REGULATION OF MACROPHAGE MIGRATION INHIBITORY FACTOR
EXPRESSION AND ITS ROLE IN STROKE AND ALZHEIMER'S DISEASE

by

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Abstract

As a pleiotropic protein, macrophage migration inhibitory factor (MIF) participates in many cellular activities including inflammatory response, energy metabolism, and apoptosis. Dysregulation of MIF has been associated with chronic inflammatory conditions, and inhibition of its activity has been proposed as a therapeutic strategy. However, MIF gene knockout shows detrimental effects under stress-induced acute conditions such as infection and ischemia/reperfusion (I/R). Compared to a large body of research regarding the role of MIF outside of the central nervous system, limited studies have been carried out to address its role in neurological conditions. Previous studies showed that MIF protects cardiomyocytes from I/R induced detrimental effects. Since strokes are detrimental to neurons in a similar way that heart attacks are to cardiomyocytes, MIF may exert similar effects to protect neurons. Therefore, we aimed to study the regulation of MIF expression and its potential role in strokes. We identified two functional cis-acting NFκB binding elements on the MIF gene promoter and demonstrated that NFκB regulates MIF expression by transcriptional activation of the MIF gene promoter via these two sites. Under hypoxic conditions, MIF gene transcription is reduced by activation of NFκB signaling, which contributes to the down-regulation of MIF expression in the ischemic territory during strokes. We further demonstrated that MIF reduces caspase-3 activation and protects neurons from oxidative stress-induced and I/R-induced apoptosis in vitro. Using a stroke model, we showed that MIF gene knockout results in elevated caspase-3 activation, exacerbates neuronal death, and accelerates infarct development. These results suggest that MIF exhibits neuroprotective effects following a stroke. As stroke increases the risk of developing Alzheimer’s disease (AD), we further evaluated whether MIF could serve as a molecular link between stroke and AD by exploring the expression profile of MIF and its role in AD. We have provided first-hand evidence suggesting that elevation of MIF expression is induced by a pathological increase of Aβ deposits at the late stage of AD, but this effect does not recover its role in mediating normal behavioral functions in AD, because it is sequestered on the Aβ deposits in a loss-of-function fashion.
Preface

One of the ongoing goals in Dr. Weihong Song’s lab is to identify the molecular link between stroke and Alzheimer’s disease (AD). A graduate student Odysseus Zis in the lab has previously showed that macrophage migration inhibitory factor (MIF) expression was temporarily upregulated in stroke patients and the brains of rats subjected to experimental stroke. Studies in Chapters 2 and Chapter 3 of this thesis follow these findings and aim to characterize the expression profile of MIF and its role during the acute phase of strokes. In Chapter 2, I designed and carried out all the experiments to study the transcriptional regulation of MIF gene expression by NFκB signaling. Part of the plasmid constructs, phMIF\text{luc} and phMIF\text{δHluc}, were cloned and functionally characterized by Odysseus Zis for his graduate study (Wang et al., 2009; Zis et al., 2014). In Chapter 3, I designed and carried out the majority of the experiments. Previous work from Odysseus Zis demonstrated that exogenous MIF protects rat primary neurons from oxygen glucose deprivation (OGD) induced apoptosis (Zis et al., 2014). He further demonstrated the protective role of exogenous MIF on rat primary neurons subjected to oxidative stress. I further confirmed his findings by showing a similar protective effect of exogenous MIF on mouse primary neurons under both OGD and oxidative stress. To study the role of MIF under oxidative stress in vitro, I established stable cell lines with exogenous MIF expression. To examine whether MIF exerts these roles in vivo, a stroke model on mice was established with the assistance of Dr. Woei-Cherng Shyu of China Medical University and Hospital, Taichung, Taiwan. Followed by establishment of the stroke model, I performed all the surgeries, and the following biochemical and histological analyses. Dr. Philip T.T. Ly helped to confirm histological staining of MIF in mouse brain. I drafted the manuscript and Dr. Yili Wu participates significantly in revising the manuscript. The procedures for obtaining embryonic mouse primary neurons and the protocol for experimental stroke on mice were approved by the University of British Columbia Animal Care Committee (protocol number: A09-0274 and A11-0025, respectively).

In Chapter 4, we first characterized the expression profile of MIF in brain tissues and cerebrospinal fluids from AD patients and in brain tissues from a double transgenic mouse model of AD, APP23/PS45. Then, we assessed the potential role of MIF in AD pathogenesis. I designed and carried out the majority of the experiments. Human tissues were used to examine the expression of MIF in patients with AD. Frozen cortical tissues were obtained from the Department of Pathology at Columbia University. Cerebrospinal fluids (CSFs) were collected in the Guangdong General hospital. Analysis of the concentration of MIF in these samples was carried out in our lab. Dr. Mingming Zhang prepared the brain lysate from AD patients and the control cases, and I performed electrophoresis and immunoblotting analysis. MIF concentration in CSFs was measure by ELISA kits with the assistance of Dr. Jiehao Zhao. I designed the behavioral tests, and bred and grouped the transgenic mice. Ms. Beibei Song helped to perform the tests. Drs. Zhe Wang and Qin Xu provided the protocols to study the interaction of MIF and Aβ. Dr. Qin Xu prepared the Aβ oligomers, Dr. Zhe Wang provided the purified GFP, and I performed the dot blot experiments.
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List of Abbreviations

Aβ                 Amyloid-β
AβDP               Aβ-degrading protease
ABC                Avidin:biotinylated enzyme complex
AD                 Alzheimer’s disease
AIF                Apoptosis-inducing factor
AMPK               AMP-activated protein kinase
ANOVA              Analysis of variance
APOE               Apolipoprotein E
APP                Amyloid-β precursor protein
APP23              Single transgenic mice carrying Swedish APP$^{K670M/N671L}$ mutation
APP23/PS45         Double transgenic mice for Swedish APP$^{K670M/N671L}$ and PS1$^{G384A}$
ARU                Animal Research Unit
ATP                Adenosine triphosphate
BACE1              β-site APP Cleaving Enzyme 1
BACE2              β-site APP Cleaving Enzyme 2
BAD                Bcl-2 associated agonist of cell death
BAX                Bcl-2-like protein 4
BBB                Blood brain barrier
Bcl-2              B-cell lymphoma 2
BID                Bcl-2 interacting domain
bp                 Base pair
BSA                Bovine serum albumin
BV-2               Mouse microglia cell line
CCR2               Chemokine receptor 2
CRE                Cyclic adenosine 3’,5’- monophosphate responsive element
CREB               cAMP response element-binding protein
CSF                Cerebrospinal fluid
CTFα               C-terminal fragment α (C83)
CTFβ  C-terminal fragment β (C89 and C99)
CXCR2  Interleukin 8 receptor
CXCR4  C-X-C chemokine receptor type 4
DBA  3,3’-Diaminobenzidine
D-DT  D-dopachrome tautomerase
DEPC  diethylpyrocarbonate
DMEM  Dulbecco’s modified eagles’ medium
DMSO  Dimethyl sulfoximine
ELISA  Enzyme-linked immunosorbent assay
EMSA  Electromobility shift assay
ERK1/2  Extracellular signal-regulated kinase 1/2
FAD  Familial AD
FBS  Fetal bovine serum
GFAP  Glial fibrillary acidic protein
GFP  Green fluorescent protein
HEK293  Human embryonic kidney 293 cell line
HIF-1α  Hypoxia inducible factor-1α
HRE  Hypoxia responsive elements
Iba-1  Ionized calcium binding adaptor molecule 1
IFN-γ  Interferon gamma
IL-1  Interleukin-1
IL-1β  Interleukin-1β
IL-3  Interleukin-3
IL-4  Interleukin-4
IL-10  Interleukin-10
I/R  Ischemia/reperfusion
ISO-1  (S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid
JNK  Jun amino-terminal kinases
LPS  Lipopolysaccharide
MAPK  Mitogen activated protein kinase
MHC  Major histocompatibility complex
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<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
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<td>N2A</td>
<td>Mouse neuroblastoma</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NEP</td>
<td>Neprilysin</td>
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<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>NMR</td>
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<tr>
<td>NR2B</td>
<td>NMDA receptor subtype 2B</td>
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<tr>
<td>Pen-2</td>
<td>Presenilin enhancer2</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
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<td>Phospholipase A2</td>
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<tr>
<td>PS2</td>
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</tr>
<tr>
<td>PV+</td>
<td>Parvalbumin positive</td>
</tr>
<tr>
<td>PVDF-FL</td>
<td>Immobilon®-FL polyvinylidene difluoride</td>
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<td>RAW264.7</td>
<td>Mouse macrophage cell line</td>
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<tr>
<td>RIPA DOC</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<td>Secretory APPα</td>
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<td>Secretory APPβ</td>
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<tr>
<td>SAD</td>
<td>Sporadic AD</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SH-SY5Y</td>
<td>Human neuroblastoma</td>
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<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
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<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt; cell</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
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<tr>
<td>TPOR</td>
<td>Thiol-protein oxidoreductase</td>
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<tr>
<td>WT</td>
<td>wildtype</td>
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To my dear grandfather

and

all my loved ones

献给我亲爱的爷爷

以及

所有爱我的和我爱的人
Chapter 1
General introduction

1.1 Stroke
Strokes resulting from disturbances of blood supply to a brain region due to either ischemia or hemorrhage usually lead to neurological and motor deficits. Stroke is the second leading cause of death worldwide (Donnan et al., 2008) and the third leading cause of death in Canada (Statistic Canada, 2014). According to the Canadian Community Health Survey, in 2009, about 1.1% of Canadians living in the community reported that they suffer from the consequences of a stroke, and this number excludes the stroke survivors who require rehabilitative care (PHAC, 2011). Stroke has a substantial economic and social impact to Canada. More than one third of stroke survivors experience a loss of function in physical and/or cognitive functions that end up requiring caregivers. This has a substantial impact on individuals, their families, and the society (Canadian Institute for Health Information, 2009). Strokes cost the Canadian economy $3.6 billion a year in health care and due to lost productivity (PHAC, 2011).

1.1.1 Ischemic cascades and neuronal death
Constant blood flow to the brain for energy and oxygen delivery is essential to maintaining normal brain functions. Compared to other organs, the brain consumes a larger amount of energy and oxygen, thus a sudden lack of blood flow will more readily induce the ischemic cascade leading to tissue damage and neurological dysfunction. Ischemic strokes account for 80% of the stroke cases, and ischemic strokes due to occlusion of the middle cerebral artery are the most common type (Moskowitz et al., 2010). It differentially affects brain regions resulting in ischemic core and penumbra. Ischemic core describes the center of the ischemic territory where the blood flow is reduced to less than 15%, while the penumbra describes the surrounding area with higher blood flow due to collateral support where cells are “at risk” but salvageable with proper intervention (Lipton, 1999; Lo, 2008). All cells in the ischemic
territory are affected by oxygen glucose deprivation (OGD), but neurons are more vulnerable than glial and vascular cells due to their higher metabolic activity and concentrations of glutamate (Choi, 1992). Cells in the ischemic core experience the most severe energy failure, and undergo necrosis within minutes after ischemic onset (Lipton, 1999). Anoxic depolarization is the major cause of neuronal necrosis characterized by cell swelling and subsequent ruptures, which results from the failure of ionic pumps that are deprived from ATP supply (Lipton, 1999). Neurons in the penumbra experience milder ischemic attacks, while the blood supply is just sufficient to maintain their basic survival needs and, thus, they are vulnerable to additional insults from the adjacent environments (Moskowitz et al., 2010). Without restoration of blood supply, ruptured cells released cell contents in the ischemic core will trigger a cascade of events in the surrounded penumbra, including glutamate receptor-mediated excitotoxicity, calcium dysregulation, oxidative stress, and inflammation, leading to delayed cell death in the penumbra and infarct expansion (Moskowitz et al., 2010). In a particular neuron, excessive extracellular glutamate, due to ruptured neurons in the ischemic core and failure of reuptake, triggers glutamate receptors activation which mediates both neuronal survival and death signals, and also allows the activation of additional mechanisms leading to cell death including the influx of excessive calcium and oxidative stress (Choi, 1992; Martin and Wang, 2010; Moskowitz et al., 2010). Additional mechanisms resulting in dysregulation of calcium, besides glutamate receptor mediated calcium influx increase, include increased release from intracellular stores (e.g., mitochondria and ER) and dysregulation of multiple ions channels such as Na\(^+\)/Ca\(^{2+}\) exchanger, acid-sensing ion channels, and transient receptor potential (TRP) channels (Broughton et al., 2009; Moskowitz et al., 2010). Oxidative stress originated from energy deprived and distressed mitochondria also contributes to penumbra neuronal death (Moskowitz et al., 2010; Niizuma et al., 2009). These events in concert determine whether the neuron undergoes apoptosis or necrosis in the penumbra (Lipton, 1999). Additionally, inflammatory cells are activated due to tissue damage and contribute to ischemic damages (Iadecola and Anrather, 2011; Jin et al., 2010).

### 1.1.2 Neuronal apoptosis during stroke

Neurons in the ischemic core are rarely salvageable due to the immediate necrosis. In contrast, neurons in the penumbra, undergo delayed cell death and are the targets for
therapeutic interventions (Lipton, 1999). In the penumbra necrosis induced by glutamate diffused from the necrotic core may be diminished by blocking the NR2B-containing NMDA receptors (Liu et al., 2007). Most of the penumbra neurons undergo apoptosis. Neuronal apoptosis under ischemic conditions follows similar cellular processes as the general apoptotic pathways, and is governed by multiple mechanisms, depending on the status of the neuron and the surrounding environments. Anti-apoptotic strategies through these mechanisms were shown to successfully reduce neuronal loss and restrict infarct expansion (Broughton et al., 2009).

As mentioned above, ischemia results in the dysregulation of intracellular calcium, which in turn activates pathological pathways leading to cell apoptosis. For example, activation of calpains by excessive Ca\(^{2+}\) results in the cleavage of Bcl-2 interacting domain (BID) to generate its truncated active form, tBID, which then translocates onto the outer membrane of mitochondria to form heterodimers with other pro-apoptotic proteins (e.g., BAX and BAD), which in turn induce the release of cytochrome c from the intermembrane space of mitochondria (Broughton et al., 2009). Oxidative stress is the cause of apoptosis following hypoxia/reperfusion in all types of cells but neurons are particularly vulnerable partially due to their limited antioxidant defense (Adibhatla and Hatcher, 2010). Reactive oxygen species (ROS) are by-products of the mitochondria-mediated ATP production during energy metabolism, and are catalyzed to non-harmful molecules by superoxide dismutases and glutathione peroxidase under physiological conditions. However, over-production of ROS, such as following ischemia/reperfusion (I/R), causes oxidative stress. It was generally thought that mitochondria is the primary source of overproduced ROS following a variety of post-ischemic stimuli, including hypoxia, excitotoxicity, and Ca\(^{2+}\) overload (Broughton et al., 2009). Recent studies also identify NADPH oxidase as another major contributor for ROS (i.e., superoxide anion) production in neurons under ischemic condition (Miller et al., 2006). This process is mediated by activation of the NMDA receptor, and may serve as a trigger of a secondary mitochondria-mediated ROS surge (Brennan et al., 2009). Pathological production of ROS by mitochondria influences the release of cytochrome c, which in turn leads to cell apoptosis (Kirkland et al., 2002). Release of cytochrome c into cytoplasm allows it to bind with apoptotic protein-activating factor-1 (Apaf-1) and pro-caspase-9 to form the
“apoptosome”, which activates caspase-9. Subsequently, caspase-9 cleaves procaspase-3 to release caspase-3 to allow the execution of apoptosis. Activation of caspase-9 and caspase-3 in the ischemic hemisphere has been observed in various stroke models (Broughton et al., 2009).

In addition to caspase dependent pathways, post-stroke neuronal apoptosis can also be mediated by caspase independent pathways following mitochondria stress. Apoptosis inducing factor (AIF) is one of such factors, and it is the best studied one. It is released via mitochondrial transition pores due to energy depletion of ATP, and translocates to the nucleus and causes DNA fragmentation, which in turn induces cell apoptosis (Cho and Toledo-Pereyra, 2008).

1.1.3 Contribution of inflammation
Post-stroke tissue damage activates immune cells that mediate inflammatory responses. During the acute phase of a stroke resident microglia are the first immune cells to respond to dying cells in the brain parenchyma. Neurons are the most vulnerable to ischemia insults. Dying neurons lose the expression of CD200 and CX3CRL1 that interact with CCR2 and CX3CR1 on microglia, resulting in activation of microglia for pro-inflammatory cytokine production (Cardona et al., 2006; Matsumoto et al., 2007). In addition to the specific signals from neurons, microglia also receive stress signals from the surrounding environments, which in turn promote their production of pro-inflammatory mediators. For example, danger-associated molecular pattern molecules (DAMPs) shed from the death cells activate TLRs on cell surface and activate the pro-inflammatory gene expression through NFkB signaling (Harari and Liao, 2010; Marsh et al., 2009). Ischemia-induced excessive extracellular glutamate also activates microglia leading to pro-inflammatory phenotype (Chapman et al., 2000). Activated microglia produce pro-inflammatory cytokines and chemokines guiding proliferation and migration of microglia and other immune cells to the lesion.

Infiltration of blood-borne leukocytes also contributes to the pathological activation of inflammation. Infiltration of leukocytes to the brain parenchyma is mediated by inflammatory signals produced by microglia/perivascular macrophages that are sent to
endothelial cells which in turn produce adhesion molecules facilitating migration of leukocytes into the brain parenchyma (Eltzschig and Eckle, 2011). Although infiltration of leukocytes can occur at the acute phase of a stroke (Iadecola and Anrather, 2011), it took hours to days to achieve massive infiltration after the stroke onset (Jin et al., 2010). Infiltration of macrophages, which mainly mediate innate immunity, appears 12 hours after stroke, the number of macrophages reaches plateau at 24 hours and throughout the next 7 days. On the other hand, infiltration of lymphocytes, the main immune cells mediating adaptive immune response, occurs 3 days after ischemic onset (Gelderblom et al., 2009; Stevens et al., 2002). Due to the early infiltration to brain parenchyma, macrophages may contribute to pro-inflammatory responses during the acute phase of a stroke.

It should be noted that pro-inflammation is a natural response of the host immune systems that are developed to sensing, reacting and cleansing the tissue damages (Chen and Nunez, 2010). However, during strokes, especially severe strokes and in subjects having a higher level of inflammatory states (i.e., patients with pre-existing systematic inflammatory conditions), inflammatory signals are amplified and overproduced, and in turn exacerbate stroke pathology. In these cases, strategies to inhibit pro-inflammatory signals will be beneficial to the stroke outcome. It has been shown that disruption of IL-1 type 1 receptor reduced multiple chemokine production and stroke damage in mice (Lazovic et al., 2005).

Microglia and macrophages play dual roles during a stroke. At the acute phase, they produce inflammatory cytokines to induce inflammation, which is detrimental to the stroke outcome, while they produce mediators (i.e., anti-inflammatory cytokines and growth factors) at the late stage of stroke to dampen down inflammation and promote tissue repair. TGF-β and IL-10 are the most studied anti-inflammatory cytokines produced after strokes that promote resolution of inflammation and are protective to surviving cells in the ischemic territory (Iadecola and Anrather, 2011). TGF-β is mainly produced by microglia and macrophages after ischemia, and exerts its anti-inflammatory role by promoting the development of T_{reg} cells (Taylor et al., 2006), which in turn produce IL-10 (Liesz et al., 2009). Besides cytokine production, microglia and macrophages are the main immune cells responsible for debris phagocytosis (Denes et al., 2007; Schilling et al., 2005). In fact, the process of phagocytosis
per se promotes secretion of TGF-β and IL-10 from macrophages (Nathan and Ding, 2010). Inflammatory cells also participate in post-stroke tissue repair by producing a variety of growth factors, such as insulin-like growth factor 1 produced by microglia, which promotes neuronal sprouting (Iadecola and Anrather, 2011).

Post-stroke infiltration of inflammatory cells persist for years (Mena et al., 2004), and dementia develops in 30% of stroke patients (Leys et al., 2005). However, the pathogenic significance of these infiltrated inflammatory cells regarding immunity and dementia remains unclear.

1.2 Alzheimer’s disease
Alzheimer’s disease (AD) is the most common neurodegenerative disorder with progressive cognitive and behavioral impairment and eventually leads to death. It is the most common cause of dementia, which refers to a series of symptoms affecting cognitive functions that are severe enough to interfere with a person’s daily life. According to the World Alzheimer Report 2012, there are more than 35.6 million people suffering from AD worldwide, and this number is projected to double every 20 years, reaching 115.4 million people in 2050 (ADI, 2010). The total estimated worldwide costs of AD and related dementia were US$604 billion in 2010. These costs account for around 1% of the world’s gross domestic product (GDP), which ranked the world’s 18th largest economy if dementia care were a country (ADI, 2010).

In Canada, the Alzheimer Society of Canada reported that the number of AD patients in 2008 was 480 thousand, or 1.5% of the Canadian population, and projected this number to over 1 million, or 2.8% of the Canadian population in 2038 (ASC, 2010). The annual total economic burden is expected to increase substantially from $15 billion in 2008 to $153 billion by the year 2038 (ASC, 2010). Besides the huge impact on the economy, this disease has imposed heavy burdens to individual families, and led to discussions of the challenges the current health care systems will face in the near future (ADI, 2013).

1.2.1 The pathophysiological characteristics of AD
Intracellular neurofibrillar tangles, extracellular neuritic plaques, and neuronal loss are considered as the three main neuropathological hallmarks of AD. Neurofibrillar tangles
(NFTs) are mainly composed of microtubule-associated protein tau in its aberrantly misfolded and abnormally hyperphosphorylated form. Mature intraneuronal NFTs extend from the soma towards distorted-appearing dendrites and the proximal segment of the axon, and are a sign of degenerating neurons (Serrano-Pozo et al., 2011). However, this pathological feature is not unique to AD, and is also observed in other forms of dementia with tauopathy, such as frontotemporal dementia with Parkinsonism linked to chromosome 17 (Hutton et al., 1998; Spillantini et al., 1998).

Extracellular neuritic plaques describe a type of amyloid plaques composed of amyloid β proteins (Aβ) and distorted neurites in the brain parenchyma. Matured neuritic plaques (i.e., dense-cored plaques) contain an amyloid core surrounded by a corona. In contrast, diffused plaques, which are another type of amyloid plaques, are associated with neurites that are free of pathological signs, and are considered as the non-pathological form of plaques (Masliah et al., 1990; Yamaguchi et al., 1988). By developing end-specific antibodies recognizing Aβ40 and 42, Iwatsubo et al. were able to show that Aβ42 stained positive for both neuritic and diffused plaques, while Aβ40 only presented with dense-cored plaques but was absent from diffused plaques (Iwatsubo et al., 1994). Histological studies have shown that diffused plaques account for the majority of the plaques in AD brains (Yamaguchi et al., 1988), and are also commonly present in cognitively intact elderly people (Dickson and Vickers, 2001). However, it has been suggested that diffused plaques might undergo evolution to neuritic plaques with the assistant by microglia in AD patients (Sheng et al., 1997). In recent years, the evolution of a plaque-like structure from purified Aβ has been well characterized (Masters and Selkoe, 2012). Assembly and dissociation of Aβ have been considered to be a highly dynamic process. It remains elusive whether Aβ composed neuritic plaques is the instigator or scapegoat in AD pathogenesis (further discussion in the following sections), but it is the feature that makes AD unique to other form of dementia and neurodegenerative diseases.

Neuronal loss in AD features atrophy in selected brain regions, including the temporal and parietal lobes, restricted regions within the frontal cortex and cingulate gyrus, hippocampus, and amygdala, which were important for memory and cognitive functions and emotional
behaviors (Wenk, 2003; West et al., 1994). Neurons in layer II of the entorhinal cortex and hippocampal CA1 neurons are particularly vulnerable (Mattson, 2004). Neuronal loss in AD mainly undergoes apoptosis. Neurons exhibiting neurofibrillary tangle pathology undergo apoptotic degeneration, as shown by the activation of caspase-3, but only partially contribute to the total neuronal loss, and other types of neuronal death also occur in neurons absent of NTFs (Gomez-Isla et al., 1997). Aβ, probably as a mixture of various species, triggers neuronal apoptosis (Mattson, 2004). Aβ induces DNA damage in cultured primary neurons leading to neuronal apoptosis (Loo et al., 1993). DNA damage and upregulation of p53 appear in the early stage of AD, and treatment with p53 inhibitor inhibits Aβ-induced neuronal apoptosis in vitro (Eckert et al., 2003; Mattson, 2000). Intracellular accumulation of Aβ results in oxidative stress by inducing mitochondrial dysfunction and leads to neuronal apoptosis in both familiar AD (FAD) and sporadic AD (SAD) (Eckert et al., 2003). Inflammatory signals also play a role in facilitating or inhibiting neuronal apoptosis and will be discussed in section 1.3.5.

Based on these classic pathological features of AD, recent studies have got into areas such as the toxic component of neuritic plaques and the mechanisms of synaptic loss that could be prior to neuronal degeneration. It has been suggested that synaptic loss predates neuronal loss, and is better correlated with cognitive function decline in AD patients (Serrano-Pozo et al., 2011).

1.2.2 The amyloid hypothesis

Since Dr. Alois Alzheimer first described the pathologic feature of AD in 1906, it was not until the middle 1980s for AD research to reach the next milestone – the discovery of the Aβ protein as the main component of neuritic plaques (Glenner and Wong, 1984; Masters et al., 1985). Shortly after this, the Aβ precursor protein, APP, was cloned and localized on chromosome 21 (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). The localization of APP on chromosome 21 and the fact that trisomy 21 syndrome (Down Syndrome) develops non-distinguishable pathology as in AD (Olson and Shaw, 1969) further demonstrated Aβ as the main character of AD pathology. Taken together with evidence showing that patients with genetic mutations on the APP gene exhibited early-onset
AD pathology (Goate et al., 1991; Hendriks et al., 1992; Mullan et al., 1992a; Mullan et al., 1992b), and these mutations altered APP processing in favor of generating more Aβ (Cai et al., 1993; Citron et al., 1992; Suzuki et al., 1994). Hardy and Higgins proposed “the amyloid cascade hypothesis” in 1992. It states that “deposition of Aβ, the main component of the plaques, is the causative agent of Alzheimer’s pathology and that the neurofibrillary tangles, cell loss, and dementia follow as a direct result of this deposition” (Hardy and Higgins, 1992). In subsequent years, more evidence has been found in supportive of this hypothesis by showing genetic factors associated with early- and late-onset of AD pointed to increased Aβ production and decreased clearance (Hardy and Selkoe, 2002). This includes that 1) mutations on early-onset AD associated genes APP and PS1/2 alter APP processing in favor of generating more Aβ peptide (Cai et al., 1993; Citron et al., 1992; Citron et al., 1997; Scheuner et al., 1996; Suzuki et al., 1994; Thinakaran et al., 1996), and 2) APOE 4ε allele, which is the major genetic risk for late-onset AD, influences Aβ clearance that leads to increased Aβ deposits (Corder et al., 1993; Polvikoski et al., 1995).

