRNA or pLVTHM plasmid (16 µl) was mixed with 2 µl of 10× buffer (New England BioLabs). MluI (1 µl) and ClaI (1 µl) (New England BioLabs) were then added, and the reaction was incubated at 37 °C for 2 hours. The Pu.1shRNA PCR product and pLVTHM plasmid reactions were then heated at 85 °C for 5 minutes. The Pu.1shRNA insert and pLVTHM backbone products with sticky ends were purified using the GE Healthcare GFX PCR and Gel
Band Purification Kit to ensure the removal of enzymes that might interfere with subsequent reactions.

### 2.16.2 Ligation and transformation reactions

The purified PCR product from the digestion and the vector DNA were ligated under the conditions described in Table 2.13. The reaction was incubated overnight at 16 °C to ligate sticky ends.

**Table 2.13: pLVTHM and Pu.1shRNA ligation reaction**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified digested DNA plasmid</td>
<td>8 µl</td>
</tr>
<tr>
<td>Purified digested shRNA sequence</td>
<td>8 µl</td>
</tr>
<tr>
<td>T4 DNA Ligase enzyme (NEB)</td>
<td>1 µl</td>
</tr>
<tr>
<td>10× Ligase buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>Nuclease free H₂O</td>
<td>to 20 µl</td>
</tr>
</tbody>
</table>

NEB, New England BioLabs.

The newly made recombinant DNA was used to transform *E. coli* bacterial cells. Homemade competent cells were pre-warmed on ice for 15 minutes, and 50 µl was aliquoted to a pre-chilled 1.5-ml tube. Recombinant DNA (10 µl) was added to the competent cells and mixed gently with the tip of a pipette. The reaction was incubated on ice for 15 minutes (for subsequent mini-preps) or for 30 minutes (for subsequent maxi-preps). The cells were then heat-shocked at 43 °C for 40 seconds and immediately placed on ice for 2 minutes.

Lysogeny Broth (LB; 250 µl) without antibiotic was added to the mixture and incubated for 1 hour at 37 °C with shaking at 250 revolutions per minute (rpm) for bacterial expansion. At the end of the incubation, the bacteria were cultured on a dish or incubated on ice until ready for
To culture the transformation reaction, 100 µl of bacteria was spread on an LB agar plate supplemented with 50 µg/ml ampicillin. The plate was incubated upside down at 37 °C for a maximum of 16 hours to prevent overgrowth of bacteria. A few colonies were selected the next day. Each colony was cultured in 3 ml of LB containing 50µg/ml ampicillin and incubated in a shaker overnight at 37 °C. The DNA was then extracted and purified from the bacteria using a Qiagen mini-prep kit according to manufacturer’s instructions. The purified product was sequenced to determine whether the Pu.1shRNA sequence was inserted in the correct orientation between the Mlu1 and Cla1 restriction sites and in-frame with the pLVTHM vector sequence. Sequencing was performed using the H1 primer (Addgene) 5’ TCGCTATGTGTTCTGGGAAA 3’, as described in the following section.

2.17 Sequencing

Each plasmid was sequenced to verify its integrity, using 200 ng of template DNA and 1 µM primer in a 10-µl reaction. Sequencing reactions were sent to the University of Manchester sequencing facility, and the results were analysed using 4Peaks software or by blasting the FASTA format in the NCBI BLAST bioinformatics website.

2.18 Statistics

All statistical analysis was performed using Excel software for data management and analysis. Any comparison made between 3 groups or more were made using ANNOVA, otherwise comparison between 2 groups were made using student’s t- test.
Chapter 3: Hoxa3 modulates gene expression in THP-1 monocytes

3.1 Introduction

_Hoxa3_ is a member of the homeobox family of transcription factors that encode master regulator proteins controlling the segmental identity along the anterior-posterior axis of embryos (Krumlauf, 1994). Later in life, _Hox_ genes, particularly from the A, B, and C clusters, are expressed in haematopoietic progenitors and are involved in the differentiation and lineage commitment of hematopoietic cells (Argiropoulos and Humphries, 2007). Enforced expression of the _Hoxa3_ gene in diabetic wounds accelerates wound closure, reduces the number of inflammatory cells recruited to or retained in the wound and promotes angiogenesis (Mace et al., 2005, 2009). Moreover, Hoxa3 promotes the differentiation of HSC/P into Gr1^+^CD11b^+^ myeloid and monocytic populations that rescue the defective angiogenic potential of diabetic wounds (Mahdipour et al., 2011).

Until now, it has not been clear whether the anti-inflammatory action of Hoxa3 was related to its differentiation potential. All previous studies have targeted the overall effect of Hoxa3 on wound healing, and on myeloid cell populations within the wound, without addressing the specific effects of Hoxa3 on each subset of leukocytes. Therefore, in this project, we focused on the effect of Hoxa3 on monocytes and macrophages because they are known to be key cell types that are involved in wound healing (Brancato and Albina, 2011; Lucas et al., 2010a). This chapter will focus on changes in gene expression in undifferentiated monocytes and macrophage-differentiated monocytes. I looked at putative target transcription factors and differentiation markers that will help to evaluate the potential of Hoxa3 to induce the
differentiation of monocytes into macrophages. We will also be looking at changes in monocytes/macrophage cell surface markers to assess the potential of Hoxa3 to induce macrophage differentiation.

THP-1 cells, a monocytic cell line, were chosen as a model to study monocyte differentiation. The THP-1 cell line was established from a human patient with monocytic leukaemia. It is widely used as a model to assess the function as well as the differentiation of monocytes and macrophages (Auwerx, 1991; Tsuchiya et al., 1980). Upon stimulation by the administration of phorbol mystrate 13 acetate (PMA), THP-1 can be differentiated into macrophage-like cells through activation of protein kinase C (Park et al., 2001; Tsuchiya et al., 1982).

In this chapter we demonstrate two methods of inducing Hoxa3 overexpression in THP-1 cells: gene transfer by nucleofection and protein delivery using protein transduction. The protein delivery method was based on previous successful approaches to overexpress Hoxb4 and Hoxa3 using a co-culture method. Here, I demonstrate the first example of successful use of protein transduction using a conditioned media enriched with Hoxa3 protein rather than using a complicated co-culture method. I aimed initially to compare the effects of Hoxa3 to Hoxc13 in THP-1 monocytes, to assess if an anterior Hox gene, located near the 3’ position of the Hox cluster (Hoxa3) shows similar or opposing actions to a posterior Hox gene located near the 5’ position of the cluster (Hoxc13) in terms of differentiation of monocytes. As we progressed with this project, we focused on the effect of Hoxa3 on monocyte differentiation without comparison to Hoxc13. My results of the effects of Hoxa3 and Hoxc13 overexpression on monocytes will be presented in this chapter.
3.2 Results

3.2.1 Differentiation of THP-1 cells into macrophage-like cells

We began our study by trying different differentiation protocols to be used in our model of THP-1 human monocytic cells. The standard protocol of differentiating THP-1 monocytes into macrophages uses 100 ng/ml PMA over 5 days (Tsuchiya et al., 1982). We used this protocol and monitored the cells’ morphology by phase-contrast imaging on a daily basis to see the difference between undifferentiated and PMA-differentiated THP-1 cells (Figure 3.1a).

Changes in the THP-1 cell morphology began on day 2 of differentiation. There was a noticeable increase in the size of the THP-1 monocytes as they began to adhere to the culture wells. These changes took place gradually until day 5 of differentiation, when most of the cells became completely adherent, forming a sheath of cells that had the morphology of native macrophages.

On day 5 of differentiation, RNA was extracted and cDNA synthesised to be used as a template for SYBR green quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to assess any changes in the differentiation markers of THP-1 macrophages compared to undifferentiated THP-1 cells. Previously published studies often used CD14 as marker to assess the differentiation potential of THP-1 from monocyte to macrophages-like cells (Park et al., 2007). Increase in CD14 mRNA or cell surface-associated protein is considered to be positive evidence that THP-1 cells have differentiated into the macrophage like phenotype.

We wanted to explore this further by looking at different markers of differentiation and investigated CD68, CD11b, and CD11c in addition to CD14. CD68 is known as pan macrophage
marker that is upregulated in human macrophages (Holness and Simmons, 1993; Ramprasad et al., 1996). CD11b, a marker of myeloid cells that is widely used in differentiation studies of THP-1 monocytes and macrophages, (Dumrese et al., 2009; Maeß et al., 2014), and CD11c, a dendritic cells marker that is also expressed in macrophages and known to be induced in PMA stimulated THP-1 cells (Chen et al., 2012). As Figure 3.1b shows, the mRNA level of all of the markers selected were significantly upregulated as a result of the 100 ng/ml PMA treatment protocol (p < 0.001, p < 0.005, p < 0.01, p = 0.06 for CD11b, CD11c, CD14, and CD68, respectively). Thus, we confirmed the successful replication of the established differentiation protocol of THP-1 monocytes into macrophage-like cells using 100 ng/ml PMA.

To examine whether using a much lower concentration of PMA can induce the macrophage phenotype in THP-1 cells, we attempted to induce differentiation with 5 ng/ml of PMA. A low PMA concentration was chosen for multiple reasons:

A high PMA concentration “100 ng/ml and above” was found to activate an isoform of protein kinase C (PKC) that can induce cell apoptosis (Lin et al., 2011).

A high concentration of the differentiation agent could obscure changes in gene expression due to induction of markers of differentiation coming from secondary stimuli such as over-expression of the Hoxa3 transcription factor.

The 5 ng/ml level of PMA was used successfully in a prior study to promote the differentiation of THP-1 cell monocytes without inducing any significant changes in gene expression (Park et al., 2007). Therefore, we applied this protocol of THP-1 cells differentiation using 5 ng/ml PMA over 3 days and monitored the morphology of the cells by taking images by phase contrast microscopy. On day 2 of differentiation, the cells began to adhere and their morphology began
to change, forming clumps. By day 3 of differentiation, most of the cells were elongated, forming the fibre-like shape that closely resembles the morphology of human macrophages in culture (Figure 3.2a). RNA was harvested on day 3 of differentiation from undifferentiated and PMA differentiated THP-1 cells to assess any changes in the CD11b, CD11c, CD14 and CD68 markers. As shown in Figure 3.2b, CD11b and CD11c mRNA levels were markedly increased (p < 0.0005) and (p<0.005), respectively.

However, CD14 did not show significant upregulation, which could be due to variations among replicates, but CD68 showed a change with borderline significance (p=0.08). Overall, the morphology of the macrophage-like cells as well as the qRT-PCR analysis suggested that 5 ng/ml PMA can be used successfully to differentiate macrophages and was used for all of the subsequent work presented in this chapter.
Figure 3.1: Differentiation of THP1 monocytes to macrophages using a high PMA protocol. THP1 monocytes were induced with 100 ng/ml PMA and a. images were taken daily to observe any changes in morphology. Images were taken at 10x (scale bar = 100μm). b. qRTPCR analysis of macrophage markers at day 5 of differentiation from undifferentiated and PMA-differentiated THP1 monocytes. *p < 0.05, **p < 0.005, ***p < 0.0005.
Figure 3.2: Differentiation of THP-1 monocytes to macrophages using a low PMA protocol.  

a. THP-1 monocytes were induced with 5 ng/ml PMA over 3 days and images were taken daily to observe any changes in the THP-1 cells' morphology. Images taken at 10x (scale bar = 100μm).  

b. qRTPCR analysis of macrophage markers at day 5 from undifferentiated and PMA differentiated THP-1 cells. **p < 0.005, ***p < 0.0005.
3.2.2 Endogenous expression of the principle transcription factors that control monocyte differentiation into macrophages

The endogenous levels of Hox transcription factors were assessed prior to over-expression analysis. *HOXB7* that is known to participate in the differentiation of monocytes, used as a positive control (Lill et al., 1995). THP-1 RNA was isolated and subsequently used as a template for cDNA synthesis. Each sample was assayed using Taqman reactions in triplicate, and gene expression was normalised to the RPL37A reference gene, which is known to be stably expressed in THP-1 cells throughout differentiation (Maeß et al., 2010). To verify the absence of genomic contamination of cDNA samples, gene expression was also investigated in THP-1 cDNA made without reverse transcriptase, i.e., THP-1 RNA. To ensure absence of reagent contamination, a no-template control using water as a template was run for each Taqman assay/probe tested.

Hox gene expression was detectable in an undifferentiated population of THP-1 cells, but not in macrophages (Figure 3.3a). *HOXC13* expression in THP-1 monocytes was significantly higher (p <0.005) relative to levels in macrophages. On the other hand, *HOXA3* was expressed at relatively low levels in undifferentiated THP-1 and minimally expressed in macrophages. *HOXB7* transcript levels were higher than *HOXA3* but lower than *HOXC13*. Altogether, our data suggest that *HOXA3* and *HOXC13* are expressed early in the differentiation process and are then downregulated in the more mature state of differentiated THP-1 cells. The expression pattern of *HOXA3* and *HOXC13* correlates with the general trend of Hox gene expression in hematopoietic cells, as they tend to be downregulated in a more differentiated state (Lawrence et al., 1996).
Myeloid-specific transcription factors PU.1/SPI-1, RUNX1, CEBPα, M-CSF and M-CSFr form a regulatory chain to promote the differentiation of monocytes (Figure 1.8).

These myeloid transcription factors were chosen as putative targets that will be investigated in Hoxa3 or Hoxc13 overexpressing THP-1 cells. Therefore, the mRNA expression levels of these myeloid genes were analysed in our model using qRT-PCR to determine their baseline levels in THP-1 cells.

The population of differentiated THP-1 cells showed higher expression of SPI-1, RUNX1, M-CSF and M-CSFr relative to undifferentiated THP-1 cells (Figure 3.3b). SPI-1 and RUNX1 expression was significantly increased in THP-1 macrophages (p<0.05 and p=0.06 respectively). M-CSF showed dramatic upregulation in THP-1 macrophages relative to undifferentiated THP-1 (p<0.0005). CEBPα and M-CSFr were not significantly changed as a result of differentiation.

In summary, these findings demonstrate that endogenous levels of myeloid transcription factors can vary between different differentiation stages of THP-1 cells, and indicate that THP-1 cells show a pattern of expression similar to human monocytes and differentiated macrophages. M-CSF and SPI-1 expression levels showed the greatest changes in their expression during the differentiation process into THP-1 macrophages (Figure 3.3b). This suggests that any change in one of these genes as a result of Hox gene over-expression may correlate with a more differentiated state of THP-1 cells.
Figure 3.3: Endogenous expression of Hox transcription factors and myeloid-specific genes in THP-1 cells. Quantitative real time PCR analysis of differentiated THP-1 cells relative to undifferentiated THP-1 cells. 

**a.** Relative expression of HOXA3, HOXC13 and HOXB7 in undifferentiated (blue) and macrophage-differentiated THP-1 cells (red). 

**b.** Relative expression of transcription factors involved in monocyte differentiation to macrophages in undifferentiated (blue) and macrophage differentiated THP-1 cells (red). All values represent expression relative to the RPL37A reference gene. Error bar = SEM, average of five qRT-PCR biological replicates.
3.2.3 Overexpression of Hoxa3 and Hoxc13 in THP-1 by nucleofection

To test the potential of Hoxa3 and Hoxc13 to promote the differentiation of monocytes, expression plasmids of Hoxa3mCherry, FlagHoxc13 or control plasmids were introduced into THP-1 cells by nucleofection. This method is based on electroporation, which creates pores in cell membranes to allow plasmids to enter the plasma membrane and ultimately the nucleus. The nucleofection method of transfection is recommended for difficult-to-transfect cell types and is believed to show high viability and less cytotoxicity than some other methods (Zeitelhofer et al., 2007). Cells to be transfected were maintained in media containing 20% human serum and transfected using a human monocyte nucleofection kit. The method used is based on the protocol recommended by (Schnoor et al., 2009) that is believed to result in high transfection efficiency and good viability of the cells.

The transfection of pmaxGFP was used as positive control to assess the success of the transfection method. GFP or mCherry positive cells were visible after 24 h but more clearly at 48 h post-transfection. The Hoxc13 expression plasmid lacks a fluorescent reporter, therefore Hoxc13 transfection efficiency was demonstrated by immunofluorescent staining using an anti-Flag antibody.

As seen in Figure 3.4, 35% of transfected cells were positive for mCherry and 60% of transfected cells were positive for Hoxa3mCherry. For GFP, around 70% of cells were positive, and 86% of cells were positive for Hoxc13 by immunofluorescent staining.

The up-regulation of Hoxa3 and Hoxc13 expression 24 h and 48 h post-nucleofection was verified using qRT-PCR that confirmed the upregulation of Hoxa3 and Hoxc13 expression post
nucleofection (Figure 3.4 c). Altogether, the images from transfected cells and qRTPCR analysis suggest successful transfection of THP-1 cells using nucleofection.

Figure 3.4: Overexpression of Hoxa3 and Hoxc13 in human monocytic cells by nucleofection. The THP-1 monocytic cell line was transfected with mCherry control, pMaxGFP control or Hoxa3mCherry and FlagHoxC13 expression constructs. a. Phase contrast and fluorescent images taken at 48 h post transfection using 10x magnification. Error bars = 100um. b. Transfection efficiency obtained as a percentage of positive cells in fluorescent images relative to the total number of cells on phase-contrast per field. c. mRNA levels of Hoxa3 or Hoxc13 in transfected THP-1 cells at 24 and 48 h post transfection compared to levels in untransfected THP-1 cells. Error bars = SEM of 3 biological replicates.
3.2.4 Effect of Hoxa3 overexpression on principle transcriptional machinery that promote the differentiation of macrophages

To evaluate whether Hoxa3 can influence the pattern of these genes in THP-1 monocytes, RNA isolated from THP-1 cells that were transfected with Hoxa3 at 24h and 48h post transfection. RNA isolated from untransfected THP-1 and mCherry transfected cells were used as a negative control. THP-1 macrophages differentiated by PMA were used as positive control to assess the changes in gene expression in response to the differentiation process.

The gene expression profile of the myeloid-specific transcription factors showed increasing levels of the SPI1 mRNA and significantly upregulate RUNX1 (p < 0.05) and MCSFr (p < 0.0005) at 24 h post-transfection with Hoxa3. At 48 h post-transfection, further up-regulation of SPI-1 levels to 1.87-fold (p = 0.08) was observed, while the RUNX1 level was down-regulated at 48 h and the levels of MCSFr remained significantly upregulated (p < 0.005) (Figure 3.5a). On the other hand, CEBPα levels did not show any significant changes in response to Hoxa3 overexpression. Altogether, the qRT-PCR data suggests that Hoxa3 can up-regulate the transcription factors that control the macrophage differentiation process including SPI1, RUNX1 and MCSFr at the mRNA levels.

To further explore the role of Hoxa3 overexpression in the macrophage differentiation process, expression levels of CSF1, which encodes M-CSF, an upstream regulator of SPI1, (Mossadegh-Keller et al., 2013), was investigated. As Figure 3.5b shows, non-transfected THP-1 cells did not show any M-CSF expression. However, in Hoxa3 transfected THP-1 cells, M-CSF levels were significantly up-regulated at 24h (p < 0.005) and at 48h (p < 0.0005). Interestingly,
the expression level of *M-CSF* in Hoxa3 transfected THP-1 cells was comparable or even higher than *M-CSF* levels in PMA induced macrophages.

Altogether, the qRT-PCR data suggests that Hoxa3 up-regulates SPI1, RUNX1 and their downstream target M-CSFr, as well dramatically upregulates *M-CSF* encoding the growth factor that initiates the differentiation of macrophages that also known as upstream regulator of SPI1. These findings suggest that Hoxa3 overexpression indeed can control the mRNA level of macrophage differentiation transcription factors.
Figure 3.5: Analysis of myeloid genes that drive the differentiation of macrophages. 

a. Relative expression of transcription factors involved in monocyte differentiation to macrophages in untransfected THP-1, mCherry transfected and Hoxa3 transfected THP-1 cells at 24 h and 48 h post transfection and in macrophage-differentiated THP-1 cells. 

b. Relative expression of the MCSF gene in untransfected THP-1, mCherry transfected and Hoxa3 transfected THP-1 cells 1 day and 2 days post transfection and in THP-1 macrophages. All values represent expression relative to the RPL37A reference gene. Error bar = SEM of three qRTPCR biological replicates *p < 0.05, **p < 0.005, ***p < 0.0005.
3.2.5 Hoxa3 overexpression affects the release of M-CSF from THP-1 cells

To investigate if the changes in expression of the *M-CSF* gene observed with Hoxa3 overexpression affect the level of the M-CSF growth factor and its release from THP-1 monocytes, ELISA using an anti-M-CSF antibody was performed. Supernatants from THP-1 cells transfected with Hoxa3 or with mCherry negative control as well from macrophage differentiated THP-1 cells as a positive control for differentiation were collected and used undiluted in this assay. As figure 3.6 suggests, Hox3 transfected THP-1 cells showed a statistically significant increase in M-CSF level in Hoxa3 transfected THP-1 compared to mCherry (p<0.05) and compared to PMA differentiated THP-1 macrophages (p<0.005). However, this change does not imply any biological relevance as it shows an increase of ~30 pg/ml. Altogether, the data suggests that THP-1 cells transfected with Hoxa3 showed significant upregulation in M-CSF level at mRNA but not at protein level.

