Molecular mechanisms underlying the neuroprotective effect of IGF-1 in neuroblastoma cells and the pro-metastatic effect of Dph3 in murine melanoma cells

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Part I: Explore the mechanism involved in IGF-1 protective effect against MPP⁺-induced apoptosis in human neuroblastoma SH-EP1 cells

ABBREVIATIONS

AD	Alzheimer's disease
AIF	Apoptosis-initiating factor
Apaf-1	Apoptotic protease activating factor 1
ATP	Adenosine triphosphate
BAX	Bcl-2 associated X protein
BBB	Brain blood barrier
BIO	6-bromoindirubin-3'-oxime
BSA	Bovine Serum Albumin
COMT	Catechol-O-methyl transferase
DA	Dopamine
DAPI	4',6-diamidino-2-phenylindole
DBS	Deep brain stimulation
DISC	Death inducing signaling complex
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ERK	Extracellular signal-regulated kinase
ESCs	Embryonic stem cells
FADD	Fas associated death domain
FBS	Fetal bovine serum
FCS	Fetal calf serum
FVM	Fetal ventral mesencephalic
GDNF	Glial-derived nerve growth factor
Grb2	Growth factor receptor binding protein 2
GSK-3 β	Glycogen synthase kinase 3 β

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HG	High glucose
HRP	Horseradish-peroxidase
hNPC	Human neural progenitor cells
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor 1 receptor
IGFBPs	IGF-binding proteins
iPS	Induced pluripotent stem cells
IP3	Inositol 1,4,5-triphosphate
IRS	Insulin receptor substrate
JNK	C-Jun N-terminal kinase
L-DOPA	3,4-dihydroxy-L-phenylalanine
LBs	Lewy bodies
LRP2	Lipoprotein receptor-related protein 2
MAO-B	Monoamine oxidases-B
MAPK	Mitogen-activated protein kinase
MEK	MAP-ERK kinase
MEKK1	Mitogen-activated protein kinase/Extracellular signal-regulated kinase kinase kinase 1
MLK3	Mixed lineage kinase 3
MPPP	1-methyl-4-phenyl-4-propionoxypiperidine
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	Mitochondrial DNA
6-OHDA	6-hydroxydopamine
PARP	Poly ADP Ribose Polymerase
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween-20
PD	Parkinson's disease

PDK1	Phospholipids-dependent kinase
PH	Plekstrin homology
PI	Phosphatidyl-inositol
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphoinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3, 4, 5 triphosphate
P/S	Penicillin/Streptomycin
REM	Rapid eye movement
SAL	Salsolinol
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	Standard error
SHC	Src homology and collagen domain protein
SNpc	Substantia nigra pars compacta
TUNEL	Terminal deoxynucleotidyl transferease dUTP nick-end labeling
UPS	Sbiquitin–proteasomal system

ABSTRACT

Parkinson's disease (PD) is mainly caused by the loss of dopaminergic neurons located in the substantia nigra pars compacta (SNpc). Thus, preventing the death of dopaminergic neurons is thought to be a potential strategy to interfere with the development of PD. The involvement of IGF-1 signaling pathways in neuronal cell survival has been identified in many cell types, but its downstream targets are frequently cell type-specific. In the present work, we studied the effect of IGF-1 on MPP⁺-induced apoptosis in human dopaminergic neuroblastoma SH-EP1 cells. We found that IGF-1 effectively protects SH-EP1 cells against MPP⁺-induced apoptotic cell death. We further delineated the underlying molecular mechanism and showed the PI3K/AKT pathway plays a central role in IGF-mediated cell survival against MPP⁺ neurotoxicity, not the mitogen-activated protein kinase (MAPK)/ERK pathway. Moreover, we demonstrated that the protective effect of AKT is largely dependent on the inactivation of glycogen synthase kinase 3 β (GSK-3 β), since inhibition of GSK-3 β by its inhibitor, BIO, could mimic the protective effect of IGF-1 on MPP⁺-induced cell death in SH-EP1 cells. Interestingly, the IGF-1 potentiated PI3K/AKT activity is found to negatively regulate the c-Jun N-terminal protein kinase (JNK) related apoptotic pathway and this negative regulation is further shown to be mediated by AKT-dependent GSK-3 β inactivation. Thus, our findings may provide a better understanding of the neuroprotective mechanism of IGF-1 on dopaminergic neuronal cell death and could hold tremendous implication for the development of therapy to arrest the progression of PD in the future.

CHAPTER 1 INTRODUCTION

1.1 Parkinson's disease

Parkinson's disease (PD), named after Dr James Parkinson who first described this disease in 1817 in a classic monograph "Essay of the Shaking Palsy", is the second most popular neurodegenerative disorder only after Alzheimer's disease (AD) [1]. PD commonly occurs in older individuals, affects at least 1% of the "over 65" population and rises to nearly 5% by age 85 [2-4]. PD can be diagnosed at any age, with a mean age of seven decades, and about 3% of cases are initially determined in individuals under their fifties [5]. Although most PD patients can survive twenty years or longer, PD is typically a chronic disease and progresses very slowly [6].

1.1.1 Clinical symptoms of PD

1.1.1.1 Motor symptoms of PD

The cardinal motor symptoms of PD include slowness or absence of voluntary movement (bradykinesia or akinesia), tremor at rest, muscle rigidity, postural instability and freezing. These symptoms are not always observed in every patient, at least in a given time frame. A number of patients also suffer from anxiety, depression, autonomic disturbances, and dementia [7]. Bradykinesia refers to slowness of movement with a progressive loss of amplitude or speed during attempted rapid alternating movement of body segments and significantly impair the daily life of PD patients since it takes much longer time for them to perform their daily activities, such as dressing or eating. Akinesia means loss of voluntary movements and comprises many clinical characters, including lack of facial expression, decreased blinking rate, reduced arm swing during walking and drooling [8-9]. Tremor at rest is the most common and easily recognized manifestation of PD. It is characterized by 4-6 Hz activity at rest in the limbs with distal

predominance. Resting tremor decreases with voluntary movement and during sleep, so typically does not impair daily life. In clinical practice, it is best observed while the patient is focused on a particular mental task [1, 8]. Muscle rigidity is characterized by the increased resistance to passive movement of a patient's limbs. It may be accompanied by pain and painful shoulder is one of the most recognizable initial symptoms of PD [10-11]. Postural instability is caused by loss of postural reflexes and the most common causes of falls of PD patients. It generally occurs at the late stages of PD after the onset of other clinical features [12]. Freezing, one of the most disabling symptoms of PD, refers to the inability to begin a voluntary movement such as walking [13].

1.1.1.2 Nonmotor symptoms of PD

In recent years, there has been an increasing interest in nonmotor symptoms of PD because their recognition is useful for the diagnosis of PD and because they are a major determinant of quality of daily life [9]. Nonmotor symptoms of PD are common features of idiopathic PD and occur across all disease stages [14]. These symptoms are often more disabling and resistant to treatment than motor symptoms [15]. Some non-motor symptoms can presage the clinical recognition of motor manifestations of PD by as much as 20 years [16-17], which confers them potential diagnostic utility in early course of PD, such as hyposmia, rapid eye movement (REM) sleep behavior disorder, constipation, and depression [18-20]. Some patients will suffer unexplained shoulder pain or fatigue before major motor symptoms emerge. On the other hand, symptoms like dementia and hallucinations occur in the late stages of disease, which is useful for distinguishing PD from other disorders [9].

1.1.2 Etiology of PD

PD is primarily a sporadic disorder, and only about 5-10% of PD patients are inherited. The opinion about the etiology of PD has remarkably changed from a purely sporadic disease to a disorder caused by both environmental and genetic factors. However, age

or aging process, as one of the factors strongly related to the onset of PD, has been overlooked and little has been done to uncover how age or aging process is involved. The role of aging or aging process in the onset of PD is suggested by its usual occurrence in late middle age and by its markedly increased prevalence at older ages. The possible contribution of aging in this illness is further supported by the increased vulnerability of dopaminergic neurons to toxic insults resulted from failure of normal cellular physiological and biochemical processes [21].

The environmental hypothesis of PD dominated much of the 20th century, especially after the discovery of MPTP which causes parkinsonism in both humans and nonhumans [22]. Environmental factors that related to the occurrence of PD are ranging from the general, such as industrialization, rural environment, well water, plant-derived toxins and bacterial and viral infection, to the specific, such as organic solvents, carbon monoxide, carbon disulfide, herbicide and pesticide [23-24].

Genetic factors have not been recognized to be contributed to PD until late 1990's. A fundamental step to the recognition of the genetic contribution was the identification of a gene named SNCA, which encodes a protein named α -synuclein, in 1997 in an Italian and three unrelated Greek families [25]. The discovery of PD genes is particularly important because these genes may provide significant insight into the disease mechanisms responsible for both genetic and sporadic PD. To 2010, 11 genes and 3 additional genetic loci have been identified to be associated with PD, and two additional susceptible genetic loci remain to be further confirmed [24].

1.1.3 Pathology of PD

Pathologically, PD is characterized, in part, by the degeneration of dopaminergic neurons in the SNpc, a midbrain structure which relays signals to the striatum for motor function coordination, and the presence of Lewy Bodies (LBs) in multiple brain areas [1].

1.1.3.1 Degeneration of dopaminergic neurons in SNpc

Loss of dopaminergic neurons in SNpc causes a consequent reduction of dopamine (DA) levels, which is a chemical messenger that transmits signals between the SNpc and the corpus striatum (Fig. 1). Approximately 50-60% of dopaminergic neurons in the SNpc are already lost and 80-85% of the dopamine content of the striatum is depleted when the first symptoms of PD become significantly visible [24]. While dopaminergic neurons in SNpc are preferentially lost in PD, they are not the only neuronal population affected. Loss of dopaminergic neurons in other areas, including locus ceruleus, dorsal raphe nuclei, nucleus basalis of Meynert and postganglionic sympathetic neurons, may contribute to many of the nonmotor features of PD [26]. Nonmotor signs of PD are the likely consequences of degeneration of both dopaminergic and nondopaminergic systems [27].

Figure 1. Schematic representation of the neuropathology of PD.

(A) Normal nigrostriatal pathway (in red). It is consisted with dopaminergic neurons whose cell bodies are located in the SNpc (arrows), and its axons and nerves terminals project from SNpc to the basal ganglia and synapse in the striatum, such as putamen and caudate nucleus (thick solid red line). (B) Diseased nigrostriatal pathway (in red). In PD, there is a significant decrease of dopaminergic neurons that project to the putamen (dashed line) and a much more modest degeneration of those that project to the caudate (thin red solid line) [1].