Ten years after the original hypothesis, Hardy and Selkoe backed up this hypothesis with updated evidence, but also listed observations showing difficulties in reconciling with the hypothesis in both mouse models and human patients (Hardy and Selkoe, 2002). The most common issue is that the plaque load does not correlate well with the level of cognitive impairment, and furthermore, which Aβ form is toxic among the various Aβ species. Detailed studies on Aβ species have provided insights into explaining the discrepancies between the above observations and the amyloid hypothesis. Firstly, the degree of dementia in AD correlates with the level of soluble Aβ species (Lue et al., 1999; McLean et al., 1999; Naslund et al., 2000; Wang et al., 1999). Transgenic mice bearing human mutant APP genes demonstrate cognitive impairments prior to plaque formation (Hsia et al., 1999; Mucke et al., 2000). A comprehensive study using microinjection method demonstrated that soluble oligomers but not the other Aβ species inhibit long term potentiation (LTP) in living rats (Walsh et al., 2002). Based on these observations, the amyloid hypothesis was refined by narrowing down the causative agent from the broad forms of Aβ proteins to the Aβ oligomer (Hardy and Selkoe, 2002).
1.2.3 APP processing and Aβ generation

The amyloid hypothesis emphasizes the importance of APP catabolism and Aβ metabolism in AD pathology. APP contains the Aβ domain that begins at the 99th amino acid from the C-terminus at Asp1 and ends at Val40 for Aβ40 or Ala42 for Aβ42, respectively. After synthesis, APP undergoes sequential proteolytic processing by secretases, including α-, β-, θ-, and γ-secretases. The first cleavage is mediated by one of the first three secretases on the N-terminus to generate various forms of secretory APP (sAPP) and C-terminal fragments (CTFs). Then, the CTFs are cleaved by the γ-secretase regardless of the first cleavage pattern. The α-secretases mediated the cleavage at the α-site (i.e., Leu17) (Esch et al., 1990; Oltersdorf et al., 1990; Sisodia et al., 1990), which precludes production of Aβ in the non-amyloidogenic pathway. Several proteases or enzymes have been reported to cleave APP at the α-site, such as “A distintegrin and metalloprotase domain” 9 (ADAM9), ADAM10, ADAM 17 (Buxbaum et al., 1998; Koike et al., 1999; Lammich et al., 1999). The β-site cleavage enzyme, beta-site APP cleaving enzyme 1 (BACE1), was identified in 1999 (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). So far, it is the only identified β-secretase in vivo. BACE1 cleaves APP at two sites at Asp1 and Glu11. Cleavage at Glu11 precludes Aβ generation, while at Asp1 site allows the pass of the intact Aβ domain on the CTFβ, C99, for further processing by the γ-secretase. The preference of these two sites determines the fate of APP processing favoring either non-amyloidogenic or amyloidogenic pathways. Due to its homology to BACE1, BACE2 was originally identified to also cleave APP (Acquati et al., 2000; Farzan et al., 2000). Later studies from our laboratory clearly demonstrated that BACE2 differs from BACE1 in transcriptional regulation and the pattern of APP processing, functioning as a novel θ-secretase (Sun et al., 2006b; Sun et al., 2005). BACE2 mainly cleaves APP at Phe20, thus precluding the production of Aβ and is not responsible for the pathogenesis of AD (Sun et al., 2006b). The γ-secretase is a multi-subunit protease complex composed of presenilin1/2 as the catalytical core, and nicastrin, Aph-1 (anterior pharynx-defective 1) and Pen-2 (presenilin enhancer 2) (De Strooper et al., 2012). Aβ is generated by its cleavage on C99.

Under physiological conditions, a vast majority of APP undergoes non-amyloidogenic cleavage mediated by α-secretases, and θ-secretase and β-secretase also contribute a minor
According to the studies by our laboratory, under the normal conditions, BACE1 cleavage of APP favors the Glu11 site, and therefore leaves only a very limited amount of APP undergoing amyloidogenic β-cleavage at the Asp1 site (Deng et al., 2013). Under the pathological conditions, Aβ production is dramatically altered, leading to AD pathogenesis. This involves in several mechanisms. In FAD patients, mutations on APP, such as the Swedish mutation, results in the cleavage preference largely shifted to Asp1, resulting in an increased C99/C89 ratio, and therefore increased Aβ production (Deng et al., 2013). Mutations in presenilin genes lead to FAD not only by affecting bulk production of Aβ, but also preferentially increasing the production of the more toxic Aβ42 (De Strooper et al., 2012). In SAD cases, increased Aβ production could be attributed to upregulation of BACE1 which leads to a bulk increase of both types of β-cleavage, as demonstrated by the work from our own lab that transcriptional upregulation of BACE1 expression by hypoxia signaling increased Aβ burden in SAD cases without AD associated mutations (Sun et al., 2006a).

1.2.4 Aβ assembly, deposition, and clearance

Unleash of monomeric Aβ from the membrane occurs both intracellularly along the secretary pathway and extracellularly into the interstitial compartments in the brain parenchyma. Once being released, monomeric Aβ is subjected to mechanisms for aggregation and clearance. It has long been known that Aβ undergoes self-aggregation in vitro (Barrow and Zagorski, 1991; Halverson et al., 1990), and an AD-linked APP mutation within the sequence of Aβ peptide accelerates its aggregation (Wisniewski et al., 1991). More advanced techniques used in recent years enable researchers to demonstrate the whole processes of Aβ assembly from monomer to oligomers to fibrils and to plaque-like aggregates in details (Masters and Selkoe, 2012). Although conditions like temperature, salts, lipids, metal ions, fatty acids, and other molecules influence the process of Aβ assembly, it seems no condition could prevent Aβ aggregation (Masters and Selkoe, 2012). Synthesis of methoxy-X04, which is a derivative of Congo red that crosses blood brain barrier, allows us to detect plaques in vivo (Klunk et al., 2002). Real-time monitoring plaque formation in an AD mouse model revealed that the emergence of a new dense core plaque is extraordinarily quick and takes less than 24 hours, followed by a rapid growth in size and stabilized 24 hours after its appearance (Meyer-
Luehmann et al., 2008). Therefore, an equally competitive clearance strategy is required to maintain the Aβ level below pathological concentration.

In the brain of a normal subject, constant production of Aβ is well balanced by an equivalent rate of removal, while in the AD brain, decreased clearance with or without increased production will lead to the accumulation of Aβ and subsequent deposition. It was shown that the overall clearance rate of Aβ rather than production was impaired in some SAD patients (Mawuenyega et al., 2010), emphasizing the importance of clearance. Due to the presence of various species of Aβ and its wide spreading, multiple mechanisms are required to eliminate Aβ under different situations. One of the mechanisms is attributed to the widely expressed Aβ-degrading proteases (AβDPs). The AβDPs define several collections of proteases which exhibit proteolytic activity in Aβ degradation, irreversibly, but are not dedicated to it. These proteases include metalloproteins (e.g., neprilysin, MMP9, and insulin-degrading enzyme) and aspartylprotease (e.g., cathepsin D) (Saido and Leissring, 2012). Due to wide expression of these proteases, they collaboratively regulate the overall presentation of Aβ – just produced monomers and perhaps some soluble oligomers are degraded along the secretary pathway; and the secreted ones are either degraded in the extracellular space and cerebrospinal fluid (CSF) presented AβDPs or following endocytosis in endosomes and lysosomes by neurons and glial cells (Saido and Leissring, 2012). However, this mechanism only partially contributes to Aβ elimination because: 1) none of the known AβDPs exhibit the capacity to directly degrade Aβ assemblies larger than the fibrils (Saido and Leissring, 2012); and 2) not all brain cells could endocytose large particles like Aβ fibrils, leaving the clearance of the plaque-like Aβ deposition to other mechanisms. Studies demonstrated that fibrillar forms of Aβ (either Aβ fibrils or the fibrillar outer layers of plaque) interact with and stimulate the phagocytosis of microglia/macrophages, although questions have been raised about whether engulfed Aβ can be degraded (Paresce et al., 1997). It should be noted that initiating phagocytosis involves in the activation of inflammatory response, thus introducing additional factors contributing to AD pathogenesis.
1.2.5 Aβ deposits induce microglia activation and neuroinflammation

It is becoming clearer that Aβ species may have different toxic effects on AD pathology. That is, soluble oligomerized Aβ may directly interact with neurons to induce neuronal toxicity (Masters and Selkoe, 2012), but Aβ fibrils and plaques may indirectly induce toxic effects to the AD brains by inducing unhealthy inflammation (Wyss-Coray and Rogers, 2012). Therefore, it is important to dissect the toxic signals that Aβ might activate in the context of a certain scenario. In the central nervous system (CNS), inflammatory response is mediated by the resident immune cells, microglia, which homogenously spread in the brain and survey the brain constantly by extending and retracting their processes (Aguzzi et al., 2013). Under normal conditions, microglia processes occasionally engulf brain tissues and transport them by retracting the processes to the cell body, possibly for digestion (Nimmerjahn et al., 2005). Perturbations to the brain, such as a laser lesion and local administration of Lipopolysaccharide (LPS) quickly activate microglia in the vicinity (<50μm), whose processes reach the lesion site within minutes after the injury (Davalos et al., 2005; Nimmerjahn et al., 2005). If the damage is not resolvable in a short term (1-2 days), microglia further away (<300μm) will be recruited to cover the damage and retained chronically, probably serving as a “seal” to prevent additional reaction, and the activity of microglia returns to basal level (Paques et al., 2010). Proliferation of microglia contributes to remaining the number of microglia for brain surveillance (Ajami et al., 2007).

Microglia may participate in AD pathogenesis in several aspects: 1) regulating growth and clearance of Aβ deposits; 2) mediating neuronal loss, and 3) influencing cognitive behaviors, and the latter two aspects may be secondary to the first. Although other factors in AD brain could activate microglia, Aβ deposits seem to be particularly potent (Wyss-Coray and Rogers, 2012). Real-time monitoring the brain of an AD mouse model revealed that microglia migrate to a plaque within 1-2 days of its formation (Meyer-Luehmann et al., 2008). Monitoring of a plaque in a one-month interval demonstrated an increased number and size of plaque associated microglia (Bolmont et al., 2008). Despite the clear evidence of recruitment of microglia to the vicinity of plaques, there are ongoing debates on the function of the recruited microglia. There are studies suggesting that the recruited microglia restrict plaque growth or even reduce plaque size by internalizing the fibrils (Bolmont et al., 2008;
Meyer-Luehmann et al., 2008). However, other studies demonstrated that microglia may not be able to degrade the internalized fibrils (Majumdar et al., 2007). This could be attributed to the effect of aging, which decreases the ability of microglia to degrade internalized fibrils due to decreased activity of NEP, an AβDP (Frenkel et al., 2013; Hickman et al., 2008).

Moreover, microglia activity contributes to toxic pool of Aβ species by turning phagocytosed Aβ aggregates into toxic Aβ oligomers and releasing them (Kiyota et al., 2009). As of now, how microglia participate in Aβ deposition and clearance during AD pathogenesis remains elusive. Nonetheless, microglia are able to switch phenotypes depending on the inflammatory signals (Aguzzi et al., 2013). Therefore, it is likely that interactions between plaques and microglia are dynamic, and are modified constantly according to the change of microenvironment due to the disease progression. Activated microglia are shown to be involved in neurodegeneration (Cardona et al., 2006), and knockout of microglial CX3CR1 prevents neuronal loss in AD brains (Fuhrmann et al., 2010). Again, inflammatory signals are likely serving as the underlying mechanisms for microglia activation and neuronal attacking, and this also explains that elevation of pro-inflammatory cytokines is often observed in the brain regions that are most vulnerable for neurodegeneration during AD pathogenesis (Rogers et al., 2007). In terms of behavior changes, microglia-mediated inflammatory signals through the TLR receptors cause depressive behavior in both normal and AD mice, and blockage or mutation of the mediating receptors eliminates the toxic effect (Hines et al., 2013; Richard et al., 2008; Tahara et al., 2006).

As mentioned above, microglia phenotypes are mainly governed by the inflammatory mediators. Under normal conditions, microglia undergo activation (by IFN-γ or IL-4 and IL-3) and deactivation (by TGF-β and IL-10) in response to an acute injury (Colton, 2009), as observed in the laser-induced acute injury. It probably also serves as a protective mechanism for the Aβ bearing normal aging. However, under the diseases condition, deactivation signals may be overwhelmed by the excessively produced pro-inflammatory signals due to the ongoing accumulation of Aβ deposits. As a result, the property of microglia changes accordingly, and in turn their interactions to the surrounding environments (e.g., plaques and neurons). Since microglia are the primary immune cells responsible for cytokine production, including pro- and anti-inflammatory cytokines as well as growth factors (Colton, 2009),
they could serve as a double-edged sword in response to stimuli depending on the
physiopathological status of the brain.

1.2.6 Risk factors for AD.
It is known that mutations in APP, PS1 and PS2 genes cause AD in an autosomal dominant
fashion. Patients who inherit these mutations display an early onset of AD before the age of
60. This form of AD is known as early-onset FAD. However, FAD is rare, and a vast
majority of AD cases are sporadic with a late onset. Genetic variances and environmental
factors act as risk factors in these late-onset SAD cases. The best known genetic variance
contributing to SAD is apolipoprotein E (APOE) ε4 (Strittmatter et al., 1993). Other than
genetic factors, environmental influences (e.g., diet), pre-existing conditions (e.g., diabetes,
stroke), and lifestyles (e.g., smoking, physical inactivity) all show association with the
development or progression of AD (Barnes and Yaffe, 2011; Mattson, 2004).

1.3 Macrophage migration inhibitory factor
Macrophage migration inhibitory factor (MIF) is a cytokine secreted from activated
lymphocytes and is among the first a few cytokines that were discovered during late 1950s to
1960s (Bloom and Bennett, 1966; David, 1966). The name of MIF was given due to its
ability to arrest macrophages/monocytes in vitro. Although there had been many predicted
functions attributed to “MIF” (David and David, 1972), due to the fact that other
endogenously secreted cytokines also exhibit macrophage arrest function, it was not until the
cloning of the MIF gene in 1989 (Weiser et al., 1989) to finally confirm and further explore
the convincing role of MIF. The following discussed aspects of MIF are based on research
conducted after its cloning. Since then, MIF has been re-discovered as a pleiotropic protein
playing vital roles in a variety of biological processes, including in immunity, oncogenesis,
and I/R. After the promising expression of MIF was found in the brain, MIF has been
predicted to contribute to neurological functions and dysfunctions.

1.3.1 Regulation of MIF gene expression
The human MIF gene, located on chromosome 22q11.23, spans 855 bp and contains 3 exons
and 2 introns. The MIF promoter is TATA-less and contains several sequences of GC (Roger
et al., 2007). The latter probably contributes to the constitutive expression pattern similar to other housekeeping genes (Zhu et al., 2008). Moreover, the MIF promoter contains several putative consensus binding sequences for transcription factors, notably AP-1, NFκB, and cAMP-response element-binding protein (CREB), indicating that the expression of MIF could be induced under specific conditions. It has been demonstrated that MIF gene expression could be transcriptionally upregulated by corticotropin-releasing factor mediated CREB in pituitary cells (Waeber et al., 1998). Our laboratory and others previously found that hypoxia inducible factor-1α (HIF-1α) could bind to the MIF promoter and upregulate MIF transcription (Baugh et al., 2006; Wang et al., 2009; Welford et al., 2006).

1.3.2 MIF expression and tissue distribution

The activity of MIF was initially identified using activated lymphocytes (Bloom and Bennett, 1966; David, 1966). Later, expression of MIF was discovered in nearly all types of circulating immune cells including macrophages/monocytes as the major sources (Calandra et al., 1994). Besides the immune system, MIF ubiquitously and constitutively expresses in most organs including kidney, liver, brain, heart, and spleen (Calandra et al., 1994; Wistow et al., 1993). In addition to the wide spreading immune cells, organ specific cells also contribute to MIF expression in many organs. Cardiomyocytes constitutively express baseline MIF in heart, and express elevated MIF after acute myocardial injury (Yu et al., 2003). Osteoblasts express MIF for bone development (Onodera et al., 1996). Synoviocytes express MIF, which is upregulated in the patients with rheumatoid arthritis (Leech et al., 1999).

In the brain, histological data showed a broad expression of MIF throughout the brain structures, including cerebral cortex, hippocampus, hypothalamus, cerebellum, and pons. In vivo analysis on brain slices showed that MIF mRNA and protein are expressed in neurons (Bacher et al., 1998; Ogata et al., 1998). Neuronal expression of MIF in the brain is in a constitutive mode, and could be induced once stimulated, for example, by LPS (Bacher et al., 1998). In addition, choroid plexus, which is responsible for CSF production, is positively stained for MIF in the epithelial and ependymal cells, while is lack of MIF mRNA expression, indicating these cells may serve as a storage of MIF for quick release once being stimulated (Bacher et al., 1998; Ogata et al., 1998). Active astrocytes also express MIF
(Ogata et al., 1998). However, MIF expression in the quiescent glial cells is scarce (Bacher et al., 1998) but could be highly upregulated in the active microglia as seen in Alzheimer’s disease (Bacher et al., 2010). MIF is also expressed in neuroprogenitor cells in the subgranular zone (SGZ) where adult neurogenesis is thought to occur (Conboy et al., 2011).

1.3.3 Structure, receptors and the signaling pathways
MIF protein contains 115 amino acids and weighs 12.6 kD. The secondary structure of MIF consists of two antiparallel α-helices and six β-sheets (Suzuki et al., 1996). This structure resembles the major histocompatibility complex (MHC) molecules. MIF crystalized as a trimer as shown by X-ray crystallography, and the tertiary structure characterized by the packing of an extended four-stranded β-sheet and two anti-parallel α-helices (Sun et al., 1996a; Sun et al., 1996b). In solutions, MIF forms dimers or stays as a monomer as demonstrated by NMR, size-exclusion chromatography, analytical ultracentrifugation (Nishihira et al., 1993; Nishihira et al., 1995; Zerovnik et al., 1999). Structural analysis reveals that MIF has a strong homology to the enzyme D-dopachrome-tautomerase, and can catalyze a similar enzyme activity, which can be inhibited by the same molecule, ISO-1 (Lubetsky et al., 2002). Although it has been hypothesized that the trimer structure of MIF might be important for receptor binding as other cytokines (e.g., TNF-TNF receptor binding) (Sun et al., 1996b), few studies have considered and analyzed in detail which form of MIF is responsible for receptor activation. Therefore, thus far, it is still a mystery whether the structures of MIF play a role in exerting its distinctive biological functions (see following sections).

It has been suggested that MIF may exert its function through autocrine and paracrine mechanisms. Newly synthesized MIF is stored inside the cells (Nishino et al., 1995), and will be released consequently to stimulation (Bernhagen et al., 1993). Secretion of MIF does not follow the classic ER-Golgi secretory pathway (Flieger et al., 2003). Secreted MIF then exerts its function through different signaling pathways. It is quickly endocytosed into the cells for the intracellular effector binding (Kleemann et al., 2000), or binds to cell surface receptors to regulate downstream signals (Bernhagen et al., 2007; Leng et al., 2003) (Figure 1.1).
Figure 1.1 MIF signaling pathways and biological functions.
(A) MIF binds to the CD74/CD44 receptor complex to activate and sustain ERK1/2 MPA kinase mediated pro-proliferative and anti-apoptotic pathways. (B) Cell signaling sending through cyclin D1 following ERK1/2 activation leads to cell cycle progression. (C) MIF maintains surface level of TLR-4 by promoting its expression through the ETS family of transcription factors. (D) MIF promotes cytoplasmic phospholipase A2 (PLA2) activated arachidonic acid release, and subsequently induces pro-inflammatory signals, such as synthesis of prostaglandins. (E) Interaction between MIF and CD74/CXCR4 complex activates JNK signaling mediated AP-1 transcription. (F) Binding of MIF to CD74 and/or CXCR4 triggers clathrin-dependent endocytosis of MIF, but it is unclear whether MIF will be released to cytoplasm after endocytosis. (G) Activation of JNK pathway is inhibited through the direct interaction between MIF and Jab-1, which follows endocytosis of MIF (I), and in turn inhibits the activation of the transcription factor AP1. (H) LPS or gram-negative bacteria activates TLR-4 and subsequently induces MIF secretion. (J) Activation of TLR-4 also activates NFkB signaling and subsequent gene transcription of pro-inflammatory cytokines. (K) Binding between MIF and CD74/CXCR4 complex triggers activation of AMPK pathway, which regulates glucose transporter translocation to the cell surface during energy deprivation. (L) MIF directly counter-acts on p53-induced apoptosis through its thiol group induced redox activity.

Cell surface receptors for MIF include CD74, CXCR4, and CXCR2. Many intracellular signaling mediated by extracellular MIF were finally connected after Leng et al. identified CD74 as the receptor for MIF, which is a MHC II-associated invariant chain (Leng et al., 2003). In some cases, CD74 requires a co-receptor, CD44 (Shi et al., 2006), to complete the downstream signal transduction (Bifulco et al., 2008; Leng and Bucala, 2006). A number of
studies regarding different biological functions of MIF have demonstrated that binding between MIF and the CD74/CD44 receptor complex activates and sustains ERK1/2 signaling (Figure 1.1A) (Bifulco et al., 2008; Calandra and Roger, 2003; Rassaf et al., 2014). Sustained ERK1/2 activation sending through cyclin D1 leads to cell cycle progression, implicating a mechanism for MIF-mediated oncogenesis (Liao et al., 2003; Swant et al., 2005) (Figure 1.1B). Another downstream signal mediated by the MIF-activated ERK1/2 signaling pathway is to maintain the surface level of TLR-4 (Figure 1.1C). This process is thought to be essential for sensing bacterial infection and inducing MIF release (Roger et al., 2001) (Figure 1.1H). It is possible that bacteria-triggered MIF release sustains ERK1/2 signaling and in turn promotes cytoplasmic phospholipase A2 activated arachidonic acid release, and subsequently induces pro-inflammatory signals, such as synthesis of prostaglandins (Mitchell et al., 1999) (Figure 1.1D). MIF-mediated sustained ERK1/2 signaling also triggers the proliferation of fibroblast-like synoviocytes in rheumatoid arthritis (Lacey et al., 2003).

Besides activation of ERK1/2 signaling, binding between MIF and the CD74/CD44 complex also triggers activation of AMP-activated protein kinase (AMPK) pathway (Miller et al., 2008) (Figure 1.1K), which is known to enhance glucose uptake (through glucose transporter-4 translocation) and stimulates glycolysis, thus limiting energy deprivation induced cell death, such as ischemia (Young et al., 2005).

MIF is also involved in the regulation of the JNK signaling pathways. Depending on the type of the cells (the main determinant of the cell surface receptors) and the upstream signal (i.e., the type of stimulation), MIF-mediated JNK pathways could either be pro-inflammatory or anti-apoptotic. It has been demonstrated that in T cell (Jurkat T cell), interaction between MIF and CD74/CXCR4 complex activates JNK signaling mediated AP-1 transcription, and results in production of CXCL8 (Lue et al., 2011) (Figure 1.1E). For cells lacking or expressing low levels of CXCR4, activation of the JNK pathway is inhibited through the direct interaction between MIF and Jab-1, which follows endocytosis of MIF, and in turn inhibits the activation of the transcription factor AP-1, and AP-1 dependent p27 degradation and G1 cell cycle arrest (Kleemann et al., 2000) (Figure 1.1G). MIF inhibiting activation of JNK-mediated cardiomyocyte apoptosis has also been demonstrated in mice following heart
I/R damage (Qi et al., 2009). It should be noted that Jab-1 is a cytosolic protein, and can be transported to the nucleus for signal transduction. Therefore, the direct binding between MIF and Jab-1 indicates that MIF is freed into cytosol for binding after endocytosis. However, the mechanism involved in MIF endocytosis is not clear. Although a recent study demonstrated that binding of MIF to CD74 and/or CXCR4 triggers endocytosis of MIF following a clathrin-dependent manner (Schwartz et al., 2012) (Figure 1.1F), we cannot rule out the possibility that this binding actually triggers activation of the receptors that transduce signals instead of through direct binding. In addition, MIF as a cytosol protein is in a free form after synthesis, perhaps serving as a potential pool to for Jab-1 binding. Unfortunately, this initial study about MIF-Jab-1 interaction did not claim which pool of MIF is responsible for the interaction, or suggest whether endocytosed MIF could possibly undergo some form of modification through the secretion-endocytosis pathway to be differentiate from newly synthesized pool. However, as far, no significant post-translational modification of MIF has been noticed (Bernhagen et al., 1994), except a covalent binding of isothiocyanates to the first proline of MIF that inhibits the tautomerase activity of MIF (Brown et al., 2009). Therefore, further studies are necessary to elucidate possible mechanisms by which MIF could distinctively exert its biological functions through multiple pathways.

MIF is also identified as a non-cognate ligand for CXCR4 and CXCR2 (Bernhagen et al., 2007). Their binding induces internalization of the receptors and functionally stimulates leukocyte chemotaxis, through which MIF mediates monocyte adhesion to cell walls in the case of atherogenesis (Bernhagen et al., 2007).

1.3.4 Biological functions of MIF

MIF is highly conservative cross species including mammals, birds, fish, parasites, and even plants (Calandra and Roger, 2003), indicating it may have important biological functions. In fact, since its discovery 40 years ago, most of the studies focused on its pro-inflammatory functions (Calandra and Roger, 2003), until recently the actions of MIF has been extended to other areas. Currently, it is widely accepted that MIF has pleiotropic functions and play important roles in many cellular processes including mediating enzyme activity (Kleemann et al., 1998), regulating cell death and survival (Hudson et al., 1999; Kleemann et al., 2000;
Mitchell et al., 2002), and facilitating energy metabolism (Benigni et al., 2000; Miller et al., 2008).

1.3.4.1 The role of MIF in innate immunity and inflammation

Immune cells, which belong to the innate immune system, are circulating in the body and ready anytime to induce host defense. The TLR4 receptor on the surface of immune cells recognizes LPS, thus playing a key role in sensing infections. Moreover, cytokines produced at the site of infection facilitate pathogen killing by inducing necessary inflammation. Studies have shown that MIF assists innate immune system in combating bacterial infection in two aspects. Firstly, constitutive expression of MIF is important in maintaining the surface level of TLR4 at the transcriptional level (Roger et al., 2001). Secondly, MIF is able to override the anti-inflammatory effect of glucocorticoid (Calandra et al., 1995), and thereby promoting the production of a panel of innate cytokines, including TNF, IFN-γ, IL-1β, IL-2, IL-6, IL-8 (Calandra and Roger, 2003). Indeed, knockout of the \textit{MIF} gene results in higher mortality in rodents following experimental infection induced by pathogen inoculations (Das et al., 2013; Flores et al., 2008; Koebernick et al., 2002; Satoskar et al., 2001). These MIF deficient mice produced significantly lower levels of innate cytokines such as IL-12 and TNF-α, and had much higher level of bacterial load after infection (Das et al., 2013; Koebernick et al., 2002).