![Figure 3.6: Analysis of M-CSF production in Hoxa3 transfected THP-1 cells.](image)

**Figure 3.6: Analysis of M-CSF production in Hoxa3 transfected THP-1 cells.** Production of M-CSF in non-transfected THP-1 cells, THP-1 cells transfected with Hoxa3 (red bar) or mCherry control (blue bar) and in PMA-differentiated THP-1 cells (black bar). Data are representative of three biological replicates. Error bars = SEM, *p < 0.05, **p < 0.005.
3.2.6 Sustained expression of Hoxa3 can upregulate macrophage differentiation markers

To further investigate the impact of Hoxa3-induced expression on the process of monocyte differentiation into macrophages, we explored macrophage differentiation markers in THP-1 cells overexpressing Hoxa3 or the mCherry control. We chose to look at the CD14, CD11b, CD11c and CD68 markers of differentiation that were previously chosen to assess the differentiation effect of PMA on THP-1 cells (Section 3.1). It is worth noting that CD11b can be controlled by the level of SPI1 in the cells (Pahl et al., 1993).

As Figure 3.7 demonstrates, Hoxa3 expression induced a general trend of upregulation in the expression levels of the macrophage differentiation markers. At the 24 h time point, the CD11b marker was significantly upregulated (p < 0.05). After culturing the cells for 48 h with Hoxa3, there was upregulation observed in CD11c expression (p = 0.08) and expression of the CD14 marker was induced at that time point (p < 0.05) as well increased trend in CD68 expression (p=0.1). Even though the expression level in CD68 in response to Hoxa3 at 48h was not significantly induced, their level was comparable to the level seen in PMA-differentiated macrophages. Therefore, Hoxa3 overexpressing monocytes show an up-regulation of the CD11b, CD14 and CD11c markers of differentiation as well as a similar level of expression of CD68 markers to levels in macrophage-differentiated THP-1 cells.
Figure 3.7: qRTPCR analysis of macrophage differentiation markers in THP-1 cells. Relative expression of macrophage differentiation markers in untransfected THP-1 monocytes, mCherry transfected, Hoxa3 transfected and in macrophage-differentiated THP-1 cells. All values represent expression relative to the RPL37A reference gene. Error bar = SEM of three qRTPCR biological replicates. *p < 0.05.
3.2.7 Effect of Hoxc13 overexpression on transcription factors and differentiation markers of monocyte/macrophage

One of the aims of this project was to investigate if homeobox genes located in the 5’ position of the cluster such Hoxc13 or Hoxa9 can have an effect similar to Hox genes located at the 3’ end of the cluster. Hoxc13 was chosen specifically to address this question as it is known to interact with proteins from the ETS transcription factor, including PU.1, and enhance target gene expression (Yamada et al., 2008). More importantly, upon activation of the CD14+ population of monocytes, HOXC13 has been shown to be significantly upregulated (Morgan and Whiting, 2008). This suggests that HOXC13 has a role in the monocyte lineage.

To investigate the effect of Hoxc13 overexpression on transcription factors, RNA was extracted from untransfected THP-1 cells, Hoxc13 transfected, mCherry control and PMA-differentiated THP-1 cells. The mRNA was reverse transcribed to cDNA to assess the expression of SPI-1, RUNX1, CEBPα, M-CSFr and M-CSF by qRTPCR. At the 24 h time point, Hoxc13 overexpression up-regulates SPI-1 and RUNX1 to levels that did not reach statistical significance. However, at 48 h post transfection, increase in SPI-1 and its upstream regulator M-CSF showed borderline significance (p=0.08), while RUNX1 and M-CSFr were significantly up-regulated (p < 0.05 and p < 0.0005, respectively) (Figure 3.8a). These data suggest that Hoxc13 transfection did not show any significant difference at 24 h but at 48 h, Hoxc13 overexpression induced the same genes that were upregulated by Hoxa3 overexpression including PU.1, RUNX1, M-CSF and M-CSFr (Figure 3.5a and Figure 3.8a).

To further verify the influence of Hoxc13 overexpression on expression of monocyte markers and to compare it to the Hoxa3 effect, the levels of CD14, CD11b, CD11c and CD68 were
assessed by qRTPCR. As Figure 3.8b demonstrates, CD11b and CD14 were up-regulated 24 h post-Hoxc13 transfection (p=0.08), whereas the levels of CD11c and CD68 did not show any observable changes. At 48 h post transfection, CD11b and CD14 were downregulated while CD11c was up-slightly upregulated, with borderline significance (p=0.07).

Our data suggest that although Hoxc13 affected the same transcription factors involved in macrophage differentiation that Hoxa3 induced, it did not show similar effect on markers of differentiation. However, it seems that more significant changes were observed as a result of Hoxa3. Therefore, we will focus in the following sections on the effect of Hoxa3 on transcription factors and monocyte differentiation, and we will present a different method of overexpression of Hoxa3 followed by gene expression and flow cytometry analysis.
Figure 3.8: qRTPCR analysis of macrophage transcription factors and differentiation markers in Hoxc13 transfected THP-1. 

**a.** Relative expression of myeloid transcription factors in THP-1 cells. Analysis done in un-transfected THP-1 monocytes, mCherry transfected, Hoxc13 transfected THP-1 monocytes and in macrophage-differentiated THP-1 cells. All values represent expression relative to the RPL37A reference gene. Error bar = SEM of three qRTPCR biological replicates. 

* p < 0.05, *** p < 0.0005.
3.2.8 Overexpression of Hoxa3 in THP-1 cells by protein transduction

The plasmid-mediated nucleofection method of delivery of transcription factors proved to be successful and gave us a clue of possible changes that can occur in THP-1 cells in response to Hoxa3 overexpression. However, for clinical applications, although gene delivery systems can be efficient, there is an accompanying risk of genetic modification due to gene integration into the host genome.

Hox proteins are able to translocate through biological membranes. This property is mediated the penetratin peptide (Derossi et al., 1994, 1996). Utilizing this unique feature of the Hox protein, Hoxb4 and Hoxa3 were delivered successfully to HSC by protein transduction using a co-culture system (Amsellem et al., 2003; Mahdipour et al., 2011).

In this chapter, we demonstrate the overexpression of Hoxa3 by protein transduction using the same principle that was used previously (Amsellem et al., 2003; Mahdipour et al., 2011), but without use of a prolonged co-culture system. Figure 3.9a illustrates the protocol that we used to generate the conditioned medium (CM) enriched with Hoxa3 or the mCherry control and the method we used to overexpress Hoxa3 in THP-1 monocytes. Hoxa3mCherry or mCherry constructs were fused to a signal peptide (SP) that is essential for the secretion of the expressed protein into the medium. 293T cells were used as host cells to generate CM enriched with Hoxa3 or the mCherry control. The expression of Hoxa3 or mCherry protein was assessed at 24 h, 48 h and 72 h post-transfection. Cells that were showing transfection efficiency higher than 70% were selected for media collection.

THP-1 cells were fed with CM enriched with Hoxa3 or mCherry to assess the effect of Hoxa3 overexpression on monocytes undergoing differentiation. Fluorescent images confirmed the
presence of Hoxa3 and mCherry in transfected 293T cells (Figure 3.9b). Phase contrast and fluorescent images from transduced THP-1 cells demonstrated the cells had normal morphology. As expected, mCherry expression in THP-1 cells transduced with Hoxa3 or with mCherry control was not detected using fluorescent images of live cells (Figure 3.9 c). This is possibly due to high levels of red background coming from the medium obscuring any positive transduction. Therefore, we conducted western blots using anti-mCherry antibody and the results confirmed the presence of Hoxa3 or mCherry within the CM, within the transfected 293T cells and also in transduced THP-1 cells. The presence of mCherry protein in THP-1 cells transduced with mCherry is unexpected and is possibly due to using whole cell lysate that cannot differentiate between proteins present within the cytoplasm and the nucleus. However, the presence of Hoxa3 but not mCherry within the nucleus compartment of the macrophage is further confirmed by immunofluorescent staining demonstrated in Chapter 4. Section 4.2.1 Figure 4.1.
Figure 3.9: Overexpression of Hoxa3 by protein transduction. 

**a.** Schematic representation of SP-Hoxa3mCherry and SP-mCherry and illustration of protein transduction protocol. 

**b.** Fluorescent image of 293T cells transfected with SP-mCherry or SP-Hoxa3mCherry. 

**c.** Bright field and fluorescent images of THP-1 cells that were treated with SP-mCherry CM, SP-Hoxa3mCherry CM or were not treated with CM. Images taken at 10x magnification (scale bar=100µm). 

**d.** Western blots for the detection of Hoxa3 or mCherry protein in the CM, in transfected 293T cells or in protein transduced THP-1 cells. mφs: macrophages.
3.2.10 Overexpression of Hoxa3 via protein transduction is a safe method

A number of studies reported the involvement of some Hox genes in acute myeloid leukemia (AML) cases by blocking the differentiation or enhancing proliferation. For example, overexpression of HOXA9, HOXA10 and HOXC6 reported in mixed lineage leukaemia cases and in AML cases (Armstrong et al., 2002). Conversely, numerous studies reported that overexpression of HOXA gene is not involved in initiation of leukemia but important in the latency of the disease (So et al., 2004). Exogenous overexpression of HOXB6 and HOXC4, in hematopoietic cells resulted in an expansion of the HSC population and early progenitors (Daga et al., 2000; Fischbach et al., 2005). Therefore, we were interested to see if overexpression of Hoxa3 by protein transduction can cause any changes to cell proliferation potential to test if administration of Hoxa3 protein into THP-1 cells can induce any leukemogenicity.

To evaluate proliferation potential, eF450 cell proliferation staining was performed. Under normal culture conditions, THP-1 cells can divide every 35 to 50 hours (Tsuchiya et al., 1980). eF450 dye can monitor cell division by binding to any protein component containing amines. As cells divide, eF450 dye will show equal distribution between the daughter cells.

A FACS analysis was performed on day 0, to assess how much of the dye was found right after labeling. Following 48 h of incubation, another measurement of the dye was taken to assess any reduction of the dye intensity corresponding to cell division. The mean fluorescent values on day 0 and day 2 measurement shows that the dye was reduced by approximately 5-folds, demonstrating active proliferation of THP-1 cells. When comparing mean fluorescent intensity...
values of Hoxa3-treated THP-1 cells to the control, there was no change in eF450 fluorescence (Figure 3.10a).

The dye dilution rate was calculated from the day 2 and day 0 measurements and values represented the mean of 2 biological repeats ± SEM (Figure 3.10b) did not show any difference between Hoxa3-treated cells and the mCherry control. In conclusion, the data from the eF450 cell proliferation dye experiments did not show any increased proliferation in THP-1 cells overexpressing Hoxa3 via protein transduction.
Figure 3.10: Effect of Hoxa3 conditioned media treatment on eF450 cell proliferation dye. THP-1 cells were labelled with eF450 dye (Materials and methods, section 2.6.3.) and assessed by flow analysis immediately (top panel: day 0) or after 2 days of incubation (bottom panel). Mean values represented below the histograms indicate fluorescent intensity showing the reduction in eF450 as a result of successive halving of the dye as the cells divides. Dye dilution rate at day 2 of incubation relative to day 0 analysis, comparing proliferation of Hoxa3 treated THP-1 cells to the mCherry control. Values are the mean of two independent biological repeats and are expressed as mean ± SEM.

To further investigate the safety aspects of overexpressing Hoxa3 by protein transduction on THP-1 monocytes, we assessed cell viability using the FAM polycaspase (FLICA) assay. This is intended to investigate whether Hoxa3’s effect on differentiation is associated with apoptosis. Under physiological conditions, hematopoietic cells undergoing apoptosis can be stimulated through activation of the cysteine-aspartic acid specific protease (caspase enzyme). The FLICA
assay measures apoptotic activity by assessing the amount of active caspase within the cells. The recognition sequence containing aspartic acid is bound to a carbonyl fluorescent reporter. A fluorescent molecule will be released once the enzyme caspase cleaves the substrate at the carboxyl end of the aspartic acid recognition sequence (Kumar, 2006).

Apoptosis assays were conducted using THP-1 cells that had been differentiated by 5 ng/ml PMA and incubated with Hoxa3, mCherry CM or not incubated with CM. Unlabelled THP-1 cells were used to set the gating for the FLICA positive cells. Interestingly, as Figure 3.11 demonstrates, THP-1 cells that had not been treated with the CM and allowed to differentiate with PMA showed a large population of FLICA-positive cells. The number of FLICA positive cells changed slightly in THP-1 cells treated with mCherry but did not increase further as a result of Hoxa3 treatment via CM for 48 h. Figure 3.11b shows representative bar graphs indicating percentages of FLICA positive cells for non-CM-treated THP-1 cells, mCherry-treated and Hoxa3-treated THP-1 cells that were differentiated with PMA for 48 h.

Taken together, these data indicate that overexpression of Hoxa3 by protein transduction did not increase the apoptosis rate beyond that of PMA-induced apoptosis. It is possible that the increased number of apoptotic cells is due to the destruction of the adherent cell sheet and the detachment of the THP-1 macrophages rather than apoptosis itself.

To determine whether the differentiation agent PMA might underlie the trend for increased apoptosis in THP-1 cells differentiating into macrophages, Hoxa3-treated THP-1 cells that were not induced by PMA were assessed for FLICA activity. In addition, THP-1 cells that were treated with different concentrations of PMA were also assessed for FLICA activity/apoptotic potential. As the data reveal, Hoxa3 treatment alone did not show any induction of apoptotic activity,
whilst treatment with Hoxa3 and 3 ng/ml PMA increased the number of apoptotic cells to approximately 70%. This number increased slightly as the PMA concentration was increased to 4 ng/ml and 5 ng/ml (Figure 3.12). Altogether, these data indicate that overexpression of Hoxa3 in undifferentiated THP-1 monocytes does not induce any apoptotic activity. Instead, it is an effect caused by adding PMA to THP-1 cells. This finding needs to be carefully considered when other conclusions are drawn from differentiating THP-1 cells.

Figure 3.11: Effect of Hoxa3 CM treatment on apoptosis of differentiating THP-1 cells. THP-1 cells were treated with Hoxa3 CM or mCherry CM for 48 h or not treated with CM, then they were labelled with FLICA according to the protocol described in Section 2.6.4. a. Flow analysis results represented by dot plots, R3 gating demonstrate the FLICA-positive population. b. Bar chart representing the percentage of FLICA positive cells. Values are the mean of two independent biological repeats and are expressed as mean ± SEM.
Figure 3.12: Effect of Hoxa3 CM treatment alone and different concentrations of PMA on apoptosis of THP-1 cells. The FLICA assay was performed on THP-1 cells that were treated with Hoxa3 CM alone or treated with Hoxa3 CM and 3 ng/ml PMA, 4 ng/ml PMA or 5 ng/ml PMA. 

a. Flow histograms represent populations that stained positive for FLICA represented by area gated under square. 

b. Bar chart represents the percentage of FLICA-positive cells comparing Hoxa3 treated THP-1 cells to Hoxa3 + PMA treated THP-1 cells. Values are the mean of two independent biological repeats and are expressed as mean ± SEM.
**3.2.11 Hoxa3 overexpression affects the gene expression profile of THP-1 monocytes differentiating into macrophages**

Gene expression analysis showed up-regulation in transcription factors of undifferentiated THP-1 monocytes as a result of Hoxa3 overexpression (Section 3.2.4). THP-1 cells also showed upregulation in macrophage differentiation marker as a result of Hoxa3 gene overexpression (Section 3.2.6). To further examine the role of Hoxa3 overexpression on THP-1 cells undergoing the differentiation to macrophages, THP-1 cells were cultured with 5 ng/ml PMA in normal growth medium and were fed with either Hoxa3 CM or mCherry control at 0 h, 24 h and 48 h. Phase contrast images were taken daily to monitor their morphology and RNA was harvested each day to assess their gene expression.

As Figure 3.13a demonstrates, there was no difference in morphology between Hoxa3-treated THP-1 monocytes and the mCherry control at the 24 h and 48 h time points. However, 72 h after initiation of differentiation, the fibre-like shape typical of macrophages was more prominent in Hoxa3 treated THP-1 monocytes. This observed change in the morphology in Hoxa3-treated PMA-stimulated macrophages demonstrates a possible further differentiation state of macrophages as a result of Hoxa3 overexpression.

Gene expression of common myeloid transcription factors or differentiation markers was assessed to investigate if any changes underlie the observed trend of changes in morphology. qRTPCR data (Figure 3.13b) revealed that M-CSF was significantly upregulated by ~2-fold on day 3 of differentiation. Surprisingly, no other major changes were observed in the pattern of expression of macrophage transcription factors. On the other hand, the gene expression profile of macrophage differentiation markers revealed that the CD68 marker of
differentiation showed a ~ 2-fold increase by day 2. This was further upregulated to ~10-fold by day 3 of differentiation (p<0.005). CD11b and CD11c were also upregulated by ~4-fold on day 3 of differentiation in Hoxa3 treated differentiated macrophages relative to the mCherry control (p<0.05; Figure 3.13c). Altogether, the phase contrast images and the gene expression profile suggests that Hoxa3 augments the PMA differentiation process of THP-1 monocytes to macrophage-like cells, possibly by increasing M-CSF transcription that upregulate CD68, CD11b and CD11c markers.
Figure 3.13: Differentiation assay with Hoxa3 overexpression. a. Phase contrast images comparing Hoxa3-treated THP-1 monocytes undergoing differentiation to mCherry control. Images display changes, taken under 10x magnification, and the area inside the red square at 72 h of differentiation, displayed at 20x. b. qRTPCR analysis of SPI-1, RUNX1, M-CSF and M-CSFr myeloid transcription factors at 24h, 48h and 72h of differentiation. Data represented as fold change of Hoxa3 treated THP-1 macrophages relative to the mCherry control. c. qRTPCR analysis of macrophage differentiation markers CD11c, CD68, CD14 and CD11b mRNA levels at 24h, 48h and 72h of differentiation. Data represented as fold change of Hoxa3-treated THP-1 macrophages relative to the mCherry control. Error bars = SEM. *p<0.05, **p<0.005
3.2.12 Protein transduction of Hoxa3 affects the release of M-CSF from differentiating macrophage-like THP-1 cells

In order to investigate if changes in M-CSF gene expression can be translated to the protein level and affect the release of M-CSF from THP-1 macrophage, ELISA was performed on supernatants from THP-1 cells transduced with Hoxa3 CM or with mCherry control at different time point. M-CSF ELISA assay revealed that Hoxa3 overexpression affected the M-CSF production from THP-1 cells and that M-CSF was gradually upregulated in Hoxa3-treated cells at 48 h and 72 h post-differentiation compared to mCherry control-treated cells but these changes did not reach statistical significance (Figure 3.14).

These data confirmed the previous finding of significant up-regulation of M-CSF expression but not protein production in undifferentiated THP-1 monocytes in response to Hoxa3. In conclusion, our data suggests that Hoxa3 can significantly increase the M-CSF at gene level but not M-CSF release from undifferentiated THP-1 monocytes as well from macrophage differentiated THP-1 cells.
Figure 3.14: Daily assessment of M-CSF production in Hoxa3 transduced THP-1 macrophages. Production of MCSF in differentiating THP-1 cells that were stimulated with PMA as well as treated with Hoxa3 (black bar) or mCherry control (white bar). Data are representative of 5 biological replicates. Error bars= SEM.
3.2.13 Effects of Hoxa3 protein transduction on macrophage surface markers of PMA stimulated THP-1 cells

After showing that Hoxa3 can modulate gene expression of macrophage differentiation markers in THP-1 monocytes and macrophages, we went on to ask if changes can occur in cell surface markers expressed on these cells corresponding to increased differentiation state of macrophages as a result of Hoxa3. To examine this, flow analysis of CD14, CD11c, CD11b and CD68 was performed daily to evaluate changes in the following cell surface markers. THP-1 cells were co-stimulated with PMA to undergo macrophage differentiation as well as stimulated with Hoxa3 or the mCherry control as indicated in Section 3.2.11. The CD14 and CD11c markers did not show any observed increase in their fluorescence beyond the isotype control (Supplementary figure 11). Therefore we were unable to evaluate these markers and focused our analysis on CD11b and CD68 markers.

In both differentiation states, THP-1 monocytes and macrophages, the cells do express the CD11b and CD68 markers. However, the intensity of fluorescence should increase more in the highly differentiated state. Therefore, we used the mean fluorescent intensity to demonstrate changes in the level of fluorescence rather than obtaining a percentage of the population that was positive for that marker.