Mitochondrial dysfunction, particularly at the level of complex I, has long been associated with the neurodegeneration of dopaminergic neurons in sporadic PD and maybe the central cause of sporadic PD [7]. This involvement was first evidenced by the fact that exposure of drug abusers to MPTP, an inhibitor of complex I of the mitochondrial electron transport chain, induced an acute and irreversible parkinsonian syndrome, almost indistinguishable from PD. Subsequently, it was demonstrated that MPTP selectively induces cell death of dopaminergic neurons in the SNpc [1, 28]. A causal connection was further supported by the finding that the activity of complex I

in the SNpc, platelets, and skeletal muscle of sporadic PD patients was reduced [29]. In addition, cytoplasmic hybrid cell lines containing mitochondria from platelets of sporadic PD patients were also demonstrated to have a decrease in the activity of complex I [30]. Also, bypassing complex I blockage protects toxin-induced dopaminergic nigrostriatal impairments in PD models [31]. Recently, the link between mitochondrial dysfunction and PD has been reinforced by the discovery that several PD genes encode mitochondrial proteins, such as Parkin, PINK1 and DJ-1 [32]. Mitochondria dysfunction leads to decreased adenosine triphosphate (ATP) production and increased generation of reactive oxygen species (ROS), which can oxidatively damage mitochondrial DNA (mtDNA), components of the respiratory chain and other mitochondrial factors, thereby starting a vicious circle between mitochondrial abnormality and oxidative stress (Fig. 2) [33]. Mitochondria not only play an important function in electron transport and oxidative phosphorylation, but they are also involved in regulating calcium homeostasis and apoptotic cell-death pathways [34].

Figure 2. Schematic presentation of the central role of mitochondria dysfunction in diverse cellular machinery that affects cell apoptosis.

Mitochondrial dysfunction impairs a number of cellular pathways, causing apoptosis. Mitochondria dysfunction increases oxidative stress directly or indirectly through free radical generated by reduced ATP production and subsequent weak excitotoxicity. Oxidative stress can impair cell survival by decreasing the function of UPS. Mitochondria also have an important function in apoptosis. Release of Cytochrome c and other ‘pro-apoptotic factors’ into the cytoplasm from mitochondria, induced by impaired calcium homeostasis, triggers a cascade of events, inducing apoptosis [34].

1.1.3.2 Lewy bodies in PD

The second pathological character of PD is the development of intracytoplasmic, protein-rich inclusions, named LBs in the surviving dopaminergic neurons. There are also aggregations in neuritis referring as Lewy neuritis. LBs are small intracellular inclusions of lipids and proteins that were first identified by haematoxylin/eosin staining (Fig. 3) [35-36]. LBs are composed of a dense granular core that is surrounded by a halo of radiating filaments. Immunostaining with specific antibodies reveals that LBs are particularly abundance with aggregated α -synuclein, but also contain numerous other proteins, including components of the UPS and molecular chaperons [37]. Besides SNpc, LBs are also observed in many other brain regions of the PD patients, including the dorsal motor nucleus of the vagus, locus ceruleus, raphe and reticular formation nuclei, thalamus, amygdale, olfactory nuclei, pediculopontine nucleus and cerebral cortex [38].

According to Braak and his colleagues' hypothesis, the formation of LBs begins at two sites and in six stages in a topographically predictable order during which components of the olfactory, autonomic, limbic and somatomotor systems become progressively impaired. In stages 1 to 2, LBs are confined in the dorsal motor nucleus of the vagus nerve and anterior olfactory structures. In stages 3 to 4, the SNpc and other nuclei of the basal mid- and forebrain undergoes initially subtle and, then, severe changes. In the final stages 5 to 6, the lesions spread to the neocortex [39]. Although LBs are thought to be a pathologic hallmark of PD, the role of LBs in the pathogenic process is still controversial. It has recently been reported that some forms of PD, for instance in some familial PD or in drug-induced parkinsonism do not have LBs [7, 40]. On the other hand, LBs is sometimes detected at autopsy in individual without symptoms of PD [41].

Figure 3. LBs are small spherical inclusions.

a, staining with haematoxylin/eosin. **b**, immunostaining with a specific antibody against α -synuclein. **c**, electron micrograph of LBs [36].

1.1.3.3 Mode of cell death in Parkinson's disease

Apoptosis refers to a specific physiological and regulated form of cell death; its deregulation causes a number of human pathologies, including neurodegenerative disease [42-43]. Cells undergoing apoptosis demonstrated several morphological features, such as condensed nuclei, DNA laddering, membrane blebbing, cytoplasmic

shrinkage, exposure of phosphatidylserine (PS) on the cell surface, and formation of apoptotic bodies [44-45]. Apoptotic cells are engulfed by phagocytes, such as macrophages or microglia cells thereby preventing inflammation [46-47]. To date, several studies have point out that apoptosis may play a key role in the neurodegeneration of dopaminergic neurons in SNpc of PD patients. Mochizuki et al had reported the observation of DNA fragments, feature of apoptosis, in SNpc of PD patients by utilizing terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) in situ. This result was further confirmed by Tatton et al using fluorescent in situ double labeling method combined with a cyanine dye that binds to DNA and by Anglade et al by electron microscopy [48-50]. Molecular markers of apoptotic pathway have also been found increased in neurons in the autopsied SNpc tissues from PD patients [51]. For example, the expression of Bcl-2 associated X protein (BAX), which is a member of the Bcl-2 protein family, was upregulated in dopaminergic neurons of PD patients [52]. In addition, there are more cleaved caspase-3-positive dopaminergic neurons in PD patients than in control [53]. Other apoptotic molecular markers that were increased in postmortem PD tissues include activated forms of caspase-9, caspase-8, and caspase-1 [54-55].

Besides the evidences from postmortem study, the role of apoptosis in PD was further supported by studies in cell culture and animal models of PD. Intracerebral injection of 6-hydroxydopamine (6-OHDA) induces apoptosis of dopaminergic neurons in SNpc of rats [56]. MPTP administration also selectively induces neurodegeneration of dopaminergic neurons and DNA fragmentation in the SNpc of mouse [57]. In cell culture models of PD, exposure to different toxins, such as dopamine, 6-OHDA, and MPTP, induces typical apoptotic morphological changes and activation of different apoptotic markers [58].

1.1.3.4 Mechanism of caspase regulation

Apoptosis is mainly mediated by caspases, a family of conserved cysteine proteases that usually cleave after an aspartate residue in their substrates [59]. At least 14

members of this family have been identified in mammals to date [60]. Caspases can be grouped into 2 subtypes, including initiator caspases, such as caspase-8 and -9, and effector caspases, such as caspase-3, and -7 [61]. Caspases are synthesized as catalytically inactive zymogens containing a prodomain, a p20 large subunit and a p10 small subunit. Activation of the zymogens by proteolytic cleavage removes the prodomain and separates the large and small subunits [62]. Apoptosis-associated caspase activation is mainly carried out through intrinsic pathway and extrinsic pathway in mammalian cells, depending on the origin of the death stimuli [63]. The intrinsic pathway, also named mitochondria pathway, is mediated by mitochondria in response to death stimuli, such as cytotoxic drugs and DNA damage. Activation of the intrinsic pathway causes a down-regulation of anti-apoptotic Bcl-2 family members, leading to the release of Cytochrome c from the mitochondria to cytoplasm. In the cytoplasm, the binding of Cytochrome c to Apaf-1 (apoptotic protease activating factor 1) induces a conformational change that allows Apaf-1 to bind to ATP/dATP and to form the apoptosome, which mediates the activation of initiator caspase-9, cleaving downstream targets, such as effector caspase-3 and -7 [64-67]. The extrinsic pathway, also named death receptor pathway, is initiated outside the cell by the binding of death ligand, such as FasL/CD95L to its corresponding death receptor, such as Fas/CD95. This binding leads to the formation of death inducing signaling complex (DISC) by clustering and recruitment of adaptor protein Fas associated death domain (FADD) and the initiator caspase-8 or -10 [68-69]. Regardless of the actual pathway to caspase activation, all pathways induce the activation of the major effector caspases, such as caspase-3, and -7, and these enzymes carry out much of the substrate proteolysis during apoptosis [70].

1.1.4 Diagnosis of Parkinson's disease

The correct diagnosis of PD is very important for prognosis and PD treatment and is essential for clinical research. Currently, PD diagnosis is mainly based on clinical criteria and there is still no effective test for diagnosis. The UK PD Society Brain Bank criteria suggested the diagnosis of PD by the presence of bradykinesia plus at

least one of muscle rigidity, tremor at rest and postural instability, with progressive disorder and DA drug response as supportive criteria [71-72].

1.1.5 Treatment of PD

At present, there are no proven treatments that can halt or even slow the progress of PD, but the symptoms of this disease can be managed. Currently available therapies for the management of PD can be divided into pharmacologic treatments and surgical interventions. Pharmacological treatments are mainly using drugs to relieve the symptoms of PD patients or to slow neuronal degeneration, thereby delaying disease progress. Systematic therapies using drugs, such as Levodopa, DA receptor agonists, and catechol-O-methyl transferase (COMT) inhibitors, can greatly improve symptoms without affecting the underlying disease state, but these drugs can neither halt nor reverse the progression of the disease and cause a lot of side effects [73]. For example, long-term levodopa treatment may induce the ‘on-off’ phenomena (fluctuations in drug response), wearing off, dose failure, akinesia and dyskinesias [74]. Recently, there has been an increasing interest in neuroprotective therapies for PD, and several drugs, such as monoamine oxidase-B (MAO-B) inhibitors, glial-derived nerve growth factor (GDNF), nicotine, and melatonin, have been proposed as candidates for neuroprotective agents for PD. Although these drugs are promising in PD treatment, their mechanisms require much effort to investigate.

Surgical interventions mainly include thalamotomy, pallidotomy, deep brain stimulation (DBS). These methods are only response to one or two symptoms of PD patients and cause many complications, such as brain haemorrhage, infarction, seizures, and even death [75-76]. Thus, novel therapeutic strategies need to be developed to resolve these issues. Recently, gene therapy and cell replacement have shown great potential in altering the course of the disease. Gene therapies are mainly using harmless viral vector to shuttle a gene into the central nervous system, modifying neurotransmitter level in basal ganglia or reducing disease progression [77].

Cell replacement therapies are mainly focused on replacing of the degenerated DA-producing neurons in the SNpc during the course of PD with “dopaminergic-like” cells derived from fetal ventral mesencephalic (FVM) tissues or from human embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells [78].

1.2 Models of PD

Animal models are important for understanding the pathology and developing novel therapies for PD. An ideal animal model should recapitulate key clinical and neuropathological characteristics of sporadic PD, such as loss of dopaminergic neurons, motor dysfunctions and the presence of LBs. Current PD models can be divided into genetic and toxin models.

1.2.1 Genetic models of PD

Although PD is primarily a sporadic disease, about 5-10% of this disease is caused by genetic mutations. Since the finding of the first PD gene, α -synuclein, in 1997, 11 genes and 3 additional genetic loci have been identified to be related to familial PD [24]. The discovery of these PD genes is very important for the development of novel genetic models that may produce further insights into the specific molecular mechanism underlying the pathogenesis of PD and provide potential therapeutic strategies for PD treatment. Currently, there are a number of genetic PD models generated either by transgenic overexpression of mutant genes for autosomal-dominant genes such as α -synuclein and LRRK2 or by knockout of mutant genes for autosomal-recessive genes such as Parkin, DJ-1 and PINK1 [79]. The discovery that the major components of LBs are α -synuclein proved the intimate relationship between familial and sporadic PD [80]. The expression level of α -synuclein is crucial for the development of PD and overexpression of WT or mutations of α -synuclein in *Drosophila* replicate many cardinal features of PD [81-82]. Although α -synuclein mutations are 100% penetrant, it is a rare cause of familial PD. LRRK2 mutations are

the most popular genetic abnormality of autosomal-dominant PD [83]. Overexpression of LRRK2 in *Drosophila* and *C. Elegans* leads to degeneration of dopaminergic neurons, but overexpression of LRRK2 in mice has minimal evidence of neurodegeneration [84-87]. Parkin was the first gene identified to be related to autosomal-recessive PD and accounted for about 50% of familial PD cases [88]. Knockout of Parkin in *Drosophila* induces severe defects in mobile ability, but no Parkin knockout mice show any behavioural abnormalities or substantial neurodegeneration [89-91]. Like Parkin, only knockout of PINK or DJ-1 in *Drosophila* will lead to behavioural deficits [92-93].