However, it should be noted although MIF is important in assisting innate immune systems through initiating the first line of host defense and promoting an appropriate level of inflammation, excessive MIF seems to break the balanced inflammatory response and shift it to over-response and leads to fatal consequences. The lethal effects of MIF have been demonstrated in studies that giving recombinant MIF to \textit{Mif}^{+/+} mice results in higher mortality due to overwhelming inflammation after injection of pathological level of LPS (Bernhagen et al., 1993), and that knockout of \textit{Mif} gene saves mice that would otherwise have been killed by overwhelming inflammation induced by the lethal dose of LPS to mimic sepsis shock (Bozza et al., 1999).
1.3.4.2 Pro-proliferation and anti-apoptotic effects of MIF

MIF exerts anti-apoptotic effects mainly through counter-acting on JNK-induced apoptosis, and negatively regulating tumor suppressor p53. JNK3 among the three JNKs (JNK1, JNK2, and JNK3), is expressed primarily in the brain, and plays a critical role in various pathological conditions (Bogoyevitch and Kobe, 2006; Leppa and Bohmann, 1999; Wagner and Nebreda, 2009). JNK3 knockout results in brain tumors and neuronal resistance to excitotoxicity (Leppa and Bohmann, 1999; Wagner and Nebreda, 2009), suggesting a pro-apoptotic role of JNK3. MIF was reported to suppress JNK activation by direct interaction with its co-activator Jab1, suggesting an anti-apoptotic role of MIF, although the paper did not specify which isoform of JNKs was studied (Kleemann et al., 2000). It has been demonstrated that knockout of MIF significantly increases MAPK kinase 4 phosphorylation, followed by activation of JNK (increased phosphorylated JNK level), and in turn BAD, and that enlarges the I/R-induced damage on the heart (Qi et al., 2009). The study also demonstrates that administration of recombinant MIF in cardiomyocytes can antagonize pharmacologically activated JNK by anisomycin (Qi et al., 2009). Additional studies clearly demonstrate cardiomyocytes undergo apoptotic pathway following I/R-induced damage (Luedike et al., 2012).

Tumor suppressor p53, under unperturbed conditions, is kept at low levels; while when stressed (e.g., hypoxia and oxidative stress), p53 is stabilized without detectable DNA damage (Lavin and Gueven, 2006). Stabilized p53 induces intrinsic apoptosis in a transcription independent manner (Niizuma et al., 2009). The anti-apoptotic role of MIF was unveiled by screening the negative regulator of p53 (Hudson et al., 1999). The following function analysis showed that MIF inhibited stress-induced p53 accumulation (Mitchell et al., 2002). However, the mechanisms by which MIF interferes with p53 are still unclear.

1.3.4.3 Modulating glucose metabolism following acute and severe stress

MIF mediates glucose metabolism by regulating insulin release and is thought to play a role in diabetic mellitus (Kleemann and Bucala, 2010; Waeb er et al., 1997). Here, we discuss the role of MIF in glucose metabolism under critical conditions, such as I/R. The role of MIF in glucose metabolism under critical situations has been demonstrated in skeletal myotubes.
after induction of severe infections, where MIF was found to mediate TNF effects to promote glycolysis, resulting in deleterious hypermetabolic stress (Benigni et al., 2000). In contrast, in the case of energy deprivation, such as ischemia, MIF was found to protect cardiomyocytes from I/R-induced damage through activation of AMPK-regulated energy metabolism (Miller et al., 2008). Activation of the AMPK pathway is known to enhance glucose uptake (through glucose transporter-4 translocation) and stimulate glycolysis in the heart, thus limiting myocardial injury (Young et al., 2005). The study by Miller et al. showed that MIF sat in the position where it controlled the activation initiation of glucose uptake. After MIF is secreted in an autocrine/paracrine fashion following stimulation, the binding between MIF and CD74/CD44 receptor complex activates the AMPK pathway (Miller et al., 2008). Later studies using both MIF agonist (Wang et al., 2013a) and using aged animal with decreased MIF expression in cardiomyocytes (Ma et al., 2010) confirm the role of MIF in energy metabolism during I/R.

1.3.4.4 Enzymatic activity of MIF

The role of MIF in the redox regulation was proposed due to its Cys57-Ala-Leu-Cys60 motif, which resembles the CXXC motif of the classical thiol-protein oxidoreductase (TPOR), such as thioredoxin (Kleemann et al., 1998). Studies have demonstrated that the two cysteines formed a disulfide bridge for exerting enzyme activity, and mutation of either Cys57 or Cys60 lost the redox activity (Kleemann et al., 1998; Nguyen et al., 2003a). In addition, the enzymatic activity of MIF was comparable with thioredoxin (Kleemann et al., 1998). The biological function of this motif in the context of redox regulation has been examined. Studies have demonstrated that the functional Cys57-Ala-Leu-Cys60 motif reduces ROS production and counteracts apoptosis induced by oxidative stress (Nguyen et al., 2003b). In addition, studies showed that MIF knockout resulted in elevated ROS level following heart I/R injury, leading to larger infarct (Koga et al., 2011).

A D-dopachrome tautomerase (D-DT) activity has also been proposed for MIF due to their sequence homology, the physiological relevance of this observed activity is currently unclear (Rosengren et al., 1996). However, it was not until recently that a number of studies were carried out to elucidate the relationship between these two proteins. A recent review
systematically compared these two proteins and summarized their similarities, including gene structure, protein structure, expression pattern, and functions in macrophage and cancer cells (Merk et al., 2012). Due to their striking similarity, treatment strategies that are under-development by targeting MIF should also take into consideration the overlapping functions from D-DT.

1.3.5 MIF in human diseases

1.3.5.1 MIF in chronic inflammatory conditions

Persistent upregulation of MIF is observed in many diseases featuring in chronic inflammation, such as rheumatoid arthritis, atherosclerosis, and AD (Bacher et al., 2010; Morand et al., 2006). In patients with rheumatoid arthritis MIF is upregulated systematically (as demonstrated by upregulation of serum MIF), as well as locally at the lesion sites (as demonstrated in synovial fluid) (Leech et al., 1999). MIF expression at the lesion sites locates in the fibroblast-like synoviocytes, endothelial cells, as well as recruited immune cells, mainly macrophages in these patients (Leech et al., 1999; Onodera et al., 1999). In addition, studies using animal models of rheumatoid arthritis induced by various methods have shown systematic and lesion-site upregulation of MIF (Ichiyama et al., 2004; Leech et al., 1998). Treatment by a specific anti-MIF antibody or knockout of MIF gene significantly reduced disease severity, indicating the involvement of MIF in the disease initiation/progression as a disease inducer/enhancer (Ichiyama et al., 2004; Leech et al., 1998; Mikulowska et al., 1997; Onodera et al., 2004).

MIF-mediated inflammatory responses may play a role in atheroma formation in atherosclerosis. Overexpression of MIF is observed in patients with atherosclerosis, and macrophages and form cells in advanced plaques are the main source (Burger-Kentischer et al., 2002). In animals, elevated MIF expression on the lesion site is observed in a rabbit model of atherogenesis induced by a cholesterol rich diet and in APOE deficient mice with spontaneous atherogenesis (Burger-Kentischer et al., 2006; Lin et al., 2000). In vitro studies also showed that oxidized low-density lipoprotein markedly upregulates MIF gene expression in macrophage cell lines (Atsumi et al., 2000). Neutralization of MIF by a specific anti-MIF antibody significantly reduced the pro-inflammatory signals locally and
systematically and alleviated the symptoms (Burger-Kentischer et al., 2006). Knockout of MIF gene significantly delayed atherogenesis and reduced atheroma lesion in low-density lipoprotein-receptor-deficient (Ldlr<sup>−/−</sup>) mice induced by an atherogenic diet, which is also accompanied by reduced smooth muscle cell proliferation (Pan et al., 2004).

In both conditions, MIF-mediated downregulation of p53 is associated with reduced apoptosis of the disease causing cells (e.g., macrophages) that over-expand at the lesion site (Leech et al., 2003; Mercer et al., 2005). MIF is also responsible for recruitment of circulating inflammatory cells to the lesion site through interaction between locally upregulated MIF and chemokine receptors on the inflammatory cells (Bernhagen et al., 2007). In contrast, little is known about the role of MIF in AD, despite previous documentation of MIF expression in activated microglia-like cells surrounding amyloid plaques (Bacher et al., 2010). However, the mechanism for sustained MIF expression is unknown.

In comparison to acute inflammation in which MIF has dual roles, under chronic inflammation, MIF has only the deleterious effects. Therefore, inhibition of MIF activity has been proposed as a therapeutic strategy to treat chronic inflammatory conditions (Greven et al., 2010; Merk et al., 2012).

1.3.5.2 MIF in cancer
Upregulated MIF expression has been demonstrated in various types of cancers, for example, prostate cancers, colon cancers, hepatocellular cancers, adenocarcinomas of the lung, glioblastomas, and melanomas (Bach et al., 2008).

MIF promotes carcinogenesis through several different mechanisms: 1) tumor genesis through p53 and ERK-MAPK-mediated anti-apoptosis and pro-proliferation; 2) facilitating angiogenesis; and 3) potentiates the foregoing events by sustaining long-term inflammation. The ability for MIF in preserving macrophage viability through p53 inhibition (Mitchell et al., 2002) and promoting cell proliferation by activation of cyclin D1 through the ERK-MAPK pathway (Mitchell et al., 1999; Swant et al., 2005; Wilson et al., 2005) may lead to tumor progression and the development of metastases (Mitchell, 2004). In addition, MIF
enhances the differentiation of endothelial cells to blood vessels (Amin et al., 2003; Chesney et al., 1999), thereby facilitating angiogenesis in many types of cancers (Bacher et al., 2003; Hira et al., 2005), which is essential for the survival of tumor cells.

### 1.3.5.3 MIF in cardiac ischemia/reperfusion

It has been reported that MIF has protective effects on cardiomyocytes following I/R *in vivo* (Koga et al., 2011; Miller et al., 2008; Qi et al., 2009). Miller et al. discovered the protective effect of MIF by activating the AMPK pathway and modulating glucose uptake (Miller et al., 2008). In addition, the protective role of MIF could be exerted by alleviating JNK-mediated apoptotic signaling in cardiomyocytes during the reperfusion period (Qi et al., 2009).

Furthermore, MIF was shown to mediate the thiol-oxidoreductase activity, through which it could reduce the level of reactive oxygen species (ROS) and the subsequent apoptosis (Koga et al., 2011). However, a detrimental role of MIF was also observed following I/R injury on the heart. Knockout of MIF reduced inflammatory signal-mediated through TLR4, and in turn reduced infarct size after a prolonged I/R injury to the heart (Gao et al., 2011).

### 1.3.5.4 Polymorphisms of MIF gene promoter and human diseases

Two polymorphisms in the MIF promoter were found to be functionally significant. These are a 5- to 8-CATT repeat on one or both alleles at position −794 (Baugh et al., 2002), and a G to C single nucleotide polymorphism at -173 (Donn et al., 2001) on the human *Mif* gene. In addition, the -173C allele is in strong linkage disequilibrium with the 7-CATT repeat allele (Donn et al., 2002). Both of these polymorphisms affect the transcriptional activity of MIF by *in vitro* assays. Lower basal and stimulated promoter activity was found in the MIF promoter construct with 5-CATT repeat (Baugh et al., 2002). A lower inflammatory response was found in patients who have 5-CATT repeat on at least one allele, and therefore have a lower disease severity in inflammatory diseases, such as rheumatoid arthritis, juvenile arthritis, and colitis (Baugh et al., 2002; De Benedetti et al., 2003; Morand et al., 2006), but are easier to suffer the *Tuberculosis* infection which requires MIF to induct an inflammatory response (Das et al., 2013). However, polymorphisms of MIF promoters did not associated with stroke or AD (Flex et al., 2004a; Flex et al., 2004b).
1.4 NFκB signaling

NFκB signaling plays an important role in gene regulation and is implicated in inflammation, oxidative stress, apoptosis and neurodegenerative disorders (Baeuerle and Henkel, 1994; Schreck et al., 1992; Stockley and O’Neill, 2007). In the CNS, NFκB signaling can be quickly induced following acute critical conditions such as brain traumas, ischemic strokes (Ridder and Schwaninger, 2009; Schneider et al., 1999), or it can be activated chronically due to the persistency of the disease condition such as Aβ production in AD (Granic et al., 2009). NFκB regulates genes involved in both pro- and anti-apoptotic processes and plays a complex role in neuronal survival/death (Mattson et al., 2000; Qin et al., 2007; Ridder and Schwaninger, 2009). Constitutive activation of NFκB signaling is often associated with chronic inflammatory diseases, such as rheumatoid arthritis (Li and Verma, 2002).

The active NFκB is a dimer composed of two of the five subunits in the NFκB family, RelA (p65), c-Rel, RelB, p105/p50, and p100/p52. The most abundant dimer in mammalian cells is composed of p65 and p50. Upon stimulation, the IκB Kinase (IKK) phosphorylates IκB, resulting in the degradation of IκB by the ubiquitin proteasome pathway. Degradation of IκB releases its binding partner NFκB, and allows it to be translocated from the cytoplasm to the nucleus where it activates/suppresses the transcription of its target genes. Active NFκB specifically recognizes a DNA sequence of 5’-GGGRNNYYCC (N and Y represent any base and pyrimidine, respectively), and modulates gene transcription by direct binding to the cis-acting elements on the promoter region of the target genes (Baldwin, 1996; Miyamoto and Verma, 1995).

1.5 Overall goal of this research

As a pleiotropic protein, MIF participates in many cellular activities and plays a role in regulating inflammatory responses, energy metabolism, and apoptosis. Upregulation of MIF levels has been associated with many chronic inflammatory conditions such as cancer, rheumatoid arthritis and atherosclerosis, and inhibition of MIF activity has been proposed as a therapeutic strategy (Bach et al., 2008; Calandra and Roger, 2003; Morand et al., 2006). In contrast, disruption of MIF expression is also problematic under stress-induced acute conditions such as infection (Koebernick et al., 2002) and heart ischemia (Koga et al., 2010;
Miller et al., 2008) – knockout of the MIF gene resulted in higher mortality (Koebernick et al., 2002) and larger infarct volumes (Koga et al., 2010; Miller et al., 2008), respectively. In contrast to a large body of research regarding the role of MIF in the systems outside the CNS, research on the role of MIF in neurological diseases is still in its infancy. Based on the evidence obtained from previous studies that 1) MIF is anti-apoptotic for most salvageable neurons after stroke undergo apoptosis, 2) MIF was transiently upregulated in stroke patients and rats subjected to experimental strokes (Wang et al., 2009), and 3) it protected cardiomyocytes following I/R, it is possible that MIF could protect neurons against strokes. In addition, a deleterious role of MIF has been implicated in chronic inflammatory conditions by sustaining the over-reactive macrophages. Since AD features in the chronic activation of microglia which are the macrophage in the CNS, it is intriguing to explore MIF could also play a role in AD. Moreover, since chronic inflammatory conditions such as atherosclerosis are a major risk factor for strokes, it raises a question about whether inhibition of MIF will put the patients with chronic inflammatory conditions at a risk of a worsened stroke outcome. It will be of interest to study whether we could control the chronic inflammatory conditions without disturbing the natural defense mechanism of MIF. The overall goal of this thesis is to investigate the expression regulation and potential role of MIF in stroke and AD.

1.5.1 To examine the regulation of MIF gene expression by NFκB signaling
MIF expresses constitutively in the brain and are tightly regulated at the transcriptional level by gene specific transcription factors under certain conditions (Baugh et al., 2006; Waeger et al., 1998; Wang et al., 2009; Welford et al., 2006). Previous studies from our lab demonstrate that the MIF level is quickly upregulated and remained highly expressed till the semi-acute phase of the stroke, and then decline to baseline level in stroke model rats (Wang et al., 2009). During stroke, nuclear translocation of NFκB p65 is observed in neurons located in the infarct, indicating an active role of NFκB in ischemic neurons (Schneider et al., 1999). Despite the fact that both NFκB activation and alteration of MIF expression are observed during the early stages of cerebral ischemia, the interaction between NFκB and MIF remains undefined. Additionally, the MIF promoter contains several putative NFκB binding sites. Taken together, we hypothesize that MIF could be transcriptionally regulated by NFκB signaling.
1.5.2 To investigate the regulation of MIF expression and its role in stroke

We have shown in Chapter 2 that NFκB regulates MIF gene expression at the transcriptional level \textit{in vitro}. Here, we aimed to study how NFκB regulates MIF gene expression following ischemic stroke \textit{in vivo} using a stroke model on mice. Moreover, we will explore the role of MIF during cerebral ischemia. It has been demonstrated that MIF plays a protective role on cardiomyocytes during heart ischemia. MIF promotes cell survival by modulating glucose uptake and metabolism during energy deprivation and suppressing oxidative stress-induced apoptosis through the redox activity in mouse models of myocardial infarction (Miller et al., 2008; Nguyen et al., 2003a; Nguyen et al., 2003b). Since cardiomyocytes in the heart and neurons in the brain share some similar characteristics such as at the end stage of differentiation and are surrounded by supporting cells including immune cells, we hypothesize that MIF may be protective for neurons during cerebral ischemia. We first proposed to study whether MIF could be protective for neurons that are challenged by stroke-like conditions \textit{in vitro}. Since several lines of evidence point an anti-apoptotic role of MIF, we next proposed to examine the pathway through which MIF could suppress apoptotic signals. Finally, we would like to compare the stroke pathology after cerebral ischemia between the MIF knockout mice and their controls to demonstrate the protective role of MIF \textit{in vivo}. The challenge to demonstrate the protective effect of MIF \textit{in vivo} is that MIF is shown to interact with inflammatory signals, which are known to be deleterious to stroke pathology. Indeed, MIF has been found to contribute to stroke pathology in mice and knockout of the \textit{MIF} gene results in smaller infarct volumes at day 7 after stroke (Inacio et al., 2011b). Although they did not observed the effect of MIF knockout on the production of a set of inflammatory cytokines during these 7 days in their follow-up study (Inacio et al., 2011a), they did demonstrate that a lack of MIF changes the phenotypes of infiltrated macrophages following a stroke (Inacio et al., 2011b). Since inflammatory signals, especially the influence from microglia/macrophages come 24 hours after stroke onset (Jin et al., 2010), we need to carefully dissect the time window that MIF preserves its protectives function. The working hypothesis is that MIF suppresses oxidative stress-induced apoptosis and is protective for mice subjected to cerebral ischemia.
1.5.3 To explore the potential role of MIF in AD

We have demonstrated in Chapter 3 that MIF plays a protective role during the acute phase of a stroke, together with the findings that MIF could result in worse stroke pathology by influencing macrophage activity during the late phase of the stroke (Inacio et al., 2011b), suggesting dual roles of MIF during a stroke depending on the time window. On the other hand, sustained expression of MIF was found to be predominantly deleterious in chronic inflammatory diseases such as rheumatoid arthritis and during atherogenesis by mediating the recruitment of macrophages to the lesion sites (Morand et al., 2006; Tillmann et al., 2013). Chronic inflammation and activation of microglia are the features of AD (Wyss-Coray and Rogers, 2012). MIF was found to be elevated in the CSFs from AD patients, as well as in the sera indicating systematic upregulation of MIF (Craig-Schapiro et al., 2011; Lee et al., 2008; Popp et al., 2009). Studies have demonstrated that stroke increases the risk of developing or accelerates the progression of AD (Leys et al., 2005; Pluta and Amek, 2008; White et al., 2002). Since MIF expression is altered in both pathological processes, it is interesting to determine whether dysregulation of MIF in stroke plays a role in AD pathogenesis. To answer this question, we will have to first understand how MIF participates in the pathology of AD. However, the study on the role of MIF in AD is extremely limited. The only available knowledge in this respective is that MIF is upregulated (Craig-Schapiro et al., 2011; Lee et al., 2008; Popp et al., 2009) and may promote toxic effects induced by Aβ in a neuroblastoma cell line (Bacher et al., 2010). Therefore, in this chapter, we aimed to characterize the expression pattern of MIF in both AD patients and a mouse model of AD, and try to explore the potential function of MIF during AD pathogenesis using animal models.
Chapter 2

Characterization of NFκB signaling regulated MIF gene expression

2.1 Introduction

MIF is ubiquitously expressed in most organs including the heart, brain, lung, liver and kidney (Bacher et al., 1998; Calandra et al., 1994; Paralkar and Wistow, 1994). In the brain, MIF is expressed in both neurons and glial cells (Bacher et al., 1998; Ogata et al., 1998). Although the TATA-less promoter of MIF contributes to its constitutive expression, it also contains gene specific binding sequences for transcription factors. Cyclic-AMP response elements (CRE) on MIF promoter was shown to directly interact with CREB for transcriptional regulation of MIF gene expression in pituitary cells (Waerber et al., 1998). Our laboratory and others identified several hypoxia responsive elements (HREs) in the MIF promoter (Baugh et al., 2006; Wang et al., 2009; Welford et al., 2006). The binding of hypoxia inducible factor-1α (HIF-1α) to the HREs in MIF promoter resulted in transcriptional upregulation in vitro and in the ischemic hemisphere of rats during stroke (Wang et al., 2009).

NFκB signaling plays an important role in gene regulations and is implicated in inflammation, oxidative stress, apoptosis and neurodegenerative disorders (Baeuerle and Henkel, 1994; Schreck et al., 1992; Stockley and O'Neill, 2007). In the CNS, NFκB signaling can be quickly induced following acute critical conditions such as brain traumas and ischemic strokes (Ridder and Schwaninger, 2009; Schneider et al., 1999), or be activated chronically due to the persistency of the disease condition such as the Aβ production in AD (Granic et al., 2009). NFκB regulates the genes involved in both pro- and anti-apoptotic processes and plays a complex role in neuronal survival/death (Mattson et al., 2000; Qin et al., 2007; Ridder and Schwaninger, 2009). Therefore, it is important to characterize the
regulation of these NFκB regulated genes to better understand their roles in diseases. Previous studies from our own lab have demonstrated that genes associated with AD pathogenesis, such as BACE1 and UCHL1, are regulated through NFκB signaling (Chen et al., 2012; Wang et al., 2011). Activation of NFκB signaling upregulates BACE1 transcription and expression, and results in an increased Aβ production, thereby contributing to AD pathogenesis (Chen et al., 2012; Sun et al., 2012; Sun et al., 2006b). In contrast, UCHL1, which promotes cell survival and reduces Aβ production, is downregulated by NFκB signaling (Wang et al., 2011; Zhang et al., 2012). A sequence analysis showed that MIF promoter contains multiple putative NFκB binding sites. However, whether these binding sites are functionally regulate MIF gene expression needs further investigation. Thus, in this chapter we will characterize regulation of MIF gene expression by NFκB signaling.

2.2 Methods

2.2.1 Generation of human MIF expression plasmids and promoter constructs

Human MIF cDNA was amplified from HEK293 cells and cloned into pcDNA4-mycHis vector to generate pMIF-mycHis mammalian expression plasmid. pMIF-Stop, expressing human MIF protein without any tag, was generated from pMIF-mycHis. Previously, we have constructed a 5’ upstream fragment of the human MIF gene promoter regions -2634 to +35 and -553 to +8 Base pair (bp) into pGL3-basic vector to generate MIF promoter luciferase report plasmid phMIFluc and HRE-deleting plasmid phMIFδHluc, respectively (Wang et al., 2009). phMIFluc was digested by KpnI to remove a fragment between two KpnI sites and the new construct phMIF-Bluc plasmid containing promoter region -2158 to +35 bp. Plasmids phMIF-Cluc and phMIF-Dluc, containing the human MIF promoter region from -553 to +35, and -194 to+35 bp, respectively, were generated by PCR amplification from phMIFluc and cloned into pGL3-basic vector at XhoI and HindIII sites. Human MIF promoter fragment of -5415 to -3090 bp was amplified by PCR with primers NheIHMIF1f (5’-ctagctagcagcagcgccttgtttctacca) and BglIIHMIF1r (5’-ggaagctctctttgtggactcctcttcata), and cloned into pGL3-basic at NheI and BglII sites to generate phMIF1-luc. Plasmid phMIF2-luc contains human MIF promoter from -3358 to +35 bp inserted between NheI and HindIII, having an endogenous BglII site located at -3355. To generate phMIF5k-luc, phMIF1-luc deected by NheI and BglII, and the fragment spanning -5415 to -3354 bp was
inserted into phMIF2-luc between *NheI* and *BglII* site. Plasmid pMIF-NFκBluc was generated by cloning the double-strand oligonucleotides (5’-ccgGGGGCTTTCCcaatGGGGCCTCCCagcaGGGAAGTTCCctg) containing three NFκB *cis*-acting elements from human MIF promoter, namely MIF-NFκB, into the pGL-pl vector (Cai et al., 2008) at *SacI* and *NheI* sites.

### 2.2.2 Cell culture and transfection

The human embryonic kidney cell line HEK293, mouse microglia cell line BV-2, mouse neuroblastoma cell line N2A, and human neuroblastoma cell line SH-SY5Y were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1 mmol/L of sodium pyruvate, 2 mmol/L of L-glutamine (Invitrogen, Carlsbad, CA, USA). Cells were cultured at 37°C in an incubator supplemented with 5% CO₂. Plasmids were transfected by either the calcium-phosphate method or lypofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacture instruction. Two micrograms of plasmid were transfected into one 35-mm tissue culture plate. Fresh medium was changed 6 hours after transfection. Cells were harvested 24 hours after transfection by centrifuge at 3,000xg for 2 minutes at 4°C followed by storage at -80°C till further analyses.

### 2.2.3 Luciferase assay

Cells were plated onto 24-well plates 24 hours prior to transfection and cultured to approximately 50% confluence before transfection. In each well, cells were transfected with 0.5 µg of plasmids using calcium phosphate transfection methods. pCMV-Rluc plasmid (1ng) expressing *Renilla* luciferase was co-transfected for normalization for the transfection efficiency. After 48 hr transfection, cells were harvested and lysed with 100 µL 1X passive lysis buffer, and a dual luciferase assay was performed as previously described (Zhou and Song, 2006). Firefly luciferase activities and *Renilla* luciferase activities were measured using the dual-luciferase reporter assay system (Promega). The firefly luciferase activity was normalized to the *Renilla* luciferase activity and expressed as relative luciferase units (RLU) to reflect the promoter activity.
2.2.4 Gel shift assay

Gel shift assay (GSA), or electrophoretic mobility shift assay (EMSA), was performed as previously described (Ly et al., 2013). Nuclear extract was fractionated from the HEK293 lysate with NFκB overexpression, as previously described (Chen et al., 2012). Oligonucleotides probes were labeled with IR700 Dye (Integrated DNA Technologies) and annealed to generate double-stranded probes at a final concentration of 0.1 pmol/µl. For competition experiments, nuclear extract was incubated with 0.5 pmol of the labeled probe and excess of unlabeled competition oligonucleotides for 20 min at 22°C. For the supershift assay, mouse anti-NFκB p65 monoclonal antibody (Sigma) was added to the above reaction and incubated for additional 20 min. The samples were analyzed on a 4% non-denaturing polyacrylamide gel, and the gel was scanned using the Odyssey scanner at a wavelength of 700 nm (LI-COR Biosciences). The sequences of the sense strand oligonucleotides to generate the double-strand probes or competitors are listed in the table 2.1.

Table 2.1 The sense strand of the DNA oligomers used for studying the interaction between NFκB and the putative consensus binding elements on the MIF gene promoter.