Although MFI values, CD68 show slight upregulation in Hoxa3-treated cells on day 2 and day 3 post-differentiation relative to the mCherry-treated control (Figure 3.15a), mean values did not suggest any significant changes in CD68 in PMA differentiated Hoxa3 treated THP-1 macrophages.
The CD11b cell surface marker showed a different trend. On day 2 of differentiation, there was a clear reduction in CD11b because of Hoxa3 treatment; but this reduction in fluorescence intensity was less pronounced at day 1 and day 3 of differentiation. Taking into consideration the gene expression profile of CD11b (Figure 3.15b), it shows that CD11b mRNA was also reduced on day 2 of differentiation but went up on day 3. These variations could explain the change in morphology in PMA differentiated Hoxa3-treated THP-1 cells on day 3 (Figure 3.13a). However, none of these changes in the CD11b level were statistically significant (Figure 3.15c). Independent of Hoxa3 treatment, CD11b and CD68 MFI peaked at day 2 of differentiation but subsequently decreased by day 3 of differentiation. This expression profile of CD11b and CD68 markers was a result of the PMA differentiation protocol.
Figure 3.15: Daily assessment of CD11b and CD68 differentiation markers in PMA-stimulated THP-1 macrophages. 

a. Representative histogram plots of CD68 in THP-1 cells that were co-stimulated with PMA and mCherry CM (upper) or with PMA and Hoxa3 CM (lower). 

b. Representative histogram plots of CD11b in THP-1 cells that were co-stimulated with PMA and mCherry CM (upper) or with PMA and Hoxa3 CM (lower). Results displayed at 24 h, 48 h and 72 h post-stimulation. 

c. Scatter plot-indicating changes in mean fluorescent intensity of CD68 (left) and CD11b (right) at 24 h, 48 h and 72 h for mCherry treated (blue line) and Hoxa3-treated THP-1 macrophages (red line). Values represent mean ± SEM (n=2).
3.2.14 Effects of Hoxa3 protein transduction on macrophage surface markers of undifferentiated THP-1 monocytes

Taking into account that Hoxa3 overexpressing THP-1 monocytes showed an expression profile of differentiation markers resembling that of PMA-differentiating macrophage profile (Figure 3.7), I hypothesized that Hoxa3 protein transduction can possibly impact the surface markers of THP-1 cells in the absence of differentiating agent. To test this hypothesis, THP-1 monocytes that were not differentiated into macrophages were treated with CM-enriched with Hoxa3 or mCherry control for 48 h. FACS analysis were then performed.

The forward and side scatter of the THP-1 population that was not treated with CM is suggestive of the presence of two distinct populations, small and large-sized THP-1 cells. However, in response to adding CM to THP-1 cells (Hoxa3 or mCherry-enriched) 50% of the smaller population moved into the population with increased forward scatter, i.e., increased size. However, Hoxa3 treatment did not induced further changes to the size of the cells (Figure 3.16a).

Flow analysis of CD68 on cells treated with Hoxa3 CM shows an increased trend in the CD68 level (Figure 3.16b: p=0.19). CD68 was shifted to the right in the presence of Hoxa3 CM on THP-1 cells suggesting that Hoxa3 alone could possibly upregulate the CD68 surface marker even in the absence of the differentiation agent PMA.

To investigate if other differentiation markers can be affected by the overexpression of Hoxa3 in THP-1 monocytes, we chose to test CD11b. Our previous qRTPCR showed an up-regulation in CD11b mRNA level as a result of Hox3 over-expression by transfection (Figure 3.7) and by
protein transduction (Figure 3.13). The flow histograms demonstrate a slight shift in the CD11b mean fluorescent intensity to the right compared to mCherry treated controls (Figure 3.17a). Interestingly, this change in CD11b fluorescence was observed in the mean (p=0.07) but not in the median (p=0.19) fluorescent intensity (Figure 3.17b). A final conclusion on the role of Hoxa3 overexpression cannot be drawn due to the inability to test the CD11c and CD14 markers (Supplementary figure 12).

Our flow cytometry analysis demonstrated that the over-expression of Hoxa3 in THP-1 in CD68 and CD11b showed increase trend even in absence of the differentiating agent. This could support the potential of Hoxa3 alone, without the differentiation agent, to perhaps change surface markers in a way that is correlated with enhanced differentiation of THP-1 monocytes.
Figure 3.16: Overexpression of Hoxa3 in unstimulated THP-1 monocytes affects the CD68 surface marker. **a.** Forward and side scatter comparing the size and granularity of untreated THP-1 monocytes to Hoxa3 or mCherry treated THP-1 cells. **b. Left:** Representative histogram plots of CD68 for isotype control (dotted line), mCherry treated THP-1 cells (green) and Hoxa3 treated THP-1 cells (red) after 48 h treatment with CM. **Right:** Bar graph indicating mean fluorescent intensity and median fluorescent intensity of CD68 in mCherry treated THP-1 (blue) and Hoxa3 treated THP-1 cells (red). Values represent mean ± SEM (n=3 biological replicates).
Figure 3.17: Overexpression of Hoxa3 in unstimulated THP-1 monocytes affects the CD11b surface marker. a. Representative histogram plots of CD11b for isotype control, mCherry treated THP-1 cells and Hoxa3 treated THP-1 cells after 48 h treatment. b. Bar graph indicating increase in mean fluorescent intensity and median fluorescent intensity of CD11b in Hoxa3 treated THP-1 monocytes compared to the mCherry control. Values represent mean ± SEM. (n=3 biological replicates).
3.3 Discussion

The development and differentiation of monocyte-derived macrophages occurs continuously throughout the life of all vertebrates. Macrophages’ function have been shown to be impaired in patients and animal models of diabetes, which is associated with serious effects on neovascularisation and wound healing.

Evidence from models of inflammation whether skin wounds or other models of infection such as parasitic infections in the gut supports the fact that inflammatory macrophages are associated with markers of immaturity such as increased Ly6C levels. However, as inflammation resolved, macrophages acquire markers of prohealing phenotype together with increase in their maturation markers such as F4/80$^{hi}$ and Ly6C$^{lo}$ (Little 2014, Crane 2014).

Overexpression of Hoxa3 transcription factor into wounds of diabetic mice reduced the total number of inflammatory cells recruited to the wound, an event that was associated with a reduction in the expression of NF-κB pathway genes (Mace et al., 2009). Until now, the exact mechanism relating to these effects of Hoxa3 on myeloid cells is not clear and whether the reduced inflammatory cell population within the wound is in part due to modulating the behaviour of these cells, promoting them into more mature, less inflammatory states.

Monocytes were chosen as the desired population for this study as they can be a model to study the differentiation process into macrophages. Upon differentiation, macrophages can become an excellent model to study a change in the phenotype of these cells due to their plasticity and ability to exhibit extreme phenotypic changes of inflammatory/M1 or anti-inflammatory/M2 as well several other sub-types in between (reviewed in Martinz F.O. and
Gordon et al., 2014). We focused on this chapter on the possible role of Hoxa3 in macrophage maturation using the THP-1 monocyte cell line that is widely used to study monocytes and macrophage function.

We begin our analysis using nucleofection as a method to overexpress Hoxa3 in our system. The qRTPCR data gave us the first insight into the possible effects of Hoxa3 on the transcriptional machinery that stimulates the development of macrophages, including up-regulation of PU.1, RUNX1 and M-CSFr. However, the production of M-CSF from THP-1 monocytes and macrophages were not upregulated, which could mean that THP-1 cells were resisting this change in M-CSF protein product in response to Hoxa3 in both stages of maturation. Interestingly, even in response to PMA, THP-1 macrophages showed significant upregulation of M-CSF in mRNA level but not at protein level supporting that this could be due to the leukemic nature of these cells. Additionally, overexpression of Hoxa3 increased the mRNA level of markers known to be up-regulated in macrophages derived from THP-1 monocytes to levels comparable to PMA-stimulated THP-1 macrophages (Figure 3.5). These findings gave us an insight into the possible role of Hoxa3 overexpression on monocytes and were suggestive that Hoxa3 may promote the development of macrophages derived from THP-1 cells.

However, the electroporation of naked DNA as method to overexpress genes for clinical use is difficult to perform (Ortiz-Urda et al., 2002). The additional risk of integration of the DNA into the genome of the host (Felgner and Rhodes, 1991) adds another factor encouraging us to look for a better method to overexpress Hoxa3 transcription factors in cells. Hox proteins possess the ability to translocate across biological membranes in an energy-free, endocytosis-free
manner due to the penetratin peptide located in the third alpha helix of these proteins (Derossi et al., 1994, 1996).

Using this property, we delivered Hoxa3 into cultured THP-1 monocytes using protein transduction. This method is a potentially safe and useful method to overexpress transcription factors in order to manipulate cells for therapeutic use, as it does not involve gene manipulation or possible damage to the cell that may be caused by other methods such as viral transduction. The passive translocation of Hox proteins was previously described with Hoxa3 and Hoxb4 using a co-culture system to deliver them into HSCs (Amsellem et al., 2003; Mahdipour et al., 2011). Here, we demonstrate for the first time the successful delivery of Hoxa3 protein into the THP-1 cell line, utilizing a conditioning media enriched with Hoxa3 rather than the more complicated co-culture system approach.

Our data demonstrate that Hoxa3 protein transduction did not change the proliferation status of the cells, as demonstrated by the eF450 cell proliferation dye. A balance between apoptosis and cell proliferation is essential to maintain tissue integrity and viability, so we also investigated the apoptosis of Hoxa3-transduced undifferentiated monocytes and differentiated macrophages. Our data suggests that Hoxa3 did not cause any cell death. However, the presence of PMA is correlated with an increased number of apoptotic cells.

One drawback of our analysis using the FLICA assay is that we did not investigate if the presence of increased numbers of apoptotic cells is due to physical detachment of adherent cells rather than due to PMA induction. Park and colleagues found that PMA induced the apoptosis of gastric cell carcinoma by activating the PKC (Park et al., 2001). This supports the fact that the increased number of FLICA-positive cells was due to the PMA treatment. The
eFluor 450 cell proliferation dye and FLICA assay demonstrate that overexpression of Hoxa3 by protein transduction did not cause any leukemogenicity nor did it affect the viability of the cells and that PMA itself could cause an increase in the apoptosis of THP-1 cells.

One study that optimised the concentration of PMA for the differentiation of THP-1 cells suggested that 5 ng/ml PMA be considered the safest possible concentration, that it is associated with dramatic changes in gene expression and induced macrophages as efficiently as higher concentrations (Park et al., 2007). We compared the low PMA concentration (5 ng/ml) to the high concentration 100 ng/ml PMA and found similar changes in morphology and in markers of differentiation that were suggestive of an efficient differentiation state in both protocols (Figure 3.1 and Figure 3.2).

Culturing THP-1 cells in the presence of Hoxa3 showed increased trend in M-CSF release from monocytes undergoing the differentiation process into macrophages (Figure 3.14). This could mean that Hoxa3 induces M-CSF, stimulating macrophage development by an autocrine action. PU.1 is known as a downstream target of M-CSF that is induced and activated by overexpressing M-CSF into HSC (Mossadegh-Keller et al., 2013). Therefore, we expected an upregulation in PU.1 expression in Hoxa3-treated macrophages undergoing the differentiation process. We found that in THP-1 cells that were transfected with Hoxa3 there was, as expected, an upregulation in PU.1 levels comparable to PMA-differentiated THP-1 macrophages (Figure 3.5). This could mean that PU.1 is induced in undifferentiated monocytes as a result of Hoxa3 overexpression, but in the presence of differentiating stimuli such as PMA, it can be suggested that Hoxa3 can no longer induce any further increase in the level of PU.1
(Figure 3.13). Further investigation of Hoxa3 and Pu.1’s roles in macrophages were carried out and are discussed in the next chapter.

Eubank and colleagues found that M-CSF could increase the production of VEGF from monocytes (Eubank et al., 2003). This would be an interesting line of investigation, to address whether the increased VEGF pathways in the Hoxa3 treated wound and resulting increased angiogenic state of the cells is due to increased M-CSF released from monocytes and macrophages within the wound.

To assess if an actual change of the cell surface markers occurred in response to Hoxa3, we have used multiple flow cytometry analysis of markers of differentiation before and after the addition of PMA. Our data showed an increased trend in the macrophage marker CD68 and CD11b in THP-1 monocytes stimulated with Hoxa3. However, in PMA stimulated macrophages Hoxa3 did not induce any observable change in CD11b and CD68 markers, suggesting that there is no further enhancement of the differentiation markers when Hoxa3 is co stimulated with PMA, which correlated with the Pu.1 data. To draw a final conclusion for the differentiation state of these cells we needed more differentiation markers. The use of CD204, CD206, and CD36 as markers to differentiate the THP-1 monocytes from macrophages will be helpful to address if Hoxa3 has the potential to differentiate monocytes into macrophages.

It would be also interesting in the future to investigate if blocking antibodies against M-CSF could inhibit the differentiation of THP-1 monocytes; this could help to reveal the mechanisms behind the Hoxa3 differentiation of THP-1 macrophages.

In summary, our data suggest that Hoxa3 has the potential to promote the differentiation of THP-1 cells into macrophages, as demonstrated by increased CD68, CD11b and increased
production of M-CSF from THP-1 monocytes and macrophages (Figure 3.18). This gives us a further understanding of the role of Hoxa3 in the monocytes to macrophage differentiation, and could help us link the role of Hoxa3 in monocytic cell development to Hoxa3’s well-established role as an anti-inflammatory transcription factor that can be used therapeutically to treat excessive inflammation in diabetic wounds.

**Figure 3.18: Proposed module for Hoxa3 mediated effects of THP-1 monocytes and macrophages.** Hoxa3 upregulates M-CSF level and production from undifferentiated THP-1 monocytes, and PMA stimulated macrophages. M-CSF is known as an upstream regulator of PU.1 and induced in undifferentiated THP-1 cells. PU.1 maintain its own expression by positive feedback forward loop. Hoxa3 treated monocytes show increased CD68 differentiation marker. Hoxa3 did not upregulate Pu.1, CD11b surface marker or CD68 cytoplasmic marker beyond their level of induction by PMA.
Chapter 4: Hoxa3 promotes the maturation of mouse macrophages and inhibits their classical activation by targeting the Pu.1 transcription factor

4.1 Introduction

Wound-site macrophages, derived mostly from circulation, play a key role in nearly all phases of wound healing. Macrophage ablation within the wound affects inflammation, angiogenesis, collagen production, re-epithelisation and results in a significant delay of healing (Leibovich and Ross, 1975; Lucas et al., 2010; Mirza et al., 2009). Macrophages have the necessary plasticity to change their phenotype in response to different environmental stimuli. In the presence of pro-inflammatory cytokines, they can be induced to M1 inflammatory macrophages that are involved in innate immunity reactions (Daley et al., 2010), characterized by the release of Nos2, TNF, IL-1, IL-6 and IL-12. As inflammation becomes resolved and in the presence of anti-inflammatory cytokines, glucocorticoids and glucose modulators, macrophages can become M2-anti-inflammatory, releasing factors such as TGF-β and VEGF (Mirza et al., 2009) and characterized by their arginase production (Briken and Mosser, 2011), mannose receptor/CD206, and scavenger receptor/CD36 (Martinez et al., 2013; Stein et al., 1992). The term ‘classically activated (M1)’ or ‘alternatively activated (M2)’ was first assigned to an in vitro macrophage derived from bone marrow or peritoneal macrophages activated through LPS and INF-γ to become M1, or through IL-4/IL-13 and blockage of INF-γ to become an M2 macrophages (Gordon, 2003). Wound macrophages do not have this extreme difference in phenotypic fate as pro- versus anti-inflammatory phenotypes. They are rather described as a broad spectrum of macrophage phenotypes predominated by inflammatory
macrophages or an M1/M2-mixed polarized state during early stages of healing switching to pro-healing/anti-inflammatory macrophages in intermediate and late stages of repair (Daley et al., 2010; Lucas et al., 2010a). Diabetes induce stable changes in myeloid cell populations within the wound affecting macrophage development and maturation from the BM and influence how they infiltrate into the wound and how they respond to signals and change their phenotype (Bannon et al., 2013; Wicks et al., 2014). This results in persistent immaturity in diabetic macrophages that may also have functional relevance to defective phagocytosis, efferocytosis (removal of apoptotic cells by phagocytosis) and bacterial killing (Davidson et al., 1984; Khanna et al., 2010). Importantly, dysregulation in the phenotype of diabetic macrophage yields prolonged pro-inflammatory macrophage that remain in the wound until the late stages of healing and ineffective switching of their phenotype to prohealing macrophage (Bannon et al., 2013; Mirza and Koh, 2011). Interestingly, this dysregulation is not only present within the diabetic wound but begins in the bone marrow in their progenitor cells and in the circulation before they are recruited to the wound (Mangialardi et al., 2012; Wicks et al., 2014, 2015). These macrophages continued to exhibit dysregulated phenotypes even when cultured outside their diabetic environment (Bannon et al., 2013). This is of particular importance to the present study as it means that functional defects in diabetic macrophages are not only caused solely by signals released from the diabetic environment, but are also because of intrinsic defects with macrophages. With this connection in mind, to effectively treat these defects, therapies need to target macrophage genes to enhance their maturation and rescue their phenotype rather than attempting to block inflammatory signals coming from diabetic wound environment. As seen in Chapter 3, the enforced expression of Hoxa3
influenced maturation of macrophage-derived monocytes in a monocytic cell line. Therefore, the aim of this chapter is to explore this phenomenon further by looking at the effect of Hoxa3 on the maturation of Diabetic (Db) and non-diabetic (ndb) bone marrow-derived macrophages. Based on the finding that overexpression of Hoxa3 within wounds of db mice limit the number of leukocytes and modulate the NF-κB pathway gene expression profile (Mace et al., 2009), a hypothesis was postulated whereby Hoxa3 may be able to mediate anti-inflammatory effects on macrophage function and phenotype. To test this hypothesis, a well-characterized model of a leptin receptor deficient mouse (db/db) was used, which is a genetically diabetic mouse module with type 2 diabetes that presents with severely impaired healing (Chen et al., 1996; Hummel et al., 1966; Wetzler et al., 2000).

Using this model we showed that Hoxa3 increased the differentiation potential of diabetic macrophages possibly by targeting the macrophages specific transcription factor Spi1, an Ets family transcription factor that is required for the generation of mature macrophages (Dahl et al., 2003; DeKoter and Singh, 2000; Henkel et al., 1996). Hoxa3 also attenuated the inflammatory response in BMDM and inhibited their Nos2, TNF and IL-12 production. Attempts to understand the mechanisms behind this effect showed that Hoxa3 interestingly reduced the endogenous levels of Spi1, also known for its ability to modify NF-κB gene expression. This fosters new insights on the influence of Hoxa3 as an activator of a known downstream effector, Spi1, during maturation and how Hoxa3 can show a repressive effect on Spi1 in the presence of inflammatory pathway modulators.
4.2 Results:

4.2.1 Enforced expression of Hoxa3 promotes the differentiation of mouse macrophages

Sustained expression of Hoxa3 significantly reduced the number of leukocytes recruited to or retained in the wound (Mace et al., 2009). The mechanisms that underscore this anti-inflammatory action of Hoxa3 on each leukocyte subpopulation, including macrophages, has not yet been determined. Protein transduction of Hoxa3 in THP-1 monocytes as well in HSC exhibited that Hoxa3 can promote the differentiation of HSC into immature progenitors and monocytes into macrophages (Mahdipour et al., 2011) (Section 3.2.6, and 3.2.14). In this chapter, the objective was to test whether overexpression of Hoxa3 in primary mouse macrophages can rescue delayed maturation of diabetic macrophages. To evaluate this potential, Hoxa3 was overexpressed by protein transduction using the same protocol illustrated earlier (Chapter 3, Section 3.2.8). Hoxa3 protein was present in the CM as demonstrated by western blot results (Figure 4.1b) and the uptake of Hoxa3 protein into targeted mouse macrophages was detected using immunoprecipitation (Figure 4.1b). This was further confirmed by confocal imaging of fixed macrophages that demonstrated the red mCherry within the nucleus of mouse macrophages transduced with Hoxa3, but not within the nucleus of mouse macrophages transduced with mCherry (Figure 4.1c,d). Following protein transduction, maturation cell surface markers were assessed by flow cytometry. F4/80 (mouse macrophage-specific marker), CD11b (common myeloid marker), CD115 (receptor for Csf1) and CD14 (LPS co-receptor) were evaluated in differentiated macrophages that were treated with Hoxa3 protein. Flow histograms represented a shift in the median fluorescent
intensity (MFI) of F4/80, CD115 and CD14 but not in CD11b markers of differentiation (Figure 4.2a). F4/80 levels showed an increasing trend in Hoxa3-treated ndb populations compared to mCherry treatment (Hoxa3 MFI=27725.5 ± 13624 versus mCherry MFI=17380.8 ±13690; P=0.2) and in the diabetic population (Hoxa3 MFI=11764 ± 7681 versus mCherry MFI=8913 ±5499; P=0.1). The effect of Hoxa3 on the CD115 marker was more pronounced and the MFI showed significant upregulation of CD115 in ndb cells treated with Hoxa3 (Hoxa3 MFI=10188.5 ± 2577 vs. mCherry MFI=6213 ± 962; P=0.05), also in the diabetic population (Hoxa3 MFI= 35915 ± 11729 vs. mCherry MFI=21824 ± 5971; P<0.1). The CD14 marker is known to be downregulated as macrophages reach maturity (Ilii et al., 1984) and was reduced in ndb macrophages treated with Hoxa3 (Hoxa3 MFI 13752 ± 4045 vs. mCherry MFI 38614 ± 17275, P<0.05) as well in a Db-derived population (Hoxa3 MFI=9177 ± 1592 versus mCherry MFI=48843 ± 22817; P<0.05). To assess if changes in the cell surface marker in Hoxa3-treated macrophages is linked to changes in gene expression encoding these markers, RNA was isolated from mouse macrophages that was treated with the Hoxa3 CM or the mCherry control. Emr1 encoding F4/80, Csf1r encoding CD115, Itgam encoding CD11b and Cd14 expression was examined using qRTPCR. Relative expression of these genes were normalised to Hsp90 and H2a acting as endogenous references within the cells. As Figure 4.2b describes, Emr1 was significantly upregulated in Hoxa3-treated ndb macrophages (p<0.05) as well as in Db-derived populations (p=0.07). The Csfr1 results also correlate with changes observed through flow cytometry and significant upregulation was seen as a result of Hoxa3 treatment of the ndb population and in the Db-derived population (p<0.05). Unexpectedly, the mRNA levels of Itgam encoding CD11b was significantly upregulated in the ndb population (p<0.05) and the discrepancy between CD11b mRNA levels
and proteins were reported previously in BMDM (Bannon et al., 2013). Conversely, CD14 was downregulated as a consequence of Hoxa3 treatment, correlating with changes in the CD14 antigen. Altogether, the macrophage differentiation markers as well mRNA expression suggests that Hoxa3 promotes the differentiation of macrophages in diabetic and ndb populations and could have the potential to rescue delayed maturation defects in the diabetic population.
Figure 4.1: Protein transduction of Hoxa3<sup>mCh</sup> in mouse macrophages. a. Schematic representation of signal peptide (SP)-mCherry control and SP-Hoxa3<sup>mCh</sup> constructs. b. Western blot detection of Hoxa3<sup>mCh</sup> and mCherry (mCh) in conditioned medium (CM, left blot) and anti-mCherry immunoprecipitated lysate (lys) from 293T cells transfected with Hoxa3<sup>mCh</sup> (right blot, left lane), mouse macrophages (mφ) treated with CM containing Hoxa3<sup>mCh</sup> (middle lane, right blot), or mCherry (mCh, right lane, right blot), respectively. c. Representative confocal image of macrophages treated with mCherry CM (top panels) or Hoxa3<sup>mCh</sup> CM (low panels) showing anti-mCherry immunolocalization (left panels), DAPI staining (middle panels) and merged channels (right panels). d. Quantification of nuclear localization of mCherry and Hoxa3<sup>mCh</sup> following treatment with the respective CM for 24h (n = 40 macrophages, Error bars=SEM, ***P<0.001).
Figure 4.2: Analysis of macrophage maturation following Hoxa3 protein transduction. a. Representative histogram plots from flow cytometry analysis of F4/80, CD115, CD11b, and Cd14 in ndb (top panels) and Db-derived (bottom panels) macrophages treated with either Hoxa3 (black shading) or mCherry control (white shading) CM for 24h. b. Quantitative RT-PCR analysis of the same markers in ndb and Db-derived macrophages treated with either Hoxa3 (black bars) or mCherry control (white bars) CM for 24h ($n = 3$, error bars = SEM, *$P<0.05$).
4.2.2 The effect of Hoxa3 on principle transcription factors that promote the maturation of mouse macrophages