1.2.2 Toxin models of PD

Toxin-generated *in vivo* and *in vitro* disease models are important in uncovering the molecular mechanism of neurodegeneration of dopaminergic neurons and in developing potential therapeutic processes. There are three main PD toxin models: 6-OHDA, rotenone, and MPTP. These toxins can selectively concentrate in the dopaminergic neurons, inducing cellular dysfunction and death.

1.2.2.1 6-OHDA model of PD

6-OHDA, shares some similar structure with DA and norepinephrine, was the first toxin model of PD associated with dopaminergic neuronal cell death in SNpc of PD patients. It was applied more than 40 years ago [94]. It is effective in rats, mice, cats, and primates. After directly injecting 6-OHDA into the SNpc of mice, this toxin accumulates in dopaminergic neurons and causes their death [95]. The advantage of this model is that animals have a quantifiable motor deficit, and this advantage makes this model particularly useful for screening drugs for PD. But this toxin does not result in the presence of LBs in the SNpc and can induce nonspecific damage to other neurons [96]. Furthermore, it is still unclear whether 6-OHDA-induced dopaminergic neurons death shares key molecular mechanisms with PD [1].

1.2.2.2 Rotenone model of PD

Rotenone is a natural compound derived from certain plant species, which has been widely used as insecticide and pesticide [97]. Rotenone is a high-affinity specific inhibitor of complex I. Low-dose administration of rotenone to rat induces selective degeneration of dopaminergic neurons and the presence of LB-like inclusions which are immunoreactive for both ubiquitin and α -synuclein [98]. Rotenone-treated animals also show motor abnormalities, such as reduced mobility, flexed posture, and rigidity, and these abnormalities can be reversed by L-DOPA [99]. However, this model suffers from much variability in the response of animals to the toxin with only 12 out of 25 rats developing lesions in the dopaminergic neurons of the SNpc [96].

1.2.2.3 MPTP model of PD

MPTP is a by-product accidentally produced by a graduate student named Barry Kidson in 1976 when he attempted to synthesize the opioid analgesic drug 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) [100]. In 1979-1982, a group of young drug abusers were diagnosed with a severe and irreversible parkinsonian syndrome in California. And subsequently, MPTP was recognised to be responsible for this new disorder. MPTP can also produce the biochemical and pathological hallmarks of PD in several other species, including non-human primates and rodents [1]. It has been reported that MPTP induces the degeneration of pigmented neurons in the SNpc of the baboons [101] and mice [57] *in vivo*. Its active metabolite, MPP^+ , produces morphological features of apoptosis in many dopaminergic cells, such as PC12 [102] and SH-SY5Y cells [3] *in vitro*. After systemic administration, MPTP crosses the blood brain barrier (BBB) and will be deprotonated to its active form, MPP^+ , by monoamine oxidase B (MAO-B) of the inner mitochondrial membrane of glial cells (Fig. 4). MPP^+ is selectively taken up by dopamine reuptake system into dopaminergic neurons and is concentrated inside mitochondria where it potently blocks mitochondria respiratory complex I, leading to cellular energy failure, reactive oxygen species (ROS) activation [2, 103-104]. The cellular energy failure and ROS production result in the activation

of mitochondrial apoptotic pathway by inducing the opening of the mitochondrial transition pore which will allow the release of Cytochrome c from the mitochondria to cytoplasm. Once inside of the cytoplasm, Cytochrome c binds with Apaf-1 and forms the apoptosome, which mediates the activation of pro-caspase-9 followed by activation of downstream caspases [105-106].

Figure 4. Schematic representation of the MPTP toxicity.

After systematic administration, MPTP crosses BBB and is converted into its active form, MPP⁺, by glial MAO mainly in glial cell. MPP⁺ accumulates in dopaminergic neurons after selectively uptake by energy-dependent dopamine uptake system. Once inside the cells, MPP⁺ inhibits the mitochondrial electron transport chain and produces oxidative stress, both inducing cell death. The mitochondrial inhibition causes a decrease in cellular ATP level, loss of mitochondrial membrane potential, abnormality of calcium homeostasis and radical formation. Oxidative stress generated directly by MPP⁺ or indirectly by MPP⁺-mediated mitochondrial inhibition provokes macromolecule peroxidation and cell death[107].

1.3 Insulin-like growth factor-1(IGF-1) signaling

IGF system is composed of three ligands which belong to the insulin-related peptide family, including insulin, IGF-1 and IGF-2, their cognate receptors which mediate the biological functions of the IGFs, as well as at least six IGF-binding proteins (IGFBPs) [108]. IGF-1 is a single chain polypeptide of 70 amino acids which has a similar structure to insulin and IGF-2 which is widely expressed in many if not all tissues, including brain [109]. It has anabolic effects on the target tissue and organs and is fundamental for normal fetal and postnatal growth, development, metabolism and apoptosis in mammals [110].

1.3.1 IGF-1 prevents apoptosis in PD models

IGF-1 may have a neuroprotective potential in PD considering that its receptors are relatively highly expressed in the SN of human brain [111] and IGF-1R^{+/-} mice show increased loss of dopamine neurons in SNpc after MPTP treatment compared to WT mice [112]. In cellular PD models, IGF-1 rescues rat cultured granule cells and human neuroblastoma SK-N-SH cells from dopamine-induced apoptotic cell death [113]. It has also been demonstrated that IGF-1 increases the survival of human neuroblastoma SH-SY5Y cells from α -synuclein cytotoxicity [114]. Furthermore, IGF-1 also salvages rat pheochromocytoma PC12 cells from apoptosis caused by 6-OHDA exposure [115].

1.3.2 IGF-1-mediated signaling pathways

The biological functions of IGF-1 are generally executed by IGF-1 receptor (IGF-1R), which is a tetrameric protein comprising of two extracellular α subunits that contain the IGF-1-binding site and two transmembrane β subunits that possess tyrosine kinase activity [116]. Recruiting of IGF-1 or IGF-2 to the extracellular α subunits of IGF1R leads to a conformational change in the transmembrane β subunits, causing trans-

autophosphorylation between the β subunits. β subunits phosphorylation which involves tyrosines 1131, 1135, and 1136 in the tyrosine kinase domain induces full activation of the kinase activity [117]. Activated IGF-1R phosphorylates additional tyrosine residues in the juxtamembrane region and carboxyl-terminal domains flanking the tyrosine kinase domain. Phosphorylated Tyr950 in the juxtamembrane area functions as a docking site for the recruitment of insulin receptor substrate 1 to 4 (IRS 1 to IRS-4) or Src homology/collagen (SHC) proteins which are responsible for activation of the phosphatidylinositol-3 kinase (PI3K) and MAPK/ERK kinase signaling pathways, respectively (Fig. 5) [118].

Docking and subsequent phosphorylation of SHC to Tyr950 of IGF-1R results in the recruitment of growth factor receptor binding protein 2 (Grb2). Grb2 binds the son of sevenless (SOS), which in turn causes GDP replacement with GTP in ras. This GTP replacement induces a conformational change in ras, promoting it to bind to a wide range of downstream effector proteins, including isoforms of the Ser/Thr kinase raf. Subsequently, GTP-bound ras activates the raf which phosphorylates the dual specificity protein kinases MEK-1/2. MEK1/2 then activates the ERK1/2 by dual phosphorylation of threonine and tyrosine on a conserved Thr-Glu-Tyr (TEY) motif in their activation loop [110]. The ras-raf-MEK-ERK pathway can also be activated via recruitment of Grb-2 to IRS-1 [119].

The second signaling pathway radiates from IGF-1R is the PI3K/AKT pathway. Binding of the p85 regulatory subunit of PI3K to IRS-1 activates its p110 catalytic subunit, which catalyzes phosphatidyl-inositol (PI) lipids at position 3 of the inositol ring to generate 3-phosphorylated forms of PI, such as phosphatidylinositol 3, 4, 5 triphosphate (PIP3) [120]. Binding of PIP3 to the pleckstrin homology (PH) domain of AKT results in the recruitment of AKT to the membrane and allows phosphorylation on Thr 308 by PH domain containing phospholipids-dependent kinase (PDK-1) and subsequent phosphorylation on Ser 473 by mTORC2 which is activated by RTK signaling through a currently unknown mechanism [121-122]. PI3K/AKT can also be

activated by direct binding of p85 to Tyr 1316 of IGF-1R [123]. Many downstream effectors of AKT are involved in regulation of gene expression, protein synthesis, metabolism, cell cycling, and cell survival [124].

Figure 5. The IGF-1 signaling pathway.

Binding of IGF-1 or IGF-2 to IGF-1R induce the autophosphorylation and activation of IGF-1R. Activated IGF-1R leads to phosphorylation of adaptor proteins belonging to the IRS family or SHC. Recruitment of the p85 subunit of PI3K to IRS-1 activates p110 catalytic subunit, leading to the generation of PIP3 and phosphorylation of AKT by PDK1. Activation of IRS and SHC also leads to activation of ERK1/2 of the MAPK cascade via the Grb2/SOS/Ras/Raf/MEK/ERK pathway [125].

1.4 SH-EP1 cells

An ideal PD cell model should possess characteristics of dopaminergic neurons, such as expressing tyrosine hydroxylase and dopamine- β -hydroxylase. Currently, PD cell models mainly include primary mesencephalic neurons, PC12 cells, MN9D cells, and SH-SY5Y cells [126-127]. These cells reproduce many characters of the dopaminergic neuron death observed in PD when treated by neurotoxins such as MPP⁺, 6-OHDA, or rotenone. SH-EP1 cells are derived from the human neuroblastoma cell line SK-N-SH, which was originally isolated from a bone marrow biopsy of a 4-year old female neuroblastoma patient in 1970 [128]. SK-N-SH cells can also give rise to SH-SY5Y cells, and moreover, SH-EP1 and SH-SY5Y cells can interconvert into each other morphologically and biochemically. Therefore, SH-EP1 cell line is an acceptable candidate for the usage as a PD model.