<table>
<thead>
<tr>
<th>Identification</th>
<th>Sequence (sense)</th>
<th>Location on MIF promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type consensus NFκB</td>
<td>5’-agttgagGGGACTTTCCcaggc</td>
<td>n/a</td>
</tr>
<tr>
<td>mutant NFκB</td>
<td>5’-agttgagGCCACTTTCCcaggc</td>
<td>n/a</td>
</tr>
<tr>
<td>MIF-NFκB-A</td>
<td>5’-gctccgGGGGCTTTCCcaagga</td>
<td>-2536 to -3527</td>
</tr>
<tr>
<td>MIF-NFκB-B</td>
<td>5’-ggccatGGGCCTCCCagctgg</td>
<td>-1027 to -1018</td>
</tr>
<tr>
<td>MIF-NFκB-C</td>
<td>5’-ggttcaGGGAAGTTCCctggat</td>
<td>-513 to 504</td>
</tr>
</tbody>
</table>

2.2.5 Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed, as described previously (Xu et al., 2012). Cross-linking between protein and chromatin was achieved by adding formaldehyde to the final concentration of 1.42% in NFκB-enriched HEK293 cells for 15 min at 22°C, and was quenched with glycine at final concentration of 125 mM for 5 min. Cells then were harvested in cold PBS and lysed with immunoprecipitation (IP) buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, NP-40 (0.5% vol/vol), Triton X-100 (1.0% vol/vol), and supplemented with protease inhibitor cocktail Complete (Roche Molecular Biochemicals). The nuclear pellets were isolated by centrifugation at 12,000g for 1 min, and resuspended with the IP buffer. The chromatin then was sheared by sonication on ice. For isolation of NFκB binding complex, the chromatin solution was incubated with a monoclonal rabbit-anti-NFκB p65 antibody (Cell
signaling, #8242) or normal rabbit serum overnight at 4°C. After IP, crosslink was reversed by boiling the sample with Chelex100 for 10 min. The supernatant containing DNA fragments was isolated by centrifugation at 12,000g for 1 min, and then used as template for PCR analysis of the chromatin fragments. The following pairs of primers, 5’- atagcctegaaggacaggag and 5’-aatccagttgccccacattt, 5’-cagctgaggaacaataccc and 5’-ctcagagagttcagggag, and 5’-agctgacagtggtaaccac anf 5’-tagtcatgcgggcaggtgaga, were used to specifically amplify fragments containing MIF-NFκB-A, B, and C, respectively. β-actin was amplified by 5’-gacaggaatcaggaagag and 5’-ttgctgatccccagctg as the internal control.

2.2.6 Semi-quantitative RT-PCR

Cell samples were homogenized with the Tri-Reagent, and total RNA was extracted following the manufactures instruction (Invitrogen). The first strand cDNA was generated from the total RNA (1μg) by the ThermoScript reverse transcriptase with Oligo(dT)20 as the primer. The resulted cDNA products were used as templates to quantify the expression of target genes by semi-quantitative PCR with Taq DNA polymerase and specific primer sets. The human MIF gene specific primers were 5’-gtagtctgacgtcagcggaggc (mMIFqRT-57F) and 5’-ctaaagctgatctgtgcctgaggc (mMIFqRT234R). The primers specifically amplifying a mouse EST were 5’-atgccgatgttcatcgtaacc and 5’-ttaggcgaaggtggttcccagc. The GAPDH gene specific primers were 5’-ccagtgagcttcccgttcagc and 5’-ccatcaccatctcagggagc. PCR products were separated on a 2% agarose gel, and the band intensity was analyzed by ImageJ.

2.2.7 Immunoblotting

Cells are harvested with cold PBS and homogenized by sonication with 5X (v/w) RIPA-DOC lysis buffer supplemented with complete mini protease inhibitor cocktail tablet (Roche Molecular Biochemicals). The samples were then centrifuged at 16,000 Xg at 4°C for 5 minutes. The supernatants were removed and added to 2X Novex® tricine SDS sample buffer (Invitrogen) followed by boiling at 100°C for 2 minutes. The samples were resolved in 12% tris-tricine gels and transferred to PVDF-FL membranes (Millipore). The membranes were blocked with 5% non-fat milk and incubated with primary antibodies for MIF (Torrey Pines Biolabs, Secaucus, NJ, USA), NFκB p65 (Cell signaling, #8242), and β-actin (Sigma,
AC-15). To detect the proteins, IDye680-labeled goat anti-rabbit and IDye800-labeled goat anti-mouse antibody were used. The blots were scanned using the Odyssey Imager (Licor).

2.3 Results

2.3.1 NFκB directly binds to its cis-acting elements on the human MIF promoter

To examine whether NFκB signaling modulates MIF gene expression, human MIF gene promoter function was analyzed. Sequence analysis revealed three putative NFκB cis-acting binding elements on the MIF promoter located at -2536 to -2527, -1027 to -1018, and -513 to -504 bp (Fig. 2.1A), suggesting MIF may be regulated by NFκB. To investigate whether these putative binding sites could bind to NFκB p65, gel shift assay was performed. Double strand oligonucleotides containing the core sequences of all three sites (MIF-NFκB) or an individual site (MIF-NFκB-A, -B, or -C) with flanking nucleotides were synthesized (Fig. 2.1A). Incubation of the wildtype NFκB probe (wt-NFκB-IRDye 700) with NFκB p65 enriched HEK nuclear extracts resulted in a shifted band composed of DNA-protein complex (Fig. 2.1B, lane 2). Competition assays demonstrated that the intensity of the shifted band was significantly reduced or abolished by adding a 5- or 50-fold molar excess of unlabeled wildtype NFκB oligonucleotide (wt-NFκB) (Fig. 2.1B, lane 3, 4), while the mutant NFκB oligonucleotide (mu-NFκB) did not compete with the probe at either concentration (Fig. 2.1B, lane 5, 6). MIF-NFκB significantly reduced the intensity, or completely abolished the shifted band at a 5- or 50-fold molar excess of the probe, respectively (Fig. 2.1B, lane 7, 8). Addition of anti-NFκB p65 monoclonal antibody further retarded the migration of the DNA-protein complex (Fig. 2.1B, lane 9), resulting in a supershifted band, which was abolished by adding 50-fold molar excess of wt-NFκB (Fig. 2.1B, lane 9, 10). This result confirmed the binding between the NFκB p65 and the probe. These data indicated that at least one of the three putative binding sites could bind with NFκB p65.
Figure 2.1 Identification of functional NFκB elements in the human MIF promoter.

(A) Schematic diagram showed the relative location of the putative NFκB binding sites on the human MIF promoter, and the corresponding sequence of the oligonucleotides. (B) and (C) Gel shift assays were performed using a wildtype NFκB consensus oligonucleotide (wt-NFκB) labeled with IRDye 700 as the probe. Lane 1 is labeled probe alone without nuclear extract. Incubation of the wildtype NFκB probe with nuclear extract resulted in a shifted band composed of DNA-protein complex (lane 2). Addition of anti-NFκB p65 monoclonal antibody in the incubation mixture as in lane 2 resulted in supershifted (B, lane 9). Competition assays were performed by adding 5- or 50-fold molar excess of unlabeled wildtype NFκB oligonucleotide (wt-NFκB) (lane 3, 4), mutant NFκB oligonucleotide (mu-NFκB) (lane 5, 6), MIF-NFκB (B, lane 7, 8), MIF-NFκB-A (C, lane 7, 8), -B (C, lane 9, 10), -C (C, lane 11, 12), and 50-fold molar excess of wt-NFκB (B, lane 10). (D) Gel shift and supershift assays were performed using MIF-NFκB-A oligonucleotide or (E) using MIF-NFκB-C oligonucleotide labeled with IRDye 700 as probes. Lane 1 is labeled probe alone without nuclear extract. Incubation of IRDye 700-MIF-NFκB-A or -C with nuclear extracts resulted in a shift band (lane 2). Addition of anti-NFκB p65 monoclonal antibody resulted a supershifted band (lane 5). Competition assays were achieved by adding 100-fold molar excess of unlabeled wt-NFκB (lane 3, 6), or the same amount of mu-NFκB (lane 4). (F) ChIP assay details described in the Material and Method section. Monoclonal rabbit-anti-p65 antibody was used to precipitate DNA fragments that could bind to NFκB p65. Target DNA fragments containing sequences corresponding to MIF-NFκB-A, -B, and -C were amplified by PCR. PCR products were resolved on 1.5% agarose gel. β-actin was amplified as control.
To determine which binding sites acts as the cis-acting element for NFκB, gel shift assays were performed with individual putative MIF-NFκB binding oligonucleotides using wt-NFκB-IRDye 700 as the probe. MIF-NFκB-A, located at -2536 to -1517 bp and MIF-NFκB-C at -513 to -504 bp, reduced the binding intensity of the probe significantly (Figure 2.1C, lane 7, 8, 11, 12), whereas MIF-NFκB-B at -1027 to -1018 bp did not compete with the probe (Figure 2.1C, lane 9, 10). This result indicated that MIF-NFκB-A and MIF-NFκB-C contained the NFκB cis-acting element capable of binding with transcription factor NFκB p65 in vitro. To further confirm the binding, MIF-NFκB-A and MIF-NFκB-C were labeled with IRDye 700 and used as probes. After incubating MIF-NFκB-A probe with NFκB p65 enriched HEK nuclear extract, a shifted band was observed (Figure 2.1D, lane 2), which was completely abolished by adding 100-fold molar excess of unlabeled wt-NFκB as the competitor (Figure 2.1D, lane 3), but not by the same amount of mu-NFκB (Figure 2.1D, lane 4). Addition of anti-NFκB p65 monoclonal antibody further retarded the shift of the DNA-protein complex (Figure 2.1D, lane 5), and the supershifted band was abolished by adding 100-fold molar excess of the unlabeled wt-NFκB (Figure 2.1D, lane 6). The same results were observed for MIF-NFκB-C (Figure 2.1E). These results clearly demonstrate that the MIF promoter contains two cis-acting elements for NFκB binding.

### 2.3.2 NFκB recognises the identified binding elements on MIF promoter under physiological conditions.

The ChIP assay was performed to examine whether NFκB could recognize the consensus sequences on the MIF promoter under physiological conditions. NFκB p65 monoclonal antibody was used to precipitate the DNA fragments that could bind to NFκB p65. DNA fragments from the immunoprecipitates pulled-down by NFκB p65 antibody were amplified by PCR using three pairs of MIF NFκB putative consensus binding site-specific primers. The results confirmed that sequences corresponding to MIF-NFκB-A and -C but not MIF-NFκB-B were amplified (Fig. 2G). β-actin, as the control for antibody specificity, could not be amplified after immunoprecipitation (Figure 2.1F). Sheared gDNA without immunoprecipitation was amplified by all the pairs of primers, indicating the target DNA sequences on the MIF promoter were intact due to their binding to NFκB p65, while after sham immunoprecipitation none of these pairs of primers amplified the targeting sequences,
indicating none of our tested DNA sequences could be precipitated nonspecifically (Figure 2.1F). Taken together, these results confirm that the two cis-acting elements MIF-NFκB-A and -C on the human MIF promoter were specifically recognized by transcription factor NFκB under physiological conditions.

2.3.3 Human MIF gene promoter is responsive to NFκB signaling activation

To investigate whether NFκB signaling regulates transcriptional activation of MIF gene expression via the cis-acting elements identified in the MIF promoter, a series of MIF gene promoter deletion plasmids were constructed and assayed. Previously, a 2.6kb human MIF promoter from -2634 to +35 bp was cloned into a luciferase reporter plasmid to generate phMIF-luc, and this promoter activity was upregulated under hypoxia (Wang et al., 2009). In the present study, MIF promoter constructs were co-transfected with an NFκB p65 expression plasmid or its backbone as the control into cells and the promoter activities were measured by luciferase assay. Overexpression of NFκB p65 significantly increased the MIF promoter activity to 1.99±0.09 folds of the control (P<0.05) (Figure 2.2A), and further confirmed by Western blot showing the luciferase protein level was increased to 1.33 ± 0.12 folds of the control (P<0.05) (Figure 2.2B and C). To further investigate the effect of NFκB signaling on the MIF promoter, plasmid phMIF-5kluc (Figure 2.2D) containing -5415 to +35 bp of the MIF promoter was co-transfected with an NFκB p65 expression plasmid. The promoter activity of phMIF-5kluc was elevated by 2.33±0.19 folds compared to control (P<0.05), similar to the effect on phMIF-luc (Figure 2.2A). To confirm the cis-acting elements’ effect, the MIF-NFκB oligonucleotides containing the binding sites (Figure 2.1A) were cloned into a modified luciferase reporter vector pGL-pl (Cai et al., 2008) to generate the plasmid pMIF-NFκB\text{Luc} (Figure 2.2E). This plasmid was created to exclude the interference from other elements with the interaction between NFκB and the MIF promoter. NFκB p65 overexpression markedly increased the promoter activity of pMIF-NFκB\text{Luc} by 33.3±1.9 folds (P<0.001) (Figure 2.2A). To determine the contribution of the individual binding sites to the promoter activity upon NFκB activation, plasmids containing MIF promoter fragments with different NFκB binding sites and/or pseudo-binding sites were generated (Figure 2.2F). Notably, overexpression of NFκB did not affect the promoter activity of phMIF-D\text{Luc} that lacked NFκB binding sites. In contrast, the promoter activities of
phMIF-luc, phMIF-Bluc, and phMIF-Cluc were significantly increased upon overexpression of NFκB p65 by 5.42±0.59, 2.91±0.24, and 1.94±0.08 folds (P<0.05) (Figure 2.2F). The results clearly demonstrate that activation of NFκB signaling enhances MIF promoter activity.

Figure 2.2 Human MIF gene promoter is regulated by NFκB signaling.  
(A) HEK293 cells were co-transfected with MIF promoter plasmids and NFκB p65 expression plasmid or its backbone. Cells were harvested with passive lysis buffer and measured for luciferase activity. The firefly luciferase activity was normalized to the Renilla luciferase activity for transfection efficiency control, and expressed as relative luciferase units (RLU) to reflect the promoter activity. The values are expressed as mean±SD, n=6. * P<0.001 relative to controls by two-way ANOVA with Bonferroni post hoc tests. (B) HEK293 cells were transfected with phMIF-luc, and were equally split into 35-mm plates 4 hours after transfection. The cells were further transfected with NFκB p65 expression plasmid or its backbone. Cells were harvested 48 hours after the second transfection, and the expression of luciferase and β-actin was assessed by Western blot. (C) Quantification of luciferase protein level. Relative intensity represents the ratio of luciferase to β-actin level expressed as mean ± SEM, n = 3. *P<0.001 relative to control by Student’s t test. (D) Restriction enzyme digestion check for phMIF 5k-luc. A 1.6k fragment was generated from an endogenous site on the promoter region of phMIF1-luc and the restriction enzyme cutting site on the vector originated from phMIF2-luc, indicating a success insertion of phMIF1-luc promoter region into phMIF2-luc vector. See the detailed cloning information of phMIF1-luc and phMIF2-luc in the materials and Methods section. (E) Restriction enzyme digestion check for pMIF-NFκB-luc. The empty vector pGL-pl and pMIF-NFκB were
digested by Sac I and Hind III, resulting in a ~140 bp and a ~190 bp fragments from the vector and pMIF-NFκB-luc, respectively. (F) Schematic diagram of deletion plasmids containing different human MIF promoter fragments in front of the firefly luciferase reporter gene. Vertical arrows indicate the starting location of the putative NFκB binding sites. The deletion plasmids were confirmed by sequencing and restriction enzyme digestion checking and digested samples were analyzed on a 0.7 % agarose gel. Vector size is 4.7 kb and the MIF promoter fragment inserts range from ~230 bp to 2.7 kb. Luciferase assay was performed as in (A). The values are expressed as mean±SD, n=6. * P<0.001 relative to controls by two-way ANOVA with Bonferroni post hoc tests.

2.3.4 Activation of NFκB signaling increases MIF expression in different cell lines

To examine the effect of NFκB signaling on endogenous MIF gene expression, an NFκB p65 expression plasmid was transfected into N2A and HEK293 cells. Twenty-four hours after transfection, cells were harvested for mRNA and protein level analysis. NFκB p65 expression significantly increased MIF expression at both the mRNA (Figure 2.3A) and protein (Figure 2.3C) levels to approximately 1.68±0.11 (P<0.05) (Figure 2.3B) and 2.42±0.36 folds of the controls (P<0.05) (Figure 2.3D) in N2A cells, respectively. The endogenous levels of MIF mRNA (Figure 2.3E) and protein (Figure 2.3G) were significantly increased in HEK293 cells after transfection with NFκB p65 expression plasmid. The level of MIF mRNA was increased to 1.29±0.08 fold of the control (P<0.05) (Figure 2.3F) and the protein level was increased to 1.70±0.25 fold of the control (P<0.05) (Figure 2.3H).

Microglia is the major immune cell type in the brain responding to stroke. Therefore, we investigated the effect of NFκB signaling activation on MIF expression in a mouse microglia cell line BV-2. NFκB p65 expression plasmid was transfected into BV-2 cells. Twenty-four hours after transfection, cells were harvested for mRNA and protein level analysis. MIF mRNA expression in BV-2 cells was significantly upregulated by 1.42±0.16 fold of the control (P<0.05) (Figure 2.3I and J); and the protein level of MIF also was elevated by 1.93 ± 0.12 folds of the control (P<0.05) (Figure 2.3K and L). Infiltration of bone-marrow macrophages into the CNS is suggested in pathological conditions including stroke and AD. Therefore, we also investigated the effect of NFκB signaling activation on MIF expression in a mouse macrophage cell line RAW264.7. LPS, a specific NFκB activator for RAW264.7 cell line through TLR4 receptor was added to culture medium (Underhill et al., 1999). After treated with 0.01 ng/ml of LPS to stimulate NFκB signaling, MIF mRNA expression in RAW cells was significantly upregulated for 1.92±0.15 folds (P<0.05) (Figure 2.3M and N);
and the protein level of MIF was also elevated by 1.79 ± 0.21 folds (P<0.05) (Figure 2.3O and P). Taken together, these results clearly demonstrate that MIF gene expression is upregulated by activation of NFκB signaling.

Figure 2.3 Regulation of human MIF gene expression by NFκB signaling in cell lines.
(A) N2A cells were transfected with NFκB expression plasmid. Total RNA was extracted from cells by Tri-reagent, and semi-quantitative RT-PCR was used to measure mouse MIF mRNA in N2A cells with mouse MIF gene-specific primers. GAPDH mRNA was used as the internal control. (B) Quantification of (A). The level of MIF mRNA was expressed as relative ratio to GAPDH. The values are shown as mean±SD, n=3. * P<0.01 relative to controls by Student’s t test. (C) N2A cells were transfected with NFκB expression plasmid. Protein levels of MIF in N2A cells were assessed by Western blot on a 12% Tris-tricine SDS-PAGE gel. MIF was detected by anti-MIF antibody and β-actin was used as internal control. (D) Quantification of (C). The level of MIF protein was expressed as relative ratio to β-actin. The values are shown as mean±SD, n=3. * P<0.01 relative to controls by Student’s t test. (E) HEK293 cells were transfected with NFκB expression plasmid or its backbone. Total RNA was extracted from cells by Tri-reagent, and semi-quantitative RT-PCR was used to measure human MIF mRNA. GAPDH mRNA was used as the internal control. (F) Quantification of the MIF mRNA level. The values are shown as mean±SD, n=3. * P<0.01 relative to controls by Student’s t test. (G) HEK293 cells were transfected with NFκB expression plasmid or its backbone. Protein levels of MIF in HEK293 cells were assessed by Western blot after being resolved on a 12% Tris-tricine SDS-PAGE gel. MIF was detected by anti-MIF antibody and β-actin was detected by β-actin antibody as internal control. (H) Quantification of MIF protein level. The values are shown as mean±SD, n=3. * P<0.01 relative to controls by Student’s t test. (I) MIF mRNA expression in BV-2 cells after transfection of NFκB expression plasmid or its backbone. Total RNA was extracted from cells by Tri-reagent, and semi-quantitative RT-PCR was used to measure mouse MIF mRNA. GAPDH mRNA was used as the internal control. (J) Quantification of MIF
mRNA level. The values are shown as mean±SD, n=3. * P<0.01 relative to controls by Student’s t test. (K) MIF protein expression in BV-2 cells following transfection of NFκB expression plasmid or its backbone. Protein levels of MIF were analyzed by Western blot on a 12% Tris-tricine SDS-PAGE gel. (L) Quantification of MIF protein level. The values are shown as mean±SD, n=3. * P<0.01 relative to controls by Student’s t test. (M) MIF mRNA expression following NFκB signaling activation in RAW 264.7 cells was assessed by addition of LPS at the concentration of 0.01 ng/ml. Total RNA was extracted from cells by Tri-reagent, and semi-quantitative RT-PCR was used to measure mouse MIF mRNA by specific primers recognizing EST of mouse MIF gene. GAPDH mRNA was used as the internal control. (N) Quantification of (M). The level of MIF mRNA was expressed as relative ratio to GAPDH. The values are shown as mean ± SD, n=3. * P<0.001 relative to controls by Student’s t test. (O) MIF protein expression following NFκB signaling activation in RAW 264.7 cells was assessed by addition of LPS at the concentration of 0.01 ng/ml. Protein levels of MIF were quantified by Western blot after being resolved on a 12% Tris-tricine SDS-PAGE gel. (P) Quantification of (O). The level of MIF protein was expressed as relative ratio to β-actin. The values are shown as mean ± SEM, n=3. * P<0.001 relative to controls by Student’s t test.

2.4 Discussion

Gene expression of MIF is constitutive under normal conditions, and is also inducible due to cis-acting elements on the MIF promoter for transcription factor binding, and many of them were shown to be functionally regulated under certain conditions (Bacher et al., 2003; Baugh et al., 2006; Roger et al., 2007; Waeber et al., 1998; Wang et al., 2009). As a pro-inflammatory cytokine, MIF was upregulated under chronic inflammatory conditions, and may play a role in maintaining the inflammatory status (Bacher et al., 2010; Morand et al., 2006). NFκB is a primary transcription factor during inflammation. In addition, a sequence analysis showed several putative binding elements on MIF promoter for NFκB, but it is not clear whether NFκB signaling activation could physiologically drive the expression of MIF. Therefore, in this chapter, we focused on characterizing the transcription regulation of MIF gene expression by NFκB signaling.

Using gel shifting assay, we first identified two functional cis-acting NFκB elements on the human MIF promoter that directly bound with NFκB p65, one was located at -513 and the other at -2536. However, the probes used in the gel shift assay was only ~20 bp, and the entire core sequences were exposed for NF-κB binding. Therefore, it remained elusive that whether the NFκB binding elements on the MIF promoter were masked under the secondary or higher structure. To resolve this question, we further studied whether NFκB could recognize its cis-acting elements on the MIF promoter under physiological condition using a ChIP assay. We demonstrated that a monoclonal antibody specifically recognizing NF-κB p65 successfully precipitated the DNA fragments containing the newly identified NFκB
binding elements. For the first time, these results clearly demonstrated that NFκB could directly bind with its cis-acting elements on the human MIF promoter. Using a luciferase assay, we investigated whether NFκB signaling could actively regulate MIF gene expression through the binding between NFκB and the MIF promoter. Our results showed that the MIF promoter activity was enhanced significantly and proportionally depending on the number of the identified effective cis-acting element(s) by activation of NFκB signaling. Taken together, our studies in this chapter demonstrated that activation of NFκB directly bound its cis-acting elements on the MIF promoter and enhanced MIF gene transcription.

NFκB signaling can be activated in response to a wide range of stimuli, including pathogens, stress signals, and pro-inflammatory cytokines such as IL-1, and cell surface receptors are responsible for the signal transduction (Li and Verma, 2002). In the current study, activation of NFκB signaling was achieved by overexpressing NFκB p65 subunit, as demonstrated in our previous studies (Wang et al., 2013b; Wang et al., 2011). By doing so, we could specifically enhance NFκB signaling and minimize the side-effects that may be induced by NFκB activators. Also, directly inducing NFκB signaling intracellularly bypasses the signal transduction mediated through cell surface receptors, so that avoids cell type dependent receptor variations induced false negative results. For example, a previous study demonstrated that in human endometriotic stromal cells IL-1β treatment significantly increased MIF mRNA expression (Veillat et al., 2009), while another study did not observe the same effect on RAW 264.7 macrophages (Calandra et al., 1994), although IL-1R and TLR share similar cytoplasmic signaling domains (Li and Verma, 2002). In our study, we used LPS to induce NFκB activation in RAW264.7, in which NFκB activation is a typical signaling pathway transduced through the cell’s surface TLRs (Underhill et al., 1999), and clearly demonstrated that MIF mRNA expression was significantly induced. These results indicate that NFκB regulated MIF gene expression could vary among different types of cells depending on the upstream signaling activation/transduction.

MIF is constitutively expressed in neurons (Bacher et al., 1998; Ogata et al., 1998). Additionally, activation of NFκB signaling has been observed in neurons under stress conditions including strokes and AD (Kaltschmidt et al., 1997; Stephenson et al., 2000). Our
results demonstrated that NFκB signaling transcriptionally regulates MIF expression in a neuronal cell line. Taken together, these results indicate that MIF expression in neurons could be actively regulated by NFκB signaling under stress conditions. Since NFκB signaling is shown to regulate genes governing both cell survival and death, it is of interest to investigate the role of neuronal MIF under these conditions.

Microglia are the immune cells in the CNS, and are constantly monitoring the status of the brain but remain quiescent under normal conditions (Aguzzi et al., 2013). They can be quickly activated once they detect emerging abnormal conditions, followed by the production of inflammatory cytokines (Kettenmann et al., 2011). Unlike in neurons, NFκB signaling is not detectable in glial cells under normal conditions (Kaltschmidt and Kaltschmidt, 2009). In concordance with this, MIF expression is not detectable in microglia under normal conditions (Bacher et al., 1998; Ogata et al., 1998). Under pathological conditions like chronic inflammation, NFκB signaling is activated in microglia (Kaltschmidt and Kaltschmidt, 2009; Li and Verma, 2002). Although evidence for induction of microglial MIF expression under pathological conditions is very limited, our result that MIF expression is upregulated following NFκB activation in a microglial cell line indeed suggests that MIF expression could be induced under pathological conditions by NFκB signaling. In fact, it has been shown that the MIF expression in microglia-like cells around amyloid plaques in an animal model of AD (Bacher et al., 2010).

2.5 Conclusion
In this chapter, we identified two functional cis-acting NFκB binding elements on the MIF gene promoter and demonstrate that NFκB regulates MIF expression by transcriptional activation of the MIF gene promoter via these two sites. Activation of NFκB signaling is able to transcriptionally enhance the expression of MIF in both neuronal and immune cells.
Chapter 3

The role of MIF during stroke

3.1 Introduction
Ischemic stroke resulting from disturbance of blood supply to the brain accounts for 80% of stroke cases and causes significant post-stroke neurological deficits and death (Donnan et al., 2008). Among a number of mechanisms involved in stroke pathogenesis, activation of the inflammatory response and the apoptotic pathways have been shown to play pivotal roles in stroke progression (Broughton et al., 2009; Iadecola and Anrather, 2011; Lipton, 1999).