The Ets transcription factor, Spi1, is a master regulator of macrophage development (reviewed in Molawi and Sieweke, 2013; Valledor et al., 1998). In fact, Spi1⁻/⁻ mice cannot produce any macrophages (Mckercher et al., 1996). Importantly, the Spi1 transcription factor is known to regulate CD11b and CD115 myeloid differentiation markers (Chen et al., 1993; Li et al., 2005; Pahl et al., 1993; Zhang et al., 1996). Based on this, it was hypothesised that Hoxa3 promoted macrophage maturation either by modulating Spi1 levels in the cell or by modulating the Spi1 regulatory genes, Cebpα and/or Runx1. To test this hypothesis, RNA was isolated from BMDM that were treated with Hoxa3mCh protein or the mCherry control for 24h and 48h and assessed for the expression of Cebpα, Runx1 and Spi1 mRNA levels. The expression level of Cebpα was not changed as a result of Hoxa3 protein overexpression (Figure 4.3a) and Runx1 levels were not significantly altered as a result of Hoxa3 treatment (Figure 4.3b). However, Hoxa3 upregulated Spi1 mRNA levels in ndb-derived macrophages after 48h (P<0.05) and in the db-derived macrophages at 24h (p<0.05) and at 48h (P< 0.005; Figure 4.4a). Further investigation into whether the changes observed in Spi1 mRNA levels were consistent with Spi1/Pu.1 protein levels in ndb and db-derived macrophages took place. Interestingly, Spi1 protein levels in ndb macrophages were increased at 24h following Hoxa3mCh treatment despite lack of significant change in mRNA at this time point. However, as expected at 48h following Hoxa3mCh treatment, a ~14.8- fold increase in Spi1 protein was identified (Figure 4.4b,d). In the db-derived macrophages treated with Hoxa3mCh, Spi1 protein levels were also enhanced by ~1.5-fold at 24h and by ~7.4-fold at 48h compared to control-treated cells (Figure 4.4c,d). Levels of
Pu.1 were quantified relative to tubulin (Figure 4.4d). These data suggest that Hoxa3 may induce Spi1/Pu.1 at the transcriptional and post-transcriptional levels in naïve macrophages. This Hoxa3-mediated upregulation of Spi1/Pu.1 proteins in macrophages may be one mechanism by which Hoxa3 promotes macrophage maturation.
Figure 4.3: Analysis of Cebpα and Runx1 expression following Hoxa3\textsuperscript{mCh} protein transduction. Quantitative qRT-PCR analysis of myeloid transcription factors, Cebpα (a) and Runx1 (b), in ndb (Ndb) and diabetic (db) macrophages treated with Hoxa3\textsuperscript{mCh} (black) or the mCherry control (white). Expression assessed at 24h (left panel) and 48h post-treatment (right panel). 24h CM: 24h condition media treatment, 48h CM: 48h condition media treatment, MPs + mCherry: macrophages treated with mCherry, MPs + Hoxa3: macrophages treated with Hoxa3.
Figure 4.4: Hoxa3 protein transduction increases Spi1 mRNA and protein levels in mouse macrophages. 

**a.** Spi1 mRNA expression after 24 or 48h of control (mCherry, white bars) or Hoxa3mCh (black bars) protein transduction in ndb or Db-derived macrophages (*n* = 3, error bars = SEM, *P* < 0.05, **P** < 0.01). 

**b.** Representative western blot analysis of Spi1 levels in control-treated (mCherry) or Hoxa3mCh-treated ndb macrophages. 

**c.** Representative western blot analysis of Spi1 levels in control-treated (mCherry) or Hoxa3mCh-treated db macrophages. 

**d.** Quantification of Spi1 protein levels from western blots after 24 or 48h treatment with mCherry or Hoxa3mCh conditioned medium (CM) in macrophages from ndb or db mice. Spi1 levels were normalized to tubulin levels on the same blot (*n* = 3, error bars = SEM, *P* < 0.05, **P** < 0.01).
4.2.3 Complex formation of Hoxa3 and Pu.1 protein

Myeloid transcription factors works in an orchestrated fashion to promote monocyte lineage commitment and macrophage fate. As outlined in the preceding text, Pu.1 plays a central role in the commitment and differentiation of macrophages. With this, Pu.1’s specific action on macrophages is highly dependent on complex formation with other transcription factors. Complex formation and co-expression of Pu.1 and Runx1 precedes the activation of Csf1R (Hoogenkamp et al., 2009) whereas the Pu.1 and Cebpα antagonistic protein-protein interaction is necessary to derive cell fate choice of macrophages over granulocytes (Dahl et al., 2003). Knowing that other homeoprotein transcription factors, such as Hoxc13, can physically interact with Pu.1 protein (Yamada et al., 2009) and that Pu.1-Hoxc13 protein-protein interactions were found to promote erythrocyte maturity (Yamada et al., 2009), it was worthwhile to begin questioning if there were any protein-protein interactions involved between Hoxa3 and Pu.1. This hypothesis was tested using an in vitro module of CoIP, where Hoxa3mCh, mCherry controls and Pu.1 were co-transfected in 293T cells. Cell lysates for Hoxa3mCh and Pu.1 co-transfection or for mCherry and Pu.1 co-transfection were used for the Immunoprecipitation reaction. As the data shows, anti-Pu.1 antibodies precipitated Hoxa3 protein in Hoxa3-Pu.1 co-transfected lysate but not the mCherry in the mCherry-Pu.1 co-transfected control (Figure 4.5a). These results were validated using anti-mCherry Ab in the immunoprecipitation and anti-Pu.1 in the western blot (Figure 4.5b). Anti-mCherry Ab showed the same and precipitated Pu.1 protein in Hoxa3-Pu.1 co-transfected lysate while the mCherry-Pu.1 co-transfected lysate did not (Figure 4.5b), suggesting that Hoxa3 and Pu.1/Spi1 proteins can physically interact. To further examine if Hoxa3 and Pu.1 protein-protein interactions can
take place in mouse macrophages, lysates from BMDMs that were treated with Hoxa3 CM or mCherry control were harvested two days following CM treatment. As described in Figure 4.5c, anti-mCherry antibodies co-precipitated Hoxa3-treated mouse macrophages but not the mCherry-treated controls. An IgG control of the same species of anti-mCherry antibody was used to show any non-specific binding, and did not show any positive reaction. Both immunoprecipitation reactions in 293T cells and mouse macrophages indicate that Hoxa3 proteins can bind to Pu.1 and may be responsible for DNA binding and for upregulation of Pu.1 mRNA and protein levels, as well as maturation of macrophages.
Figure 4.5: Hoxa3 and Pu.1 proteins associate in the cell. a. Expression plasmids for Pu.1 and mCherry control (lane 2) or Pu.1 and Hoxa3<sup>mCh</sup> (lane 3) were introduced into 293T cells. Whole cell lysate was extracted 48h post-transfection and immunoprecipitated with Dsb-x labeled anti-Pu.1 Ab. Then, western blot analysis of Hoxa3<sup>mCh</sup> was performed with anti-mCherry antibodies. b. Reciprocal Hoxa3 and Pu.1 protein-protein interactions where lysate from 293T cells co-transfected with Pu.1 and mCherry control or Pu.1 and Hoxa3<sup>mCh</sup> were immunoprecipitated with Dsb-x anti-mCherry, and western blot analysis of Pu.1 was performed with anti-Pu.1 antibody. c. Association between endogenous Pu.1 from mouse macrophages and exogenous Hoxa3 from protein transduction. At day 5 of differentiation of BMDM, mouse macrophages were treated with Hoxa3<sup>mCh</sup> or mCherry control CM, lysates were extracted at day 7 of differentiation and after being treated with CM for 48h and subjected to immunoprecipitation with Dsb-x labeled anti-mcherry or IgG control.
4.2.4 Hoxa3 overexpression inhibits inflammatory phenotype in LPS-induced macrophages

Excessive inflammation is a serious condition that can complicate diabetic wound healing. Studies in diabetic mouse and rat models suggest that macrophage as well as inflammatory cell activation is aberrant and leads to a prolonged M1 inflammatory profile even in late stages of healing (Bannon et al., 2013; Miao et al., 2012; Mirza and Koh, 2011). Administration of Hoxa3 into a wound of diabetic mice reduced inflammatory cell number within the wound, an event associated with inhibition of the NF-κB gene pathway (Mace et al., 2009). Based on this, it was hypothesized that Hoxa3 anti-inflammatory action can affect macrophage activation and may reduce the inflammatory phenotype in diabetic macrophages. After confirming that Hoxa3 can rescue maturation defects in macrophages, we then tested whether Hoxa3 can also rescue defects in macrophage activation and function. To evaluate this, BMDM from db and ndb mice were treated with Hoxa3mCh or with mCherry control and were either left in a non-activated state or were classically activated by LPS and INF-γ. Initially, possible changes in gene expression in non-activated and activated macrophages treated with Hoxa3 were examined by looking at Inflammatory M1 markers, including Nos2, Tnf, Ccl2 and CD86. All inflammatory markers tested showed a general reduction as demonstrated in (Figure 4.6). Hoxa3 elicited a reduction in Nos2 mRNA level in ndb macrophages (Figure 4.6a; p = 0.07) and a significant reduction in Tnf, CD86 and Ccl2 mRNA levels in ndb and db macrophages (Figure 4.6b-d; p<0.05). These results indicate that Hoxa3 anti-inflammatory action on leukocytes within the wound can also affect ex vivo macrophages derived from db and ndb mice.
To further explore the role of Hoxa3 overexpression on classically activated macrophages, supernatants were collected from non-activated macrophages as well as from classically activated macrophages treated with Hoxa3\textsuperscript{mCh} or the mCherry control to determine the nitric oxide production by Greiss assay as well as IL-12 and TNF inflammatory cytokines by ELISA. In agreement with the qRT-PCR data, Nitric oxide release was significantly reduced in ndb and db population treated with Hoxa3 (Figure 4.7a; p<0.05). The anti-inflammatory action of Hoxa3 was further substantiated by IL-12 cytokine levels that were diminished by Hoxa3 at a rate of \(~4\) folds in ndb macrophages (p=0.06) and by \(~2\) folds in db-derived populations (Figure 4.7b; p=0.06). TNF levels were also significantly lowered in CA macrophages treated with Hoxa3 in ndb macrophages (Figure 4.7c; p<0.005). In db-derived populations, a reduction was also noted, but there was quite a variation among replicates that affected significance (Figure 4.7d). Altogether, these data suggest that Hoxa3 can limit the inflammatory action of LPS and INF-\(\gamma\) as demonstrated by reduced M1 markers as well cytokine release from macrophages. This points to that Hoxa3 not only targeting inflammatory cells recruited to the wound, but also targeting macrophage populations derived \textit{ex vivo} and lessening their inflammatory phenotype, even with the presence of strong inflammatory mediators, like LPS and INF-\(\gamma\). These results can be tested within macrophages in the wound and may have a therapeutic effect on targeting inflammatory macrophage populations within the wound.
Figure 4.6: Analysis of inflammatory markers in Hoxa3\textsuperscript{mCh} and mCherry treated macrophages from ndb and db mice. Relative expression (RE) of Nos2 (a), TNF-\(\alpha\) (b), \(Cd68\) (c) and Ccl2 (d) relative to the Hsp90 reference gene from CA m\(\phi\) treated with mCherry CM (white) or with Hoxa3\textsuperscript{mCh} CM (black). (n=5, Error bars=SEM, *p<0.05).
Figure 4.7: Analysis of inflammatory cytokines in Hoxa3^{mCh} and mCherry treated macrophages from ndb and db mice. a. Nitric oxide release from CA m\phi treated with mCherry CM (white) or with Hoxa3^{mCh} (black) b. IL-12 production from CA macrophages treated with mCherry CM (white) or with Hoxa3^{mCh} (black). c-d. TNF production from CA m\phi treated with mCherry CM (white) or with Hoxa3^{mCh} CM (black) in ndb (c) and db (d) mice. (n=3, Error bars=SEM, *p<0.05, **p<0.005).
4.2.5 Possible mechanism of Hoxa3-mediated anti-inflammatory effect on M1 macrophages

In spite of the well-established role of Spi1 transcription factors in macrophage maturation, the biological role of Spi1 in the phenotype of fully matured macrophage is not yet fully clear. The level of Spi1 gene expression can determine cell fate choice between B-lymphocytes or macrophages. Low levels of Spi1 can promote differentiation into B-lymphocytes while high levels associated with macrophage differentiation (DeKoter and Singh, 2000). This suggests a possible important link between innate and adaptive immunity by the Spi1 gene. In addition, genome wide analysis has further demonstrated the importance of Spi1 in initiating inflammation and inflammatory response in macrophages (Ghisletti et al., 2010; Natoli et al., 2011). Accordingly, conditional deletion of one allele of Spi1 attenuated inflammatory response in pulmonary macrophages (Karpurapu et al., 2011). In this model, NF-κB activation was modulated, thereby reducing IL-6, TLR4, TNF and NOS expression and secretion (Karpurapu et al., 2011). Based on these studies, and knowing that Spi1 is an identified target of Hoxa3, we wanted to find out what effect Hoxa3 might have on Spi1 expression in inflammatory macrophages.

As a first step in this analysis, the mRNA levels of Spi1 were looked at in classically activated (LPS+ INF-γ), alternatively activated (IL-4 + anti-INF-γ) and non-activated ndb and db macrophages. Interestingly, it was found that Spi1 in classically activated macrophages was significantly increased in ndb macrophages (p<0.05) as well as in the db-derived populations (p<0.005; Figure 4.8a). The upregulation in Spi1 transcript was not observed in alternatively activated macrophages or in non-activated macrophages, indicating that this was specific to
the classically activated pathway. Increased Spi1 levels as a result of inflammatory stimulus by LPS and INF-γ supports the previously described data in Spi1 deficient mice that exhibited a diminished inflammatory response (Karpurapu et al., 2011). Following this, we examined changes in Spi1 levels as a result of Hoxa3 pre-treatment in inflammatory induced macrophages from ndb and db mice. Interestingly, the qRTPCR data shows that Hoxa3 pre-treatment reduced the level of Spi1 expression in inflammatory/M1 macrophages in ndb-derived populations (p=0.08; 4.8b). This decrease was even more evident in the db-derived population (p<0.05; 4.8c).

To further explore if changes observed in Spi1 mRNA levels can be translated into Pu.1 proteins, western blots using anti-Pu.1/Spi1 antibody was performed on cell lysates taken from classically activated macrophages pre-treated with Hoxa3mCh or with mCherry controls. As anticipated, Pu.1 protein was downregulated in CA macrophages derived from ndb mice, and similar results in the db-derived population (Figure 4.8d). Quantification of the changes in the Pu.1 protein concentrations relative to tubulin shows that these changes in Pu.1 were significant in the ndb-derived macrophages (p<0.05) and even more significant in the db-derived CA macrophages (p<0.005; Figure 4.8e). As well, these changes are in agreement with Spi1 mRNA expression in CA macrophages and points to the fact that even though Hoxa3 can upregulate the Pu.1 levels in macrophages undergoing maturation (Figure 4.4), in terminally differentiated macrophages, the effect of Hoxa3 can be the opposite, particularly when macrophages are induced to the M1 phenotype by LPS and INF-γ signals.
To examine if the inhibition of Pu.1 in classically activated macrophages is the specific mechanism whereby Hoxa3 attenuates the inflammatory response from classically activated macrophages, a construct in which Pu.1 is inserted into a pCMV-Flag2 vector was used. The Flag-tagged Pu.1 facilitated its detection separately from endogenous Pu.1. BMDMs differentiated with M-CSF were subjected to overexpression of Hoxa3mCh or mCherry CM (Section 2.3.2) as well as overexpression of Flag2-Pu.1 plasmid or Flag2 empty vector (Figure 4.9a) (as demonstrated in Section 2.3.3) using Amaxa nucleofection. The co-overexpression of Hoxa3 and Pu.1 as well the control groups is arranged according to one of the following 4 groups: (Hoxa3mCh + Pu.1), (Hoxa3mCh + empty vector), (mCherry + Pu.1), and (mCherry + empty vector). The success of transfection is demonstrated by upregulation of Spi1 mRNA levels in mouse macrophages detected 24h following transfection using qRT-PCR analysis. Spi1 levels were increased by 5.4 folds upon transfection with 2µg of Pu.1 plasmid (p<0.005; Figure 4.9b). Similarly, upon transfection with 4µg of Pu.1 plasmid, there was dramatic upregulation in Spi1 mRNA concentrations by 25.8 folds (p<0.005; Figure 4.9b). More specifically, the presence of Flag-Pu.1 within macrophages was confirmed with western blots using anti-Flag. As demonstrated in Figure 4.9c, Flag protein detected at ~43 kDa was apparent with 2µg, 4µg and 5µg plasmids but showed highest protein level in the 4 µg transfected macrophages. Based on quantitative RT-PCR analysis as well western blots, transfection with 4µg of Flag2-Pu.1 is the optimum concentration for nucleofection to achieve maximum expression. Pu.1 overexpressing macrophages were classically activated with LPS and INF-γ. Then, the expression levels of Nos2, Tnf-α, IL-12 and Ccl2 being tested in macrophages co-expressed with Hoxa3 CM and the Pu.1 gene. As the qRT-PCR data suggest, while the overexpression of Pu.1
affects CA mCherry treated macrophages and significantly upregulates inflammatory markers, Nos2 (p<0.005), IL-12 (p=0.09), Tnf-α (p<0.08) and Ccl2 (p<0.05; Figure 4.10 a-d), the (Pu.1 +Hoxa3) treated macrophages failed to show similar response. This suggests that Hoxa3 inhibits Pu.1-mediated effects on inflammatory macrophages, or in other words, the effect of inflammatory transcription factor, Pu.1, was suppressed by the anti-inflammatory action of Hoxa3. This was investigated a level further by Griess reaction and ELISA assay to examine Nos production, IL-12 and TNF release from inflammatory macrophages. Nitric oxide production was upregulated in mCherry macrophages transfected with Pu.1, but their production remained elevated, even in Hoxa3-treated mφ co-transfected with Pu.1 (Figure 4.10e). TNF-α ELISA data reveal that, in mCherry-treated cells, the presence of Pu.1 did not show any notable change in TNF production. However, in Hoxa3-treated macrophages the level of TNF was significantly reduced (p<0.05; Figure 4.10f) and this inhibition was no longer apparent when Pu.1 is co-expressed with Hoxa3 (p=0.09: Figure 4.10f). Conversely, IL-12 release did not show any significant change in the presence or absence of Pu.1 (Figure 4.10g). Altogether, the qRTpCR, Greiss and ELISA data suggested that Hoxa3 can stop the inflammatory action of Pu.1 at mRNA but not at protein level. In other words, Hoxa3 may inhibit inflammatory macrophages by mechanism involves competing with inflammatory transcription factor, Pu.1. However, we cannot determine if Hoxa3 is dominant over Pu.1 because of the discrepancy between the data at mRNA and protein/cytokine production. This finding uncovers some important mechanism behind the anti-inflammatory action of Hoxa3 on macrophages that could also be applicable to anti-inflammatory action of Hoxa3 during wound healing.
Figure 4.8: Analysis of Pu.1 levels in activated and non-activated macrophages. a. Quantitative RT-PCR analysis of mφ transcription factor Spi1 in non-activated (NA; blue), classically activated (CA; red) and alternatively activated (AA; green) ndb (left panel) and db (right panel) macrophages. (n= 6, Error bar= SEM) b. Quantitative RT-PCR analysis of mφ transcription factors Spi1 in NA, CA and AA macrophages that were pre-treated with mCherry control (blue) or with Hoxa3mCh (red) in ndb macrophages. c. Quantitative RT-PCR analysis of mφ transcription factor Spi1 in NA, CA and AA macrophages that were pre-treated with mCherry control (blue) or with Hoxa3mCh (red) in db macrophages (n=6, Error bar = SEM). d. Representative western blot analysis of Pu.1 levels in classically activated mφ that were treated with Hoxa3mCh or with the mCherry control. Data represents lysates from ndb (left) and db (right) macrophages. e. Fold change of Pu.1 levels in CA Hoxa3mCh treated macrophages relative to mCherry controls and all values normalized to tubulin.
Figure 4.9: Transfection of Flag2-Pu.1 in mouse macrophages. a. Schematic representation of pCMV-Flag2-Pu.1 and pCMV-Flag2 constructs. b. Quantitative RT-PCR analysis of mφ transfected with empty vector and transfected with 2µg and 4µg of Flag-Pu.1 construct. Values are relative expression of Pu.1 gene relative to Hsp90 endogenous reference gene. Error bar= standard deviation c. Western blot detection of Flag2-Pu.1 in lysates from mouse mφ transfected with 2µg, 4µg and 5µg of pCMV-FLAG2-Pu.1 vector. Tubulin was used as the endogenous control.
Figure 4.10: Analysis of pro-inflammatory (M1) markers in CA macrophages from db mice overexpressing Hoxa3 and Pu.1 transcription factors. a-d. Relative expression (RE) of Nos2, Tnf-α, IL-12 and Ccl2 to H2A reference gene from classically activated mφ treated with mChery CM and transfected with control (left blue) or with Pu.1 (left orange) and from CA mφ treated with Hoxa3 CM and transfected with control (right blue) or with Pu.1 (right orange) (n=6, error bars=SEM). e-g. Production of nitric oxide, TNF-α and IL-12 from CA mφ treated with mCh CM and transfected with control (left blue) or with Pu.1 (left orange) or CA mφ treated with Hoxa3mCh CA and transfected with control (right blue) or Pu.1 (right orange) (n=3, error bars=SEM, *p<0.05, **p<0.005).
4.3 Discussion