1.5 Aims of the study

As already mentioned, PD is characterized by the degeneration of dopaminergic neurons in the SNpc and MPTP selectively induces apoptotic cell death in dopaminergic neurons through its active form MPP⁺ and causes parkinsonisms in various species including human [1]. In the SNpc of PD patients, the expression of IGF-1R is upregulated, implying the potential implication of IGF-1 signaling in PD [111]. Although the involvement of IGF-1 signaling pathways in cell survival has been identified in many cell types, but its downstream targets are frequently cell type-specific. Thus, it is worthwhile to determine the protective mechanism of IGF-1 in a MPP⁺-generated PD model using human neuroblastoma SH-EP1 cells. To this end, the first aim of our study is to check the effect of IGF-1 on MPP⁺-induced cell death in SH-EP1 cells. The biological effects of IGF-1 are mainly mediated by the PI3K/AKT pathway and MAPK/ERK pathway, which lead to the second aim of our study - to

determine the contribution of PI3K/AKT and MAPK/ERK pathways in the neuroprotection mechanism, i.e., which pathway is responsible for the protective effect of IGF-1 against MPP⁺-induced neurotoxicity. In our previous study, JNK pathway has been shown to contribute to the neurotoxicity of MPP⁺ in SH-EP1 cells [129]. Moreover, IGF-1 has been demonstrated to be able to block the activation of JNK [130]. Thus, our third aim will be investigating whether there is any crosstalk between the IGF-1 signaling and JNK. It is hoped that these results would eventually produce an effective cure for Parkinson's disease, especially the use of IGF-1 for patients in the early stage of Parkinson's disease as a cure or to retard its progression.

CHAPTER 2 MATERIALS AND METHODS

2.1 Reagent

IGF-1, MPP⁺, Akti, a specific inhibitor of AKT, 6-bromoindirubin-3'-oxime (BIO), a specific inhibitor of GSK-3 β , SP600125, a specific inhibitor of JNK1/2 and 4, 6 diamidine-2-phenyllindole dihydrochloride (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). LY294002, a specific inhibitor of PI3K, was from Cell Signaling Technology (Danvers, MA, USA). U0126, a specific inhibitor of MEK was obtained from Millipore (Billerica, MA, USA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA, USA).

2.2 General buffers and solutions

Table 1. General buffers and solutions

10 $\times$ PBS (phosphate buffer saline)	80 g NaCl, 2 g KCl, 14.4 g Na ₂ HPO ₄ in 1 liter ddH ₂ O (distilled, deionized water), pH7.4
1 $\times$ PBS-T	1 $\times$ PBS, 0.1% Tween-20
Lysis buffer	20 mM Tris-HCl, pH 6.5, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na ₃ VO ₄ , 1mM phenylmethylsulfonyl fluoride, supplemented with complete protease inhibitor
4 $\times$ Stacking buffer	0.5 M Tris-HCl, pH 6.8, 0.4% SDS
4 $\times$ Resolution buffer	1.5M Tris-HCl, pH 8.8, 0.4% SDS
10% ammonium persulfate (APS)	10 ml ddH ₂ O containing 1 g APS
10 $\times$ SDS-PAGE	0.25 M Tris-HCl, 1.9 M glycine, 1%SDS

Running buffer	
5× SDS Loading buffer	250 mM Tris-HCl, pH 6.8, 10% SDS, 30% glycerol, 5% β -mercaptoethanol (or 0.5 M DTT (Dithiothreitol)), 0.02% bromophenol blue
10× Transfer buffer	250 mM Tris base, 1.92 M glycine
1× Transfer buffer (1 L)	100 ml 10× Transfer buffer, 100 ml methanol, 800 ml ddH ₂ O
Blocking buffer	1×PBS-T with 5% non-fat milk

2.3 Medium for cell culture

Dulbecco's modified Eagle's medium (DMEM)-high glucose (HG), supplemented with 100 IU/ml penicillin, 100 μ g/ml streptomycin, 10% heat-inactivated fetal calf serum (FCS) for DMEM-HG.

2.4 Cell culture

Human neuroblastoma cell line SH-EP1 (gift from Dr. Evelyne Goillot, Laboratoire d'Immunologie, Centre Leon Berard) was cultured in DMEM-HG supplemented with 10% fetal calf serum (FCS), 100 UI/mL penicillin and 100 μ g/mL streptomycin in a humidified atmosphere containing 5% CO₂ in air at 37°C. For cell passage, cells were washed with PBS and incubated with 0.25 tripsin until cells detached from the bottom of the cell culture flask. Culture medium was then added to stop trypsinization. The cells were pipetted up and down gently and split in 1:10 in fresh culture medium.

2.5 Drug treatment

The culture medium was changed to DMEM-HG without serum 16 h before

experimental treatments, to reduce constitutive activity of kinases. The selected concentration of MPP⁺ (Sigma, St. Louis, MO, USA) was based on our previous study, which was the most effective concentration in killing SH-EP1 cells [129]. Cells were incubated with different inhibitors in the concentration indicated in the results for 1 h prior to MPP⁺ or MPP⁺ plus IGF-1 (Sigma, St. Louis, MO, USA) treatment.

2.6 Cell Viability Assay

Cell viability was examined using crystal violet (Sigma-Aldrich, USA) staining as described previously [131]. In brief, 2×10^4 cells were seeded on 96-well plates. Following overnight incubation, cells were washed with fresh medium without serum and treated with reagents. All drugs were diluted in the same medium. After 24 h treatment, the plate was stained with 0.5% crystal violet in 20% methanol for 20 min at room temperature and then washed with tap water. After incubation in room temperature for 20 min, crystal violet in stained cells was dissolved with 20% acidic acid, and measured at a wavelength of 570 nm with Tecan (Männedorf, Switzerland). Absolute reading values were normalized by scaling to the mean of SH-EP1 culture grown in DMEM-HG alone (defined as 100%). At least three independent experiments were performed in triplicate.

2.7 DAPI staining of SH-EP1 cells

SH-EP1 cells were seeded on coverslips coated with 0.1% poly-L-lysine (PLL) in 12-well plates. After being treated with MPP⁺ or MPP⁺ plus IGF-1 for 24 h, cells were washed with PBS and fixed with 4% cold paraformaldehyde for 15 min. SH-EP1 cells were washed with PBS for 3 times, and then incubated in 4, 6 diamidine-2-phenyllindole dihydrochloride (DAPI) (Sigma-Aldrich, USA) solution (5 µg/ml) for 15 min. After that, the cells were washed with PBS for 3 times before examining by Zeiss Axiovert LSM-510-META-inverted microscope (Carl Zeiss, Thornwood, NY,

USA). The incidence of apoptosis was analyzed by counting 500 cells and determining the percentage of apoptotic cells.

2.8 Western blot

After treatment, cells in 60cm dishes were washed twice with cold 1×PBS and then lysed in ice-cold lysis buffer and centrifuged at 14,000g for 20 min at 4°C. The proteins in the supernatant were collected and the concentration was assessed using a Protein Assay Kit II (BioRad, Hercules, USA). After incubating at 100°C for 5 min, 40 µg of protein samples was resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, CA, USA). The membranes were blocked with PBS-T (0.1% Tween-20 in PBS) containing 5% non-fat milk, and then incubated with different primary antibodies at 4 °C overnight (Table 2). After washing with 1×PBS for 30min, the membranes were further incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h in room temperature with gentle agitation. The membrane was then stringently washed and subjected chemiluminescence detection using Pierce's West Pico Chemiluminescence substrate (Pierce, IL, USA), followed by exposing to X-ray film (Kodak) and developing with Kodak X-OMAT ME processor. In some cases, we quantified immunoblots by measuring the immunoreactive protein band density with the software ImageJ 1.41.

Table 2. Antibodies used for Western Blot

Antibody	Company	Dilution factor
Mouse anti β -actin	Sigma-Aldrich, USA	5000
Rabbit anti cleaved caspase-9	Cell signaling technology, USA	1000
Rabbit anti cleaved caspase-3	Cell signaling technology, USA	1000

Rabbit anti cleaved PARP	Cell signaling technology, USA	1000
Rabbit anti phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell signaling technology, USA	2000
Mouse anti phospho-AKT (Ser473)	Cell signaling technology, USA	1000
Rabbit anti AKT	Cell signaling technology, USA	2000
Rabbit phospho-GSK-3 α/β (ser21/9)	Cell signaling technology, USA	1000
Rabbit anti GSK-3 β	Cell signaling technology, USA	2000
Mouse phospho-JNK1/2	Cell signaling technology, USA	1000
Rabbit anti ERK2	Santa Cruz Biotechnology, USA	2000
Rabbit anti JNK1	Santa Cruz Biotechnology, USA	2000
Goat anti rabbit IgG conjugated with HRP	Sigma-Aldrich, USA	2000
Goat anti mouse IgG conjugated with HRP	Sigma-Aldrich, USA	1000

2.9 Statistical analysis

Data were expressed as mean $\pm$ standard error (SE) values. The group means were compared by analysis of variance, and the significance of differences was determined by post hoc testing using Bonferroni's method. Differences were considered significant at $p < 0.05$.

CHAPTER 3 RESULTS

3.1 IGF-1 protects SH-EP1 cells from MPP⁺-induced apoptotic cell death

Human neuroblastoma SH-EP1 cells were treated with MPP⁺ to induce cell death as we described previously [129]. The protective effect of IGF-1 against MPP⁺ was assessed by cell viability assay using crystal violet staining. In cells treated with MPP⁺, the cell viability was about 34% of the control, whereas IGF-1 greatly increased the survival of SH-EP1 cells in a dose-dependent manner. The maximum protective effect occurred at 200 ng/ml of IGF-1 (Fig. 6A). The protective effect of IGF-1 was observed at a concentration as low as 10 ng/ml, while 200 ng/ml IGF-1 increased the cell survival to 59% ($P < 0.01$, compared to the treatment with MPP⁺ alone) of the control and further increasing IGF-1 concentration to 400 ng/ml only slightly increased cell survival, but the increase was not significant compared with the survival at 200 ng/ml of IGF-1 ($P > 0.05$) (Fig. 6A). Consistent with the cell survival data, DAPI staining also showed that IGF-1 could markedly reduce the number of MPP⁺-induced apoptotic nuclei in SH-EP1 cells (Fig. 6B-6E). Since MPP⁺ induces mitochondria dysfunction and release of cytochrome c to trigger apoptosis [132-133] and the activation of caspase-9 and caspase-3 are the major downstream events of the mitochondrial apoptotic pathway [134], we determined their activation in MPP⁺ treated SH-EP1 cells in the presence or absence of IGF-1 (200 ng/ml). As shown in Fig. 6F, MPP⁺ caused a significant activation of caspase-9 and caspase-3 16 h or 24 h post-treatment and these activations were greatly attenuated by IGF-1. Moreover, cleavage of the caspase substrate poly (ADP-ribose) polymerase (PARP), a biochemical feature of apoptosis, was also assessed. Cleavage of PARP was also observed within 16 h and 24 h of treatment with MPP⁺, but the cleavage was significantly inhibited in the presence of IGF-1 (200 ng/ml) (Fig. 6F). These results suggest that IGF-1 promotes SH-EP1 cells

survival by preventing apoptosis induced by MPP⁺.

Figure 6. IGF-1 promotes SH-EP1 cells survival against MPP⁺ neurotoxicity.

A: IGF-1 protects SH-EP1 cells from MPP⁺-induced cell death in a concentration-dependent manner. Cells were treated with MPP⁺ or MPP⁺ plus IGF-1 for 24 h. Cell viability was assessed by cell survival assay. Data are expressed as percent of values in untreated control cultures, and represent the mean $\pm$ S.E. of three experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$, compared with MPP⁺-treated cells. Apoptosis was detected by DAPI staining, B: control, C: 2 mM MPP⁺, D: 2 mM MPP⁺ plus IGF-1 (200 ng/ml). Scale bar: 50 μ m, yellow arrows indicate apoptotic bodies. E: IGF-1 reduced the activation of caspase-9, caspase-3 and cleavage of PARP. Cells were treated with MPP⁺ or MPP⁺ plus IGF-1 for 16, 24 h. Samples were assessed by western blot with antibody against the cleaved caspase-9, caspase-3 and PARP. Three independent experiments were done which yield similar results, and a representative blot is shown. In all blots, expression of β -actin (ACTB) is shown as a protein loading control.