Ischemic stroke differentially affects brain regions and results in an ischemic core and penumbra. Neurons in the ischemic core are rarely salvageable due to their immediate necrosis. In contrast, neurons in the penumbra experience a relatively milder ischemic insult and undergo delayed cell death and are the targets for therapeutic intervention (Lipton, 1999). In the penumbra, neurons can undergo both necrosis and apoptosis. Necrosis induced by glutamate diffused from the necrotic core can be diminished by blocking a specific subtype of NMDA receptors (Liu et al., 2007). Most of the neurons in the penumbra undergo apoptosis. Anti-apoptotic strategies including inhibition of Apaf-1 (involved in intrinsic apoptosis), expression of dysfunctional FasL (involved in extrinsic apoptosis), and knock-down of AIF (involved in caspase-independent apoptosis) were shown to successfully reduce neuronal loss and infarct volume (Broughton et al., 2009).

Ischemic stroke-induced acute brain damage rapidly activates inflammatory responses, leading to upregulation and release of pro- and anti-inflammatory factors, such as tumor necrosis factor-α (TNFα), IL-1β, IL-6, IL-10, IL-20 and TGF-β (Iadecola and Anrather, 2011). In the brain, the cytokines are produced not only by infiltrating immune cells, but also by resident brain cells including neurons and glial cells (Amor et al., 2010). As one of the major consequences of ischemic stroke, inflammation amplifies stroke pathology regardless of the triggering stimuli (Xiong et al., 2011; Ziegler et al., 2011), and anti-inflammatory
approaches are beneficial in reducing infarct volume and promoting cell survival (Lazovic et al., 2005; Zhang et al., 2003). This highly suggests that inflammation plays a key role in stroke-induced progressive brain damages.

MIF, a 114 amino-acid protein, was first identified as a pro-inflammatory cytokine (Bernhagen et al., 1993; Calandra and Roger, 2003), and later was found to function as a pleiotropic protein involved in many cellular activities, such as regulating cell death and survival (Hudson et al., 1999; Kleemann et al., 2000; Mitchell et al., 2002). The role of MIF during ischemia, either being protective by suppressing apoptosis or detrimental by promoting inflammation, is not well characterized. A study on intestinal ischemia demonstrated that lack of MIF gene expression significantly suppressed circulating inflammatory cytokines and reduced lethality in mice (Amaral et al., 2007). MIF has been shown to promote cell survival by modulating glucose uptake and metabolism during energy deprivation in mouse model of myocardial infarction (Koga et al., 2010; Miller et al., 2008). MIF has also been shown to inhibit the transcription of tumor suppressor p53 and negatively regulate JNK signaling, thereby preventing immune cells, such as macrophages from undergoing apoptosis, growth arrest, and cellular senescence (Hudson et al., 1999; Kleemann et al., 2000; Mitchell et al., 2002). In addition, MIF possesses redox activity through its Cys-Xaa-Xaa-Cys group, suppressing oxidative stress-induced apoptosis (Nguyen et al., 2003a; Nguyen et al., 2003b). These studies suggest that MIF could be protective during ischemia. However, there have been limited studies on the role of MIF in brain disorders. We recently reported that MIF is dysregulated during ischemic stroke in both patients and a rat stroke model in a temporal manner (Wang et al., 2009). MIF was found to contribute to stroke pathology in mice and neuronal death in vitro following oxygen-glucose deprivation (Inacio et al., 2011a) and knockout of the Mif gene results in smaller infarct volume at 7 days after stroke by promoting the macrophage and microglia response (Inacio et al., 2011b), suggesting a detrimental role of MIF in cerebral ischemia. The same group also concluded that MIF was not involved in promoting inflammatory response during these 7 days (Inacio et al., 2011a), leaving an open debate regarding the role of MIF in regulating neuronal death/survival in the central nervous system.
We have shown in Chapter 2 that NFκB regulates MIF gene expression. In addition, during stroke, nuclear translocation of NFκB p65 is observed in neurons located in the infarct, indicating an active role of NFκB in ischemic neurons (Schneider et al., 1999). Although both NFκB activation and alteration of MIF expression are observed during the early stages of cerebral ischemia (Huang et al., 2003; Wang et al., 2009), the interaction between NFκB and MIF and its role in cerebral ischemia remain undefined. In this chapter, we aimed to study how NFκB regulates MIF gene expression following ischemic stroke in vivo. We will further explore the role of MIF during the early phase of stroke.

### 3.2 Methods

#### 3.2.1 Animals and genotyping

Animal experiment protocols were approved by The University of British Columbia Animal Care and Use committee. *Mif*<sup>+/−</sup> mice on the BALB/c (generation N10) background as described previously (Mizue et al., 2005) were obtained from Jackson Laboratory. All the mice were allowed access to water and food *ad libitum*. All mice were genotyped at the beginning of weaning and at the time of euthanasia. At 3 weeks of age, mice were anesthetized with isoflurane and ear-marked. At the time of euthanasia, a piece of the ear was also harvested. The tissue was digested in 200μL of lysis buffer (10 mM Tri-HCl pH8.0, 10 mM EDTA pH8.0, 150 mM NaCl, 0.5% SDS) with 3μL of 10 μg/mL Proteinase K (New England Biolabs) at 55°C overnight. The next day, samples were centrifuged and DNA was precipitated with 0.7X volume of isopropanol. DNA was pelleted by centrifugation at 16,000xg for 15 min, washed twice with 70% ethanol, air dried, and re-spended in sterile de-ionized water. The genomic DNA was subjected to PCR to amplify MIF gene with MIF KO WT F-ACCACGTGCTTAGCTGAGCCA/MIF KO WT R-CCGCAAGCCAAGGAAGCAGAA, and disrupted MIF gene using MIF KO F-TCCCGGACCAGCTCATGACTTTTA/MIF KO Neo R-GTTGGCCGCTACCGGTGGATGT.

#### 3.2.2 Middle cerebral artery ligation (MCAI).

Male mice, aged between 10 to 12 weeks, were subjected to transient distal middle cerebral artery ligation (tMCAI) surgery (Chen et al., 1986) with modification. Briefly, following
isolation of right common carotid artery (rCCA), the mouse was put on a stereotaxic frame (KOPF) and positioned on its right side for MCA ligation. A skin incision was made between the right eye and ear, and the muscle fibers were separated and retracted to expose the skull surface. The right MCA (rMCA) was easily visualized through the skull and a 1mm burr hole was opened by a dental drill around the rMCA to expose the rMCA for ligation with a 10-0 suture. Quickly after ligation of rMCA the rCCA was occluded by a non-traumatic micro-clamp (S&T Instrument). A total of 1 ml saline was injected subcutaneously before and after the surgery. The mouse was laid on a warm blanket to recover. After transient rMCA and rCCA ligation for 2 hours, the suture and micro-clamp were removed, and the mice were allowed for reperfusion. Control mice underwent similar surgical procedures except that the rMCA and rCCA were not ligated.

3.2.3 Sample collection and TTC staining.
A pilot study was carried out to determine the sampling strategy. Following the tMCAI procedure, mice were sacrificed at 0, 2, 8, or 22 hours after reperfusion. The freshly dissected brains were placed on a brain slice matrix, and coronal sections were made to generate 1mm thick slices beginning from the anterior edge of the cerebral cortex. The brains slices were incubated in 1% 2,3,5-Triphenyl-tetrazolium chloride (TTC) solution in PBS for 10min at 37°C. After incubation, the slices were fixed in cold 4% paraformaldehyde (PFA) solution, and scanned for subsequent analysis. ImageJ was used for infarct area determination. Focal ischemia resulted in tissue infarction and was illustrated by the complete loss of TTC staining in contrasting to the surrounding dark-red stained viable tissue. The infarcted area was restricted to the cerebral cortex supplied by MCA at 24 hours post ischemia. The rest of the cerebral cortex supplied by anterior/posterior cerebral arteries was not affected following the tMCAI procedure. Brain tissues from the ischemic and contralateral non-ischemic area were dissected for protein expression analysis. For the sham-operated mice, equivalent areas were dissected. All samples were stored in -80°C before biochemical analysis.
3.2.4 Immunohistology, immunoblot analysis and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

The 4% PFA-fixed brain samples were prepared for either cryosectioning at 20μm thickness or paraffin-embedded sectioning at 5 μm thickness. TUNEL assay was performed on the 5 μm-thick slices following the manufacture’s instruction (Promega). For immunostaining, the brain slices were permeabilized in 0.3% Triton X-100 in PBS (PBS-Tx) for 30 min and blocked with 5% BSA in PBS for 1 hour at 22˚C. Next, the slices were incubated with primary antibodies in PBS with 1% BSA at 4°C overnight. After rinsing with PBS-T 3 times, the slices were applied with biotinylated swine anti-rabbit secondary antibodies in PBS-T with 1% BSA for 1 hour at 22˚C, and visualized by the ABC and DAB method. For immunoblotting, brain tissue and cells were lysed with the RIPA-DOC buffer. The lysates were resolved by gel electrophoresis, and the protein was analyzed by Western blot. The primary antibodies were recognized by IRDye 800CW-labelled goat anti-mouse or IRDye 700CW-labeled goat anti-rabbit, and were visualized and analyzed using the Odyssey system (LI-COR Biosciences). The primary antibodies were: anti-cleaved caspase-3 (Cell signaling) for immunohistology, anti-NFκB p65 subunit (Cell signaling technology, #8242) for immunohistology and immunoblotting, anti-MIF antibody (Torrey Pines Biolabs, Secaucus, NJ, USA) for immunoblotting, and anti-β-actin antibody (Sigma, AC-15) for immunoblot, used as a loading control.

3.2.5 Stable cell lines, human MIF purification, and ELISA

Stable cell lines expressing exogenous human MIF were generated. HEK293 or SH-SY5Y cells were transfected with phMIF-stop plasmid. The cells were then maintained in Zeocin (Invitrogen) to screen for positive colonies. Positive colons on HEK293 and SH-SY5Y background cells are named HMS and SYMS, respectively. To obtain human MIF protein for primary neuronal treatment, pMIF-mycHis was transfected into HEK293 cells and cell lysate was prepared under undenatured condition. MycHis tagged MIF protein was purified using Nickel-NTA magnetic agarose beads following the manufacture’s protocol (Qiagen). The identity of the purified protein was verified by Western blot, and the purity was assessed by Coomassie Blue staining following gel electrophoresis. MIF concentrations in culture media
were measured by human MIF ELISA kit (R&D systems) following the manufacture’s instruction. Culture medium were diluted 5 times prior to the assays.

3.2.6 Primary neuronal culture, in vitro I/R treatment and MTS assay
Primary neuronal cultures were derived from E14 mice embryos as previously described (Sun et al., 2006b). Ten day-old cultures of mouse neurons were subjected to 50 μM H2O2 for 16 hours or “in vitro ischemia/reperfusion” treatment. The latter was achieved by incubating primary cultures under OGD condition for 2 hours followed by normal incubation condition for additional 16 hours. For cells undergoing OGD, the cells were cultured with either fresh Neurobasal media (Invitrogen) for control cells or glucose-free Neurobasal media (Invitrogen) for OGD treatment under hypoxic condition (2% oxygen complemented with nitrogen). Both Neurobasal media were supplemented with B27 (Invitrogen) and L-glu (Invitrogen). The cells were treated with purified mycHis tagged human MIF protein or vehicle solution during the entire experimental periods. Cell viability was assessed using the MTS assay following the manufactures instruction (Promega). Cell viability was calculated by dividing the absorbance of treated or untreated cells by the average absorbance of non-treated (control cells) and expressed as a survival ratio.

3.3 Results
3.3.1 Downregulation of MIF expression in infarct regions after cerebral I/R
Previously we showed that MIF gene expression was altered in patients suffering stroke and in the brains of stroke model rats (Wang et al., 2009). Here, we further studied the spatial alteration of MIF expression, and focused on the brain region undergoing acute infarction within 24 hours after focal ischemia induced by the tMCAI procedure. Since the right MCA was ligated with additional ligation of the right CCA, the blood supply was impaired in the entire right hemisphere, and this stroke model precisely produced an infarct restricted to the MCA supplied cortical region. Mice aged between 10 to 12 weeks were subjected to the tMCAI procedure and reperfused for 4 or 22 hours. At 22 hour post reperfusion, a clear infarction in the MCA supplied cortical region was obtained, as assessed by TTC staining (Figure 3.1A). The MCA territory and cortical peri-MCA area were defined as described in Figure 1A. The brain tissue in the respective areas, along with its counterpart in the
unaffected hemisphere (as control) were dissected for MIF protein analysis. At 6 hour after the onset of ischemia, MIF expression in the MCA territory was significantly reduced to 0.80 ± 0.07 fold on the ischemic hemisphere compared to the unaffected counterpart (P<0.05) (Figure 3.1B and C). At 24 hours after the onset of ischemia, brain samples collected from the MCA supplied territory were assessed for protein expression even though significant tissue decomposition had occurred. Significantly lower expression of MIF to 0.75 ± 0.10 fold (P<0.05) reduction in the ischemic hemisphere was also observed (Figure 3.1D and E). The level of β-actin, which served as the internal control, was significantly reduced (Figure 3.1D), despite the same amount of protein loaded, confirming severe decomposition of the parenchyma. MIF protein expression was also examined in the cortical peri-MCA territory. In contrast to the MCA territory, neither at 6 hours (Figure 3.1F and G) nor at 24 hours (Figure 3.1H and I) post-ischemia was there an effect on protein expression in the cortical peri-MCA area (P>0.05).

Figure 3.1 Reduced MIF expression in the infarct-targeted area after cerebral focal ischemia in mice. BALB/c mice were subjected to 2 hour right MCAI followed by 4 or 22 hours of reperfusion or a sham procedure on the right hemisphere. The brain was freshly cut into 1mm slices and subjected to TTC staining or protein expression analysis. (A) 22 hours after reperfusion, infarcted tissue restricted to the MCA supplied cerebral cortex was clearly distinguished by pale TTC staining and defined as the MCA territory (area 1), and with the cortical peri-MCA area (area 2). (B) 6 hours or (D) 24 hours after tMCAI, the MCA territory was lysed by RIPA-Doc buffer with 1% SDS. The lysate was resolved on a 12% Tris-tricine SDS-PAGE gel. MIF was detected by anti-MIF antibody and β-actin was detected by β-actin antibody as control. (C) and (E) Quantification of (B) and (D), respectively. The ratio of MIF to β-actin was further normalized to unaffected hemisphere in shamed mice. Values were expressed as mean±SD, n = 6 for sham and 7 for MCAI, * P<0.05, by two-way ANOVA test with Bonferroni post hoc tests. (F) 6 hours or (H) 24 hours after tMCAI, the lysates of peri-MCA territory were subjected to 12% Tris-tricine SDS-PAGE. MIF and β-actin were detected by their respective antibodies. (G) and (I) Quantification of (F) and (H) respectively. The ratio of MIF to β-actin was normalized to unaffected hemisphere in shamed mice. Values were expressed as mean±SD, n = 6 for sham and 7 for MCAI, P>0.05, by two-way ANOVA with Bonferroni post hoc tests.
3.3.2 NFκB downregulates MIF gene transcription under hypoxia

A significant increase in nuclear NFκB and the activation of the NFκB signaling pathway within the infarcted area flowing stroke have been observed, suggest that NFκB may be involved in MIF dysregulation in the MCA territory (Schneider et al., 1999; Stephenson et al., 2000). To investigate this issue, we first examined the expression of NFκB p65 in the brain following stroke. Compared with the peri-MCA area, nuclear p65 in the MCA territory was increased (Figure 3.2A), indicating that NFκB was activated in this region.

We have shown that both hypoxia (Wang et al., 2009) and activation of NFκB signaling alone result in upregulation of MIF gene expression. It remained intriguing as to why MIF expression was downregulated in the MCA territory 6 hours after the onset of stroke, when massive tissue decomposition has yet to occur. A previous report showed that, although the CRE on the MIF promoter was responsive to CRF-mediated MIF gene upregulation (Waeber et al., 1998), it also is involved in MIF gene downregulation under hypoxic conditions (Baugh et al., 2006). Given that the HREs on the MIF promoter were 33 and 43 bp upstream of MIF-NFκB-A and -C, respectively (Figure 3.2B), we examined the effect of NFκB signaling on MIF promoter activity under hypoxia, which better mimics the condition of NFκB activation following ischemia. The promoter activity of phMIF-luc was elevated by either hypoxia (2.66±0.20 folds of the control, *P*<0.05) or transfection of NFκB p65 alone (5.66 ±0.17 folds of the control, *P*<0.05), whereas HREs-deficient plasmid phMIFδH-luc responded to NFκB p65 overexpression with a 10.43±0.20-fold increase in activity (*P*<0.05), but did not respond to hypoxia (Figure 3.2C). Successful induction of signaling pathways were confirmed by enhanced promoter activities of positive control plasmids pEpoE-luc by hypoxia and pNFκB-luc by NFκB (data not shown). To assess the effect of hypoxia on NFκB signaling-mediated MIF promoter activity, cells co-transfected with the NFκB p65 expression plasmid and MIF promoter constructs were subjected to hypoxia treatment. Dual activation of hypoxia and NFκB signaling resulted in significant reduction of the luciferase activity of phMIF-luc to 0.86±0.03 of the control (*P*<0.05), while the elevation of the promoter activity of phMIFδH-luc by NFκB signaling was not affected by additional activation of the hypoxia signaling pathway (Figure 3.2D). These data indicate that induction of HIF-1α by hypoxia does not directly affect the binding of the *cis*-acting elements in the
MIF gene promoter to NFκB and that the reduction of promoter activity under dual signals most likely was due to the interaction between the HRE and NFκB binding motif on the MIF promoter.

We further examined the effect of NFκB signaling on endogenous MIF expression under hypoxic conditions. Dual activation of hypoxia and NFκB signaling was achieved by overexpressing NFκB p65 expression plasmid under hypoxic condition. MIF mRNA (Figure 3.2E) and protein (Figure 3.2G) expression was reduced significantly to 0.80±0.05 fold (Figure 3.2F) (P<0.05) and 0.16±0.01 fold (Figure 3.2H) (P<0.05), respectively. Taken together, these results indicate that activation NFκB signaling under hypoxic condition contributes to downregulation of endogenous MIF gene expression.
NFkB Downregulates MIF gene transcription under hypoxia.

(A) Brain slices from BALB/c mice subjected to 2h-tMCAI followed by 2 hour reperfusion were stained for NFkB p65. In the MCA territory, p65 exhibited nuclear localization (solid arrow heads), while in the cortical peri-MCA region, p65 diffused in the cytoplasm (empty arrow heads). Scale bar, 20 μm. (B) Schematic diagram showing the location of HREs and NFkB binding sites on the MIF promoter. (C) HEK293 cells were transfected with MIF promoter plasmids and subjected to hypoxia treatment, or co-transfected with NFkB p65 expression plasmid. Cells were harvested with passive lysis buffer and measured for luciferase activity. The firefly luciferase activity was normalized to the Renilla luciferase activity for transfection efficiency control, and expressed as relative luciferase units (RLU) to reflect the promoter activity. The values are expressed as mean±SD, n=6. * P<0.001 relative to controls by two-way ANOVA with Bonferroni post hoc tests. (D) HEK293 cells were co-transfected with NFkB p65 expression plasmid with additional hypoxia treatment, and promoter activities were evaluated as in (C). The values are expressed as mean±SD (n=6). * P<0.01 relative to controls by two-way ANOVA with Bonferroni post hoc tests. (E) HEK293 cells were transfected with NFkB expression plasmid and subjected to hypoxia (2% of oxygen balanced by nitrogen). Total RNA was extracted from cells by Tri-reagent, and semi-quantitative RT-PCR was used to measure MIF mRNA with MIF gene specific primers. GAPDH mRNA was used as the internal control. (F) Quantification of (E). The values are shown as mean ± SD, n=3. * P<0.001 relative to controls by Student’s t test. (G) HEK293 cells were transfected with NFkB expression plasmid subjected to hypoxia (2% of oxygen balanced by nitrogen). Protein levels of MIF were assessed by Western blot on a 12% Tris-tricine SDS-PAGE gel. (H) Quantification (G). The values are shown as mean±SD, n=3. * P<0.01 relative to controls by Student’s t test.
3.3.3 MIF protects neurons from oxidative stress- and ischemia/reperfusion-induced apoptosis in vitro

Our results suggested that activation of NFκB signaling under hypoxic conditions contributes to downregulation of MIF expression in the MCA territory during the acute phase of stroke. To further determine the role of MIF in stroke, we first assessed the function of MIF on cultured primary cortical neurons under stroke-like conditions. Stroke-like conditions were induced in cultured mouse cortical neurons by an in vitro model of I/R. The primary neuronal culture was challenged in glucose-free media under hypoxic condition for 2 hours (ischemic period), the culture medium then replaced by normal medium, followed by 16 hours of incubation under normoxic conditions (reperfusion). In vitro I/R resulted in significant loss of neurons, 88.08±2.93% viability (P<0.01), measured by a MTS assay (Figure 3.3B). The effect of MIF was assessed by adding purified human MIF protein (Figure 3.3A). Treatment of cortical neurons with purified human MIF at a concentration of 200 ng/ml during “ischemic” and “reperfusion” periods completely rescued the detrimental effect induced by in vitro ischemic/reperfusion with cell viability of 100.04%±3.34% (P<0.001) (Figure 3.3B). These results suggest a protective role for MIF on neurons under in vitro ischemia-reperfusion-like conditions.

Oxidative stress contributes to cell death induced by ischemia, particularly following reperfusion (Niizuma et al., 2009). To determine the effect of MIF on oxidative stress-induced neuronal death, primary cultures of cortical neurons isolated from mouse embryos were treated with H2O2. H2O2 treatment of cortical neurons resulted in a significant decrease in neuronal viability to 67.52±1.18% versus control (P<0.01), as assessed by the MTS assay (Figure 3.3C). When cortical neurons were incubated with purified MIF at a concentration of 200 ng/ml, H2O2-induced cell death was significantly prevented and cell viability was increased to 90.06±1.03% relative to control (P<0.01) (Figure 3.3C). These results clearly demonstrate that MIF rescues neuronal loss following stroke-like conditions in vitro, and may be particularly protective against reperfusion-induced oxidative stress.
Figure 3.3 Purified human MIF protein rescued neuronal death following in vitro I/R and oxidative stress.

(A) Human MIF was purified from HEK293 cells overexpressing MIF-mycHis by Nickel-NTA magnetic agarose beads under a non-denaturing condition. The purified human MIF was resolved on 12% Tris-tricine SDS-PAGE gel. The protein was assessed by Coomassie blue staining. The identity of the protein was verified by Western blot by anti-MIF antibody. (B) *In vitro* I/R was achieved by challenging the primary neuronal culture in glucose-free media under hypoxic conditions for 2 hours (ischemic period), followed by 16-hour incubation under normoxic conditions after medium change to normal medium (reperfusion period). Purified human his-tagged MIF (200ng/ml) or vehicle were added in the medium during the entire experiment. Cell viability was assessed by using MTS assays. Survival ratio represents the average absorbance to control absorbance. Values represent mean± SD, n=10. *P<0.01 by Student’s *t*-test. (C) Oxidative stress was induced by 50μM of H$_2$O$_2$ for 16 hours in the normal medium. Purified human his-tagged MIF (200ng/ml) or vehicle were added in the medium during the entire experiment. Cell viability was assessed by using MTS assays. Survival ratio represents the average absorbance to control absorbance. Values represent as mean± SD, n=6, *P<0.001 by Student’s *t*-test.

3.3.4 MIF inhibits activation of caspase-3 and reduces oxidative-induced apoptosis

To study the effect of MIF on neuronal apoptosis, HEK293 and SHSY5Y were transfected with a human MIF expression plasmid to generate HMS (Figure 3.4A) and SYMS stable cell lines (Figure 3.4C). HMS and SYMS robustly express human MIF protein 2.46±0.14 fold (Figure 3.4B) and 7.02±0.40 fold (Figure 3.4D) compared to control in cell lysates detected by Western blot. MIF levels in culture medium of these cell lines, as detected by ELISA, were also significantly increased from 18.5±1.11 to 54.30±3.29 ng/ml in SYMS (*P<0.01) and 32.00±2.36 to 384.39±20.05 ng/ml in HMS (*P<0.01) stable cells, respectively (Figure 3.4E). To further examine the protective role of MIF, TUNEL assay was performed. H$_2$O$_2$
treatment at a concentration of 50 μM induced significant cell death in HEK293 cells (Figure 3.4F), resulting in 12.81±1.01 fold increase of TUNEL-positive cells (\(P<0.01\)) (Figure 3.4G). In contrast, overexpression of MIF in HMS cells (Figure 3.4F) blocked the H₂O₂-induced cell death (Figure 3.4G). H₂O₂ treatment at a concentration of 100 μM resulted in complete detachment of HEK cells from the culture dish 24 hours after treatment, whereas 33.44±2.52% HMS cells survived after treatment, and 7.82±1.70% of the surviving cells were TUNEL positive (Data not shown). Our results clearly demonstrate that MIF is essential in combating oxidative stress-induced cell death and promoting cell survival under such conditions.

MIF exhibited strong rescue effects on neurons subjected to H₂O₂ treatment. Besides, H₂O₂-induced oxidative stress triggers activation of the caspase cascade. We therefore sought to investigate whether MIF’s protective effect was mediated through the caspase signaling pathway. SYMS and SH-SY5Y cells were treated with H₂O₂ for 16 hours, after which caspase-3 cleavage was analyzed by Western blot. In the control groups, no cleaved form could be detected in both cell lines (Figure 3.4H), whereas H₂O₂ treatment markedly induced the cleaved active form of caspase-3 in both cell lines (Figure 3.4H). However, cleaved caspase-3 was 38.40±6.45% (\(P<0.01\)) lower in SYMS than in SH-SY5Y control cells. Furthermore, addition of the MIF inhibitor, ISO-1, significantly increased the level of the cleaved caspase-3 by 1.36±0.05 fold in SHSY5Y and 2.47±0.12 fold in SYMS (\(P<0.001\)) (Figure 3.4I).
Figure 3.4 MIF protected neurons by inhibiting caspase-3 activation.

(A) Stable cell line HMS was generated by transfection of a human MIF expression plasmid pMIF-Stop mammalian expression plasmid into HEK293 and stable colonies were selected by Zeocin. The lysate was resolved on a 12% Tris-tricine SDS-PAGE gel. MIF and β-actin were detected by anti-MIF and anti-actin antibodies. (B) Quantification of MIF expression in HMS cell lysates. Values represent mean±SD, n=3, * P<0.01 relative to HEK293 controls by Student’s t test. (C) Stable cell line HMS was generated by transfection of a human MIF expression plasmid pMIF-Stop mammalian expression plasmid into SHSY5Y and stable colonies were selected by Zeocin. The lysate was resolved on a 12% Tris-tricine SDS-PAGE gel. MIF and β-actin were detected by anti-MIF and anti-actin antibodies. (D) Quantification of MIF expression in SYMS cells. Values represent mean±SD, n=3. * P<0.01 relative to SHSY5Y controls by Student’s t test. (E) Secreted MIF in the media was measured by ELISA. Values represent mean±SD, n=3. * P<0.001 relative to background cells by Student’s t-test. (F) HEK293 and MIF stable HMS cells were treated with 50 μM H2O2 for 24 hours in sodium pyruvate lacking medium, followed by TUNEL assay (green channel). DAPI (blue channel) is used for the nuclei staining. Scale bar 100 μm. (G) Quantification of the cells number identified by DAPI staining and apoptotic cells identified by TUNEL labeling by ImageJ. Signals were averaged from 5 random-selected views. Values represent as mean±SD, n=3, * P<0.01 relative to cells without H2O2 treatment by two-way ANOVA with Bonferroni post hoc tests. (H) SHSY5Y and MIF stable SYMS cells were treated with 200 μM H2O2 for 16 hours. The MIF inhibitor, ISO-1, was added at the concentration of 50μM to block the effect of MIF. Cells were lysed by Chap cell extract buffer. The lysate was resolved on a 12% Tris-tricine SDS-PAGE gel, and the protein level was analyzed by Western blot. An anti-caspase-3 antibody recognizing both pro- and cleaved caspase-3 was used. (I) Quantification of (H). The ratio of the cleaved form of caspase-3 to β-actin level was analyzed. Values represent as mean ± SD, n=3, *P<0.01 by two-way ANOVA with Bonferroni post hoc tests.
3.3.5 Disruption of MIF gene expression accelerates neuronal death within the MCA territory

To examine the effect of MIF on neuronal apoptosis during the acute phase of stroke in vivo, TUNEL assays were performed on MIF-knockout (Mif\(^-/-\)) and wildtype (Mif\(^{+/+}\)) mice subjected to experimental stroke. The results showed that Mif\(^-/-\) mice developed TUNEL positive cells in the MCA territory as early as 6 hours after the onset of ischemia, and nuclei in this area appeared condensed (Figure 3.5A). In contrast, in Mif\(^{+/+}\) mice, nuclei in the MCA territory displayed healthy appearance, and no TUNEL positive cells appeared (Figure 3.5A).