In many tissues including skin and other models of inflammation, early inflammatory stages are associated with increased number of ‘less mature’ monocytes and macrophages, with shift to ‘more mature’ population as the inflammation resolve and healing progress to proliferative phase (Crane et al., 2014; Koh and DiPietro, 2011; Little et al., 2014). Studies using animal models and human samples revealed that diabetes could negatively impact inflammatory cells maturation and function. It affects how these cells mature from the BM, how they respond to signals and infiltrate to the wound and how they function within the wound (Bannon et al., 2013; Gaudreau et al., 2007; Khanna et al., 2010). Recent observations from our lab shows that macrophages isolated from the BM of diabetic mice do express much lower levels of F4/80 maturation markers and increased concentrations of CD11b (Bannon et al., 2013). This is consistent with myeloid cell persistent immaturity within diabetic mice. The latest observation from our lab presented that suppression of Cebpα levels in Gr1+ population of diabetic mice is one example of intrinsic defects in transcription factors critical for myeloid cell differentiation and cell fate selection (Wicks et al., 2015). It appears that targeting the immature population in NOD2 diabetic mouse modules using GM-CSF not only increased the population of mature dendritic cells but also rescued diabetes (Gaudreau et al., 2007). Db-derived Gr1+CD11b+ are abundant in the circulation as well as in the wound but are dysfunctional (Mahdipour et al., 2011). The maturation of diabetic Gr1+CD11b+ populations were abnormally skewed toward monocytes and their proliferation potential was also defective (Mahdipour et al., 2011). Although environmental stimuli in the diabetic wound plays a role in directing macrophages
to abnormally increased inflammatory phenotypes, evidence has shown that intrinsic factors play an equally important part in these defects. Abnormal macrophage polarisation occurring in diabetic macrophages is a problem that is based within the BM and appears even when BMDM from db mice are cultured outside their diabetic environment (Bannon et al., 2013). Importantly, Hoxa3 and Hif1 are essential pro-angiogenic transcription factors that were also found to be downregulated within wound resident cells (Mace et al., 2005, 2007). However, overexpression of Hoxa3 in the diabetic wound accelerated the overall healing process, reducing excessive inflammatory cells within the wound, and rescuing their differentiation and proliferation defects in Gr1+CD11b+ cells (Mace et al., 2005, 2009; Mahdipour et al., 2011). Based on these findings, we aimed to test the therapeutic potential of Hoxa3 on immature dysfunctional macrophages. Addressing diabetic macrophage intrinsic factors that cause maturation and polarisation defects would have better therapeutic outcomes compared to targeting abnormal inflammatory signals coming from the wound that themselves cause these defects. In Chapter 3, the applicability and safety of using protein transduction as a method to overexpress Hoxa3 in THP-1 cell lines was tested. In this Chapter, the same principle of Hoxa3 protein transduction on an ex vivo culture model of BM-derived macrophages from diabetic and ndb mice was used. BM cells were differentiated specifically to macrophages under the control of M-CSF. They were also subjected to treatment with CM overexpressing Hoxa3 during the last day of differentiation to explore the effect of Hoxa3 on macrophage maturation. The major conclusion from these analyses is that Hoxa3 elevated the differentiation of macrophages, particularly from diabetic mice possibly via increasing endogenous Spi1 in macrophages (Figure 4.11). Spi1 transcription factor that have direct role
in macrophage lineage commitment, development, maturation, and survival (Dakic et al., 2005; Henkel et al., 1996) was significantly upregulated at the mRNA and protein level. These findings support the hypothesis that Hoxa3 rescues at least some of the maturation defects in diabetic myeloid cells. Testing if Hoxa3 mediated changes in maturation markers of macrophages by targeting Pu.1 was attempted. Spi1shRNA sequence was cloned into a pLVTHM vector to knock down the expression of Spi1 in Hoxa3-induced macrophages. Cloning of Pu.1shRNA into pLVTHM vector was successful (supplementary Figure 13, 14 and 15). However, Spi1 knock down using viral transduction was attempted and due to time constrains was not completed (supplementary figure 16). Thus providing a platform for ongoing experiments to make use of the successfully cloned Pu.1shRNA in the pLVTHM plasmid. A further Spi1 target, CD115, encoded by the Csf1R gene (Zhang et al., 1994c), a receptor critical for macrophage maturation (Anderson et al., 1999), was upregulated at both the transcript and cell surface marker level, thus supporting the hypothesis of Hoxa3-mediated maturation by upregulating endogenous Spi1 levels in mouse macrophages. As Pu.1 is known to bind the human CD11b/Itgam promoter (Pahl et al., 1993), we expected that an effect on CD11b concentrations would be present as a consequence of Hoxa3 treatment. Surprisingly, the level of CD11b surface marker remained unchanged, potentially meaning that just small subset of Pu.1 targeted genes are affected by Hoxa3. During haematopoiesis, many of the biological functions mediated by Pu.1 transcription factors are achieved through protein-protein interactions and synergistic complex formation with other myeloid regulatory factors, like Cebpα, RUNX1, GATA-2 and IRF8 (Li et al., 2005; Sharf et al., 1997; Walsh et al., 2002; Zhang et al., 1996a). The promoter and enhancer element of Csf1R contains binding sites for Pu.1,
Runx1 and Cebpα and Cebpβ. The adjacent binding sites giving possible clues for physical interactions between these proteins. They also cooperate functionally to initiate the expression of Csf1R, and thereby promote the proliferation and differentiation of macrophages (Li et al., 2005; Zhang et al., 1996). The Tsuchida group have made intensive investigations into finding the physical and functional relationship between the Ets transcription factor, Pu.1, and other homeobox and non-homeobox proteins (Yamada et al., 2008, 2009; Yamamoto et al., 1999). They showed that the homeobox protein, Hoxc13, can physically interact with Pu.1 protein and that their binding affects the maturity of erythrocytes using an erythroleukemia cell line model (Yamada et al., 2008). This observation posed the further question of whether there is the possibility of protein-protein interactions between Hoxa3 and Pu.1 protein. This gap in knowledge was beginning to be addressed using a co-immunoprecipitation assay, which suggested that Hoxa3 and Pu.1 could physically interact in vivo and on the surface of mouse macrophages. Whether this interaction is essential for the upregulation of macrophage markers is yet to be determined.

4.3.1 Hoxa3 and macrophage polarisation

Diabetes majorly impacts macrophage polarisation during the healing process (Bannon et al., 2013; Khanna et al., 2010; Miao et al., 2012). While normal macrophages undergo phenotype switching from proinflammatory during the inflammatory phase to prohealing macrophages during the healing phase, diabetic wounds fail to appropriately switch to prohealing macrophages and show more restricted pro-inflammatory programme even in the late stages of repair (Bannon et al., 2013; Miao et al., 2012; Mirza and Koh, 2011). In this chapter, it was
demonstrated that sustained expression of Hoxa3 in macrophages significantly alters their inflammatory phenotypes in response to LPS and INF-γ. Hoxa3 protein transduction not only downregulates the expression of M1 markers, *Nos2, Tnf, IL-12, Ccl2* and *CD86*, but also suppresses the production of TNF, IL-12 cytokine and nitric oxide synthase activity from classically activated macrophages. This in line with previous reports demonstrating a role of Hoxa3 in inhibiting TNF levels in the wound and inhibiting inflammation (Mace et al., 2009). Neutralising TNF in the wound improves the whole the repair process and inhibits the excessive inflammatory responses of macrophages (Goren et al., 2007). Attenuating the inflammatory phenotype of ndb and diabetic macrophages means that Hoxa3 can re-program those macrophages irrespective of strong inflammatory stimuli i.e. LPS and INF-γ and possibly mediate a macrophage phenotypic shift to pro-healing/M2 macrophages. Pu.1 transcription factors have well-established roles not only in macrophage maturity, but also in promoting function in fully matured macrophages. Deletion of the Pu.1 gene affects both innate and adaptive immunity, controlling the expression of pattern recognition receptors, such as TLR2 (Haehnel et al., 2002) and TLR-4 (Rehli et al., 2000) and was found to mediate the NF-κB pathway (Karpurapu et al., 2011). Based on these findings, we questioned whither mechanism behind Hoxa3 mediated anti-inflammatory effects on macrophages involved Pu.1. This newly identified downstream target of Hoxa3 during macrophages maturation in the present work, could also be involved in the macrophage activation process.

Therefore, Pu.1 transcripts and proteins were evaluated with different activation stimuli and were found to be upregulated in classically but not alternatively activated macrophages. This was not surprising, as functional inactivation of Pu.1 in peritoneal and alveolar macrophages
attenuated the inflammatory response (Karpurapu et al., 2011). The relationship between inflammation in wound healing and Pu.1 was previously demonstrated in Pu.1 null mice. Those mice healed faster and without scar formation, believed to be the consequence of a lack of neutrophils and macrophages (Martin et al., 2003). However, recent observations show that the role of Pu.1 in inflammation is not exclusive to simply reducing the number of inflammatory cells but also by controlling inflammatory response and activating NF-κB genes, including Nos2, TNF, IL-6, and cyclooxygenase2 (COX-2) (Karpurapu et al., 2011). Interestingly, it was found that as a result of Hoxa3 treatment, classically activated diabetic macrophages showed reduced Pu.1 transcription and protein concentrations, suggesting a mechanism for a Hoxa3 anti-inflammatory response. To address that, M1 markers in classically activated Db-derived macrophages that overexpressed both Hoxa3 and Pu.1 were examined. These macrophages revealed that Hoxa3-mediated downregulation of Tnf, Nos2, Ccl2 and IL-12 expression were achieved by reducing Pu.1 levels (Figure 4.12). This interesting finding precipitated a change of perspective with respect to Hoxa3 and Pu.1 transcriptional relationships to macrophages. This means that during maturation, enforced expression of Hoxa3 acts as an activator for the Pu.1 gene that subsequently upregulates maturation markers. However, in fully matured macrophages and when Hoxa3 is expressed during LPS and INF-γ stimulation, the action of Hoxa3 on Pu.1 is reversed, acting as a suppresser and thus inhibiting inflammatory programming. In summary, it was indicated in this Chapter that the Hoxa3 transcription factor could rescue macrophage maturation and inhibit excessive M1 activation, a secondary feature that is responsible for delayed healing of diabetic wounds. It
was also indicated that a mechanistic role exists for Pu.1 in mediating macrophage maturation as well as in promoting inflammatory responses in M1 macrophages.

4.3.2 Hoxa3 as a trans-activator and trans-suppressor of Pu.1

Hoxa3 act as a “master control” transcription factor that sets the transcriptional machinery of other transcription factors into action to mediate biological function. Hoxa3 can control the same gene via activation or repression, depending on the situation and presence of other co-stimulatory molecules. A possible model that could explain the upregulation and downregulation of Pu.1 in macrophages as a result of Hoxa3 is that the function of Hoxa3 as transcriptional activator or transcriptional repressor is context dependent. While bone marrow cells undergo the process of differentiation by M-CSF, Hoxa3 mediates this process by activating the level of the differentiation-specific transcription factor, Pu.1, and the subsequent maturation markers. As the culture model becomes inflammatory, Hoxa3 can exert its anti-inflammatory function in macrophages by targeting and suppressing endogenous Pu.1 levels as well inflammatory target genes and cytokines. The mechanism behind this regulatory function and how Hox proteins can switch on and off the same target gene is largely unknown. Evidence from more than 20 years ago supports the hypothesis that Hox protein can act both as transcriptional activator and suppressor. Pinsonneault et al. (1997), hypothesised a model for Hox protein functional specificity. In which the original suppressor function Hox protein, when bound as monomer, was lost and instead became an activator when extradenticle (Exd), a Hox gene cofactor in Drosophila, binds as a heterodimer (Pinsonneault et al., 1997).
Another dimension of Hox genes transcriptional specificity is regarding the DNA consensus site that the Hox protein can bind to. In the T(T/A)AT site, the presence or absence of G in position number 5 can differentiate between an activator or a suppressor motif. Six2 gene, a downstream target of Hoxa2, contains two binding sites within its promoter, one for Hoxa2 to bind as a suppressor containing G at position number 5 and a position for an activator Hoxa2 that contains T at that site (Kutejova et al., 2008).

It is also important not to forget that many of the Hox DNA binding sites on the promoter are also associated with the Pbx/Meis binding site. A group of genes known as Hox co-factors, consist of Three-amino-acid-loop-extension (TALE) proteins (Moens and Selleri, 2006). Thus, the presence or absence of co-factors in switching off, or switching on Pu.1 must be considered.

All these theories put forward the importance of an in vivo model where the Hoxa3 and Pu.1 transcriptional and non-transcriptional relationship is well studied to fully appreciate the functional aspects of Hoxa3 on macrophages. Effectively, it would be of particular interest in the future to elucidate cooperative interactions between Hox and Ets transcription factors. More specifically, between Hoxa3 and Pu.1 which themselves play roles in differentiation and inflammation, two important functions that are deregulated in diabetes.
Figure 4.11: Hoxa3 promotes the differentiation of Db-derived BM cells into macrophages by enhancing the level of Pu.1. BM-derived cells under normal situations can be differentiated ex vivo to macrophage specific pathways by adding M-CSF into the media. However, BM-derived macrophages from diabetic mice, even after completion of differentiation, show altered expression in maturation markers, such as F4/80, CD115 and CD11b (left panel). In the presence of Hoxa3 condition medium, the expression of F4/80 and CD115 is restored (right panel), thereby rescuing defective maturation in diabetic macrophages.
Figure 4.12: Hoxa3 diminishes the classical activation of macrophages through repressing Pu.1 levels. The master regulator of macrophages, being transcription factor, Pu.1, contains binding sites for inflammatory transcription factor (NF-κB) (Natoli et al., 2011). a. Under normal conditions, Pu.1 opens the chromatin for trans-activators, like NF-κB and its downstream target, to set an inflammatory response in motion. b. In the presence of Hoxa3, known for inhibitory action of the NF-κB gene and its downstream targets, it may remove the binding of activated enhancers of NF-κB, thereby inhibiting the inflammatory genes, Nos2, TNF and Ccl2.
Chapter 5: Hoxa3 promotes the M2-prohealing macrophage phenotype by activation of the Stat6 pathway

5.1 Introduction

In humans and mammals, the wound healing process can be subdivided into three consecutive overlapping phases – inflammation, cell proliferation and tissue remodelling (Gurtner et al., 2008; Singer and Clark, 1999). The successful transition from one phase to another largely depends on differentiation and activation of cells infiltrating or residing in the wound, including macrophages (reviewed in Koh and DiPietro, 2011). Macrophages infiltrate into the wound around day two post-injury, and their number begin to rise to approximately to reach 40 fold of their starting number at around day 7 of healing (Daley et al., 2010). During the early inflammatory phase, macrophages can respond to inflammatory signals coming from apoptotic cells, inflammatory modulators and macrophage themselves in an autocrine manner to obtain an inflammatory phenotype (M1). They can actively be involved in inflammation and releasing cytokines, like TNF, IL-12 and NOS (Daley et al., 2010; Eming et al., 2007; Koh and DiPietro, 2011). As the healing progresses, with proliferation and formation of new blood vessels, macrophages have the plasticity to change their phenotype and begin to acquire markers for reparative macrophages, such as Arg1 (Briken and Mosser, 2011) and mannose and scavenger receptors (Martinez et al., 2013; Stein et al., 1992) that play crucial roles in successful healing. Essentially, this simplified version of switching from M1 during the inflammatory phase to M2 during healing phase does not really reflect macrophage polarisation in vivo, but is better described as a continuum of phenotypic changes whereby by
mixed polarised M1/M2 macrophages, predominated by M1 macrophages early in healing, switch to mainly reparative macrophages during mid-to-late stages (Bannon et al., 2013; Daley et al., 2010; Lucas et al., 2010a). The integral function of M2 macrophages in wound healing is primarily attributed to growth factor secretion, including VEGF, TGF-β, IGF and PDGF, that governs angiogenesis, myofibroblast differentiation, collagen deposition and wound closure (Leibovich and Ross, 1975; Lucas et al., 2010a; Mirza et al., 2009b). The mechanism behind the phenotypic change of macrophages from an M1 inflammatory to M2 pro-healing during skin repair has begun to be revealed only very recently. The involvement of IL-4, IL-13 and IL-4Rα in wound macrophages was largely assumed until findings in wound sections double labelled with F4/80 and IL-4Rα together with qRTPCR analysis of IL-4Rα in F4/80+CD11b+ sorted specific populations from the wound confirmed the presence of IL-4Rα positive population that increased during mid- stages of repair (Knipper et al., 2015).

In Chapter 4, it was demonstrated that Hoxa3 can suppress inflammatory macrophage markers and cytokines, suggesting a potential rescue of the chronic inflammatory phenotype of macrophages in diabetes. In this chapter, investigation of the macrophage phenotype was continued, and we looked at the effect of Hoxa3 on alternative activation of mouse macrophages using BMDM from ndb and db mice. BMDM were treated as demonstrated earlier with condition media overexpressing Hoxa3 or the mCherry control (Section 2.3.2) and were activated using IL-4 and a blockade of INF-γ (Section 2.7).

IL-4-mEDIATE JANUS KINASE - SIGNAL TRANSDECUR AND ACTIVATOR OF TRANSCRIPTION FACTOR 6 (JAK-STAT) signalling (Figure 5.1) (Goenka and Kaplan, 2011; Hebenstreit et al., 2006). Stimulation of IL-4 receptor (IL-4R) by IL-4 cytokine entails dimerisation of the α subunit of the receptor and
mediate their phosphorylation by janus kinase enzymes (JAKs). As a result, monomeric Stat6 binds to phosphorylated IL-4Rα and subsequently result in Stat6 dimerisation, phosphorylation and translocation into the nucleus where it can upregulate M2 genes, including Mrc1 and Ym1 (Figure 5.1). IL-4 knockout mouse models cannot develop prohealing/M2 macrophages (Brombacher et al., 2009). Similarly, Stat6 knockout mouse models showed more pronounced phenotypes, including loss of M2 cytokine production and inability to differentiate into M2 macrophages (Kaplan et al., 1996).