3.2 IGF-1-promoted cell survival is mediated by the PI3K/AKT pathway, not MAPK/ERK pathway.

It has been reported that the protective effects of IGF-1 are mainly mediated by the activation of its two important downstream signaling pathways: the PI3K/AKT pathway and the MAPK/ERK pathway [122]. To determine whether IGF-1 protects SH-EP1 cells from MPP⁺-induced apoptosis through these pathways, we examined the activation of AKT and ERK in SH-EP1 cells upon IGF-1 treatment. Cells were treated with MPP⁺ or IGF-1 plus MPP⁺ for 15 min, 1, 4, and 24 h, and cell extracts were subjected to western blot analysis to detect phosphorylated AKT (pAKT, active form). AKT was phosphorylated at Ser473 when cells were treated with IGF-1 and MPP⁺, whereas treatment with MPP⁺ alone did not induce the phosphorylation of AKT. The activation of AKT quickly reached the peak level in 15 min and sustained to 24 h after IGF-1 treatment (Fig. 7A). To further assess the contribution of PI3K/AKT pathway in IGF-1-promoted cell survival, we preincubated SH-EP1 cells with LY294002 or Akti for 1h before the treatment with MPP⁺ or MPP⁺ plus IGF-1. Cell survival was assessed

24 h after treatment. LY294002, a specific PI3K inhibitor that acts on the ATP-binding site of the enzyme, or Akti, a specific inhibitor of AKT, acts on the pleckstrin homology (PH) domain of AKT, suppressed the activation of AKT (Fig. 7C and 7D) and partially, but significantly, abolished the protective effect of IGF-1 against cell death induced by MPP⁺ (Fig. 7B). As control, LY294002 or Akti alone did not significantly affect the cell survival of untreated SH-EP1 cells. In addition, these two inhibitors also abolished the reduced caspase-9, caspase-3, and PARP cleavage mediated by IGF-1 as shown in Fig. 7C and 7D. These results indicate that IGF-1-mediated cell survival against MPP⁺ neurotoxicity is dependent on the PI3K/AKT pathway.

Figure 7. IGF-1-dependent inhibition of apoptosis induced by MPP⁺ in SH-EP1 cells is through the PI3K/Akt pathway.

A: Treatment with MPP⁺ or MPP⁺ plus IGF-1 induced a rapid and sustained AKT activation in SH-EP1 cells. Cells were incubated with MPP⁺ or MPP⁺ plus IGF-1 for a time course as indicated, samples were examined by western blot with antibody against phosphorylated AKT (S473). B: In the presence of LY294002 (20 μ M) or Akti (5 μ M), cells were cultured with MPP⁺ or MPP⁺ plus IGF-1 for 24 h. Cell survivals were determined by cell survival assay. Data are from three repeated experiments. ** P <0.01, compared to cells treated with MPP⁺ plus IGF-1. In the presence of LY294002 (C) or Akti (D), cells were incubated with MPP⁺ or MPP⁺ plus IGF-1 for 16 h, protein extracts were analyzed by western blot with antibodies against phosphorylated AKT (S473) or cleaved caspase-9, caspase-3, and PARP. Three independent experiments were done which yield similar results, and a representative blot is shown. In all blots, expression of AKT or β -actin (ACTB) is shown as a protein loading control.

As MAPK/ERK pathway is another important downstream signaling pathway involved in IGF-1-mediated cell protection, we tested the effect of IGF-1 on ERK activation and its involvement in IGF-1-promoted cells survival against MPP⁺-induced neurotoxicity. As shown in Fig. 8A, ERK was activated by MPP⁺, and the peak phosphorylation of ERK occurred within 15 min after MPP⁺ treatment. Phosphorylation level then dropped to baseline at 1 h, followed by significant increase at 24 h. The same pattern of ERK phosphorylation was also observed in SH-EP1 cells treated with IGF-1 plus MPP⁺. In contrast, treatment with IGF-1 also resulted in phosphorylation of ERKs, but the activation of ERKs was transient and slight (Fig. 8B). To further check the possible involvement of ERK in IGF-1-enhanced cell survival, we pretreated SH-EP1 cells with U0126, a specific inhibitor of MEK1 which is the upstream effector of ERK1/2, for 1h before the treatment with MPP⁺ or IGF-1 plus MPP⁺, and cell survival was determined after 24 h. As expected, U0126 completely blocked ERK1/2 activation. However, the inhibitor had no effect on IGF-1-enhanced cell survival ($P>0.05$, compared with SH-EP1 cells treated with MPP⁺ plus IGF-1) (Fig. 8C). Furthermore, it had no influence on the reduced caspase-9, caspase-3, and PARP cleavage mediated by IGF-1 as shown in Fig. 8D, indicating that activation of ERK is unlikely to be involved in IGF-1-enhanced cell survival in SH-EP1 cells.

Figure 8. MAPK/ERK pathway is not involved in IGF-1-mediated protection against MPP⁺ neurotoxicity in SH-EP1 cells.

A: SH-EP1 cells were treated with MPP⁺ or MPP⁺ plus IGF-1 for a time course as indicated, samples were assessed by western blotting with antibody against p-ERK1/2. B: SH-EP1 cells were treated with IGF-1 for a time course as indicated, samples were assessed by western blotting with antibody against p-ERK1/2. C: In the presence of MEK1 inhibitor, U0126, cells were cultured with MPP⁺ or MPP⁺ plus IGF-1 for 24 h. Cell survivals were determined by cell survival assay. Data are from three repeated experiments. ** $P < 0.01$, compared to MPP⁺-treated SH-EP1 cells. D: In the presence of U0126, cells were incubated with MPP⁺ or MPP⁺ plus IGF-1 for 16 h, protein extracts were analyzed by western blot with antibodies against p-ERK1/2 and cleaved PARP. Three independent experiments were done which yield similar results, and a representative blot is shown. In all blots, level of total ERK2 is shown as a protein loading control.

3.3 IGF-1-mediated cell protection is dependent on the inhibition of GSK-3 β by PI3K/AKT pathway.

GSK-3 β is a multifunctional serine/threonine kinase that was first isolated as enzyme capable of phosphorylating and inactivating the enzyme glycogen synthase [135]. It plays a key role in numerous signaling pathways, including cellular response to Wnt,

receptor tyrosine kinases, G-protein-coupled receptors and functions in a wide range of cellular processes, ranging from glycogen metabolism, to cell cycle regulation, proliferation and apoptosis [136]. GSK-3 β is unusual in that it is constitutively active in cells and is mainly regulated by inhibition of its activity which can be achieved through phosphorylation of its serine residues (Ser9) by AKT [137-138]. To test the possibility that IGF-1 could induce the phosphorylation and inactivation of GSK-3 β in SH-EP1 cells, we examined the phosphorylation of GSK-3 β by western blot in SH-EP1 cells after incubation with MPP⁺ or MPP⁺ plus IGF-1 in indicated time. Compared with MPP⁺-treated cells, MPP⁺ plus IGF-1-treated cells had a significant increase in phosphorylation of GSK-3 β (Fig. 9A). To check whether IGF-1-induced inactivation of GSK-3 β is mediated by PI3K/AKT pathway, we preincubated SH-EP1 cells with LY294002 or Akti for 1 h before treatment with MPP⁺ or MPP⁺ plus IGF-1. As shown in Fig. 9B and 9C, the phosphorylation of GSK-3 β induced by IGF-1 was counteracted by LY294002 or Akti, indicating that IGF-1 induced inhibition of GSK-3 β was mediated by the PI3K/AKT pathway.

GSK-3 β has pro-apoptotic roles in PC12, Rat-1 cells, and cerebellar granule neurons, as its inhibition protects cells against apoptotic stimuli [139-140]. To further determine whether inactivation of GSK-3 β by PI3K/AKT pathway contributes to the survival of SH-EP1 cells against MPP⁺ neurotoxicity, we pretreated SH-EP1 cells with the GSK-3 β inhibitor BIO prior to the addition of MPP⁺. Cell survival was examined 24 h later. As expected, BIO could partially confer cell protection against MPP⁺ insults in SH-EP1 cells ($P < 0.01$, compared to SH-EP1 cells treated with MPP⁺ alone) (Fig. 9D). As control, BIO alone did not significantly influence the cell survival of untreated SH-EP1 cells. Consistently, MPP⁺-induced cleavage of caspase-9, caspase-3, and PARP was also inhibited by BIO (Fig. 9E).

Figure 9. AKT-mediated survival of SH-EP cells is dependent on the inhibition of GSK-3β.

A: Cells were treated with MPP⁺ or MPP⁺ plus IGF-1 for a time course as indicated, samples were assessed by western blotting with antibody against phosphorylated GSK-3β (Ser9). In the presence of LY294002 (B) and Akti (C), cells were treated with MPP⁺ or IGF-1 plus MPP⁺ for 4 h. Cell extracts were analyzed by western blot with antibodies against p-GSK-3β. D: In the presence of GSK-3β inhibitor, BIO (0.5 μM), cells were incubated with MPP⁺ for 24 h. Cell survival was examined with cell survival assay. Data are means ±S.E of three replicate values in 3 separate experiments. ** *P* < 0.01, compared to MPP⁺-treated SH-EP1 cells. E: In the presence of BIO (0.2, 0.5 and 1 μM), SH-EP1 cells were incubated with MPP⁺ for 16 h, and then samples were examined by western blot with antibody against cleaved caspase-9, caspase-3, and PARP. Three independent experiments were done which yield similar results, and a representative blot is shown. In all blots, staining for total GSK-3β or β-actin (ACTB) was used as a loading control.

3.4 IGF-1-promoted cell protection is mediated by the inhibition of JNK activation induced by MPP⁺ via GSK-3 β inhibition by AKT.

JNK is a subfamily of the MAPK superfamily and has three isoforms (JNK1, 2 and 3), with slicing variant [141]. JNK activation is regulated by a MAPK module (MAP3K $\rightarrow$ MAP2K $\rightarrow$ MAPK) through sequential protein phosphorylation [142]. So far, two MAP2Ks (MKK4 and MKK7) for JNK have been identified [143-144]. MKK4 and MKK7 are dual-specificity protein kinases and phosphorylate JNK at Thr183 and Tyr185, leading to its activation [145]. In our previous study, JNK pathway has been demonstrated to be involved in the neurotoxicity of MPP⁺ [129]. In human embryonic kidney 293 and L929 cells, activation of AKT by IGF-1 has been shown to block JNK activation and thereafter preventing apoptosis [146]. IGF-1-mediated AKT activation has also been demonstrated to protect human vascular smooth muscle cells and isolated human islets from apoptosis via JNK inactivation [130, 147]. On the other hand, in mouse embryonic fibroblast, IGF-1 was found to activate JNK and then promote cell proliferation [148]. Thus, it is interesting to determine whether there was any crosstalk between IGF-1-induced PI3K/AKT pathway and JNK pathway in SH-EP1 cells. First, we examined the activation of JNK upon MPP⁺ insults. As expected, JNK1/2 activation was strongly induced by MPP⁺ (Fig. 10A and 10B). Next, we compared the activation of JNK1/2 in response to MPP⁺ or MPP⁺ plus IGF-1 and found that MPP⁺-induced JNK activation was strongly inhibited by IGF-1 (Fig. 10A and 10B). Meanwhile, we found that the inhibition of JNK by IGF-1 was dependent on PI3K/AKT pathway, because either LY292004 or Akti could reverse such inhibition.