At 8 hours after ischemia onset, the number and area of TUNEL positive cells were significantly increased in Mif\(^-/-\) mice, while scarce TUNEL positive cells developed in Mif\(^{+/+}\) mice (data not shown). The difference between Mif\(^-/-\) and Mif\(^{+/+}\) mice is less evident at 24 hour after stroke, 54.65±9.17\% in Mif\(^{+/+}\) vs. 65.58±6.75\% in Mif\(^-/-\) mice (P>0.05) (Figure 3.5B).

We have shown that MIF protects neurons from oxidative stress-induced apoptosis by inhibiting activation of caspase-3 in vitro. To examine whether MIF could exert the same effect in vivo following experimental stroke, Mif\(^-/-\) and Mif\(^{+/+}\) mice were subjected to tMCAI, and the cleaved form of caspase-3 was determined histologically 2 hours after reperfusion. To avoid batch-to-batch variation, immunostaining was performed side by side for Mif\(^{+/+}\) and Mif\(^-/-\) mice. Sample images were taken from the peri-MCA region and MCA territory for evaluation of caspase-3 activation (Figure 3.5C). In the peri-MCA region, both wildtype and knockout mice displayed weak background caspase-3 signal (Figure 3.5C, upper panels). In the MCA territory, cleaved caspase-3 was detected in both Mif\(^-/-\) and Mif\(^{+/+}\) mice, and Mif\(^-/-\) mice displayed stronger staining signals (Figure 3.5C, lower panels). Taken together, these results demonstrate that MIF negatively regulates caspase-3 and thus protects against neuronal death during the acute phase of stroke; disruption of MIF gene expression within the MCA territory accelerates neuronal death and infarct development.

Focal ischemia-induced infarction was routinely examined to assess stroke outcome. Since MIF promoted neuronal survival, we assessed whether it affected infarct formation in vivo. Mif\(^{+/+}\) and Mif\(^-/-\) mice were subjected to tMCAI. At 4, 10 and 24 hours after the onset of
ischemia, TTC staining was performed to assess infarct development during the acute phase of stroke (Figure 3.5D). Loss of TTC staining was not significant at 4 hours after stroke onset in both MIF wildtype and knockout mice. At 10 hours, impaired cell activity/incomplete infarction in the MCA-supplied region was observed, which was indicated by partial loss of TTC staining. Mif−/− mice exhibited a significantly greater loss of TTC staining, including the development of a complete infarct, by 8 hours compared to the wildtype mice (Figure 3.5D). The infarct volume was 6.51±1.48% for the wildtype mice and 9.07±1.32% for MIF-knockout mice (n=4, P<0.05). However, both Mif+/+ and Mif−/− mice exhibited complete loss of TTC staining 24 hours after stroke onset and there was no difference in terms of the infarct volume (Figure 3.5D). The infarct volume was 9.91±0.69% and 9.13±0.71% for wildtype and knockout mice, respectively (n=3, P>0.05). These results indicate that MIF deficiency results in loss of cell function at an early stage following stroke and accelerated development of an infarct. Interestingly, the protective effect of MIF in the MCA territory in MIF+/+ mice diminished during later phases of infarct development.

Figure 3.5 MIF reduced cell death and infarct development during stroke.
Wildtype and MIF-knockout mice were subjected to 2h-tMCAI followed by 2, 4, 8 or 22 hours reperfusion on the right hemisphere. The brain was freshly cut into 1mm slices and subjected to TTC staining. (A) Wildtype (Mif+/+) and MIF-knockout (Mif−/−) mice were subjected to 2 hour-tMCAI followed by 4 or 22 hours reperfusion,
and the brain slices were analyzed for cell apoptosis by TUNEL assay (green channel). The nuclei were stained by DAPI (blue channel). Representative images were taken from the same location in the MCA territory. (B) Average counts of the nuclei and TUNEL positive cells from 5 selected views and were quantified by ImageJ, and the percentage of TUNEL signal was presented. Values represent as mean±SD, n=3. P>0.05 by Student’s t-test. (C) Cleaved caspase-3 was determined histologically in wildtype and MIF-knockout mice 4 hours after 2 hour-tMCAl. Sample images were taken from the cortical peri-MCA region and MCA territory. Activation of caspase-3 was indicated by positive staining of the cleaved form of caspase-3. Arrows point to caspase-3 positive staining cells. Scale bar, 20 μm. (D) Wildtype and MIF-knockout mice were subjected to 2 hour-tMCAl followed by 2, 8 or 22 hour reperfusion on the right hemisphere. The brains were freshly cut into 1mm slices and subjected to TTC staining.

3.4 Discussion

MIF is expressed in many organs including the brain, and is involved in a variety of biological processes as a pleiotropic protein. It plays fundamental roles in inducing inflammatory responses and regulating cell death and survival (Calandra and Roger, 2003; Hudson et al., 1999; Kleemann et al., 2000; Mitchell et al., 2002). Stroke is detrimental to neurons in a similar way as a myocardial infarct is to cardiomyocytes. However, the effect of MIF on neurons experiencing ischemia remains elusive. A recent study showed that Mif−/− mice had smaller infarcts compared to wildtype mice following focal ischemia (Inacio et al., 2011b). We previously demonstrated altered MIF expression following stroke with initial upregulation and eventual downregulation (Wang et al., 2009), suggesting the expression pattern of MIF could affect its function. In the present study, we examined the temporospatial expression of MIF and its role during stroke. We adapted a stroke model that produced infarction in the frontal and parietal cortex, which is mainly supplied by the MCA. Infarction induced by this stroke model is highly reproducible. Compared to other focal stroke models, such as proximal middle cerebral artery occlusion (MCAo) in which the developed infarct spreads throughout cerebral cortex, this model produces smaller infarct restricted to the MCA supplied cerebral cortex, providing us an opportunity to assess the expression and function of MIF in a relatively homogenous cell group, especially for neurons. Our results demonstrate that MIF expression is selectively downregulated in the infarcted MCA territory, while it is not affected in the posterior cerebral artery/anterior cerebral artery-supplied peri-MCA area, despite its direct connection to the infarcted MCA territory. Our observations are consistent with a previous study showing that MIF is expressed in the peri-infarct area up to 72 hours after stroke, and diminishes in the ischemic core 3 hours after ischemia (Inacio et al., 2011b).
Both hypoxia and NFκB signaling pathways are activated following stroke in the infarct area (Schneider et al., 1999; Stephenson et al., 2000; Zhang et al., 2010). In previous studies, activation of NFκB signaling within the area that later became infarct was shown by EMSA using brain lysates, and nuclear localization was observed histologically (Schneider et al., 1999; Stephenson et al., 2000). Stephenson et al. (2000) further attributed early activation of NFκB signaling to neuronal rather than glial cells (Stephenson et al., 2000). In this study, we confirmed nuclear localization of NFκB in the MCA territory, and it was mainly diffuse in the adjacent peri-MCA area. Hypoxia activates HIF-1α, which mediates the upregulation of MIF gene expression via interaction with HREs on the MIF promoter (Baugh et al., 2006; Wang et al., 2009; Welford et al., 2006). In Chapter 2, we identified two functional cis-acting NFκB binding elements in the MIF promoter through which NFκB signaling regulates MIF gene expression by transcriptional activation of the MIF promoter. We found that activation of either hypoxia or the NFκB pathway alone drives upregulation of MIF gene transcription. However, NFκB signaling activated under hypoxic conditions leads to an inhibition of MIF gene expression. Since deletion of HREs in the MIF promoter eliminated the direct effect of HIF-1α on promoter activity and preserved the ability of NFκB to upregulate gene expression during hypoxia, it was likely that there could have been an interaction between HRE and NFκB binding elements on the MIF promoter under hypoxic conditions. Previous studies demonstrated that HIF-1α was significantly induced in the infarct area 1 hour after stroke onset, peaked at 12 hours, and gradually declined thereafter (Zhang et al., 2010) and a significant upregulation of NFκB signaling was also observed in a similar time window (Stephenson et al., 2000). Furthermore, it was reported that under hypoxia, the induction of a HIF-1α mediated HRE reporter gene occurred before NFκB signaling-mediated NRE reporter gene (Nakayama, 2013), indicating that activation of HIF-1α precedes that of NFκB signaling. Taken together, it is likely that in the infarcted area, rapid activation of HIF-1α contributes to the initial upregulation of MIF, and subsequent activation of NFκB signaling suppresses MIF expression thereafter. Activation of NFκB signaling after stroke is evident despite the controversial issue as to whether it is protective and deleterious to stroke outcome (Ridder and Schwaninger, 2009; Schneider et al., 1999). It is plausible that MIF may serve as a downstream effector responsible for synchronizing the upstream regulatory signals such as NFκB signaling, and thereby determining cell fate. Our results suggest that MIF expression is
tightly regulated by NFκB and hypoxia, and could serve as an effector that directly regulates cell survival during the acute phase of stroke.

Inflammation and apoptosis are two key therapeutic targets to reduce stroke-related neurological dysfunction. Interestingly, the function of MIF has been implicated in both of these processes, being anti-apoptotic but pro-inflammatory. MIF exhibits strong anti-apoptotic properties by suppressing p53 activity by transcription inhibition and negatively regulating JNK signaling (Hudson et al., 1999; Kleemann et al., 2000; Mitchell et al., 2002). Under ischemic conditions, MIF is protective by stimulating AMP-activated protein kinase during I/R-induced energy deprivation in the heart (Miller et al., 2008). However, the main effector of this pathway, GLUT4, only has been shown to function in the cerebellum (Bakirtzi et al., 2009). Recently, a similar study in heart ischemia observed increased infarction in Mif−/− mice, and suggested a protective effect of MIF via reduction of oxidative stress-induced ROS accumulation and cytochrome c release (Koga et al., 2011). Oxidative stress induced by massive ROS generation also occurs after stroke and is one of the major insults leading to neuronal apoptosis (Niizuma et al., 2009). Neurons undergoing apoptosis during stroke are often the target for therapeutic interventions. Cell apoptosis under stroke conditions may be activated through caspase-dependent pathways involving the dysfunction of mitochondria (Broughton et al., 2009). It was reported that post-ischemic reperfusion resulted in the overproduction of ROS in mitochondria (Szeto, 2006), thereby affecting the release of cytochrome c and leading to caspase-3 activation. The role of MIF in reducing ROS levels has been observed during heart ischemia (Koga et al., 2011), and it was suggested that MIF’s CXXC motif (Cys57-Ala-Leu-Cys60) plays a role in redox balance under stressed conditions (Nguyen et al., 2003a; Nguyen et al., 2003b). In our study, we clearly demonstrate that stably expressing MIF in a human neuronal cell line reduced caspase-3 cleavage induced by H2O2 treatment, and this effect was diminished after addition of ISO-1, a MIF inhibitor (Al-Abed et al., 2005). The effect of MIF expression on inhibition of caspase-3 activation and reduction of TUNEL staining under oxidative stress confirmed the protective role of MIF. We also observed these characteristics of MIF in vivo. In MIF knockout mice, caspase-3 activation in the infarct area was markedly increased when compared to wildtype mice.
An anti-apoptotic role for MIF has also been suggested in suppressing JNK activation by direct interaction with its co-activator Jab1 (Kleemann et al., 2000). Expression of MIF in neurons enables its autocrine ability for subsequent signal transduction. It also has been reported that knockout of JNK3, which, among the three JNKs (JNK1, JNK2, and JNK3) is expressed primarily in the brain, contributes to neuronal resistance to excitotoxicity (Leppa and Bohmann, 1999; Wagner and Nebreda, 2009). We observed a protective effect of MIF in reducing NMDA-induced excitotoxicity in primary neuronal cultures (data not shown).

Furthermore, we showed that MIF exhibited a strong effect in combating oxidative stress induced by H₂O₂. Additionally, we observed faster neuronal apoptosis in Mif⁻/⁻ mice at 6 and 8 hours after stroke onset and deletion of MIF gene accelerated infarct development during the acute phase of stroke. It is worth noting that PV+ interneurons are the neurons most resistant to ischemic cell death (Lipton, 1999). Interestingly, several independent studies investigating, respectively, cerebral distribution of MIF (Zhang et al., 2014), the function of MIF in stroke (Inacio et al., 2011a; Inacio et al., 2011b; Inacio et al., 2011c), and hypertension (Li et al., 2006; Li et al., 2008; Sun et al., 2007; Sun et al., 2004) reported that MIF was expressed in parvalbumin positive (PV+) interneurons. Taken together, the data clearly supports a neuroprotective role for MIF during stroke.

Following the acute phase of stroke, the area of the infarct gradually extends to adjacent areas, which lasts for several days. This late phase process is slower when compared to the infarct development during the acute phase of stroke, which suggests that different mechanisms may be involved between acute and late phase infarct development. Interestingly, a recent study assessed the infarct volume at day 7 following transient MCAo and found that Mif⁺/⁺ mice developed slightly larger infarcts when compared to Mif⁻/⁻ mice (Inacio et al., 2011b). Although no differences in cytokine production was found between the Mif⁺/⁺ and Mif⁻/⁻ mice during these 7 days (Inacio et al., 2011a), the results indeed demonstrated that MIF-mediated the response of macrophages/microglia to ischemia (Inacio et al., 2011b). The role of MIF in initiating and sustaining inflammatory responses has been well characterized outside of the central nervous system. It has been shown that constitutive expression of MIF is essential to combat common infections and knockout of the Mif gene
can result in higher mortality to certain infections (Koebernick et al., 2002) but protection from excessive host inflammation (Calandra et al., 1995). A protective effect of MIF has been attributed to its unique ability to override the anti-inflammatory effects of glucocorticoids (Calandra et al., 1995) and thereby promoting the production of pro-inflammatory cytokines (Calandra et al., 1995; Koebernick et al., 2002). Additionally, constitutive expression of MIF maintains the surface expression of TLR4, which in turn is responsible for sensing infection and initiating a host defense response (Calandra and Roger, 2003). These characteristics of MIF suggest that MIF has both an “anti-stress” role and is necessary to activate the first line of host defense. In contrast, under chronic inflammatory conditions, such as rheumatoid arthritis, atherosclerosis, and Alzheimer’s disease, MIF is persistently upregulated (Bacher et al., 2010; Morand et al., 2006). Recruitment of macrophages is involved in the pathology of rheumatoid arthritis and atherosclerosis, and was shown to be mediated by the interaction between MIF expressed at the lesion site and the chemokine receptors on inflammatory cells (Bernhagen et al., 2007). Although the mechanism for sustained MIF expression is unknown, MIF has appeared to have a predominant deleterious effects in chronic inflammation in comparison to the effect of MIF during acute inflammation. Similar mechanisms may explain the late-phase infarct development. Upregulation of MIF expression in the peri-infarct area in response to the acute stress induces a macrophages/microglia response, which in turn contributes to a larger infarct volume by prolonging infarct development. MIF could play dual roles following ischemia depending on the time window. It is worth noting that a recent study on heart ischemia took into consideration the severity of the ischemic insult and assessed infarct volume during both early and late phases of infarct development. It reported that mice lacking MIF expression developed larger infarcts following mild ischemic insult (15 min occlusion) during the early phase (4 hours after the ischemic insult) of infarct development, while this protective effect of MIF was not observed at 24 hours following an ischemic insult induced for 30 min (Koga et al., 2011). This observation is in concordance with our results, and is likely due to the loss of MIF expression.

To date, inhibition of MIF has been suggested in many therapeutic strategies, especially in inflammation-induced diseases. Our results show that downregulation of MIF by NFκB
signaling accelerates neuronal apoptosis during stroke, suggesting that use of MIF inhibitor may reduce the already narrow therapeutic window following stroke, and confer a higher risk of ischemic damage. Our study indicates that MIF protects neurons against ischemia and a strategy to control MIF expression at a therapeutic level could have beneficial effects for stroke patients.

3.5 Conclusion

In this chapter we demonstrate that MIF reduces caspase-3 activation and protects neurons from oxidative stress-induced and in vitro ischemia/reperfusion-induced apoptosis. Furthermore, we demonstrate that disruption of MIF gene expression results in elevated caspase-3 activation and exacerbates neuronal death and increased infarct development during stroke in Mif−/− mice in vivo. Our results suggest that MIF exhibits neuroprotective qualities following stroke and may be an important molecule for preserving a longer time window for stroke treatment.
Chapter 4

Association of MIF with Aβ plaques potentially inhibits the cognitive function of MIF in Alzheimer’s disease

4.1 Introduction

The current understanding of the role of MIF during AD pathology is extremely limited. Searching on the Pubmed using phrases “Alzheimer’s disease” and “MIF” yields only 6 research articles. Three of them demonstrated upregulated MIF levels in biological fluids including CSFs and sera collected from AD patients (Craig-Schapiro et al., 2011; Lee et al., 2008; Popp et al., 2009); one found no changes in MIF expression in the brain of an AD mouse model (Mehlhorn et al., 2000); and one studied the role of CD74 in AD (Bryan et al., 2008), which was identified as the receptor of MIF (Leng et al., 2003). Only one of the articles briefly assessed the function of MIF, and demonstrated the effect of the MIF inhibitor, ISO-1, on Aβ-induced toxicity in a neuroblastoma cell line (Bacher et al., 2010).

In contrast, there is a large body of research reported central roles of MIF in many other diseases. As a pleotropic protein, MIF has been shown to be beneficial when expressed by functional cells, for example, promoting cardiomyocytes survival during cardiac I/R by inhibiting apoptosis, reducing ROS production and regulating glucose metabolism (Koga et al., 2011; Luedike et al., 2012; Miller et al., 2008). In Chapter 3, we also demonstrated a protective role of MIF in suppressing oxidative stress-induced caspase-3 activation. In AD pathogenesis, neuronal death through apoptotic pathways is observed, and there is evidence suggesting that oxidative stress originated from dysregulation of mitochondria functions serves as a cause of apoptosis (Eckert et al., 2003). Therefore, MIF may be essential for
neuronal survival during AD pathogenesis. In contrast, MIF plays a deleterious role when overexpressed by immune cells, resulting in excessive inflammation in chronic inflammatory diseases in the systems outside of the CNS (Morand et al., 2006). It is well established that Aβ deposits induce chronic neuroinflammation in AD, which features in activation of resident microglia, as well as infiltration of peripheral macrophages (Rubio-Perez and Morillas-Ruiz, 2012). However, whether MIF plays a role in initiating and/or maintaining the inflammatory status in the CNS is unknown. Collectively, it is intriguing to investigate how MIF would participate in these seemingly paradoxical roles during AD pathogenesis.

Animal models are powerful tools to study human diseases. In the AD field, transgenic mice bearing human APP and/or PS transgenes with various mutations are widely used to study Aβ related AD pathology. In our own lab, we use APP23 and PS45 double transgenic mice to study AD pathogenesis and drug development (Ly et al., 2013). Studying the function of MIF in AD is still in its infancy and no yet transferred to animal models. Due to the strong implication of MIF’s role in AD pathology, we would like to take an advantage of the AD model mice to study the potential role of MIF during AD pathogenesis. Moreover, clinical trials are undertaken to evaluate whether inhibition of MIF would be beneficial in chronic inflammatory diseases. Since these diseases are associated or coexisted with AD, animal models could provide first-hand evidence whether inhibition of MIF would affect AD progression.

In this chapter, we aimed to first investigate the regulation of MIF expression during AD pathogenesis in human patients and in a mouse model of AD. Then we will study how the expression pattern of MIF affects the functional performance under AD pathogenesis.

4.2 Methods

4.2.1 Human brain tissues

Frozen control and AD human cortices were obtained from the Department of Pathology, Columbia University. These samples were used to examine the expression of MIF by immunoblotting experiments.
Table 4.1 Human brain tissues for analysis of MIF expression in Alzheimer’s disease.

<table>
<thead>
<tr>
<th>Code</th>
<th>Group</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Use</th>
<th>Brain Area</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1751</td>
<td>AD</td>
<td>M</td>
<td>76</td>
<td>IB</td>
<td>Fc</td>
<td>Columbia University</td>
</tr>
<tr>
<td>1780</td>
<td>AD</td>
<td>M</td>
<td>72</td>
<td>IB</td>
<td>Fc</td>
<td>Columbia University</td>
</tr>
<tr>
<td>4556</td>
<td>AD</td>
<td>F</td>
<td>70</td>
<td>IB</td>
<td>Fc</td>
<td>Columbia University</td>
</tr>
<tr>
<td>4693</td>
<td>AD</td>
<td>F</td>
<td>70</td>
<td>IB</td>
<td>Fc</td>
<td>Columbia University</td>
</tr>
<tr>
<td>4854</td>
<td>AD</td>
<td>M</td>
<td>54</td>
<td>IB</td>
<td>Fc</td>
<td>Columbia University</td>
</tr>
<tr>
<td>794</td>
<td>Control</td>
<td>F</td>
<td>51</td>
<td>IB</td>
<td>Fc</td>
<td>Columbia University</td>
</tr>
<tr>
<td>1170</td>
<td>Control</td>
<td>M</td>
<td>58</td>
<td>IB</td>
<td>Fc</td>
<td>Columbia University</td>
</tr>
<tr>
<td>1226</td>
<td>Control</td>
<td>M</td>
<td>23</td>
<td>IB</td>
<td>Fc</td>
<td>Columbia University</td>
</tr>
<tr>
<td>1441</td>
<td>Control</td>
<td>M</td>
<td>51</td>
<td>IB</td>
<td>Fc</td>
<td>Columbia University</td>
</tr>
<tr>
<td>4263</td>
<td>Control</td>
<td>M</td>
<td>61</td>
<td>IB</td>
<td>Fc</td>
<td>Columbia University</td>
</tr>
</tbody>
</table>

Abbreviations: AD, Alzheimer’s disease; M, male; F, female; IB, immunoblotting; Fc, frontal cortex

4.2.2 CSF collection

CSF samples were obtained from patients visiting the Guangzhou General Hospital. The control group recruited patients who had no history or evidence of cognitive decline. CSFs were taken from them by lumbar puncture under anesthesia when they had surgery for other diseases. CSFs from patients with AD, mild cognitive impairment (MCI), and vascular dementia (VD) were collected for neurological diagnosis.

Table 4.2 Human CSF samples for analysis of MIF concentration.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sample size (female/male)</th>
<th>Age (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30 (14/16)</td>
<td>61.2±9.6</td>
</tr>
<tr>
<td>AD</td>
<td>28 (14/14)</td>
<td>69.4±6.8</td>
</tr>
<tr>
<td>MCI</td>
<td>10 (4/6)</td>
<td>68.9±9.6</td>
</tr>
<tr>
<td>VD</td>
<td>17 (8/9)</td>
<td>66.3±10.2</td>
</tr>
</tbody>
</table>

4.2.3 Animals

Animal experiment protocols were approved by The University of British Columbia Animal Care and Use committee. APP23 transgenic mice carry human APP751 cDNA with the Swedish double mutation at positions 670/671 (KM→NL) under control of the murine Thy-1.2 expression cassette (Sun et al., 2006a). PS transgenic mice carry human presenilin-1 cDNA with the G384A mutation (Qing et al., 2008). Mif−/− mice on the c57/BL6 background were generated by breeding Mif−/− mice on BALB/c background with c57/BL6 mice. Mif−/− mice on BALB/c have been described in Chapter 3. All mice were allowed to access water and food ad libitum. APP23/PS45 double transgenic mice were bred by cross APP23 with PS45 mice. APP23/MIF+/− mice were bred by cross APP23 with Mif−/− mice on c57/BL6 background. Positive pups were determined by genotyping.
4.2.4 Genotyping

All transgenic mice were genotyped at the beginning of weaning and at the time of euthanasia. At 3 weeks of age, mice were anesthetized with isoflurane and ear-marked. At the time of euthanasia, a piece of the ear was also harvested. The tissue was digested in 200μL of lysis buffer (10 mM Tri-HCl pH8.0, 10 mM EDTA pH8.0, 150 mM NaCl, 0.5% SDS) with 3μL of 10 μg/mL Proteinase K (New England Biolabs) at 55°C overnight. The next day, samples were centrifuged and DNA was precipitated with 0.7X volume of isopropanol. DNA was pelleted by centrifugation at 16,000xg for 15 min, washed twice with 70% ethanol, air dried, and re-suspended in sterile de-ionized water. The genomic DNA was subjected to PCR to amplify human APP using Thy1E2F-CACCACAGAATCCAGTCGG/app1082R-CTTGCAGTTCTGGCCTCTTCC and human presennilin 1 with PS1F-CAGGTGCTATAAGGTCAT and PS1R-ATCACAGCCAAGATGAGC. Hemizygous knockout of MIF gene was confirmed by MIF KO F-TCCCAGGACCAGCTCATGACTTTTA/MIF KO Neo R-GTTGGCGCTACCAGGTGGATGT.

4.2.5 Immunoblotting

Frozen human cortices (~10mg) or mouse cortical tissues were homogenized by sonication with 5X (v/w) RIPA-DOC lysis buffer supplemented with complete mini protease inhibitor cocktail tablet (Roche Molecular Biochemicals). The samples were then centrifuged at 16,000 Xg at 4°C for 30 minutes. The supernatants were removed and added to 2X Novex® tricine SDS sample buffer (Invitrogen) followed by boiling at 100°C for 2 minutes. The samples were resolved in 12% tris-tricine gels and transferred to PVDF-FL membranes (Millipore). The membranes were blocked with 5% non-fat milk and incubated with primary antibodies for MIF (Torrey Pines Biolabs, Secaucus, NJ, USA) and β-actin (Sigma, AC-15). To detect the proteins, IDye680-labeled goat anti-rabbit and IDye800-labeled goat anti-mouse antibody were used. The blots were scanned using the Odyssey Imager (Licor).