Our results showed consistent upregulation of the M2 markers of macrophages, including Ym1, Mrc1 and TGF-β, as well as increased production of TGF-β as a result of Hoxa3 CM treatment. In addition, we observed an upregulation in arginase expression and production from BM-derived as well as wound-derived macrophages. This means that Hoxa3 not only augments the alternative activation of primary macrophage cultured ex vivo, but also promotes the prohealing phenotype of macrophage within the diabetic wound during the healing process. The mechanism behind this activation demonstrates that Hoxa3 can target and phosphorylate Stat6, to be discussed further in this chapter.
Figure 5.1: IL-4/Stat6 pathway in mediating the prohealing macrophage phenotype. The M2 macrophage differentiation pathway initiated by binding of IL-4 cytokine to IL-4 receptor (IL-4R) composed of IL-4Rα and IL-4Rγ subunits. This leads to phosphorylation of the α subunits of the receptor by janus kinase (JaK) enzyme and binding of the Stat6 monomer to phosphorylated IL-4Rα. Subsequently, Stat6 will be dimerised and phosphorylated by the action of the JaK enzyme. Phosphorylated Stat6 translocates into the nucleus and acts as a transcription factor, upregulating IL-4 downstream targets, including arginase1 (Arg1), macrophage mannos receptor (Mrc1) or CD206 and Ym1.
5.2 Results

5.2.1 Hoxa3 promotes M2-prohealing macrophage polarisation in ex vivo cultured macrophages derived from the BM

M2-prohealing macrophages are a major source of growth factors, such as TGF-β and VEGF, which contribute to tissue repair and wound healing (Lucas et al., 2010a; Mirza et al., 2009b). Previous work from our group has shown that enforced expression of Hoxa3 upregulates Tgf-β in myeloid cells (Mahdipour et al., 2011) suggesting it may promote prohealing phenotype of the macrophages. Therefore, it was hypothesised that Hoxa3 may promote the M2 profile of macrophages as part of their anti-inflammatory pro-angiogenic role. To test this, we continued assessment of the Hoxa3mCh-treated macrophage profile by looking at markers of prohealing macrophages in alternatively activated and non-activated macrophages. In brief, BMDM from db and ndb mice treated with Hoxa3mCh or mCherry controls were serum starved for at least 6 hours followed by treatment with IL-4 and anti-INFγ and compared to non-activated controls.

We initially analysed whether there were any aberrant differences in the markers of prohealing macrophages as a result of Hoxa3 treatment by measuring the level of Arginase enzyme and TGF-β production. Consistent with the anti-inflammatory phenotype generated by Hoxa3, the urea levels, a product of arginase, was significantly increased (p<0.05) in ndb-derived populations and showed a trend of enhancement in the db-derived population (p=0.13; Figure 5.2a). Similarly, TGF-β production significantly rose in alternatively activated ndb- (p<0.05) and db-derived macrophages (p=0.06) along with a strong trend towards
upregulation in non-activated macrophages (p=0.09; Figure 5.2b). This suggests that Hoxa3 can upregulate arginase and TGF-β production. The data also suggest that even in the absence of IL-4 cytokine, Hoxa3 has the potential to upregulate TGF-β production in macrophages. Furthermore, this corroborates previous findings from our laboratory (Mahdipour et al., 2011). To further evaluate if Hoxa3 can indeed promote a prohealing profile in macrophages, investigation of the expression level of Arg1, TGF-β, Mrc1 and Chi3l3, or Ym1 markers of prohealing macrophages was performed. RNA was isolated from db and ndb macrophages that were treated with Hoxa3 mCh CM or mCherry controls 24h following their activation with IL-4 and anti-INF-γ, and the results were compared to a non-activated negative controls. The data indicates that there was a general trend of upregulation in Arg1, TGF-β, Mrc1 and Chi3l3 markers. Significant upregulation was noted in ndb-derived macrophages in Mrc1 (p=0.07; Figure 5.2e) and Chi3l3 (p=0.06; Figure 5.2f). In db-derived macrophages, the expression levels of Mrc1 and Chi3l3 were significantly increased (Figure 5.2e,f; p<0.05). Taken together, the data suggest that Hoxa3 augment the polarisation of M2 macrophages, and this finding is in agreement the anti-inflammatory effects of Hoxa3 on myeloid cells. This could be therapeutically relevant as Hoxa3 may have the potential to rescue dysregulated macrophages in a diabetic wound by inhibiting their excessive inflammation and mediating their switch to prohealing macrophages.
Figure 5.2: Analysis of prohealing (M2) macrophage markers in Hoxa3- or mCherry-treated BM-derived macrophages from ndb and db mice. 

a. Urea production (arginase assay) in non-activated (NA) or alternative activated (AA) macrophages from ndb or db mice treated with mCherry (white bars) or with Hoxa3mCh (black bars).

b. TGF-β production from NA or AA macrophages from ndb or db mice treated with mCherry (white bar) or Hoxa3mCh (black bar).

c-f. Relative expression (RE) of M2-prohealing markers in mouse macrophages, Arg1, Tgfβ1, Mrc1 and Chi3l3(Ym1) in ndb and db to Hsp90 (reference gene) (n = 5, *p<0.05).
5.2.2 Hoxa3 can reduce the number of Nos2+ macrophages and increase the number of Arg1+ macrophages in a diabetic wound

The findings described here and in Chapter 4 demonstrate that Hoxa3 protein transduction not only suppresses inflammatory markers and cytokines in diabetic macrophages, but also promotes the production of arginase and TGF-β, which could imply there is potential for Hoxa3 to rescue the dysregulated phenotype of diabetic macrophages. Therefore, it was desired to test whether enforced expression of Hoxa3 could influence the macrophage phenotype during the diabetic wound healing process. Previous treatment of Hoxa3 plasmid into the wound of diabetic mice showed a promising response, including an accelerated healing process, reduced excessive recruitment of leukocytes in the wound and improved angiogenesis (Mace et al., 2005, 2009; Mahdipour et al., 2011). These in vivo studies did not address the individual effects of Hoxa3 on macrophage phenotypes in a db wound. Thus, we continued the analysis by investigating the effect of Hoxa3 on macrophage phenotype using sections from Day 7 wounds of diabetic db/db mice treated with Hoxa3 or control plasmids. The effect of Hoxa3 on normal wounds was not examined because previous studies showed enforced Hoxa3 expression had no effect on the healing of normal wounds (Mace et al., 2005). Day 7 of healing represents mid stage of proliferative phase in which most macrophages acquiring the pro-healing phenotype (Daley et al., 2010). Wounds from diabetic mice had been previously treated, processed and stored by Dr Kimberly Mace as described in (Mace et al., 2009). Five µm sections were cut using a microtome, and sections were stained with haematoxylin and eosin (H&E) to select the best section that would be used for Immunofluorescent staining and to locate the area within the section that would be chosen for data analysis and quantification (Figure 5.3a). Wound
sections were triple-labelled with antibodies against Mac3/CD107b (macrophage marker), Nos2 (inflammatory marker) and Arg1 (pro-healing marker). Analyses were made in granulation tissue, adipose tissue and peri-wound dermis as portrayed in Figure 5.3a. A relatively large percentage of Mac3⁺Nos2⁺Arg1⁻ cells (pro-inflammatory) were present in control-treated diabetic wounds, specifically 52% (Figure 5.3 b,d). However, when wounding was induced with Hoxa3 treatment, this percentage went down to 31.4% (Figure 5.3b,d; p=0.1). Conversely, the percentage of Mac3⁺Nos2⁻Arg1⁺ was 24% cells in control-treated diabetic wounds but increased to 49% in Hoxa3-treated wounds (Figure 5.3b,d; p<0.05). An insignificant change was observed in the Mac3⁺Nos2⁺Arg1⁺ population demonstrating that Hoxa3 did not affect the mixed phenotype populations of the wound. Secondary-only controls show the absence of non-specific antibody binding (Figure 5.3c).

Although the immunofluorescence data does not provide a complete analysis of macrophage phenotypes within the wound, together, these data support the conclusion that Hoxa3 promotes the switch from pro-inflammatory to pro-healing macrophages within a diabetic wound.
Figure 5.3: Forced expression of Hoxa3 in vivo inhibits M1-like polarisation and promotes M2-like polarisation in wounds of db mice. a. Representative sections from day 7 wound of db mice showing anatomy of the wound, (scale bar: 1 mm). b. Immunofluorescent detection of Mac3 (mφ marker), Nos2 (pro-inflammatory marker) and Arg1 (pro-healing marker) at day 7 of the wound in db mouse treated with empty vector control (top panel) or with Hoxa3 expression plasmid (bottom panel) (scale bar: 100 μm) c. Immunofluorescence negative controls (secondary antibody for Mac3 (left), Nos2 (middle), or Arg1 (right). d. Quantification of Nos2^Arg1^, Nos2^Arg1^ or Nos2^Arg1^ cells in Immunofluorescent sections from controls (white bar) or Hoxa3 (black bar)-treated db wounds, *p<0.05, n=5. es: eschar, ep: epidermis, d: dermis, gr: granulation tissue, ad: adipose tissue, m: muscle.
5.2.3 Hoxa3 increases the production of VEGF and TGF-β in a diabetic wound

The previous analysis showed that Hoxa3 can promote the M2-like phenotype of macrophages within the wound of diabetic mice as assessed by an increased number of Arg1^+ macrophages at day 7 post-injury. However, reliance on Arg1, a hallmark of alternatively activated macrophages, to establish macrophage phenotypic switching from pro-inflammatory to prohealing is not sufficient, as arginase can also be detected in classically activated mouse macrophages and its expression is not reliable in human macrophages (Brancato and Albina, 2011). Therefore, the expression of VEGF and TGF-β from mouse macrophages within Hoxa3-treated wounds was evaluated and compared to control-treated Day 7 wounds. VEGF is a potent angiogenic factor that modulates the neovascular process during the proliferative stage of healing and promotes the formation of granulation tissue within the wound (Johnson and Wilgus, 2014; Olsson et al., 2006). TGF-β is also a critical factor for wound healing that mediates tissue repair and regeneration by controlling myofibroblast differentiation and collagen deposition along with wound closure (Tomasek et al., 2002). Evidence from macrophage depletion analyses in wounds showed that VEGF and TGF-β were the main factors that were reduced by macrophage-depleted wounds, impacting the healing process (Lucas et al., 2010a). Effectively, in diabetic wounds, as a result of impaired phenotype of macrophages, the expression of these factors was reduced which dramatically impacted the overall healing process and complicated chronic wounds (Koh and DiPietro, 2011).

Therefore, the expression of VEGF from macrophages was tested within the wound to detect if Hoxa3 treatment also affected this growth factor. Diabetic wound sections were double labelled with antibodies against the macrophage marker anti-Mac3 and the angiogenic marker
anti-VEGF, and counterstained with DAPI to permit quantification of the cells that were postively stained. VEGF is a secreted factor that is not only produced from macrophages within the wound but also secreted from skin epithelial cells and vascular endothelial cells (Nogami et al., 2007). Wound sections that were immunoflourescently stained with Mac3 and VEGF were examined for staining in granulation tissue as well in adipose tissue, both known to be an abundant source of macrophages. Sections stained only with the secondary antibody were used as a negative control for background staining of Mac3 and VEGF, neither of which exhibited any positive staining (Figure 5.4b). Immunoflourescent labeling for Mac3 did not show any significance difference in the number of macrophages in Hoxa3-treated Day 7 wounds compared to controls (Figure 5.3a). VEGF+ cells trended towards an increase in Hoxa3-treated wounds but did not reach statistical significance (Figure 5.4a). However, double labeling for VEGF and Mac3 antibodies indicated a significant elevation in the number of Mac3+VEGF+ double-labelled macrophage populations in diabetic wounds. The data here provide an evidence for Hoxa3 increasing the number of VEGF-producing macrophages without modifying the total number of macrophages in Day 7 diabetic wounds, thus changing macrophage phenotype into an angiogenic healing state.

To evaluate the level of TGF-β in wound macrophages, sections from Day 7 diabetic mouse wound that were either treated with Hoxa3 or control, immunofluorescently labeled with anti-Mac3 (macrophages marker) and anti-TGF-β (tissue regeneration /M2 macrophage marker). Secondary antibody staining for Mac3 and TGF-β showed the background level of staining for both antibodies and did not reveal any non specific staining (Figure 5.5b). As the data demonstrate, Hoxa3 expression increased the secretion of TGF-β into the wound, particularly
into granulation tissue where most angiogenesis take place (Figure 5.5a). Upon quantification, the number of TGF-β+ cells in Hoxa3 treated sections were significantly increased (Figure 5.5a,c). However, double labeling of Mac3+TGF-β+ sections did not show a substantial increase in the number of macrophages that express or secret TGF-β into the wound (Figure 5.5a,c). This could mean that Hoxa3 is increasing TGF-β secretion into the wound not necessarily the macrophage specific TGF-β but possibly from other sources such as platelets and keratinocytes. Together these findings suggest that Hoxa3 increases TGF-β secretion into the wound, a potent growth factor for angiogenesis, proliferation of extracellular matrix and collagen deposition.
Figure 5.4: Enforced expression of Hoxa3 in vivo increased VEGF+ macrophages in day 7 db wounds. a. Representative images of immunofluorescent detection of DAPI (blue), Mac3 (mφ marker; green), VEGF (angiogenesis growth factor; red) and Mac3+VEGF+ (VEGF secreted specifically by macrophages; yellow) in day 7 wounds of db mice treated with empty vector controls (top panel), Hoxa3 expression plasmids (bottom panel) images taken at 20x (scale bar: 10 μm.) b. Immunofluorescence-negative controls (secondary antibody). c. Quantification of Mac3+, VEGF+ and Mac3+VEGF+ double positive cells in IF sections from control (white bar) or Hoxa3 (black bar)-treated db wounds, *p<0.05 n=10.
Figure 5.5: Enforced expression of Hoxa3 in vivo increased TGF-β secretion in day 7 db wounds. 

a. Representative image of immunofluorescent detection of DAPI (blue), Mac3 (mφ marker; green), TGF-β (growth factor secreted by macrophages; red) and Mac3⁺TGF-β⁺ (TGF-β secreted specifically by macrophages; yellow) in day 7 wounds of db mice treated with empty vector controls (top panel) and with Hoxa3 expression plasmids (bottom panel). Images were taken at 10x (scale bar: 10 μm).

b. Immunofluorescence-negative controls (secondary antibody).

c. Quantification of Mac3⁺, TGF-β⁺ and Mac⁺TGF-β⁺ double positive cells in IF sections from control (white bar) or Hoxa3 (black bar)-treated db wounds, *p<0.05 n= 4 samples, 3 fields/samples n=12
5.2.4 Possible mechanism of M2 macrophage phenotype mediated by Hoxa3 overexpression

5.2.4.1 Impact of Hoxa3 on macrophages’ M-CSF expression

Quantitative RT-PCR analysis and ELIZA of THP-1 monocytes that were transfected with Hoxa3 plasmid or transduced with Hoxa3 CM revealed that Hoxa3 increased endogenous M-CSF levels in THP-1 monocytes (Sections 3.2.5 and 3.2.12). Based on the fact that some groups identify the M-CSF as an M2 stimulus (Figure 1.2), and on the data generated from Chapter 3, (Figure 3.5b, Figure 3.6 anf Figure 3.14), we hypothesised that increasing endogenous M-CSF level could be one mechanism whereby Hoxa3 promotes the M2-prohealing phenotype of macrophages. To evaluate this hypothesis, the level of M-CSF in activated macrophages was assessed. Macrophages from db and ndb mice that were CA, AA or non-activated were evaluated for their levels of M-CSF expression. The data shows that there was a general trend of increased expression of M-CSF in ndb macrophages than in diabetically-derived populations (Figure 5.6c). However, analysis of variance (ANOVA) statistical analysis did not confirm any statistical significance. Then, macrophages that were treated with Hoxa3^{mCh} CM or mCherry for 48h were either classically activated, alternatively activated or left in a non-activated state. Quantitative RT-PCR analysis performed on ndb and db macrophages did not demonstrate any upregulation in M-CSF levels in alternatively activated macrophages as expected. Instead, there was a slight but insignificant reduction of M-CSF in NA, CA and AA macrophages treated with Hoxa3 in ndb populations (Figure 5.6a). Similar results obtained from db-derived populations but showing a borderline significance in M-CSF level in CA and AA macrophages were seen
(Figure 5.6b). This could mean that Hoxa3 inhibits M-CSF, a known upstream regulator of Pu.1 that was also inhibited in CA macrophages, thus supporting the mechanism established previously (Figure 4.12) for the Hoxa3 anti-inflammatory effect on CA macrophages. However, these data did not suggest any mechanism for induced AA phenotype in Hoxa3-treated macrophages. Altogether the above data showing that AA macrophages utilised in these experiments: 1) are regulated by a mechanism that does not involve M-CSF; and 2) that Hoxa3 augmented the AA pathway but not by inducing M-CSF in mouse macrophages.
Figure 5.6: Analysis of M-csf levels in activated and non-activated macrophages. a. Relative expression (RE) of mφ growth factor, $M$-csf, in non-activated (NA; blue), classically activated (CA; red) and alternatively activated (AA; green) ndb (left side) and db (right side) macrophages. (n=3, error bars= SEM)  

b-c. Relative expression (RE) of mφ growth factor, $M$-csf, in NA, CA and AA macrophages that were pre-treated with the mCherry control (blue) or with Hoxa3$mCh$ (red) in ndb (b) or db (c) macrophages. c. (n= 6, Error bar= SEM)
5.2.4.2 Impact of Hoxa3 on the IL-4/Stat6 pathway in alternatively activated macrophages treated with Hoxa3

Although IL-4 and IL-13 are the principal stimulators of the alternatively activated macrophages through IL-4Rα, the presence of IL-4Rα in wound macrophages was assumed until recently proven in IL4Rα⁻/⁻ mice and IL4Rα⁻/⁻ myeloid cells population (Knipper et al., 2015). Increased IL-4Rα was evident in F4/80⁺CD11b⁺ macrophages that appeared in mid-stage of repair, a stage whereby most macrophages acquire the prohealing phenotype (Knipper et al., 2015). Therefore, the objective was to uncover the mechanism of Hoxa3 mediated-M2-phenotype by looking at their influence on IL-4Rα in the different states of activation. To investigate this question, firstly we assessed the response of IL-4Rα in different activation states in macrophages irrespective of their treatment with CM from Hoxa3 or mCherry. Macrophages from db and ndb mice were classically activated, alternatively activated or non-activated and were evaluated for their level of IL-4Rα expression. The analysis of IL-4Rα expression although suggest a difference in the trend of IL-4Rα among the three activation status, analysis of variance (ANNOVA) statistical analysis did not reveal any statistical significance (Figure 5.7a). Then BM derived macrophages that were pre-treated with Hoxa3mCh or mCherry control CM for 48h were classically activated, alternatively activated or non-activated to determine influence of Hoxa3 on IL4Rα marker during different stages of activation. Quantitative RT-PCR performed on ndb and db mice showed that Hoxa3 upregulates the IL-4Rα expression in the AA macrophages of ndb mice (Figure 5.7; p=0.08). Interestingly, this upregulation was also evident in the classically activated but not in non-activated ones. (Figure 5.7b). Similar trends were obtained in the db macrophages (Figure
Altogether, the IL-4Rα mRNA data suggest that Hoxa3 may influence the alternative activation pathway of macrophage by increasing IL-4Rα levels.

We next wanted to evaluate if Stat6, a downstream target of IL-4Rα was influenced by overexpression of Hoxa3 in alternatively activated mouse macrophages. Analysis of our pre-existing microarray data comparing Hoxa3 treated wounds to controls at Day 4 of healing showed that Stat6 was among those target genes significantly upregulated as a result of Hoxa3 overexpression (Figure 5.7a). As myeloid cells represent a large proportion of the cell population in wounds at this time point, this makes the case for Stat6 being a downstream target of Hoxa3 in wound macrophages. To investigate if Hoxa3 promotes signalling through the Stat6 pathway, to promote the M2 phenotype, the transcript and protein levels of Stat6 was examined following IL-4 stimulation and compared to untreated controls. Hoxa3mCh-treated macrophages and controls were induced with IL-4 and anti-INFγ as described earlier (Section 2.7) and RNA was harvested 24h following activation to assess the level of Stat6 transcription in response to Hoxa3. As figure 5.8b suggests, Stat6 levels were significantly elevated in ndb macrophages in response to Hoxa3 treatment (p<0.05) as well in the db-derived populations (p=0.09), indicating that Hoxa3 not only upregulates Stat6 in the wound, but also in alternatively activated murine macrophages. As demonstrated in the IL-4 signalling pathway (illustrated in Figure 5.1), only phosphorylated Stat6 protein can translocate into the nucleus and act as a transcription factor and upregulate M2 target genes. Therefore, we wished to test whether Hoxa3 could influence Stat6 phosphorylation (pStat6), as well as total Stat6 protein levels in alternatively activated mouse macrophages. Based on the transient nature of a phosphorylation event, protein lysates from macrophages that were stimulated
with IL-4 were harvested 15 minutes after stimulation. The detection of Stat6 and pStat6 (Tyr461) were determined through western blot analysis. Hoxa3-treated macrophages also showed an increase in total Stat6 protein in ndb and db macrophages (Figure 5.8c). Importantly, the data highlights an influence of Hoxa3 on Stat6 phosphorylation and demonstrates that Hoxa3 can increase pStat6 concentrations in ndb- and db-derived macrophages stimulated with IL-4. The data from western blotting correlates positively with Stat6 mRNA levels and with data from wound-derived Stat6 concentrations, confirming Stat6 as one of Hoxa3’s direct or indirect downstream targets. Altogether, the data provide evidence for Hoxa3 upregulating IL-4Rα expression, its downstream target Stat6 at transcriptional and post-transcriptional level, and Stat6 phosphorylation, a further step in alternative pathway of macrophage activation.
Figure 5.7: Analysis of IL-4Rα level in activated and non-activated macrophages. a. Quantitative RT-PCR analysis of macrophages receptor IL-4Rα in NA (blue), CA (red) and AA (green) macrophages from ndb (left panel) or db (right panel). One-way ANNOVA represent no statistical significance b-c. Quantitative RT-PCR analysis of macrophages receptor IL-4Rα in NA (left panel), CA (middle panel) and AA (right panel) macrophages that were pre-treated with mCherry control (blue) or with Hoxa3mCh (red) in ndb (b) or db (c) macrophages (n= 3, Error bar= SEM).
Figure 5.8: Contribution of Hoxa3 to Stat6/pStat6 M2 activation. 

a. Fold change of Stat6 in Hoxa3- or control-treated whole wounds based on microarray database (n=3, error bars= SEM, *p<0.05). 

b. Fold change of Stat6 in macrophages stimulated with 20ng/ml IL-4 (right) normalised to Stat6 levels in macrophages not stimulated with IL-4 (left). H2α and Hsp90 were used as endogenous housekeeping genes and values represent mean ± SEM of n=5 biological replicates (*p<0.05, n=5, error bars= SEM). 

c. Western blots of total protein lysate from macrophages pre-treated with Hoxa3 or mCherry controls that were stimulated with IL-4 cytokines for 15 minutes or non-IL-4-stimulated mφs. Results reveal the level of total Stat6 protein (top lane), phosphorylated Stat6 (middle lane) or tubulin loading control (bottom lane). Data were obtained from ndb (left) or db macrophages (right).
5.3 Discussion

The alternatively activated macrophages often referred to as “wound-healing macrophages”, can mediate critical regenerative processes, including neovascularization and wound closure. Wound healing macrophages are identified by their expression of factors that mediate angiogenesis and tissue repair, such as arginase, Ym1, TGF-β, mannose receptor/CD206, as well as VEGF (Gordon, 2003; Martinez et al., 2009a; Raes et al., 2002). Under pathological conditions, such as diabetes, the cytokines released by the cells infiltrating and residing in the wound together with intrinsic factors from myeloid cells themselves are dysregulated, affecting macrophage phenotype within the wounds. Macrophages then continue to produce inflammatory factors along with TNF, IL-12 and NOS even at Day 10 of healing (Koh and DiPietro, 2011), while VEGF and TGF-β growth factor production are diminished and insufficient to promote angiogenesis and wound closure efficiently (Koh and DiPietro, 2011).