Previous studies have demonstrated that activation of GSK-3 β preceded the activation of JNK by directly phosphorylating and subsequent activating mixed lineage kinase 3 (MLK3), an upstream effector of JNK, and GSK-3 β inhibitor, kenpaullone, attenuated the kinase activity of MLK3 and JNK induced by NGF deprivation in PC12 cells [149]. To evaluate the contribution of GSK-3 β inactivation to AKT-mediated JNK inhibition,

we examined the effect of BIO on MPP⁺-induced activation of JNK. As expected, we found that BIO reproduced the effect of IGF-1 and strongly inhibited MPP⁺-induced JNK activation (Fig. 10C), indicating that AKT-dependent JNK inhibition is mediated by GSK-3 β inactivation. To further delineate the detailed mechanism underlying GSK-3 β inactivation mediated JNK inhibition, we examined the effect of BIO on MKK4 and MKK7, the direct upstream effectors of JNK [145]. As shown in Fig. 10C, MPP⁺ only induced the activation of MKK4, not MKK7, and this activation is strongly suppressed by BIO, suggesting that GSK-3 β inactivation blocked MPP⁺-induced JNK activation through inhibiting MKK4 activity.

Figure 10. AKT blocked the activation of JNK induced by MPP⁺ through inactivation of GSK-3 β .

In the presence of LY294002 (A) and Akti (B), cells were treated with MPP⁺ or IGF-1 plus MPP⁺ for 4 h. Cell extracts were analyzed by western blot with antibodies against phosphorylated JNK. Densitometry is shown below as the ratio of p-JNK1/2/JNK1. Data are from three independent experiments with similar results and are means $\pm$ S.E. ** $P < 0.01$, compared to SH-EP1 cells treated with MPP⁺ alone. # $P < 0.05$, ## $P < 0.01$, compared to SH-EP1 cells treated with MPP⁺ plus IGF-1. Staining for total JNK1 was used as a loading control. C: Cells were pretreated with BIO for 1 h before treatment with MPP for 4 h, samples were assessed by western blot with antibodies against phosphorylated MKK7, MKK4 and JNK. Staining for total MKK7, MKK4 and JNK1 was used as a loading control.

To investigate the significance of JNK activation in MPP⁺-induced apoptosis in SH-EP1 cells, we preincubated SH-EP1 cells with JNK specific inhibitor SP600125 prior to MPP⁺ treatment. As shown in Fig. 11A, SP600125 partially suppressed MPP⁺-induced apoptotic cell death in SH-EP1 cells (* $P < 0.05$, compared with MPP⁺-treated cells). As controls, SP600125 alone did not significantly affect the cell viability of untreated SH-EP1. In addition, we found that JNK inhibitor could block the cleavage of apoptotic marker caspase-9, caspase-3, and PARP (Fig. 11B). These results indicated that inactivation of JNK is required for IGF-1 mediated protective effect against MPP⁺-induced cell death of SH-EP1 cells.

Figure 11. JNK inhibitor, SP600125, protects SH-EP1 cells from MPP⁺-induced apoptosis.

A: In the presence of SP600125 (30 μ M), cells were incubated with MPP⁺ (2 mM) for 24 h. Cell survival was examined with cell survival assay. Data are means $\pm$ S.E of three replicate values in 3 separate experiments, * P < 0.05, compared with MPP⁺-treated cells. B: In the presence of SP600125 (30 μ M), cells were incubated with MPP⁺ (2 mM) for 16 h, samples were examined by western blot with antibodies against cleaved caspase-9, caspase-3, and PARP.

CHAPTER 4 DISCUSSION AND CONCLUSION

4.1 Discussion

PD is the second most popular progressive neurodegenerative disease in the world only after Alzheimer's disease [150]. The major clinical motor features of PD are mainly induced by the degeneration of dopaminergic neurons in the SNpc of PD patients [10]. Human postmortem material, animal models, and cell culture models have provide important evidences that apoptosis is involved in the loss of dopaminergic neurons in the SNpc of patients [151-153]. MPTP is a neurotoxin that induces a syndrome that mimics the core neurological symptoms of PD in humans and causes dopamine neuronal apoptosis in SNpc of PD patients, nonhuman primates and mice [1]. Thus, rescuing neuronal apoptosis induced by MPP⁺ neurotoxin, the active form of MPTP, with pro-survival factors might present a potential therapeutic strategy for PD. In the present study, we demonstrated that IGF-1 protects SH-EP1 cells from MPP⁺-induced cell death through the activation of PI3K/AKT signaling pathway. Treatment of SH-EP1 cells with IGF-1 activates AKT, leading to the inhibition of GSK-3 β and subsequent inactivation of JNK. As a result, the inhibition of JNK by AKT-dependent GSK-3 β inactivation leads to the attenuation of MPP⁺-induced apoptosis. Our results may provide a new insight into signaling mechanisms mediated by IGF-1 in cell survival against MPP⁺-induced apoptosis and may have significance in the future PD therapy development.

4.1.1 IGF-1 protects SH-EP1 cells against MPP⁺-induced apoptosis

IGF-1 and its receptor, IGF-1R, are expressed in many different brain regions from early stages of embryogenesis and throughout life and play a crucial role in the normal development of brain by regulating glial and neuronal cell differentiation, proliferation

and survival [154]. IGF-1 is a potent neural survival factor that has been widely used to protect neural cells from apoptosis in several neurodegenerative models, including PD. For instance, in a rat model of PD, transplantation of human neural progenitor cells (hNPC) overexpressing of IGF-1 to the right striatum of rats confers a neuroprotective effect against the loss of dopaminergic neurons induced by 6-OHDA [155]. Additionally, IGF-1 rescues human dopaminergic neuroblastoma cells SH-SY5Y from apoptosis induced by salsolinol (SAL), a dopamine-derived tetrahydroisoquinoline, or by osmotic stress [156-157]. Moreover, IGF-1 promotes survival of rat heochromocytoma PC12 cells against apoptosis caused by MPP⁺ [158]. In accordance with these results, we showed here that IGF-1 significantly increased the survival of SH-EP1 cells against MPP⁺ induced cell death. DAPI staining also demonstrated that IGF-1 could markedly decrease the number of MPP⁺-induced apoptotic nuclei in SH-EP1 cells. Furthermore, IGF-1 significantly inhibits the activation of caspase-9 and caspase-3, as well as PARP cleavage. These results indicate that IGF-1 protects SH-EP1 cells by preventing apoptosis induced by MPP⁺.

4.1.2 MAPK/ERK is not involved in IGF-1-mediated protection against MPP⁺-induced apoptosis

The protective effects of IGF-1 are mediated by binding to its receptor, IGF-1R. Once IGF-1 binds to IGF-1R, IGF-1R initiates its downstream signaling pathways, such as MAPK/ERK pathway or PI/3K/AKT pathway [122]. MAPK/ERK pathway activation by growth factors, hormones, and neurotransmitters, play an important role in many physiological progresses of the cells, such as proliferation, survival, differentiation, development, and stress response [159]. However, the role of ERK activation in neuronal cell survival is still controversial. MAPK/ERK pathway can either enhance the neuronal cell survival or induce apoptotic cell death, depending on the specific cell types and insults. ERK activation has been reported to protect cultured rat cerebellar granule neurons against apoptosis induced by trophic factor deprivation through a dual mechanism that involve direct phosphorylation and inhibition of the pro-apoptotic

protein BAD and the expression of CREB-dependent prosurvival genes [160]. In addition, activated ERK attenuated the cell death of cultured rat cortical neurons caused by DNA damage-inducing drugs, cisplatin [161]. Moreover, nerve growth factor (NGF)-induced sustained ERK activation promotes PC12 cell survival by inducing the expression of micro-RNA miR-221 and 222 which results in down-regulation of the mRNA level of pro-apoptotic BH3-only protein Bim [162]. However, ERK activation has also been demonstrated to promote neuronal cell death. For example, activated ERK contributes neural apoptosis in cerebellar granule neurons induced by low concentration (5mM) of potassium (K^+) through promoting plasma membrane damage [163]. Delayed and persistent ERK activation is also observed to promote cell death of HT-22 hippocampal-derived neurons and primary cultured cortical neurons caused by glutamate-induced oxidative toxicity [164]. Furthermore, sustained activation of ERK plays a death-promoting role in NO-induced neural cell death in rat cultured primary midbrain cells in glutathione depletion conditions [165]. In our present study, we demonstrated that complete inhibition of ERK did not attenuate the protective effect of IGF-1 against MPP⁺-induced apoptotic cell death and have no influence on the reduced caspase-9, caspase-3, and PARP cleavage mediated by IGF-1. This result indicates that MAPK/ERK may not be involved in the anti-apoptotic effects of IGF-1 against MPP⁺ neurotoxicity. The biological significance of MAPK/ERK pathway primed by IGF-1 in SH-EP1 cells is not clear, and a further study will be needed to clarify this issue.

4.1.3 The protective effect of IGF-1 against MPP⁺-induced apoptosis in SH-EP1 cells is mediated by PI3K/AKT pathway

The second pathway induced by IGF-1 is the PI3K/AKT pathway and many studies have implicated the PI3K/AKT pathway is one of the major anti-apoptotic pathways in cells which is turned on by of a variety of growth factors under various stresses [166-168]. Activation of PI3K/AKT pathway by IGF-1 produces an anti-apoptotic signal in rat cerebellar granule cells through phosphorylation of FKHRL1, a member of Forkhead family, and subsequent suppression of FKHRL1-dependent transcription of

death genes [169]. PI3K/AKT pathway is also involved in heregulin (a member of neuregulin family) mediated survival of C6 glioma cells and primary cultures of oligodendrocytes against apoptosis induced by growth factor deprivation [170]. Moreover, PI3K/AKT pathway activated by brain-derived neurotrophic factor (BDNF) prevents cultured rat hippocampal neurons from apoptotic cell death caused by glutamate toxicity through inhibiting the activation of MAPK/ERK pathway [171]. Lastly, PI3K/AKT pathway protects PC12 cells from apoptosis induced by serum withdrawal or UV irradiation by negatively regulating the activation of JNK pathway through inducing the expression of JNK-interacting protein-1 (JIP-1, a scaffold protein that inhibits JNK) [172]. In an attempt to determine the downstream signaling events underlying the neuroprotective mechanism of IGF-1, we assessed the activation of AKT upon IGF-1 treatment. We found that IGF-1 induced a potent and sustained activation of AKT in SH-EP1 cells. The PI3K inhibitor, LY294002, or AKT inhibitor, Akti, abolished the activation of AKT as well as the anti-apoptotic effects of IGF-1. Interestingly, in the presence of LY294002 or Akti, MPP⁺-induced cell apoptosis were significantly enhanced in SH-EP1 cells treated with or without IGF-1. This suggests that inhibitors strongly reduced the neuroprotection resulting from basal level and IGF-1 induced PI3K/AKT activities. Taken together, our results indicated that the PI3K/AKT signaling pathway is responsible for the protective effect of IGF-1 against MPP⁺-induced apoptosis in SH-EP1 cells.