4.2.6 Immunohistology

Half brains were fixed in 4% PFA in PBS. Fixed brains were either dehydrated in 30% sucrose solution followed by cryosectioning at 30μm thickness or prepared for paraffin-
embedded sectioning at 5μm thickness. For immunohistochemistry, the brain slices were incubated with 3% hydrogen peroxide in PBS for 10 min, permeabilized in 0.3% Triton X-100 in PBS (PBS-Tx) for 30 min, and blocked with 5% BSA in PBS for 1 hour at 22°C. Next, the slices were incubated with primary antibodies at 4°C overnight. After rinsing with PBS-Tx for 3 times, the slices were applied with secondary antibodies for 1 hour at 22°C, followed by 30-min incubation with avidin-biotin-peroxidase complex (ABC, Vector Laboratories). Color development was achieved by the DAB (Vector Laboratories) method. After rinse with ddH2O, brain sections were subjected to additional hematoxylin staining to visualize nuclei. The sections were observed under traditional microscopy. For Aβ plaque detection, the procedure for secondary antibody incubation was omitted, and the slices were proceeded for ABC incubation and color development. The number of plaques were quantified manually following qualification under 40X magnification. The area of the plaques was quantified using ImageJ. Images were taken from 10 matching areas (5 slices with 540μm intervals for each mouse) between APP23 and APP23/MIF+/- transgenic mice. All the images used for plaque quantification were taken at the same time with the same exposure level. The color images were first transformed to 8-bits images, set at the same threshold followed by auto-quantification of the area. For immunofluorescent staining, the brain slices were permeabilized in PBS-Tx for 30 min followed by sequential incubation with primary and fluorescent labeled secondary antibodies as above. After rinse with PBS, brain sections were coverslipped using the VECTASHILD® mounting medium with DAPI (Vector Laboratories), and observed under fluorescent microscopy. The primary antibodies are: rabbit anti-MIF antibody (Torrey Pines Biolabs), mouse anti-GFAP antibody, biotinylated 4G8 antibody, rabbit anti-Iba-1 (DAKO). Secondary antibodies are: biotinylated swine anti-rabbit IgG (DAKO) for immunohistochemistry, Alexa 488 labeled goat anti-rabbit IgG (Invitrogen), Alexa 594 labeled goat anti-rabbit IgG (Invitrogen), Alexa 488 labeled goat anti-mouse IgG (Invitrogen), and Alexa 594 labeled goat anti-mouse IgG (Invitrogen) for immunofluorescent staining. Primary and secondary antibodies were diluted in PBS with 1% BSA.
4.2.7 Cell culture, Aβ oligomer preparation, ELISA and LDH assay

The mouse microglia cell line BV-2, mouse macrophage cell line RAW264.7, and human neuroblastoma cell line SH-SY5Y were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1 mmol/L of sodium pyruvate, and 2 mmol/L of L-glutamine (Invitrogen, Carlsbad, CA, USA). Cells were seeded onto 96-well plates and cultured at 37˚C in an incubator supplemented with 5% CO2. Aβ oligomers were prepared as previously described with modification (Fa et al., 2010; Stine et al., 2003). Briefly, synthetic Aβ1-42 was dissolved in 1,1,1,3,3,3-Hexafluoro-2-Propanol (HFIP, Fluka), vacuum dried, and dissolved in DMSO as a 5mM stock. Aβ oligomer was prepared by diluting the stock Aβ in sterile PBS to 100μM and incubated at 4°C for 12 hours. The oligomers were further diluted to 10μM by culture medium to treat the cells. LPS was used as positive control for MIF secretion, and was used at the concentration of 100 ng/mL. 16 hours after Aβ treatment, culture medium was collected and centrifuged at 3,000xg for 2 min at 4˚C prior to assays. MIF concentrations in culture media were measured by human (R&D systems) or mouse (Mybiosource) MIF ELISA kit following the manufacture’s instruction. Culture medium were diluted 5 and 2 times prior the assays to measure human and mouse MIF concentration, respectively. To assess cell membrane integrity, LDH assay (Promega) was performed using the same batch of culture medium.

4.2.8 Dot blot assay

To prepare the membrane for the dot blot assay, 2 μL of oligomerized Aβ peptide (100 μM) or purified green fluorescent protein (GFP) protein (approximately 50 μM) were spot on a nitrocellulose membrane, and were led dry. The membrane was then blocked in 0.3% BSA in PBS for 1 hour at room temperature prior to incubation with mixed proteins of purified hMIF and GFP at the concentration of approximated 5 μM at 4˚C for overnight. The membrane was then washed and immunoblotting (section 4.2.5) was performed to detect MIF and GFP. The primary antibody to detect MIF was a monoclonal anti-MIF antibody (D-2, Santa-Cruz). The primary antibody to detect GFP was a polyclonal anti-GFP antibody.
4.2.9 Fractionation of the mouse brain.

Four-month old APP23/PS45 and wildtype (as controls) mice were euthanized. Brains were dissected, weighed, and briefly rinsed with cold PBS. The cortical tissues were dissected and homogenized in 5x PBS with 0.5% Triton-100 (v/w) supplemented with 2x protease inhibitor cocktail Complete (Roche Molecular Biochemicals) using a Dounce glass homogenizer. The homogenates were incubated on ice for 10 min before centrifuge at 16,000xg at 4°C for 15 minutes. The resulted layers were collected separately for further analysis. The supernatants were collected and subjected to ultracentrifugation at 100,000xg for 1 hour at 4°C, and the second supernatants were collected and labeled as S’. To detect the level of MIF and Aβ, homogenates from each layer were subjected to sonication in sufficient amount of the RIPA-DOC buffer to completely dissolve proteins. The same amount of protein was loaded for gel separation and protein level detection. The gel electrophoresis was performed using 16% Tris-tricine gel followed by protein analysis by Western blots. The primary antibodies used to detect Aβ, MIF and β-actin are 6E10, anti-MIF (Torrey Pines Biolabs, Secaucus, NJ, USA), and anti-β-actin (Sigma, AC-15) antibodies, respectively.

4.2.10 Morris water maze test

The Morris water maze test was performed as previously described (Bromley-Brits et al., 2011). Briefly, the test was performed in a 1.5-meter diameter pool with a 10-cm diameter platform placed in the southeastern quadrant of the pool. The procedure consisted of one day of visible platform tests and four days of hidden platform tests, plus a probe trial 24 hours after the last hidden platform test. In the visible platform test, mice were tested for 5 continuous trials with an inter-trial interval of 60 minutes. Mouse behavior including distance traveled and escape latency was automatically video-recorded by automated video tracking (ANY-maze, Stoelting). The tests were performed on APP23/MIF^{+/−} mice, and APP23 mice, which were negative littler mates of APP23/MIF^{+/−} mice. The tests were performed at the ages between 13 to 14 months. Only male mice were used for tests. Briefly, the first day of the test was a visible platform test, followed by 4 days of hidden platform testing, and a probe trial on the last day. Escape latency, distance traveled, and the number of times passing through the removed platform (probe trial) were recorded.
4.3 Results

4.3.1 Upregulation of MIF expression in AD patients.

Postmortem brain tissues collected from AD patients and age-matched controls were assayed by immunoblotting to evaluate the expression of MIF (Figure 4.1A). Expression levels of MIF were significantly elevated to 1.58±0.14 fold, compared to the controls (1.00±0.14 folds) (P<0.05) (Figure 4.1B). Since samples were collected from postmortem brains, this result revealed upregulation of MIF expression at the end stage of AD pathology. Proteins that are aberrantly expressed in CSF have been used as biomarkers for diagnostics of many CNS diseases. In order to assess the regulation of MIF in patients at earlier stages of AD pathology, the MIF level in CSF collected from control subjects and patients diagnosed with MCI and AD was measured. CSFs from patients with VD was also collected for MIF concentration measurements. Our results showed that the MIF concentration in the CSFs was significantly upregulated in the patients diagnosed with AD (14.62±1.15 ng/ml) compared to the control subjects (10.07±0.60 ng/ml) (P<0.05) (Figure 4.1C). However, the concentration of MIF in MCI and VD patients did not differ from the control subjects (9.89±1.48 and 9.86±0.83 ng/ml, respectively) (P>0.05), and were significantly lower compared to AD patients (P<0.05) (Figure 4.1C). Notably, the CSF concentration of MIF was elevated approximately 1.4 fold in AD patients compared to the control subjects, which was similar as the fold increased in the brain tissue, indicating the level of MIF in CSF could be an indicator of the level of MIF in the brain tissue. Collectively, our results demonstrated that MIF expressed at different levels at the early and late stage of AD, and that the elevation of MIF is a characteristic of the late stage AD.
4.3.2 Upregulation of MIF expression during AD pathology.

To determine the change of the MIF expression during AD pathology, we measured the expression of MIF in the brain tissue from APP23/PS45 double transgenic mice at different ages. APP23/PS45 mice have an accelerated AD-like pathological progression compared to APP23 mice. By the age of 3 months, APP23/PS45 mice have developed significant amount of plaques and demonstrated memory loss in behavioral tests. We first assessed the plaque formation in APP23/PS45 mice at different ages to confirm the stage of AD pathology. Brain slices obtained from APP23/P45S mice were stained with thioflavin S and 4G8 to identify plaques. At 1.5 months of age, no evident plaque was observed in the entire brain, while positive thioflavin S staining was detected in the neurons in the cortical layers (Figure 4.2A,a, arrows), and 4G8 staining confirmed the neuronal expression rather than the extracellular Aβ plaques (Figure 4.2A,b, arrows). In 2-month old mice, a few plaques appeared in the cortical and hippocampal regions and were scattered in the cortical layers (Figure 4.2B, hollow arrow heads). As expected, the number of plaques significantly increased at 3 months of age in all brain regions as indicated by both thioflavin S and 4G8
staining (Figure 4.2C, hollow arrow heads). MIF expression levels in cerebral parenchyma were also measured using the same group of mice. Immunoblotting results showed that MIF protein level in the brain tissue was similar between wildtype (WT) and APP23/PS45 mice at the age of 1.5 months (1.00±0.06 vs 0.96±0.04 folds, P>0.05), as well as at 2 months (1.00±0.03 vs 1.02±0.02 folds, P>0.05) (Figures 4.2D, E, G, H). However, at 3 months of age, MIF expression in the APP23/PS45 mice increased to 1.37±0.05 fold (P<0.05) of the WT mice at the same age (Figures 4.2F and I). We also measured the MIF level in CSFs from the AD mice, but failed due to the insufficient collection of CSFs. Our results demonstrated that upregulation of MIF in the APP23/PS45 mice occurred at the late stage of the disease when large amount of amyloid plaques had been formed. This was in line with the results observed in human patients.

Figure 4.2 Amyloid plaque development and MIF expression in APP23/PS45 mice during AD progression.
APP23/PS45 double transgenic mice and the wildtype controls were euthanized at the ages of 1.5, 2 and 3 months. Half of the brain was fixed and cryosectioned for plaque assessment, and the other half was used for MIF expression evaluation. (A, B and C) Thioflavin S and 4G8 stained representative brain sections of the 1.5-
(A), 2- (B) and 3- (C) month old APP23/PS45 mice (APP/PS). Solid arrows point to thioflavin S and 4G8 stained cortical layers on the cortical region. Hollow arrows point to thioflavin S and 4G8 stained neuritic plaques. (D, E and F) Brain tissues were lysed in RIPA-DOC buffer, and an equal amount of protein was resolved on a 12% tris-tricine gel. MIF was detected by an anti-MIF antibody, CTFs were detected by an C20 antibody to confirm the expression of the transgene, and β-actin was detected by the β-actin antibody serving as the loading control. (G), (H) and (I). Quantification of (D), (E) and (F), respectively. The ratio of MIF to β-actin was normalized to wildtype mice. Values were expressed as mean± SEM, n = 4~8 for wildtype mice and 4~10 for APP23/PS45 mice. (G) and (H) * P>0.05 by Student’s t tests. (I) ** P<0.05 by Student’s t tests. Scale bar, 500µm.

4.3.3 MIF secretion by a neuronal cell line after Aβ stimulation

It has been well documented that Aβ activates NFκB signaling in neurons and glial cells (Bonaiuto et al., 1997; Chen et al., 2005; Kaltschmidt et al., 1997). In Chapter 2 we demonstrated that MIF is transcriptionally upregulated by NFκB signaling in neuronal, microglia and macrophage cell lines. Here, we further demonstrated that MIF upregulation in the brain is accompanied with a significant increase of Aβ deposition. Therefore, we believe that all the above cells may be responsible for MIF upregulation in AD brains. Since MIF is a secretory protein, and exerts functions via autocrine/paracrine mechanisms, we are interested in whether Aβ could also trigger MIF secretion, thereby affecting cell functions. Microglia and infiltrated macrophages are closely associated with Aβ deposits in AD brains. Therefore, we first tested whether MIF could be secreted after Aβ treatment on a mouse microglia and mouse macrophage cell lines, BV-2 and RAW 264.7, respectively. LPS was used as a positive control because it has been showed to trigger MIF secretion in RAW 264.7 (Calandra et al., 1994; Flieger et al., 2003). A baseline expression of MIF was detected in the medium from RAW 264.7 at the concentration of 11.73±1.54 pg/mL, while it was not detectable in that from BV-2 (Figure 4.3A and B). After the LPS treatment, the concentration of MIF significantly increased in the culture medium to 18.83±0.88 ng/mL for RAW 264.7 (P<0.05) and 14.35±0.86 ng/ml for BV-2 (Figure 4.3A and B). However, Aβ treatment did not significantly alter the concentration of MIF in the medium from RAW 264.7 (9.43±1.24 pg/mL) compared to controls but was significantly lower than that from LPS treatment (P<0.01) (Figure 4.3A). To our surprise, Aβ treatment did not raise the concentration of MIF above detection limit in the culture medium from BV-2 (Figure 4.3B). LDH assays were performed to examine the cell membrane Integrity that affects the release of MIF. Our result demonstrated that Aβ treatment did not resulted in cell membrane leakage in either cell lines (Figures 4.3D and E). In contrast, LPS treatment resulted in cell membrane damage in
4.89±0.35% and 7.15±0.22% of RAW 264.7 and BV-2, respectively (P<0.05) (Figures 4.3D and E), albeit the chance was slim that leaked MIF resulted in doubling MIF concentration in culture medium. The next candidate for MIF secretion is the neuron. We investigated whether Aβ could serve as a trigger for MIF release in SH-SY5Y, and LPS was used as a control. The baseline release of MIF to the culture medium from the SH-SY5Y was also observed at the concentration of 26.98±0.91 ng/mL (Figure 4.3C). In contrast to the reduction of MIF secretion in RAW264.7 and BV-2, LPS treatment slightly reduced MIF release in SH-SY5Y to the concentration of 24.36±1.12 ng/mL, compared to controls (P<0.05) (Figure 4.3C). In addition, LPS treatment did not affect the cell membrane integrity of SH-SY5Y as shown by the LDH assay (Figure 4.3F). The Aβ treatment on SH-SY5Y cells induced a significant increase of MIF concentrations in the culture medium to 32.97±0.79 ng/mL (Figure 4.3C), and the increase was not due to the cell membrane leakage as shown by LDH assay (Figure 4.3F). Taken together, our result suggested that Aβ triggered MIF secretion by neurons.

Figure 4.3 MIF secretion after amyloid beta stimulation.
Cells were seeded on seeded onto 96-well plates, and cultured 24 hours prior to treatment. Aβ treatment was achieved by adding medium diluted Aβ_{1-42} oligomer stock solution (100μM in sterile PBS) at the final concentration of 10μM. LPS at the concentration of 100 ng/mL was used as a positive control for MIF secretion. 16 hours after treatment, the culture medium was collected and centrifuged prior to analysis. (A), (B) and (C). Culture media collected from RAW 264.7, BV-2 SH-SY5Y cell lines, respectively, were measured for MIF concentrations by ELISA. Values represent the mean±SEM, n=4. * P<0.05 relative to controls by one-way ANOVA with Newman–Keuls post hoc tests. # P<0.05 relative to LPS by one-way ANOVA with Newman–Keuls post hoc tests. (D), (E) and (F). The same batch of media from RAW 264.7, BV-2 SH-SY5Y cell lines, respectively, were subjected to LDH assay to evaluate the cell membrane integrity. Values represent the mean±SEM, n=4. * P<0.05 relative to controls by one-way ANOVA with Newman–Keuls post hoc tests.
4.3.4 MIF deficiency affects learning and memory.

The above results suggested that neurons were responsible for the MIF secretion that was triggered by the Aβ deposits. Although it is debatable whether Aβ deposits serve as a cause of cognitive decline during AD pathogenesis, inhibition of Aβ production and plaque formation have been shown to successfully mitigate AD pathology-induced cognitive deficits (Ly et al., 2013; Qing et al., 2008; Richard et al., 2008). Since the lack of MIF was shown to impact on cognitive performance (Conboy et al., 2011), we hypothesized that Aβ-triggered MIF secretion could serve as a compensatory mechanism to assist cognitive performance during AD pathology. To test whether MIF participates in cognitive performance during AD pathogenesis, we examined whether MIF deficiency could affect learning and memory in AD model mice. APP23/MIF+/- and APP23 mice were subjected to memory function assessment by the Morris water maze at the age between 12 to 13 months. In the visible platform tests, APP23/MIF+/- and APP23 mice had similar escape latencies (40.5±3.0 s and 42.8±4.6 s, P>0.05) (Figure 4.4A) and path length (7.7±0.7 m and 8.7±0.7 m, P>0.05) (Figure 4.4B), indicating that hemizygous knockout of MIF did not affect mouse mobility or vision. In the hidden platform test, APP23/MIF+/- mice showed significant memory impairment on the 5th day of the test. The escape latency on the last day of the hidden platform test was significantly longer than in APP23 mice (28.1±2.9 vs 15.7±1.4 s, P<0.05) (Figure 4.4C). The APP23/MIF+/- mice also swam significantly longer distances to reach the platform as compared to APP23 mice (4.3±0.5 vs 2.6±0.1 m, P<0.05) on the 5th day of hidden platform test (Figure 4.4D). We also examined whether hemizygous knockout of MIF also affected swimming speed in APP23 transgenic mice. Our results demonstrated that the swimming speed of APP23/MIF+/- mice did not differ from that of APP23 mice on each day of the test, but significantly slowed down on the 3rd, 4th, and 5th days compared to the 1st day (Figure 4.4E). In the probe trial on the last day of testing, the platform was removed. No differences were detected between APP23/MIF+/- and APP23 mice for the number of times the mice passed through the position of the previous platform (Figure 4.4F) or the time spent and distance traveled in the 3rd quadrant (Figures 4.4G and H), where the hidden platform was previously placed. These results indicated that MIF deficiency did not alter reference memory in APP23 transgenic mice, but affected spatial learning during the hidden platform training and induced depressive behavior after repeated exposure to the same environment.
Figure 4.4 MIF deficiency potentially affects memory function in APP23 transgenic mice.

A Morris water maze test consists of one day of visible platform trials and four days of hidden platform trials, plus a probe trial 24 hours after the last hidden platform trial. Animal movement was tracked and recorded by the ANY-maze tracking software. APP23/MIF+/- and APP23 mice (APP/MIF+/- and APP, respectively) at the age of 12 to 13 months were subjected to the Morris water maze test. (A) During the first day of visible platform tests, the APP23/MIF+/- and APP23 mice exhibited a similar latency to escape onto the visible platform. *P>0.05 by Student’s t test. (B) APP23/MIF+/- and APP23 mice had similar swimming distances before escaping onto the visible platform. *P>0.05 by Student’s t test. (C) In hidden platform tests, mice were trained with five trials per day for four days. APP23/MIF+/- mice showed a longer latency to escape on to the hidden platform on the 5th day. *P<0.05 by Two-way ANOVA with Bonferroni post hoc tests. (D) APP23/MIF+/- mice had a shorter swimming length before escaping onto the hidden platform on the 5th day. *P<0.05 by Two-way ANOVA with Bonferroni post hoc tests. (E) Swimming speed was not different between APP23 and APP23/MIF+/- mice. *P>0.05 by two-way ANOVA. APP23/MIF+/- mice swim significantly slower at the 3rd, 4th, and 5th day of the test compared to the 1st day. #P<0.05 compared to the 1st day for the APP23/MIF+/- group by one-way ANOVA followed by Bonferroni tests. (F) On the last day of testing, no statistical difference was detected between APP23/MIF+/- and APP23 mice for the number of times the mice passed through the position of the platform. *P>0.05 by Student’s t test. (G) APP23/MIF+/- and APP23 mice spent similar time in the 3rd quadrant, where the platform previously located. *P>0.05 by Student’s t test. (H) APP23/MIF+/- and APP23 mice swam a similar distance in the 3rd quadrant. *P>0.05 by Student’s t test. All the values are expressed as mean±SEM. N=6 for each group.
4.3.5 MIF expression is associated with Aβ plaques.

Since our results suggested that sufficient MIF is necessary for normal cognitive performance, it seems controversial that AD patients with increased MIF levels still demonstrated memory loss. In order to address this discrepancy, we thoroughly analyzed the expression patterns of MIF in the brain. Immunohistology was performed on brain sections from APP23/PS45 mice using a specific anti-MIF antibody. The pattern of MIF staining demonstrated as spotted clusters in both the hippocampal region and cerebral cortex (Figure 4.5A), which remarkably resembled that of 4G8 stained Aβ plaques. Next, we performed the double staining of MIF and 4G8 on an adjacent brain section (5μm) to the one used for the DAB staining. The fluorescent signals for MIF as pointed by white arrows (Figure 4.5B) were confirmed by the DAB staining of MIF as pointed by black arrows (Figure 4.5A). The double staining of MIF and 4G8 revealed the colocalization of MIF and Aβ plaques (Figure 4.5B). Interestingly, two patterns of association between MIF and Aβ plaques were observed. Besides the colocalization as shown in Figure 4.5B,b, some MIF staining exhibited a pattern that embraced the dense core of the Aβ plaques in hippocampal (Figure 4.5B,a, insert) as well as in cortical (Figure 4.5C) regions. There were a few cells stained positive for MIF (arrow heads, Figure 4.5B inserts), and we did further analysis to identify the cell type.
Figure 4.5 Colocalization of MIF and amyloid plaques in APP23/PS45 mice.

(A) and (B) Fixed brains were prepared for paraffin-embedded sectioning at 5μm thickness. MIF was detected by a specific anti-MIF antibody and visualized by ABC and DAB methods. Nuclei were stained by hematoxylin. The sections were observed under microscopy. Arrows point to MIF expression, which resembles the expression pattern of Aβ plaques. Scale bar: 100μm. (C) Fixed brains were dehydrated in a 30% sucrose solution and embedded in O.C.T. for cryosectioning at 30μm thickness. MIF was detected by a polyclonal MIF antibody and Alexa 488 labeled goat anti-rabbit IgG secondary antibody; and neuritic plaques were detected by a monoclonal 4G8 antibody and Alexa 594 labeled goat anti-mouse IgG secondary antibody. Nuclei were stained with DAPI, and brain sections were observed under fluorescent microscopy. Arrows point to colocalization of MIF and plaques. Arrow heads point to MIF expression cells. Scale bar: 100μm in B, and 25μm in inserts and C.

Many studies have demonstrated that microglia and astrocytes accumulate around amyloid plaques. Since MIF expression was tightly associated with amyloid plaques, we intended to see whether glial cells were responsible for the plaque-associated MIF expression. Double staining of MIF and GFAP was performed on brain sections from APP23/PS45 mice at the age of 7 months (Figure 4.6A). A similar MIF expression pattern was observed, confirming the characteristics of MIF expression in the AD brains – enriched at the vicinity of amyloid plaques. Active astrocytes stained with GFAP were prevalent in the entire brain section.
without particular enrichment. At the site of MIF enrichment, MIF expression overlapped with GFAP staining, although the highest expression of GFAP was neither in close vicinity of nor colocalized with MIF (arrows, Figure 4.6A). Due to the lack of available antibodies, double staining for colocalization study was not performed for MIF and microglia. Instead, amyloid plaques were stained with 4G8 to locate the possible expression of MIF, and the Iba-1 antibody was used to detect microglia (Figure 4.6B). Our results demonstrated that Iba-1 positive microglia were in closer vicinity to the amyloid plaques than astrocytes, suggesting highly possible overlapping of microglia and MIF expression. In addition, morphology of MIF expression cells (arrow heads, Figure 4.6A) was very similar to Iba-1 stained microglia (arrow heads, Figure 4.6B).

Figure 4.6 MIF expression overlaps but is not restrict inside of the vicinity microglia and astrocytes.

Fixed brains were dehydrated in a 30% sucrose solution and embedded in O.C.T. for cryosectioning at 20 μm thickness. (A) MIF was detected by a polyclonal MIF antibody and Alexa 488 labeled goat anti-rabbit IgG secondary antibody; and active astrocytes were detected by a monoclonal GFAP antibody and Alexa 594 labeled goat anti-mouse IgG secondary antibody. Nuclei were stained with DAPI, and brain sections were observed under a fluorescent microscopy. Arrows point to GFAP positive cells. Arrow heads point to possible MIF expressing cells. Scale bar: 200μm, and 50μm in inserts. (B) Neuritic plaques were detected by a monoclonal 4G8 antibody and Alexa 488 labeled goat anti-mouse IgG secondary antibody; and microglia were detected by a polyclonal Iba-1 antibody and Alexa 594 labeled goat anti-rabbit IgG secondary antibody. Nuclei were stained with DAPI, and brain sections were observed under fluorescent microscopy. Arrow heads point to possible MIF expressing cells. Scale bar: 50μm.
4.3.6 MIF interacts with Aβ and is enriched in the fractions Aβ aggregates.

Our results suggested possible association of MIF with amyloid plaques in the extracellular space. However, one could not rule out the possibility that the presence of MIF was merely due to constant secretion by the vicinity cells. In order to examine whether MIF is associated with plaques in the extracellular matrix, we first examined whether MIF could interact with Aβ using a dot blot assay. Oligomerized Aβ and purified GFP were spot on a membrane and incubated with the protein mixture of purified MIF and GFP. The GFP spotted on the membrane served as the control for non-specific binding by MIF, and the GFP in the protein mixture served as the control for non-specific binding by Aβ. The result demonstrated that MIF interacted with oligomerized Aβ, but neither did MIF nor Aβ interacted with GFP, indicating a specific binding between oligomerized Aβ and MIF (Figure 4.7A). We then studied whether MIF and Aβ were associated in the AD brain by exploring whether MIF and Aβ present at the same density fraction. Brain tissues were obtained from APP23/PS45 mice and homogenized in PBS with 0.5% Triton-100. The homogenates were fractionated under 16,000xg for 15 minutes at 4°C. Four layers, supernatant (S), pellet layer 1 (P1), pellet layer 2 (P2), and pellet layer 3 (P3) were formed. The supernatants were collected and subjected to ultracentrifugation at 100,000xg for 1 hour at 4°C, and the second supernatants were collected and labeled as S’. Immunoblots were performed to locate the presence of Aβ enriched fractions. To study the potential association between plaques and MIF, immunoblots were performed to detect the presence of Aβ and the level of MIF in each collected fraction. Our results demonstrated that Aβ was not detectable in the S’ fraction, but was detected in P1, P2 and P3 fractions (Figure 4.7B). Concentrations of MIF were similar between controls and the APP23/PS45 mice in the supernatant, indicating AD pathology did not increase the level of free MIF in the soluble fraction (0.96±0.14 fold of the control, \( P>0.05 \)) (Figure 4.7B and C). Interestingly, soluble MIF tended to be enriched in the P1 fraction (1.60±0.31 fold of the control, \( P>0.05 \)) and was significantly enriched in the P2 fraction (1.92±0.21 fold of the control, \( P<0.0 \)), where Aβ aggregates presented (Figure 4.7B and C). However, the level of MIF in the P3 fraction, which was mainly composed of cell debris, did not differ between control and APP23/PS45 (0.93±0.23 fold of the control, \( P>0.05 \)) (Figure 4.7B and C). Taken together, these results are supportive that MIF could bind with Aβ containing plaques in AD brains.
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Figure 4.7 MIF is enriched at the density layers with the presence of amyloid plaques.