Hoxa3 is an intrinsic factor that is expressed within hair follicles, endothelial progenitor cells and normal wound microvessels and suppressed in diabetic wounds (Mace et al., 2005). Enforced expression of Hoxa3 in wounds of diabetic mice has been observed to reduces the number of inflammatory cells infiltrating the diabetic wound, improving angiogenesis and accelerating healing (Mace et al., 2005, 2009). Here, it is demonstrated that Hoxa3 administered by protein transduction in ex vivo cultured macrophages and by gene transfer in in vivo wounds promotes the M2/pro-healing phenotype of macrophages. Hoxa3 increased TGF-β and arginase transcriptional and post-transcriptional levels in macrophages that...
cultured from diabetic and non-diabetic mouse BM cells. More importantly, this work shows arginase, VEGF and TGF-β are increased at Day 7 of diabetic wound healing, suggesting the functional importance of Hoxa3 in elevating these deficient factors in the wounds of diabetic mice and the potential of Hoxa3 in treating dysregulated macrophage phenotypes. The importance of arginase comes from its role in producing collagen from arginine (Munder, 2009). Arginase competes with inducible NOS to produce urea and ornithine from arginine. Ornithine also gives rise to proline, a precursor in collagen formation mediating tissue repair (Albina et al., 1990). In the absence of arginase, arginine can be degraded by iNOS to nitric oxides, a product that mediates inflammatory response and microbial elimination (illustrated in Figure 5.9) (Rath et al., 2014). One limitation of this study is that arginase is not highly expressed in human macrophages; rather it was expressed and released from human neutrophils. This is suggestive of the fact that neutrophils may replace the role of macrophages in regards to arginase production in human wounds (Munder et al., 2005).
Figure 5.9: The metabolism of arginine by arginase and iNOS enzymes. Inflammatory and pro-healing macrophages, respectively, can control the balance between iNOS and Arg1 enzymes within the wound. During the early stages of healing, inflammatory signals, such as LPS, INF-γ or TNF from inflammatory macrophages can mediate the release of iNOS that catabolises arginine to nitric oxide (NO). During the intermediate or later stages of healing and in the absence of inflammatory mediators, pro-healing macrophages can participate in arginine metabolism into urea and ornithine. The latter gives rise to proline, a substrate that can be involved in collagen production and tissue repair for mediating wound closure. This process is modulated by negative feedback inhibition of Arg1 by the iNos enzyme while iNos negatively regulated by increased Arg1 concentrations.
5.3.1 Administration of M2-macrophage into wounds as mean to improve healing

The use of macrophage ablation mouse models clearly demonstrated the role of macrophages during wound healing process as their absence affected angiogenesis, reduced tissue regeneration and collagen deposition (Goren et al., 2009a; Lucas et al., 2010; Mirza et al., 2009). On one hand, the elimination of macrophages is detrimental to healing, but the dysregulation of macrophages accompanied by abundance in their numbers in diabetic wound also derails the healing process. One would expect that if db wounds are deficient in pro-healing/M2 polarised macrophages, then administration of these macrophages into wounds could rescue their diabetic phenotype. Surprisingly, a study published in the last few years has described that the addition of M2 polarised macrophages early in the healing did not improve the healing of diabetic wounds, and rather prompted a delayed healing response and negatively impacting wound closure (Jetten et al., 2014b). If from one perspective, macrophage deficiency is detrimental to healing, it is puzzling then how the addition of M2 macrophages can delay the healing response. The author argued that these macrophages retain their alternative activation phenotype within the wound until day 15 after their administration by measuring Arg1 and Ym1 markers, and confirmed that they did not adopt the inflammatory phenotype of diabetic wounds. However, these exogenously administered macrophages seemed to be non-functional as the cytokine level did not differ in mice treated with M2 macrophages (Jetten et al., 2014b). This could be explained by the fact that adding pro-healing macrophages too early in the healing process could retard the normal rhythm of healing and inhibit the standard inflammatory process from taking place. Therefore, preventing initial inflammatory responses that are necessary to initiate next phase of healing
could further retard phenotypic changes in macrophages within the diabetic wound. Along these lines, attempts to rescue the absence of pro-healing macrophages should not be mediated by adding these deficient cells that could exacerbate chronic wounds. Ultimately, to overcome the dysregulated macrophage phenotype, the excessive inflammatory macrophage phenotype must be inhibited, and the pro-healing macrophage phenotype must be augmented. The present study has supplied evidence that Hoxa3 is a suitable candidate to treat dysregulated macrophage phenotypes and that it seemed to overcome this obstacle by acting as an anti-inflammatory transcription factor as well as a pro-angiogenic pro-healing factor. Sustained expression of Hoxa3 in diabetic wounds downregulates Nos2+ macrophages and upregulates the pre-existing pro-healing phenotype in diabetic wounds as indicated by Arg1+Mac3+ double-positive populations. This function fulfilled by Hoxa3 transcription factors combats both forms of dysregulation of diabetic macrophage phenotypes.

5.3.2 The effect of Hoxa3 on VEGF and TGF-β

In the study presented here, Hoxa3 gene transfer into diabetic wound was shown to increase VEGF production from macrophages by day 7 of healing. This finding is in agreement with the angiogenic potential of Hoxa3 in the literature. Hoxa3 transgenes increased endothelial cell migration and angiogenesis as potent as VEGF-induced angiogenesis in immortalised HMEC in vitro and in chick chorioallantoic membrane (CAM) in vivo (Mace et al., 2005). Microarray analysis of whole wound reviled that Hoxa3 upregulates VEGF signalling pathway downstream target genes (Mace et al., 2009). VEGF is well known as a powerful angiogenic factor that promotes neovessel formation by controlling the migration and recruitment of endothelial
cells as well keratinocytes into granulation tissue (Eming et al., 2007; Li et al., 2006). Keratinocytes are also known as a source of VEGF production, though VEGF induction in keratinocytes is a process controlled by macrophages and TGF-β (Frank et al., 1995). VEGF promotes wound vascularisation by several mechanisms that directly or indirectly involve endothelial cells and their precursors (Eming et al., 2007). The increased migration of keratinocytes and endothelial cells were also found in Hoxa3-treated diabetic wounds through mechanisms involving upregulation of uroplasminogen activator receptor (uPAR) and matrix metalloproteinase protein 14 (MMP14) (Mace et al., 2005). Thus, it is likely that Hoxa3 functions through a mechanism involving MMP14 and uPAR, mediating cell migration and thereby increasing VEGF production and angiogenesis, though this is yet to be determined.

Hoxa3 protein transduction of BMDM enhances TGF-β mRNA expression and the release of its protein product. TGF-β is a growth factor that is produced in many cells within the wound, including platelets and epidermal cells, though macrophages are the main source of TGF-β within wounds (reviewed in Rodero and Khosrotehrani, 2010). TGF-β released into the wound acts centrally in collagen deposition by favouring the differentiation of fibroblasts into myofibroblasts (Lijnen et al., 2003). In addition, TGF-β can play several positive roles in tissue regeneration, such as promoting angiogenesis (reviewed in Penn et al., 2012). Secreted TGF-β released in inactive form that is coupled with latent associated peptide (LAP) or with latent TGF-β binding protein (LTBP) that permits binding to the extracellular matrix (Khalil, 1999). Physiologically, mature TGF-β can be separated from its bound protein and activated through several mechanisms, such as proteolysis by metalloproteinases (Khalil, 1999). Here, the problem of the TGF-β-LAP complex overcame by acidification followed by neutralisation of the
culture supernatant prior to detection by ELISA (Section 2.8.2). However, the anti-TGF-β Ab used for detection within the wound can detect both monomeric and heterodimer versions - it cannot differentiate between active or inactive forms of TGF-β within the wound. We previously found that diabetic Gr1^+CD11b^+ populations of leukocytes present with deficiency in TGF-β levels (Mahdipour et al., 2011) likely one of the causative factors for dysregulated macrophage phenotypes. However, low TGF-β concentrations were rescued by Hoxa3 overexpression in Gr1^+CD11b^+ population (Mahdipour et al., 2011) paralleling the data presented in this work. The importance of TGF-β as a growth factor that mediates wound closure is also implicated in the regulation of the macrophage phenotype. TGF-β is the main stimulus that drives the differentiation of macrophage subpopulations, identified, as mentioned earlier, as M2c or TGF-β-secreting macrophages, also called deactivated macrophages (Martinez et al., 2009a). M2c macrophages also function in the healing response, such as through resolving inflammation and extracellular matrix remodelling (Lu et al., 2013). TGF-β could be involved in the formation of this macrophage subtype by autocrine or paracrine means, and maybe associated with a mechanism of Hoxa3 anti-inflammatory action on macrophages and leukocytes in diabetic wounds. This hypothesis still necessitates investigation in order to link the suppression of inflammatory cell influx to increased TGF-β production in Hoxa3 treated db wounds.
5.3.3 Mechanism of Hoxa3 associated effects on macrophage polarisation to the M2 phenotype

In attempts to resolve the mechanism underlying Hoxa3-mediated increases in M2 target genes, including Arg1, Ym1 and Mrc1, it is worthwhile to explore two pathways. Joshi and colleagues (2014) and others found that M-CSF differentiated macrophages show M2-like gene expression and that GM-CSF-derived macrophages drive their M1-like phenotype (Fleetwood et al., 2007; Joshi et al., 2014; Lacey et al., 2012). Therefore we put forward the hypothesis that Hoxa3 induction of M2 phenotype in mouse macrophages is possibly mediated through increasing endogenous M-CSF and secretion. An idea proposed based on the data generated from THP-1 cell lines whereby Hoxa3 upregulates the expression and release of M-CSF from THP-1 monocytes thereby promoting their differentiation. However, this does not seem to be the case for primary mouse macrophages, as the data discussed here does not signify any upregulation in M-CSF in Hoxa3-treated mice macrophages as far as was observed. Such changes were not even detected in response to alternative activation. In the recent experimental guidelines addressing the problem of misuse of terminology and the standards needed to be followed regarding macrophage activation, one of the suggestions was to avoid the use of M1 and M2 macrophage nomenclature from GM-CSF differentiated and M-CSF differentiated macrophages as a consequence of the lack of in vivo evidence that those macrophages truly represent the two phenotypic forms (Murray et al., 2014). Therefore, we investigated the other well characterised and well described pathway responsible for moderating alternative macrophage activation involving signalling through the IL-4 receptor to Stat6 (Hebenstreit et al., 2006; Maier et al., 2012). The importance of IL-4rα not only as
being the receptor that bound IL-4 and IL-13 to promote M2 macrophages but as marker involved in fibrotic tissue formation by targeting Relmα (Knipper et al., 2015). In this project, qRT-PCR analysis showed increased trend of IL-4Rα transcripts. However, we could not evaluate the level of IL-4Rα surface marker as the (anti-CD124/IL-4Rα) antibody did not show any increased fluoresce beyond the isotype control (Supplementary figure 17). Notably, from the current data, Hoxa3 has been demonstrated to stimulate the pro-healing phenotype in macrophages in vitro possibly through modulation of an IL-4rα downstream target, Stat6. IL-4-stimulated macrophages that were also treated with Hoxa3 exhibit an increase not only in Stat6 transcription but also in phosphorylated Stat6. This therefore confirms the involvement of Hoxa3 in the IL-4/Stat6 signalling pathway for mediating the upregulation of Stat6 targets, including Mrc1, Ym1 and Arg1. The upregulation of the Stat6 gene by Hoxa3 has been previously observed in microarray data generated from Hoxa3-treated diabetic wounds (Mace et al., 2009). It would be of particular importance in the future to evaluate Stat6 knockdowns in wound macrophages and test if Hoxa3 no longer mediates the M2 phenotype.

The upregulation of pStat6 in Hoxa3-treated BM-derived macrophages opens up the possibility to another level of regulation by Hox genes, including non-transcriptional activity. The non-transcriptional effects of homeoproteins acting on kinase pathways have already been described for the intestinal-specific gene, Cdx2. Gue et al., (2012) recognised that Cdx2 interferes with cyclin-dependant kinase through interactions with β-catenine and revealed that regions within the homeoprotein responsible for such interactions are located upstream to the homeodomain structure (Guo et al., 2010). Prochantz et al. (2004) proposed that homeoproteins could act as signalling molecules. The identification of highly conserved
sequences within the DNA binding domain of the homeoprotein structure, “the pentratin peptide”, that modulates the secretion and internalisation of homeoproteins precedes and complements the hypothesis that homeoproteins can have non-transcriptional effects via behaving as signalling molecules (Joliot and Prochiantz, 2004; Prochiantz and Joliot, 2003).

Engrailed protein (En), a non-Hox homeoprotein in Drosophila, is an example of a homeoprotein that has intracellular as well as extracellular effects on decapentaplegic (Dpp), a downstream target of En (Layalle et al., 2011). The transcriptional relation between homeobox and Stat transcription factors has been demonstrated previously in a model for breast cancer. Enforced expression of Hoxa1 upregulated the transcriptional activity of Stat3 and Stat5, both responsible for cell survival and growth, increasing oncogenicity of human immortalised mammary epithelial cells (Mohankumar et al., 2008). Together, this evidence provides support for the notion that the interaction between Stat and homeoproteins can synergistically affect multiple cell types to control cell differentiation and/or phenotype.

To summarise, the data presented in this chapter suggests that Hoxa3 has the potential to promote alternative activation of macrophages in culture and increase the markers of pro-healing macrophage in the wounds. Thus, this provides substantial evidence that Hoxa3 can potentially treat dysregulated macrophage phenotypes in diabetic wounds. These changes are mediated by upregulation of Stat6 transcription and phosphorylation, a downstream target of the IL-4 receptor, thus providing a further understanding of the mechanisms that augment the prohealing phenotype of macrophages. In essence, these findings provided a better picture of the role of Hoxa3 in rescuing the diabetic phenotype of macrophages by inhibiting the excessive inflammatory phenotype and augmenting the deficient pro-healing phenotype. This
enabled us to learn about the specific effects of Hoxa3 upon macrophage subpopulations of leukocytes in addition to the previously recognised role of Hoxa3 in inhibiting all leukocytes within the wound. Hoxa3 as healing factor plays different roles, targeting multiple downstream pathways to correct the deregulation in leukocytes that contribute to chronic wound pathology. It would be beneficial in the future to use a standardised amount of purified Hoxa3 protein in the wounds to address whether it can be safely applied to rescue diabetic macrophage phenotypes. Experiments with purification of Hoxa3 proteins in our laboratory have begun to take place and, in the future, will mostly utilise Hoxa3-treated wounds with purified proteins in order to fully explore the therapeutic potential of Hoxa3 in healing and in regulating hematopoietic cell populations in wounds.
Chapter 6: Final Discussion

6.1 Overview

This thesis focused on studying the influence of Hoxa3 on macrophage differentiation and polarisation and delineated how Hoxa3 signals through monocyte/macrophage transcription factors, growth factors and signalling pathways to mediate the differentiation of M2 pro-healing macrophages. Identifying these changes that Hoxa3 can cause in macrophage can rescue dysregulation in myeloid cell phenotype including macrophage immaturity and persistent inflammatory phenotype associated with diabetes. In order to do so, I established a method to overexpress Hoxa3 protein in human monocytes and in mouse macrophages. This was followed by assessing whether Hoxa3 can influence the differentiation of monocyte-derived macrophages and BMDM. Using protein transduction of Hoxa3-enriched conditioned medium allowed me to overexpress Hoxa3 in monocytes or BMDM in culture without genetic modification.

Using the Hoxa3 over-expression system that we developed, I found that Hoxa3 promotes macrophage differentiation to the pro-healing state. Hoxa3 reduced the levels of the inflammatory markers Nos2, Ccl2, TNF and IL-12 and increased the level of the pro-healing markers Arg1, TGF-β and CD206. Furthermore, I confirmed that Stat6, a downstream effector in the IL-4 signalling pathway, was upregulated and underwent increased phosphorylation in Hoxa3-treated macrophages. Thus, these findings outline the mechanism whereby Hoxa3 mediates this M2 macrophage phenotype.
Hoxa3 did not affect macrophage proliferation or apoptosis. Hox proteins are known to be required for the proliferation and differentiation of haematopoietic cells (Argiropoulos and Humphries, 2007).

However, their uncontrolled expression can be a predisposing factor for establishing some types of leukaemia (Alharbi et al., 2013; Lawrence et al., 1996). In this thesis, I showed that there was no change in the proliferation of Hoxa3-treated THP-1 cells. I also found that the apoptosis that occurred in Hoxa3-treated cells was likely due to PMA co-stimulated with Hoxa3 that mediated the adherence and differentiation of THP-1 monocytes into macrophage-like cells. Other members of our group investigated cell survival and colony-forming assays to determine the safety and efficacy of applying Hoxa3-enriched conditioned medium on human monocytes isolated from volunteers and did not observe any adverse effects of using Hoxa3-enriched medium on the cells. Therefore, our work confirms the safety and applicability of using protein transduction to therapeutically treat dysregulated in myeloid cell populations.

To test macrophage differentiation and phenotype, primary BM macrophages derived from ndb and db mice enabled the observation of a better response regarding cell differentiation, and allowed the generation of mature macrophages that could be classically or alternatively activated to assess any changes in markers or cytokines corresponding to the inhibition or enhancement of each pathway. Investigations carried out on THP-1 monocytes provided a clue to which downstream target may be affected and revealed that Hoxa3 can increase the expression of monocyte differentiation markers while increasing M-CSF production. Nevertheless, studies on primary mouse macrophages allowed us to identify the potential role
of Hoxa3 overexpression in promoting the differentiation of diabetic macrophages. In addition, we identified Pu.1 as a Hoxa3 downstream target as well as a binding partner that possibly plays a direct role in the differentiation potential of Hoxa3.

Lastly, analyses of wounds from diabetic mice showed that Hoxa3 also promoted macrophages within the wound to switch their phenotype from persistent proinflammatory to pro-healing macrophages that produced more Arg1 and VEGF. Enhancement of the Arg1 and VEGF production from wound macrophages together with increased total TGF-β secretion within the wounds is a promising finding for the future of diabetic wound healing. These findings provide mechanistic insight into the accelerated wound closure that was previously detected in Hoxa3-treated diabetic wounds (Mace et al., 2005).

In the following section, I will discuss the significance of using Hoxa3 to promote macrophage differentiation and phenotype, the involvement of Pu.1 and Stat6 in macrophage differentiation and polarisation, and how Hoxa3 protein can be used in cell-based therapy. An outline of the limitation of this work and the future direction is also included.

**6.2 The significance of Hoxa3 in macrophage differentiation and phenotype**

Much of our understanding of the role of Hox-3 paralogue group, comprised of Hoxa3, Hoxb3, and Hoxd3, is as important mediators of angiogenesis and wound repair (Hansen et al., 2003; Mace et al., 2005, 2009). However, these genes also play important role in haematopoiesis (reviewed in (Mahdipour and Mace, 2011). Figure 6.1 illustrates the involvement of Hox-3 genes in myeloid cell development. Hoxb3 is involved in HSC self-renewal and directs lineage
commitment to myeloid cells (Antonchuk et al., 2001; Sauvageau et al., 1995). Hoxa3 and Hoxb3 both act as repressors of lymphoid lineages (Figure 6.1) (Ko et al., 2007).

**Figure 6.1: The involvement of Hox-3 genes in myeloid cell development.** CLP: common lymphoid progenitor, CMP: common myeloid progenitor, CFU-GM: colony forming unit—granulocytic monocytic.