4.1.4 PI3K/AKT-dependent survival of SH-EP cells is mediated by phosphorylation and subsequent inactivation of GSK-3 β

Several AKT substrates have been reported in recent years, such as Bad, caspase-9 and GSK-3 β [121, 173]. GSK-3 β , originally identified as an enzyme capable of regulating insulin-dependent glycogen synthesis, is a multifunctional serine/threonine kinase expressed in all eukaryotes [136]. GSK-3 β plays a key role in a number of cellular processes, including glycogen metabolism, protein synthesis, insulin signaling, Wnt and Hedgehog signal transduction, cell proliferation, embryonic development and apoptosis [174-175]. GSK-3 β is constitutively active under resting conditions and is

primarily regulated by inhibition of its activity which can be achieved through phosphorylation of a serine residue in the N-terminus of the protein by AKT [137]. GSK-3 β has been shown to be involved in MPP⁺-induced mitochondrial dysfunction in mouse brain in vivo and immortalized murine cortical neurons (TSM1) or primary cultures of mouse neuronal cells in vitro, and blockage of GSK-3 β activation by pharmacological inhibitors or siRNA-promoted downregulation protects dopaminergic neurons from MPP⁺-mediated neurotoxicity by inhibiting mitochondrial membrane potential changes and subsequent activation of caspase-9 and caspase-3 [176]. In addition, inhibition of rapid activation of GSK-3 β by its selective inhibitors, indirubin-30-oxime and AR-A014418, protects dopaminergic neurons from apoptosis, restores the striatal dopamine level and reduces behavioral impairments of mice induced by MPTP [177]. Moreover, treatment with a GSK-3 β inhibitor, lithium, could produce neuroprotective effects against neurotoxicity induced by MPP⁺ in PC12 cells or in MPTP-induced striatal dopaminergic neurodegeneration and dopamine depletion in a mouse model of PD [178-179]. Consistent with these reports we showed here that IGF-1-mediated inactivation of GSK-3 β protects SH-EP1 cells from MPP⁺-induced cell death. This conclusion was supported by two evidences. First, GSK-3 β was phosphorylated and inactivated in response to IGF-1 stimulation and the inactivation is reversed when SH-EP1 cells were pretreated with LY294002 or Akti. Second, BIO, a GSK-3 β inhibitor, mimicked the protective effect of IGF-1 against MPP⁺-induced SH-EP1 cell death and blocked MPP⁺-induced caspase-9, caspase-3, and PARP cleavage. Our findings further confirmed the conclusion that inactivation of GSK-3 β plays an important role in reducing MPP⁺-induced neurotoxicity.

4.1.5 Inhibiting the activation of JNK by IGF-1-activated PI3K/AKT pathway protects SH-EP1 cells from apoptosis induced by MPP⁺

JNK, a member of the MAPK family, is activated by cytokines or in response to environmental stress [180]. JNK is an established mediator of stress-induced apoptosis and has been reported to be involved in the neurodegenerative processes in PD pathogenesis, and blockage of JNK activity may represents a potential strategy for

preventing loss of dopaminergic neurons in PD [181-182]. In vivo, JNK inhibitor, SP600125, attenuates neural apoptosis of dopaminergic neurons and partly restores the reduced DA level induced by systematic administration of MPTP by inhibiting JNK-mediated c-Jun phosphorylation [183]. In addition, overexpression of JNK binding domain (JBD) of JIP-1 in mice blocks JNK activation and protects dopaminergic neurons from apoptosis caused by MPTP [184]. Furthermore, inhibiting the activation of JNK by overexpression of parkin protects human neuroblastoma SH-SH5Y cells from apoptosis induced by DA or 6-OHDA [185]. In the present study, we found that IGF-1-promoted cells survival is executed by inhibition of JNK activation, as suggested by the observation that MPP⁺-induced JNK activation was suppressed by IGF-1 and such suppression could be reversed by LY294002 or Akti. Moreover, JNK inhibitor, SP600125, attenuates MPP⁺-induced apoptotic cell death in SH-EP1 cells and significantly reduces the cleavage of caspase-9, caspase-3, and PARP, providing a further evidence to support the protective effect of JNK signaling in MPP⁺-induced apoptosis in SH-EP1 cells.

4.1.6 Inactivation of GSK-3 β by PI3K/AKT pathway blocks MPP⁺-induced activation of JNK

Although many reports have been shown that inhibition of GSK-3 β suppresses PD mimetics, such as MPTP/MPP⁺, 6-OHDA, rotenone, induced neuronal apoptosis in several cell types, the mechanism about how GSK-3 β inactivation facilitates cell survival is still not fully understood [177, 186-187]. It has been demonstrated that GSK-3 β inhibition attenuated MPP⁺-induced apoptosis in SH-SY5Y cells by reducing hyperphosphorylation of Tau, along with decreased levels of accumulated α -Synuclein [188]. GSK-3 β inhibition has also been reported to protect rat cerebellar granule neurons from neuronal apoptosis induced by trophic factor withdrawal by suppressing the translocation of Bax, a member of pro-apoptotic Bcl-2 family, to mitochondria and blocking its conformational activation [189]. Lastly, inhibition of GSK-3 β activity by GSK-3 β inhibitors or GSK-3 β specific shRNA protects HT-22 hippocampal-derived

neurons from radiation-induced apoptosis through blockage accumulation of p53, a pro-apoptotic tumor suppressor protein, by phosphorylation of its specific E3 ubiquitin ligase MDM2 [190]. In the present study, we found that GSK-3 β inhibition can promote cell survival via inhibiting JNK activation, as supported by the observation that BIO reproduced the inhibitory effect of IGF-1 on JNK activation induced by MPP⁺. Our results also showed that BIO suppressed the activation of JNK by attenuating the kinase activity of MKK4. Similarly, it had been reported that GSK-3 β functions as a natural activator of Mitogen-activated protein kinase/Extracellular signal-regulated kinase kinase kinase 1 (MEKK1), an upstream activator of MKK4 and JNK, and GSK-3 β inactivation induced by insulin suppressed the activity of MEKK1/MKK4/JNK signaling, promoting the cell survival of neuroblastoma B103 cells [191]. Interestingly, MPTP induce the activation of MKK4 in nigrostriatal neurons in vivo and MPP⁺ induced the activation of MKK7 in PC12 cells[192-193], but our results demonstrated that MPP⁺ only induced the activation of MKK4, not MKK7, suggesting that MPP⁺ induced activation of MKK4 or MKK7 is depending on the cell type or biological context. Taken together, these data suggest that the inhibition of MPP⁺-induced MKK4 and JNK activation is a downstream event of AKT/GSK-3 β signaling and may add more weight to GSK-3 β blockage in the treatment of PD.

4.1.7 Limitations of IGF-1 in PD treatment and possible solution

Although IGF-1 is a promising candidate in neuroprotection, its application in neurological disorder treatment is still limited. The main obstacle is the delivery of IGF-1 because most IGF-1 forms an inactive complex with IGF binding proteins (IGFBPs), thus greatly reducing its circulating concentration of active units to a significant low level, making circulating IGF-1 does not cross BBB efficiently, even though IGF-1 can cross BBB indirectly by a transcytosis process involving low-density lipoprotein receptor-related protein 2 (LRP2) and the IGF1 receptor [194-196]. The best strategy to improve the efficiency of crossing BBB is to identify small peptides mimicking IGF-1 which can activate IGF-1R and induce its biological

activity. To achieve this purpose, we may adopt a two-step screening method involving antibody selection and IGF-1R binding. First, we will screen synthetic peptide library with an antibody specifically recognizing the active site of IGF-1. Then we will screen the selected peptides by binding to the extracellular domain of IGF-1R to confirm the selected peptides can bind to the IGF-1R. Our strategy will greatly reduce the opportunity of selecting peptides that non-functionally bind to IGF-1R.

4.1.8 A working model for the MPP⁺-mediated apoptotic and IGF-1-mediated survival signaling pathway

Based on our current data, we proposed a working model of MPP⁺-induced cell death and IGF-1-mediated antiapoptotic signaling through JNK (Fig. 12). Previous report has demonstrated that MPP⁺ induced the activation of GSK-3 β [176]. Thus, in the treatment with MPP⁺, the activated GSK-3 β will then trigger the activation of MKK4, JNK and promote subsequent apoptotic cell death of SH-EP1 cells. In contrast, in the presence of IGF-1, the PI3K/AKT pathway will be activated. Consequently, activated AKT will block the activity of GSK-3 β by phosphorylating its Ser9. Phosphorylation of GSK-3 β by AKT will down regulate the activity of MKK4 and JNK and thus promotes cell survival against MPP⁺ neurotoxicity.

Figure 12. Working model for the MPP⁺-mediated apoptotic and IGF-1-mediated survival signaling pathway.

In conclusion, our data show that IGF-1 provides a strong protection against MPP⁺-mediated SH-EP1 cells death through the PI3K/AKT pathway, not the MAPK/ERK pathway. Moreover, PI3K/AKT pathway dependent SH-EP1 cells survival is mediated by inactivation JNK via AKT-dependent GSK-3 β inhibition. Thus, our results may provide a new insight in the treatment of PD by targeting GSK-3 β to block the pro-apoptotic JNK Signaling.

4.2 Conclusion

PD is primarily caused by the degeneration of dopaminergic neurons in the SNpc of PD patients. There are no proven treatments that can arrest or reverse the progression of PD, hence it is urgently required to derive specific neuroprotective strategies to

effectively delay or stop the disease progression. However, this step will only be attained if the neuroprotective molecular mechanisms are better understood. In our present study, we employed MPTP model, a well established PD model, to study the neuroprotective mechanism of the growth factor IGF-1 in SH-EP1 neuroblastoma cells. Herein, we demonstrated that IGF-1 effectively protects SH-EP1 cells from cell death by inhibiting MPP⁺-induced apoptosis. Through western blots and cell viability assays, PI3K/AKT pathway was shown to be necessary for the IGF-1-conferred neuroprotection, whereas MAPK/ERK, the other signaling pathway activated by IGF-1, was shown not to be involved in MPP⁺-induced cell death. It was also demonstrated that AKT exerted its protective effect through phosphorylation and subsequent inactivation of GSK-3 β . Finally, we found interplay between PI3K/AKT and JNK inhibition and this interplay was shown to be mediated by AKT-dependent GSK-3 β inactivation. Our findings not only suggest new insights into the neuroprotective mechanisms of IGF-1 in the PD progression but also provide a potential possibility of using IGF-1 in the future PD treatment.