(A) 0.2 nmol of oligomerized Aβ peptide approximated 0.1 nmol of purified GFP protein were spot on a nitrocellulose membrane, and the membrane was incubation with mixed proteins of recombinant hMIF and purified GFP at the concentration of approximated 5 μM at 4°C for overnight. The membrane was then subjected to immunoblotting to detect MIF and GFP by a monoclonal anti-MIF antibody (D-2, Santa-Cruz) and a polyclonal anti-GFP antibody, respectively. The red channel detects IR-dye labeled goat anti-rabbit antibody, and the green channel detect IR-dye labeled goat anti-mouse antibody. (B) Brains from four months old APP23/PS45 and wildtype (as controls) mice were homogenized in 5x PBS with 0.5% Triton-100 (v/w) and centrifuge at 16,000xg at 4°C for 15 minutes. The supernatants were collected and subjected to ultracentrifugation at 100,000xg for 1 hour at 4°C, and the second supernatants were collected and labeled as S'. Homogenates from each layer were further dissolved in RIPA-DOC buffer followed by brief sonication. The same amount of protein was loaded on 16% and 12% Tris-tricine SDS PAGE gels for Aβ and MIF separation, respectively. Aβ was detected by a 6E10 antibody, MIF was detected by anti-MIF antibody, CTFs were detected by a C20 antibody to confirm the expression of the transgene, and β-actin was detected by β-actin antibody serving as the loading control. S', supernatant after ultracentrifugation; P1, pellet 1; P2 pellet 2; P3, pellet 3. (C) Quantification of samples from (B). Values were expressed as mean±SEM, n = 3. * P<0.05 by Student’s t test.
4.4 Discussion

As a unique hallmark of AD pathology, Aβ containing neuritic plaques participates in inducing inflammatory response, which contributes to AD pathogenesis. The ongoing production of Aβ leads to the chronic inflammation that is characterized by activation of microglia cells and increased production of inflammatory mediators in the brain, such as pro-inflammatory cytokines, chemokines, macrophage inflammatory proteins, and prostaglandins (Rubio-Perez and Morillas-Ruiz, 2012). MIF as a pro-inflammatory cytokine has been shown to be upregulated systematically in circulation and locally at lesion sites in many chronic inflammatory diseases, such as rheumatoid arthritis, and plays central roles in the disease initiation and progression (Morand et al., 2006). It remains unclear whether MIF expresses and functions similarly in the CNS during AD pathogenesis. Previous studies have demonstrated that MIF levels were upregulated in sera and CSFs from AD patients, indicating that MIF may also be upregulated systematically and locally in the brain (Bacher et al., 2010; Craig-Schapiro et al., 2011; Lee et al., 2008; Popp et al., 2009). In the present study, we further examined MIF expression regulation during AD pathogenesis by characterizing the level of MIF in demented (including AD and VD) patients and in an animal model of AD. We demonstrated that at the late stage of AD pathology, upregulation of MIF was evident in both AD patients (brain tissues and CSFs) and 3-month-old APP23/PS45 mouse brains with substantial Aβ deposition. Notably, in patients with MCI, which is a condition commonly leading to AD, the MIF level was not altered in CSFs, so as it was in brain tissues from 2-month-old APP23/PS45 mice with much fewer Aβ deposition than 3-month-old mice, suggesting that MIF probably is not immediately upregulated at the early stage of AD pathology. This result is in line with a previous study showing that among a series of pro-inflammatory cytokines including MIF, only the production of IL-1β was induced in 14-month old APP single transgenic mice, which developed a few Aβ deposits (Mehlhorn et al., 2000). Our study also demonstrated that the MIF level was not induced in patients with VD (Figure 4.1C), a form of dementia related to AD but with distinctive mechanisms, indicating that elevation of MIF in the CSF possibly is specific to patients with dementia due to AD. This result was in line with our further experiment that MIF was released by neuronal cells upon the stimulation of Aβ, but not by LPS, a specific
inflammatory inducer for immune cells (Figure 4.3C). This suggests that MIF release could be a defense mechanism by neurons, and its upregulation was AD-specific, and distinct from other type of dementia, such as VD.

Although a previous study had observed MIF expressing cells surrounding Aβ plaques in APP transgenic mice, neither the cell identity was confirmed, nor was MIF expressed in vicinity to Aβ plaques (Bacher et al., 2010). In the present study, we clearly demonstrated that MIF expression immediately surrounds Aβ plaques, and is directly binding with Aβ. This result is consistent with a previous study which demonstrated that MIF was one of the three proteins in the brain lysate (glutamine synthesis and hemoglobin α-chain are the other two) that can be pulled down by affinity chromatography of Aβ42 (Oyama et al., 2000). It is well known that Aβ42 undergoes self-aggregation, and is important for Aβ plaque development. Protein associated with Aβ may affect fibril formation and deposition. For example, cystatin C has been reported to associate with Aβ42 to inhibit the formation of amyloid fibrils (Sastre et al., 2004). Interestingly, a recent study screened 190 analyses and suggested cystatin C and MIF as biomarkers complemented to the current biomarkers in CSF for the improvement of AD diagnostics (Craig-Schapiro et al., 2011). In addition, cystatin C and MIF share a similar tertiary structure with 4-strand β-sheet, and both undergo self-aggregation amorphologically or to filaments at a physiological condition and are amyloidogenic at lower pH in vitro (Lashuel et al., 2005; Levy et al., 2006). Because MIF binds to Aβ directly and colocalizes with Aβ plaques, it is intriguing whether MIF will affect the amyloidogenesis of Aβ. Further studies may be carried out in this regard.

It has been suggested that MIF facilitates defending mechanisms under stressful conditions by promoting cell survival in the systems outside the CNS. During heart ischemia, MIF acts in an autocrine fashion and signals through the CD74/44 receptor complex to promote cell survival by temporarily maintaining energy homeostasis; knockout of MIF thus results in the larger ischemic damage (Miller et al., 2008). In the CNS, as we demonstrated in Chapter 3, maintaining MIF expression is important in defending cerebral ischemia by reducing oxidative stress-induced caspase-3 activation in neurons during the acute phase. Presumably,
maintaining MIF expression is also important for neurons to defend Aβ-induced toxicity. However, there is limited research carried out to investigate the effect of MIF in Aβ-induced toxicity in neurons, except for one study demonstrating a protective effect of a MIF inhibitor on Aβ-induced toxicity in SH-SY5Y cells (Bacher et al., 2010). In contrast to their results that Aβ-induced massive damage in SH-SY5Y cells, we did not observe such damage in our study. Instead, we clearly showed secretion of MIF from SH-SY5Y cells after treatment of Aβ. It has been shown that inhibition of p53 attenuates Aβ-induced neuronal apoptosis (Culmsee et al., 2001), and MIF can directly inhibit p53 activation (Hudson et al., 1999). We hypothesize that secretion of MIF precedes cell damage, and MIF released under the autocrine fashion, in turn, activates cell survival signals. Perhaps, only when a cell is compromised to a degree that production of MIF cannot counteract Aβ-induced toxicity do cells start to die. Further studies on the effect of MIF on Aβ-induced neuronal toxicity should be carried out to address this issue.

It should be noted that MIF acts in an autocrine fashion and interacts with its cell surface receptors to transduce its signals; therefore, secretion of MIF to the extracellular space is necessary for MIF to exert its cellular functions. It has been suggested that MIF is constitutively expressed and is perhaps constantly secreted by neurons in the brain (Bacher et al., 1998; Ogata et al., 1998). However, at the late stage of AD, a large portion of the extracellular MIF is sequestered by Aβ plaques, as we demonstrated, and perhaps has no functions any more, which suggests that MIF is necessary for cognitive performance during AD pathogenesis, and that upregulation of MIF may supplement the functional pool of MIF. This also explains why MIF upregulation is observed at the late stage of AD with significantly increased Aβ deposition.

To our surprise, the typical immune cells, microglia and macrophages that are sensitive to LPS did not respond to Aβ treatment regarding MIF secretion, which emphasized the neuronal specificity on MIF secretion following Aβ stimulation. However, that is not to say that immune cells do not secret MIF. Our study did not provide direct evidence on the role of MIF in chronic inflammation due to AD pathology, and we could not rule out its general effect in inflammatory processes. Pro-inflammatory cytokines are known to trigger secretion
of MIF from macrophages (Calandra et al., 1994), and such cytokines are predominantly produced in the late stage of AD (Rubio-Perez and Morillas-Ruiz, 2012). Therefore, it is likely that immune cells also contribute to upregulation of MIF in the late stage of AD. At this stage, overproduced MIF could still be beneficial in promoting neuronal survival if it can be received by neurons; on the other hand, however, the pool of MIF produced by immune cells may locally promote their own survival and proliferation, which in turn produce and release more MIF, leading to a vicious circle as seen in chronic inflammatory diseases in the peripheral.

Inhibiting MIF for peripheral inflammatory diseases has been suggested. MIF deficiency has a negative influence on learning and memory during AD pathogenesis (Figure 4.4), which implicates that such interventions on the patients who are under the risk of or experiencing early AD pathology should be applied with caution. Nevertheless, we could not rule out the possibility that the potential pro-survival and pro-inflammatory functions of MIF counteract each other during AD pathogenesis, and inhibition of one would reveal the other. Therefore, it will be of importance to dissect the MIF signal complex so that the deleterious effects could be inhibited without affecting the beneficial ones.

4.5 Conclusion
Studies in this chapter provide an overview of the expression and the potential roles of MIF in AD pathogenesis. We demonstrate specific upregulation of MIF in CSFs and brain tissues due to neuronal supply at the late stage of AD pathogenesis, and suggest that the level of MIF in the CSF could serve as an indicator of the AD stage at which global inflammation has occurred in the brain. In addition, hemizygous knockout of MIF in APP mice results in depressive behavior which affects memory task performance during AD pathogenesis. We also for the first time demonstrated the direct association between MIF and Aβ histologically and biochemically. Taken together, our data provided first-hand evidence of the expression profile of MIF and its effect on AD pathology. Our data suggested that upregulation of MIF is induced by the pathological increase of Aβ deposits at the late stage of AD pathology, but did not recover its role in mediating normal behavior functions in AD, because it is sequestered on the Aβ deposits in the loss-of-function fashion.
Chapter 5

Conclusions and future directions

5.1 General discussion

The overall goals of this thesis were to characterize regulation of MIF expression and explore
its potential role in stroke and AD. Previous work from our lab demonstrated that MIF is
deregulated in serum from stroke patients and in the brain of rats subjected to experimental
stroke (Wang et al., 2009). Specifically, MIF is upregulated at the early phase of the stroke in
the infarct surrounding area, indicating that MIF may have an active role during a stroke.
MIF plays a protective role in cardiomyocytes following cardiac I/R through multiple
mechanisms (Miller et al., 2008; Qi et al., 2009; Takahashi et al., 2001), but it is not clear
what the role MIF plays in neurons following cerebral I/R, the most common form of strokes.
In 2011, the first three papers about the role of MIF in stroke were published by Inacio et al.
(Inacio et al., 2011a; Inacio et al., 2011b; Inacio et al., 2011c). All three studies found a
deleterious role of MIF in stroke, and suppressed MIF expression (achieved by environment
enrichment housing) or genetic deletion of MIF alleviated stroke pathology in terms of the
infarct volume, but the underlying mechanism was poorly addressed. The question is how the
predicted protective mechanisms of MIF could exacerbate stroke outcomes.

Both hypoxia and NFκB signaling pathways are activated following a stroke in the infarct
area (Schneider et al., 1999; Stephenson et al., 2000; Zhang et al., 2010). In Chapter 3, we
confirmed nuclear localization of NFκB in the MCA territory, and it mainly diffused in the
adjacent peri-MCA area. Previous work from our lab and others demonstrated that hypoxia
activated HIF-1α, which mediates upregulation of MIF gene expression via interaction with
HREs on the MIF promoter (Baugh et al., 2006; Wang et al., 2009; Welford et al., 2006).
Here, in Chapter 2 and Chapter 3, we further identified two functional cis-acting NFκB
binding elements on the MIF promoter, through which activation of NFκB signaling
regulates MIF gene expression by transcriptional activation of the MIF promoter. We found
that activation of either hypoxia or the NFκB pathway alone drives upregulation of MIF gene transcription. However, activation of NFκB signaling under hypoxic conditions leads to an inhibition of MIF gene expression. Activation of NFκB signaling after a stroke is evident despite the controversial conclusions as to whether it is protective and deleterious to stroke outcome (Ridder and Schwaninger, 2009; Schneider et al., 1999). It is plausible that MIF may serve as a downstream effector responsible for synchronizing the upstream regulatory signals, such as NFκB signaling, and in turn determine the cell fate. Our results suggest that MIF expression is tightly regulated by NFκB and hypoxia signaling, and could serve as an effector that directly regulates cell survival during the acute phase of strokes.

According to studies on stroke pathology, multiple events including cell death, inflammatory response, and damage repair occur orderly and/or simultaneously to contribute to stroke pathology. Growing evidence has suggested that a same molecule could exert a “biphasic nature” in regulating stroke pathology (Lo, 2008). For example, MMPs damage the blood brain barrier, cause edema and neuronal death during the acute phase of a stroke, but promote neurovascular remodeling in peri-infarct areas during stroke recovery, and if inhibited exacerbate stroke outcomes (Lo, 2008). Likewise, MIF as a pleotropic protein has been suggested to be both anti-apoptotic and pro-inflammatory. In chapter 3, we demonstrated that MIF indeed played anti-apoptotic role during the acute phase of strokes by inhibiting caspase-3 activation. Due to this effect, knockout of MIF results in accelerated cell death and larger infarct volumes at 10 hours after strokes. However, due to the downregulation of MIF within the MCA territory, as demonstrated in Chapter 3, the protective effect gradually diminished and at 24 hours after ischemia onset and made no difference in the infarct volume between MIF−/− mice and wildtype mice. This result actually indicates that infarct evolution may be accelerated by some mechanisms in the wildtype mice or slowed down in the MIF−/− mice. An important event during this time window (i.e., between 12 to 24 hours after stroke) is that infiltration of macrophages has occurred (Jin et al., 2010). Interestingly, Inacio et al. observed increased immunoreactivity of galectin-3 but not CD68 (a general marker for macrophages) in the infiltrated monocytes/macrophages in MIF−/− mice (Inacio et al., 2011b). Galectin-3 as a marker for active microglia/macrophages is found to be chemotactic to circulating monocytes/macrophage and potentiates IL-1β production after LPS stimulating
Although Inacio et al. did not find the difference in the production of pro-inflammatory cytokines between MIF\textsuperscript{-/-} mice and the wildtype controls, we could not rule out its role in mediating inflammatory responses. In fact, in heart ischemia studies, MIF was found to be a ligand of CXCR2 and CXCR4, and was suggested to have a chemotactic property to directly mediate migration of immune cells expressing these receptors after ischemic attack (Bernhagen et al., 2007). Therefore, MIF may influence inflammatory responses without activation of cytokine production after ischemic attack. It is likely that lack of MIF expression reduced infiltration of monocytes/macrophages, which in turn limits infarct evolution by suppressing immune responses. Taken together, these mechanisms reconcile the seemingly discrepancies regarding the role of MIF during strokes, and suggest that MIF plays dual roles depending on the “time window” during stroke pathology.

In contrast to the role of MIF during acute stress, sustained expression of MIF was found to be predominantly deleterious in chronic inflammatory diseases such as rheumatoid arthritis by mediating the recruitment of macrophages to the lesion sites (Morand et al., 2006). Chronic inflammation and activation of microglia are the features of AD, and Aβ deposits are blamed to be the inducer of the chronic inflammatory responses (Wyss-Coray and Rogers, 2012). Despite numerous evidence showing the fundamental role of MIF during inflammatory responses, studies of MIF in AD is still in its infancy, and only a few studies demonstrated that MIF was elevated in the CSFs from AD patients, as well as in the sera indicating systematic upregulation of MIF (Calandra et al., 1994; Leng and Bucala, 2006; Popp et al., 2009). In Chapter 4, we confirmed that MIF is elevated in the CSF and brain parenchyma in AD patients. More importantly, neuronal MIF secretion induced by Aβ possibly serves as a stress defense mechanism in neurons since insufficient expression of MIF will result in depressive behaviors in AD. However, upregulation of MIF does not recover its role in maintaining normal behavior in AD presumably because it is sequestered by Aβ deposits in a loss-of-function fashion. The result that there is a physical association between MIF and Aβ also correlates well with the observation that parenchymal expression of MIF is upregulated only when plaques are substantially increased during late stage of AD pathogenesis. The findings in Chapter 4 further advanced our understanding of the role of MIF during AD pathogenesis. However, important questions still remain. For example, we
suggest that Aβ triggered MIF secretion could serve as a mechanism to recover the loss of function due to the sequestering effect by Aβ. However, it could also serve as a mechanism to attenuate neuronal apoptosis as previously suggested for cardiomyocytes in heart ischemia (Qi et al., 2009). Another example is whether the direct binding between Aβ and MIF influence the evolution and/or metabolism of Aβ plaques, which is known to potentiate chronic inflammatory response by several mechanisms (Wyss-Coray and Rogers, 2012).

5.2 Novelty and significance
MIF was rediscovered to be a pleiotropic protein after being cloned in 1989. It exerts pro-inflammatory properties and is also shown to be anti-apoptotic through a variety of mechanisms. During I/R, limiting MIF activity demonstrated opposite effects to the ischemic tissues. The first significance of this thesis is that we found the missing piece of the puzzle, so that the apparent discrepancies are reconciled in the case of cerebral I/R.

First of all, we for the first time demonstrated the anti-apoptotic role of MIF during cerebral ischemia at the acute phase of strokes. The importance of this finding is that it emphasizes the intrinsic anti-stress property of MIF. This property of MIF probably allows the ischemic tissue to “hold” for the restoration of blood supply. Another significant finding is that MIF expression is quickly downregulated in the ischemic territory due to the activation of NFκB signaling. Therefore, MIF could only provide temporary protection to the neurons in the ischemic territory. As a result, the protective effect is transient and may be overlooked. As we demonstrated, at 24 hours after ischemia onset, the protective effect of MIF is diminished. It is worth mentioning that there are studies characterizing the role of MIF at the late stage of a stroke, which demonstrated that knockout of MIF resulted in slightly smaller infarct volumes at 7 days after ischemia onset, and the authors attributed the detrimental effect of MIF to its role in modulating activity of monocytes/macrophages (Inacio et al., 2011b). Taken together, a complete profile of the roles of MIF during stroke has been established.

Discrepancies regarding the role of MIF during I/R have been suggested in previous studies on different tissues. A study on intestinal ischemia demonstrated that the lack of MIF gene expression significantly suppressed circulating inflammatory cytokines and reduced lethality.
in mice (Amaral et al., 2007). In contrast, MIF deficiency led to larger infarct volumes following cardiac I/R due to the loss of protection by MIF-mediated anti-apoptosis, suppressing oxidative stress, and promoting energy metabolism (Koga et al., 2011; Miller et al., 2008; Qi et al., 2009). The differences between the studies are that they overlooked the influence due to the expression pattern of MIF, and that they focused on different time windows of the entire processes during and after I/R. Based on the above theory, we suggest that for a stroke, it is highly likely that, these discrepancies will also be reconciled as we found in the case of cerebral ischemia if the above mentioned two factors are considered.

Additionally, this thesis provides valuable first-hand evidence regarding the expression regulation of MIF and its role during AD. Specifically, we pointed neurons as the contributor of upregulated MIF potentially as a self-defense mechanism. This surprising finding breaks the traditional thoughts regarding immune cells-mediated MIF expression during inflammation. Additionally, this finding emphasizes the role of MIF in the functional cells, which could be different to its role in the surrounding supporting cells and infiltrated immune cells. Therefore, future studies should separate the effects of MIF that are contributed from different compartments of the tissue.

5.3 Implications and future directions
5.3.1 Is MIF beneficial or detrimental?
It is getting clear that one should take into consideration the context to evaluate the role of MIF during different physiological and pathological conditions. In the case of cerebral I/R, MIF plays a dual role depending on the stage of I/R, perhaps the serenity as well. It is likely that constitutive expression of MIF in neurons is essential for a neuron to “hold” a proper oxidoreductive (as we have demonstrated) and metabolic homeostasis (as suggested from the cardiac I/R studies) for a short period of time. If the ischemic attack is minor and endogenous store of MIF is sufficient to combat the oxidoreductive reactions, neurons will be saved once blood supply is restored. In contrast, if occlusion of blood vessels is long enough to induce cell death and subsequent cell death-induced inflammation, and during which MIF loses its protective effects due to loss of expression, its detrimental role in the inflammatory response will be highlighted.
Currently, since the available MIF inhibitors demonstrated non-selective inhibition of the activity of MIF, it is important to determine the time when inhibition of MIF becomes necessary. The question becomes how to determine such time window during which one of MIF’s roles dominates the other. Based on our current findings that endogenous MIF-mediated anti-apoptotic effect is diminished in the ischemic territory by 24 hours after the ischemic onset, introducing MIF inhibitors may provide beneficial effect in limiting additional growth of infarct facilitated by inflammatory responses. Strategies like the MRI may be used to determine the penumbra and infarct in stroke patients. On the other hand, whether enhancing MIF expression in neurons would be beneficial to stroke outcomes should be examined as well.

In the case of AD, the dilemma is that MIF provides beneficial effects in terms of facilitating normal cognitive behaviors, and also presumably for neuronal survival. Hypothetically, constitutive expression of MIF could also facilitate microglia to quickly respond to newly formed Aβ deposits, and in turn initiate migration and restriction to the deposits. On the other hand, it is highly likely to be able to potentiate chronic inflammation by promoting survival of microglia that should have otherwise undergone apoptosis, although this hypothetic effect needs further investigation – this can be achieved by measuring the level of inflammatory cytokines and the degree of microglia activation between APP23 and APP23/MIF+/− mice. Additional studies should be carried out to study the role of MIF during the early stages of AD when MIF upregulation is not yet detected so that a complete picture of the function of MIF can be drawn to evaluate the balance between the deleterious and beneficial effects of MIF during different stages of AD pathogenesis, and to set out the plan for controlling its activity.

5.3.2 Can we differentiate the beneficial from the detrimental effect of MIF?

The current strategies for inhibiting the activity of MIF are achieved mainly by sequestering its oxidoreductive activity. Such inhibitors include ISO-1 and p425. The disadvantage of these inhibitors is that they have broad effects on the activities of MIF that could mediate different biological processes. Therefore, the current strategies set for MIF inhibition requires accurate targeting on the inflammation predominant events during the disease cascades.
Additionally, since MIF possesses beneficial effects, an ideal treatment strategy regarding controlling MIF activity would be to preserve the beneficial effects and, at the same time, inhibit the detrimental ones. In this thesis, we demonstrated that MIF is beneficial in attenuating caspase-3 activation induced apoptosis. Previously, studies on cardiomyocytes demonstrated that MIF provides protective effects by multiple mechanisms (Koga et al., 2011; Miller et al., 2008; Qi et al., 2009). Clearly, maintaining MIF expression is beneficial in functional cells. On the other hand, studies from the chronic inflammatory conditions suggested that MIF expression in immune cells is deleterious to disease outcomes probably due to its role in promoting pro-inflammatory cytokine production (Morand et al., 2006). Taken together, it is likely that maintaining MIF expression in functional cells (e.g., neurons) and meanwhile inhibiting it in immune cells will probably achieve a win-win result in I/R. However, a general difficulty in manipulating the effect of a ligand like MIF is that it may act on multiple receptors and cells and, therefore, it is difficult to specifically deliver a signal to the targeting cells. A recent study suggested that the detrimental effect of MIF was mainly mediated through the CXCR2 receptors on the immune cells, while on the functional cells, the beneficial effects were achieved through both CXCR2 and CD74 receptors (Liehn et al., 2013). These results provide an opportunity to develop strategies targeting the receptors of MIF mediating detrimental cellular signals in immune cells without affecting its beneficial effect on functional cells. Future studies focusing on how MIF interacts with its receptors to induce a certain biological effect should be characterized in order to better targeting the detrimental effect of MIF.

5.3.3 Will MIF serve as a molecular link between stroke and AD?

It has long been acknowledged that strokes increase the risk of development of AD (Kalaria, 2000; Leys et al., 2005). Previous studies from our lab and others suggested that long lasting ischemia and hypoxia following strokes affect APP processing leading to increased production of Aβ by increasing the gene expression of BACE1, and in turn increasing the risk of AD (Sun et al., 2006a; Tesco et al., 2007). In this thesis, we attempted to establish a molecular link between stroke and AD through the function of MIF, because MIF expression may also be affected by post-stroke long-term hypoxia. Therefore, we aimed to examine how deregulation of MIF could affect AD pathogenesis. In this thesis, we demonstrated the direct
interaction of MIF and Aβ, and suggested that this binding may affect the function of MIF in mediating normal cognitive functions. Additionally, we demonstrated that the plaque-associated MIF expression resembles the morphology of microglia, as also suggested by a previous study (Bacher et al., 2010). However, additional studies need to be carried out to investigate the influence of microglial MIF in several aspects. Firstly, since MIF is not expressed in the quiescent microglia, it will be important to investigate the mechanism underlying this upregulation of microglial MIF. Studies in this thesis suggested that NFκB signaling, which is induced following stimuli, may serve as one of the reasons. Secondly, although we suggested that the function of MIF in mediating normal cognitive behaviors may be inhibited due to binding with Aβ, we cannot rule out the possibility that it would be able to exert the chemotactic function as previously suggested (Bernhagen et al., 2007) and serve as a mechanism for the recruitment of immune cell to Aβ plaque. Thirdly, MIF may also affect cell proliferation as it has been demonstrated for fibroblasts (Lacey et al., 2003; Mitchell et al., 1999). Therefore, future studies focusing on these aspects will provide a better understanding of the role MIF during AD and how stroke will influence AD progression.

5.4 Conclusion remarks

In conclusion, by carefully characterizing the regulation of MIF expression and its role during the acute phase of a stroke, we were able to demonstrated the protective role of MIF during strokes, which reveals one of the dual roles of MIF following cerebral I/R. MIF exhibits neuroprotective effects following strokes and may be an important molecule for preserving a longer time window for stroke treatment, suggesting that the use of an MIF inhibitor may reduce the already narrow therapeutic windows following strokes, and confer a higher risk of ischemic damage. In addition, we provided first-hand evidence of the expression profile of MIF and its effect on AD pathology, which suggests that MIF upregulation is induced by pathological increase of Aβ deposits at the late stage of AD, but this effect did not recover its role in mediating normal behavior functions in AD, because it was sequestered by the Aβ deposits in the loss-of-function fashion. Future studies focusing on characterizing MIF-mediated microglial activity on Aβ aggregation and metabolism will be able to provide a better understanding of AD pathogenesis.
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