Previously in our lab, we found that Hoxa3 overexpression in the HSC/P population promoted the differentiation of these cells into Gr1^+CD11b^+ cells that include monocytes and granulocytes, but interestingly favoured the granulocyte over monocyte fate (Mahdipour et al., 2011). Evidence from my study supports that Hoxa3 protein transduction also has the potential to differentiate monocytes, increasing their endogenous M-CSF levels and inducing macrophage maturity, particularly diabetic macrophages. Our data from the BMDM outline the importance of Hoxa3 in rescuing diabetic macrophages from the persistent immature phenotype and increasing the F4/80 and CD115 maturation markers and endogenous Pu.1
level. This implies that these macrophages must have endogenous levels of Hoxa3 that allowed for their proper differentiation. The deficiency of Hoxa3 in diabetic wound must have affected the level of Hoxa3 in macrophages thus impaired their differentiation. The data generated from the BMDM and wound-derived macrophages demonstrate that Hoxa3 is influenced not only in the macrophage differentiation process but also their phenotypic switch to the M2 fate, a process that is defective in diabetes (Bannon et al., 2013; Mirza and Koh, 2011). The findings in this thesis provide evidence that Hoxa3-enriched conditioned medium and transient overexpression of Hoxa3 plasmid into wounds can rescue diabetic macrophages from persistent inflammation not only by inhibiting inflammatory markers and cytokines but also by enhancing the release of growth factors needed for neovascularisation and for maintaining effective wound repair and regeneration. The positive influence of Hoxa3 on the myeloid cell population we obtained in this study together with the previously identified role of Hoxa3 in wound cell migration and recruitment (Mace et al., 2005, 2009; Mahdipour et al., 2011) supports the concept of the ‘cell reprogramming effect’. In essence, Hoxa3 can correct myeloid cell dysregulation whether it involves the differentiation process or the phenotypic changes of leukocytes in the wound needed to take place for normal progression of the healing process from the inflammatory to reparative stage. The upregulation of differentiation markers in Hoxa3-treated macrophages might be a direct consequence of differentiation to the M2 pro-healing phenotype. It is assumed that macrophage differentiation and polarisation are intertwined processes, but this requires laboratory investigation. It would be interesting to investigate the maturation markers in classically activated macrophages and compare them to those in alternatively activated macrophages as well wound-derived macrophages early in
healing compared to late stages of healing. This would help determine the extent maturation affects macrophage phenotypic changes. Based on the data presented in this thesis, I expect M2 polarised macrophages to be more mature while inflammatory macrophages may show less maturation ability. This hypothesis would also align with the diabetic macrophage phenotype that exhibits persistent immaturity and persistent inflammation (Bannon et al., 2013; Koh and DiPietro, 2011; Miao et al., 2012; Wicks et al., 2014, 2015). It would also be compelling to allow Hoxa3-treated macrophages to differentiate further, beyond day 7, and to observe whether they can acquire alternative activation markers without any stimulating cytokines. Non-activated macrophages treated with Hoxa3 for 48 h and differentiated only up to day 7 did not show any significant changes in their classical and alternative markers (Supplementary Figure 18).

The data we obtained in this thesis from BMDM on macrophage differentiation and polarisation are limited to the fact that we performed gene expression, ELISA and FACS studies on all macrophages that we had in culture. Thus, the data did not differentiate between macrophages that had taken up the Hoxa3 protein into their nuclei and the ones that did not. Although it is believed that direct protein therapy can target all cells exposed, it would be ideal in the future to use cell-specific separation of Hoxa3-treated cell populations to reduce variability and to ensure that analysis and all downstream applications are based solely on treated macrophages. This could largely improve the quality of data and statistical significance.
6.3 Proposed mechanisms for Hoxa3-mediated effect on macrophage maturation and polarization

The data reported in Chapter 3 and Chapter 4 resolved some of the mechanisms for the effect of Hoxa3 on macrophage development and polarisation. The increased maturation markers of BMDM are possibly mediated via upregulation of the following: Csf1, Spi1 and Csf1r. Spi1 is a critical ETS family transcription factor for macrophage development, and the phenotype of a Spi1-deficient mouse model revealed an absolute requirement for Spi1 in macrophage differentiation (McKercher et al., 1996; Scott et al., 1994). The importance of Spi1 in macrophages stems also from the fact that other cell types such as B lymphocytes, T lymphocytes and fibroblasts can be reprogrammed to macrophages as a result of enforced expression of Spi1 in these cells (Laiosa et al., 2006; Xie et al., 2004). Importantly, a recent finding shows that Spi1 levels can be induced by overexpression of CSF1 in HSC (Mossadegh-Keller et al., 2013). This supports the data generated from THP-1 cells, in which Csf1 was induced together with Spi1 in THP-1 monocytes that showed a borderline increase in CD11b and CD68 markers (Figure 3.17). If we link these data to the data generated from BMDM, we can postulate that Hoxa3 increases the regulatory circuits that control macrophage differentiation by inducing Csf1, which induces Spi1 that ultimately leads to the upregulation of Csf1r/CD115 (Figure 6.2). The expression level of Spi1 in haematopoietic cells is critical for cell fate choice and commitment toward lymphoid cells or macrophages (Mak et al., 2011). Higher expression levels of Spi1 induce HSC to differentiate along macrophage lineages, whereas low levels push the cell fate towards lymphocytes (DeKoter and Singh, 2000). Although we did not see any increase in Csf1 expression in mouse macrophages and this was
limited to data generated from THP-1 cells, which could be a result of culturing the BM cells in medium containing L929 cells that secrete M-CSF. Thus, exogenous M-CSF from culture conditions could modify endogenous CSF1 gene expression. Importantly, we also report that in an inflammatory context, where macrophages are co-stimulated with Hoxa3 together with inflammatory mediators, Hoxa3 downregulates Spi1 (Figure 6.2). This was proposed as a mechanism that complements the previously found role of Hoxa3 in NF-κB pathway genes that were all downregulated in response to Hoxa3 overexpression in wounds (Mace et al., 2009). This is because Spi1 known as a positive regulator of NF-κB target genes including TNF, IL-6 and Nos2 (Karpurapu et al., 2011). As a result of overexpressing both Hoxa3 and Spi1 in macrophages, this anti-inflammatory effect of Hoxa3 on the expression of TNF, IL-12, Ccl2 and Nos2 was lost, confirming that Hoxa3 can diminish the inflammatory effect of mouse macrophages by downregulating Spi1 levels.

The data from Chapter 5 provides evidence that pro-angiogenic anti-inflammatory Hoxa3 induces the reparative phenotype of macrophages. Reparative macrophages are also referred to as anti-inflammatory due to their role in parasitic infection and immunoregulatory roles (Martinez et al., 2009a; Noël et al., 2004). The Hoxa3-treated M2 macrophages showed higher Arg1, CD206 and Ym1 expression as compared to controls and expressed more Arg1 and TGF-β in wound derived cells. Reparative macrophages are so-named because the growth factors they release mediating pro-healing functions involving neovascularisation, collagen deposition and wound closing (Brancato and Albina, 2011; Rodero and Khosrotehrani, 2010). In this connection, wound-derived macrophages that were treated with Hoxa3 showed more VEGF⁺-producing macrophages which correlates with the angiogenic role of Hoxa3 identified in other
cell types (Mace et al., 2009). Attempts we made to resolve the mechanisms of the involvement of Hoxa3 in mediating the M2 phenotype of macrophages suggests Stat6 and phospho-Stat6 as possible targets of Hoxa3 to augment the alternative activation of macrophages. We demonstrate in this thesis the novel involvement of Hoxa3 in the IL-4 signalling pathway. This finding also points out to the Hoxa3’s non-transcriptional effects, such as inducing Stat6 phosphorylation.

6.3.1 The relation between Hoxa3 and the ETS transcription factor Spi1

The Hox family of transcription factors has previously been shown to physically and functionally interact with proteins from the ETS family of transcription factors. In Chapter 4, we reported that exogenous Hoxa3 protein forms a complex with endogenous Spi1 protein in mouse macrophages. The next obvious question to ask is whether this protein–protein interaction is essential for the function of Hoxa3 observed in macrophage maturation and phenotype. Yamada and colleagues reported evidence supporting the premise that HOXC13 physically and functionally interacts with SPI1 and that their interaction is involved in the differentiation of erythroid myeloid leukaemia cells (Yamada et al., 2008). Other members of the Hox protein family such as HOXA10 also upregulate SPI1 transcriptionally and forms complexes with SPI1 and SPIB-B proteins (Yamada et al., 2009). Most of these studies utilised Immunoprecipitation together with GST pull-down and they determined that binding took place via the ETS domain of SPI1 and the homeodomain of the Hox protein. Knowing that the homeodomain protein contains the helix-loop-helix that is the essential structure for protein–protein interactions further support this finding (Aravind et al., 2005). We predict that based
on these analyses, the homeodomain is likely the binding domain of Hoxa3 that binds to the ETS domain of the Pu.1 protein.

These studies also formed a mutual conclusion that Pu.1 was the DNA binding motif and that the Hox protein acted as a cofactor (Yamada et al., 2008, 2009). Therefore, we can postulate that Hoxa3 is likely acting as a cofactor in the context of Hoxa3-Spi1 gene regulation for macrophage differentiation and phenotype, thereby attracting co-activators or co-suppressors depending on the context to switch Spi1 activity on or off rather than binding the DNA of Spi1 and its downstream targets such as Csf1r.

**Macrophage dysregulation in diabetes**

![Diagram showing the proposed model for Hoxa3 effect on the differentiation of mouse macrophages](image)

**Figure 6.2: Proposed model for Hoxa3 effect on the differentiation of mouse macrophages.**
The difference in differentiation and phenotype between normal macrophages (left) and diabetic macrophages (right). Culturing diabetic macrophages in Hoxa3-enriched medium induced differentiation possibly by increasing endogenous Spi1 that ultimately upregulates the M-CSF/CD115 marker and F4/80, thereby rescuing diabetic maturation defects.
6.4 The future of using Hoxa3 protein in diabetic wound therapy

The problem of diabetes and wound repair, as discussed earlier, is when it complicated to chronic non-healing wounds. The challenge of diabetic foot ulcers which comprise about 25% of cases of diabetic complications (Singh et al., 2005) form a major burden for patients, health care professionals and the economy. In parallel to standard care, options available for treating are growth factor application, enzymatic debridement and bioengineered skin grafts. The application of a single factor addresses only one part of the healing process. For example, VEGF, a potent angiogenic agent, can rescue angiogenesis (Galiano et al., 2004). Blocking excessive inflammatory signals is another approach for treating the chronic inflammation aspect (McKelvey et al., 2012). However, in both situations, there remain some unaddressed problems within the wound and it is possible that these approaches cannot achieve full recovery. The promising role of Hoxa3 in wound repair comes from the fact that a single application can target several pathways and can cause several effects without the need to apply a cocktail of growth factors (Figure 6.2, Figure 6.3). The therapeutic potential of Hoxa3 on diabetic wounds was first investigated using the topical application of Hoxa3 on diabetic wounds that lacked endogenous Hoxa3. This was associated with increased angiogenesis and increased endothelial cell and keratinocyte migration into the wound and decreased total leukocytes infiltrating to or retained in the wound (Mace et al., 2005, 2009).
Similar to any homeoprotein, Hoxa3 contains a cell-penetrating peptide within its homeodomain structure enabling it to translocate across biological membranes (Derossi et al., 1994). This adds an additional benefit to the therapeutic application of Hoxa3. Both Hoxa3 and Hoxb4 were successfully used in protein transduction using a co-culture method (Amsellem et al., 2003; Mahdipour et al., 2011). The result of these studies allowed the expansion of HSC population (Amsellem et al., 2003) and provided the first evidence of the differentiation potential of Hoxa3 (Mahdipour et al., 2011). A recent study showed for the first time the therapeutic use of Hoxa3 in bilayered skin grafts composed of a top epidermal layer of keratinocytes overlaid on a bottom layer of fibroblasts that constitutively expressed Hoxa3 (Kuo et al., 2013). The authors depended on the ability of Hoxa3 to translocate after secretion from the fibroblasts to the keratinocyte layer within the construct, and this Hoxa3-secreting bioengineered graft showed a survival benefit over non–Hoxa3-secreting grafts. Importantly, the positive effects that Hoxa3 can establish for the overall healing process, including reduced wound contraction, enhanced angiogenesis and reduced inflammatory cell markers, was achieved with this therapeutic approach (Kuo et al., 2013). It would be interesting in the future to determine the applicability and efficacy of Hoxa3 skin constructs in treating leukocyte deregulation and maturation defects in chronic wounds.

The overexpression of Hox genes such as \textit{HOXB8} and \textit{HOXB6} increase the proliferation of progenitor cells and has been reported in myeloproliferative disorders and acute myeloid leukaemia (Fischbach et al., 2005). The enforced expression of Hoxb4 induces HSC expansion without promoting any leukaemic changes and has been reported as a safe therapeutic tool for clinical use (Amsellem et al., 2003; Sauvageau et al., 1995). This motivated us to examine
the proliferative potential of Hoxa3 and the safety of applying Hoxa3 to ex vivo cultured BMDM and human monocytes. Although the use of conditioned medium enriched with Hoxa3 was an advantage over using gene therapy, it was limited by the fact that Hoxa3 was not standardised in each conditioned medium used. We are aware that the amount of Hoxa3 secreted into the medium was in the range of 20–200 ng and we only used CM from 293T cells that exceeded 70% transfection efficiency. However, we still need to determine the exact amount of Hoxa3 that macrophages can take up before its effects become evident. Hoxa3 purification has begun to take place in our lab, to be tested on macrophage differentiation and polarisation.

Altogether, this thesis supports the previously identified anti-inflammatory pro-angiogenic role of Hoxa3 in the wound and shows that Hoxa3 diminishes the inflammatory phenotype of macrophages and augments the pro-healing phenotype by secreting the angiogenic growth factors TGF-β and VEGF not only in BMDM but also in diabetic wounds. Attempts to explore the mechanism behind this effect suggest that the promotion of the pro-healing macrophage phenotype was likely achieved by increasing Stat6 transcription and phosphorylation, a downstream effector of IL-4 signalling. Our data suggest that inhibiting inflammatory macrophages and augmenting the pro-healing fate is likely achieved by two independent mechanisms targeting Spi1 and Stat6, respectively (Figure 6.3). Attempts made to resolve these mechanisms were performed only in the BMDM model and need to be verified using wound-derived macrophages. Furthermore, our data support the idea that Hoxa3 induces cell differentiation along the monocyte-macrophage pathway; such findings were also identified in diabetic macrophages, supporting the premise that Hoxa3 can rescue the persistent immaturity of diabetic macrophages.
The Spi1 shRNA construct I made needs to be utilised to confirm the proposed hypothesis that Hoxa3 mainly promotes macrophage maturation by targeting Spi1, a master regulatory gene for macrophage maturity. In conclusion, Hoxa3 targets several pathways to serve these main processes: cell differentiation, anti-inflammation and angiogenesis (Figure 6.2 and Figure 6.3). We have a promising candidate for the treatment of non-healing wounds and we would like to see in the future the comprehensive evaluation of the topical application of Hoxa3 protein to murine wounds before proceeding to human clinical trials.
Figure 6.3: Proposed model of Hoxa3 effect on macrophage phenotype. Hoxa3 rescued the aberrant phenotype in diabetic macrophages favouring M2 pro-healing macrophages in macrophages derived from BM as well d7 diabetic wounds. Increased Stat6 phosphorylation in Hoxa3 macrophages can explain these changes and suggest a possible mechanism. As expected, Hoxa3 also reduced the excessive inflammatory macrophage phenotype, reducing most M1 markers and cytokines. Spi1, which plays a pivotal role in NF-κB activation, was downregulated in these cells, suggesting that Hoxa3 acts by targeting Spi1 levels to reduce inflammation.
References:


Supplementary Figure 1: Map of pcDNA3.1 Flag-Hoxc13. This construct used to overexpress Hoxc13 in THP-1 cells.
Supplementary figure 2: Map of pcDNA3.1 mCherry. This construct used as control in transfection of THP-1 cells.

Supplementary figure 3: Map of pcDNA3.1 Hoxa3 mCherry. This construct used to overexpress Hoxa3 by transfection in THP-1 cells.
Supplementary figure 4: Map of pCMV-Sport6 vector. This vector used to overexpress Pu.1 in 293T cells for Co Immunoprecipitation assay.
Supplementary figure 5: Map of pSec Tag2 mCherry. This construct used as control in protein transduction.

Supplementary figure 6: Map of Hoxa3 mCherry in pSecTag2A. This construct used to overexpress Hoxa3 in THP-1 cells and BMDM using protein transduction.
Supplementary figure 7: Map of pFlagCMV vector. This vector used to overexpress Flag-Pu.1 in co transfection of Hoxa3 and Pu.1 in BMDM using nucleofection transfection.
Supplementary figure 8: Map of pLVTHM vector.
This vector used to knock down Pu.1 using Pu.1shRNA BMDM using viral transduction.
Supplementary figure 9: Map of psPAX2 vector.
This vector used as packaging vector in Pu.1shRNA viral transduction.
Supplementary figure 10: Map of pMD2.G vector.
This vector used as envelop expressing plasmid in Pu.1shRNA viral transduction.
Supplementary Figure 11: CD14 and CD11c in PMA differentiated THP-1 cells. CD14 and CD11c antibody was not detected on the surface of differentiated THP-1 cells and analysis could not be done using these two antibodies. D1+ PMA, day 1 treated THP-1 cells with PMA; D2+ PMA, Day 2 treated THP-1 cells with PMA.
Supplementary Figure 12: CD14 and CD11c in undifferentiated THP-1 monocytes. a. Mean and median values for CD14 and CD11c antibody showing no observed change in the fluorescence from un-labeled cells (no antibody) and isotype control. b. Bar chart showing no observed change in CD14 (left) and CD11c antibody (right) between Hoxa3 and mCherry treated cells.
Supplementary Figure 13: Amplification of Spi1shRNA with pLVTHM primers. a. Annotated sequence of Pu.1shRNA sequence. b. Sequence of PLVTHM forward and reverse primer annotated to clarify Mlu1 and Cla1 digestion sites. c. Gel electrophoresis showing the result of amplification of Pu.1shRNA sequence with PLVTHM primers showing bands of Pu.1shRNA at expected size 200bp. NTC: negative control.
Supplementary figure 14: Sequencing of ligation product of Spi1shRNA and pLVTHM vector.

GGCGGGACACCCAGCGCGCTGCGCCCTTGGCAGGAAGATGGCTGTGAGGGACAGGGGA
GTGGCGCCCTGCAATATTTGCATGTCGCTATGTGTTCTGGGAAATCACCATAAACGTGAAAT
GTCTTTGGATTTGGGAATCTTATAAGTTCTGTATGAGACC
GGCG
CGCTGGAGCTCAGCTGGATG
TAGTGAAGCCACAGATCTGAGGCTG
GAGCTCTGGCTAAGCTAGGGGAACCCACTGCTAAAGCTCAATAAAGGTTCTGGAGTTGTGCT
TCAAGTATGCTGTTGGCCCGCTCTGGTGTAAGCTAGATCCCTCAGACCCCTTT
AGTCAGTGTTGAAAATCTCTAGCAGCATCTAGAATTAGCTGTATCTAGTGCACCC
AAAATCGTATGTTAGTACATAAGATGTTATTGATTAATTGTAGCCGCGTTCTAACGAC
ATGTACAAGCCTAATTGTTGAGCATCTGGCTTTACTGAAGCAGACCCCTATCATCTCTCTCG
ACTGCCGTCAGAGTGCAGGTTTGAGACGATCTGTGATCGAGCCCTCTCTCGTAAGCTGCTCT
CCCGAAAAATGGTCAGCGAACCAATCACGAGGCTCATCGACTCCAGACCTCTACGCCGA
CGATCGGTGCTGCCAGCCGCTGCCAGGGCCACCTACGCCGTCGTCGACCGCTATATCGCGAC
ATCCCGAATGGGAAGATCGGCGTCCTGGCCTCTGGCTAGGCGGCTTGTCCGCGTGA
TGGTGCGAGGCCCCTGTNCGGGGGACTGTTGGGCGCCCATCTCCTGATGCACATCTCTTG
GGCGGCCGGTCTCAACGCGCTCAA

Mlu
Cla1
Forward sequence for Spi1sh
Reverse sequence for Spi1sh
PLVTHM vector
Loop
Extra sequence
Supplementary Figure 15: Confirmation of successful cloning of Spi1shRNA in pLVTHM by BLAST. i. Alignment of sequence from ligation product to sequence of pLVTHM from add gene matched part (i) and (iii) leaving a unmatched 200bp. ii. Sequence represent the 200bp part of sequencing result of ligation product of PLVTHM to spi1shRNA aligned to Spi1shRNA sequence in supplementary figure 3a (ii).
Supplementary Figure 16: Spi1 knockdown in BMDM using viral transduction. a. Phase contrast, fluorescent image and merged image of 293T cells transfected with PLVTHM control (top panel) or with Pu.1shRNA construct. Scale bar: 100µm b. Flow sorting results of BMDM that express GFP-positive cells 48h following the viral transduction of the PLVTHM virus (control; top) or Spi1shRNA virus (bottom). Flow histograms represent no expression of GFP in cell transduced with pLVTHM control or with Spi1shRNA cells.
Supplementary Figure 17: Flow cytometry histograms of IL-4Rα (CD124) antibody
Supplementary Figure 18: Analysis of M1 and M2 markers in Hoxa3- or mCherry-treated BM-derived macrophages that were not activated. a, b. M1 markers in non-activated macrophages treated with Hoxa3 (black) or mCherry control (white) from ndb (a) or db (b) mice. c, d. M2 markers in non-activated macrophages treated with Hoxa3 (black) or mCherry control (white) from ndb (c) or db (d) mice.