Part II Molecular mechanisms underlying the pro-metastatic effect of Dph3 in murine melanoma cells

ABBREVIATIONS

AIF	Apoptosis-initiating factor
ALM	Acral lentiginous melanoma
<i>blastacidin</i> +	Blasticidin-resistant gene;
CMV	Cytomegalovirus promoter
DelGEF	Deafness Locus Associated Putative Guanine Nucleotide Exchange Factor
Dph3	Diphthamide synthesis 3
eEF-2	Eukaryotic elongation factor 2
FAK	Focal adhesion kinase
GNAQ	Guanine nucleotide-binding protein Q
Kti11	<i>Kluyveromyces lactis</i> killer toxin insensitive 11
LMM	Lentigo maligna melanoma
LTR	Long terminal repeat
MM	Malignant melanoma
MMP-2	Matrix metalloproteinase-2
MT1-MMP	Membrane type 1-matrix metalloproteinase
NM	Nodular melanoma
Ovca1	Ovarian cancer-associated gene 1 protein
pA	Poly(A) signal sequence
PTEN	Phosphate and tensin homologue
3'RACE	3' Rapid Amplification of cDNA Ends
5'RACE	5' Rapid Amplification of cDNA Ends
RanGEF	Ran Guanine nucleotide Exchange Factor
RGP	Radial growth phase
Rib	Ribozyme sequence

SA	Splicing acceptor
SD	Splicing donor
SSM	Superficial spreading melanoma
VGP	Vertical growth phase

ABSTRACT

Melanoma is the most dangerous skin cancer due to its highly metastatic potential and resistance to chemotherapy. Currently, there is no effective treatment for melanoma once it is progressed to metastatic stage. Therefore, further study to elucidate the molecular mechanism underlying the metastasis of melanoma cells is urgently required for the improvement of melanoma treatment. In the present study, we found that dipthamide synthesis 3 (Dph3) is involved in the metastasis of B16F10 murine melanoma cells by insertional mutagenesis. We demonstrated that Dph3 disruption impairs the migration of B16F10 murine melanoma cells. The requirement of Dph3 in the migration of melanoma cells was further confirmed by gene silencing with siRNA *in vitro*. In corresponding to this result, overexpression of Dph3 significantly promoted the migratory ability of B16F10 and B16F0 melanoma cells. Moreover, down regulation of Dph3 expression in B16F10 melanoma cells strikingly inhibits their cellular invasion and metastasis *in vivo*. Finally, we found that Dph3 induces the activation of the AKT signaling pathway. We further confirm the involvement of AKT pathway in Dph-3-promoted melanoma migration and invasion by incubation of B16F10 cells with LY294002, an AKT inhibitor, or overexpression of D2AKT in B16F10 cells. To conclude, our findings suggest a novel mechanism underlying the metastasis of melanoma cells which might serve as a new intervention target for the treatment of melanoma.

CHAPTER 5 INTRODUCTION

5.1 Melanoma

Melanoma, one of the three most popular human skin cancers, arises from malignant transformation of neural-crest derived melanocytes in the skin [197]. Melanocytes are specific pigment-producing cells mainly located in the basal layer of the epidermis, hair bulb, eyes, ears, and meninges [198]. Melanocytes produce and transfer melanin to surrounding keratinocytes, thereby determining the degree of pigmentation of the skin and protecting skin from sun-induced damage by absorbing UV radiation [199]. UV radiation, especially UVB which is a minor component of sunlight, is the most important environmental factor causing melanoma. UVB is shown to induce DNA damage, upregulate gene expression or suppress immune reaction [200]. Melanoma nearly exclusively affects white populations, whereas its incidence is very low in populations of Asian origin, and people of African descent are virtually completely unaffected by melanoma [201-202]. Although melanoma only accounts for 4% of all skin cancer, it is the most deadly one and is responsible for 80% of deaths of skin cancers due to its rapid growth and highly metastatic potential [203]. Melanoma incidence has been increasing at an alarming rate in most countries with white population over the last 50 years; currently, the average lifetime risk of developing melanoma has reached 1 in 50 in many western countries [204]. Most melanoma patients can be successfully treated by surgical resection of the tumor at the early stage of development and 80% of cases are treated in this way. However, there is still no effective treatment for melanoma currently once it is progressed to metastatic phases and the treatment efficiency of metastatic melanoma has not been significantly improved over the past 50 years, with a median survival rate of 6 months and 5-year survival rate of less than 5% [197, 205-207].

5.1.1 Melanoma progression

According to the Clark model of melanocytes progression to melanoma based on clinical and histopathological features (Fig. 13), there are 5 distinct stages in the evolution of melanocytes to malignant melanoma based on histological criteria [199, 208]: (1) common acquired and congenital benign nevi with structurally normal melanocytes. Benign nevi rarely develop to cancer because their growth is limited probably due to oncogene-induced senescence. (2) dysplastic nevi with structural and architectural atypia. Dysplastic nevus may develop from a preexisting melanocytic nevus or as a new lesion. (3) radial growth phase (RGP) melanoma, an intra-epidermal lesion that can grow laterally and remain largely confined to the epidermis. RGP melanoma is considered to be at the early stage of primary melanoma development without metastatic capacity. (4) vertical growth phase (VGP) melanoma that infiltrates and invades the dermis as a large cluster of cells. VGP melanoma is believed to have malignant potential and metastatic competence. (5) metastatic melanoma. The principle organs of melanoma metastasis are lungs, brains, skin and liver [203]. Individual melanomas may skip steps in their progression process, appearing without identifiable intermediate lesions.

Figure 13. Clark model of melanoma Progression.

Melanoma arises from melanocytes that normally located in the epidermis' basal layer. In the early stages of melanoma development, benign nevi occur with increased number of melanocytes. The development of nevi is a relatively frequent event but the transition of nevi to more malignant disease is relatively rare. In addition, some dysplastic melanocytes do not form nevi but directly progress to metastatic melanoma. RGP melanoma is considered as the primary malignant stage. In the VGP, melanoma cells show pagetoid spread and have penetrated the basement membrane[199].

5.1.2 Genetic alterations in melanoma

Melanoma tumorigenesis, as a multistep progress, requires a sequential accumulation of genetic alterations that culminate in cellular transformation and the key genetic mutations are summarized in the table 3 below. The common genetic alterations in sporadic and familial melanomas consist of activating mutations in RAS/RAF/MEK/ERK pathway, and inactivating changes in CDKN2A and PTEN tumor suppressors [199].

Table 3. Selected genetic alterations in melanoma

Gene	Alteration	Frequency	Pathways affected by alteration	References
Kinases or signaling factors				

BRAF	Mutated	50-70%	MAPK	[209]
NRAS	Mutated	15-30%	MAPK	[209]
AKT3	Amplified	25%	Cell cycle	[210]
KIT	Mutated	1% overall	MAPK/PI3K	[211]
CDK4	Mutated or amplified	5%	Cell cycle	[212]
Transcription factors				
MITF	Amplified	20%	Melanocyte lineage and cell cycle	[213]
MYC	Amplified	20%	Cell cycle	[214]
Tumour suppressors				
CDNK2A	Mutated or deleted	30-70%	Cell cycle	[215]
p53	Mutated or deleted	10%	Cell cycle	[216]
PTEN	Mutated or deleted	30-50%	PI3K	[217]
APAF-1	Mutated	40%	Cell cycle	[218]

5.1.2.1 RAS/RAF/MEK/ERK pathway

The most popular genetic alteration in melanoma is the abnormal activation of the RAS/RAF/MEK/ERK pathway which is a key regulator of melanoma cell proliferation and survival. Hyperactivation of ERK is found in up to 90% of human melanoma cases [219]. Activation of this pathway is mainly achieved by gain function mutation in components of the MAPK pathway including NRAs, BRAF, KIT, and guanine nucleotide-binding protein Q (GNAQ) [209, 211, 220-221]. NRAS and BRAF mutations are associated with majority of melanomas, with NRAS counting for 15-30% and BRAF counting for 50-70% of all melanomas, respectively. The most popular replacement is leucine for glutamine at position 61 (Q61L) for NRAS and glutamic acid for valine at position 600 (V600E) for NRAF [209]. ERK can also be activated by growth factors or mutational activation of growth factor receptors [222-223].

5.1.2.2 PTEN/PI3K/AKT pathway

Another important signaling pathway involved in melanoma progression is the Phosphate and tensin homologue (PTEN)/PI3K/AKT pathway. PTEN is a tumor suppressor gene which is found to be deleted and mutated in 30-50% melanoma cases [224-225]. PTEN encodes a lipid and protein phosphatase and negatively regulate the activity of PI3K pathway by inhibiting the phosphorylation of AKT by dephosphorylating and inactivating PI3K at the inner cellular membrane, and inactivation of PTEN by mutation, deletion, or epigenetic silencing results in constitutive activation of this pathway [226]. AKT plays a critical role in melanoma by promoting proliferation and cell survival and hyperactivation of AKT is found in up to 60% of melanomas [210]. Besides loss of PTEN, AKT can also be activated by PI3K mutation which has been found in 3% of metastatic melanomas or through the activation of NRAS [199, 227]. Of the three isoforms of AKT, AKT3 is preferentially activated in 43-67% of sporadic melanomas and is responsible for the reduced sensitivity of cells to apoptotic stimuli and progression to a more aggressive tumor [210]

5.1.2.3 The CDKN2A tumor suppressive pathway

CDKN2A is composed of 4 exons and encodes for two distinct proteins, p16^{INK4a} (exons 1 α , 2, and 3) and p14^{ARF} (also called p19^{Arf} in mouse) (exons 1 β , 2, and 3) by means of a different promoter and through use of alternative reading frames [228]. 25-40% of familial melanoma cases bear an inactivating mutation in CDKN2A which is achieved by mutation, deletion or promoter methylation [229]. Both p16^{INK4a} and p14^{ARF} are potent tumor suppressor genes with distinct but equally important roles in cell cycle and apoptosis regulation [230]. By binding to the cyclin-dependent kinases CDK4 and CDK6, p16^{INK4a} inhibits its interaction with cyclin D, thereby blocking G1/S cell cycle check point through inactivation of the retinoblastoma tumor-

suppressor gene (Rb) [231]. p14^{ARF} deficiency abolishes oncogene-induced senescence and increases susceptibility to melanoma transformation through controlling the level of the tumor suppressor protein p53 by promoting its ubiquitination and subsequent degradation via binding and inactivating the nuclear protein MDM2 [232-233].

5.2 Tumor Metastasis

Tumor metastasis, the spread of malignant tumor cells from a primary site to distant tissues and their subsequent growth into tumor, is the most life-threatening factor for most cancer patients and is responsible for 90% death of cancer patients [234-235]. Researchers have been studying the tumor metastasis process for over 100 years now, and are just slowly beginning to understand the underlying mechanisms of how metastatic cells rise from the primary tumors, as well as why certain types of cancer have a preference to metastasize to particular organs.

5.2.1 “Seed” and “Soil” hypothesis

In 1889, after scrutinizing over 900 autopsy records of patients with different primary cancers, Stephen Paget noticed a non-random pattern of metastasis to visceral organs and bones. He proposed that the outcome of metastasis was not a random process, but rather that certain disseminated tumor cells (the “seed”) have specific preference to certain organs (the “soil”) and metastasis formed only when the seed and soil were compatible [236]. His proposal was well supported by the fact that mechanical arrest of disseminated tumor cells in the capillary bed of distant organs was indeed important, but subsequent proliferation and growth resulting in a secondary lesion were influenced by the specific organ cells [237].

5.2.2 Metastasis progresses

Tumor metastasis consists of a series of sequential, interrelated biological processes that can be envisioned a succession of cell biological changes (Fig. 14), including cancer cells separating from original tumor, local invasion through surrounding tissues, intravasation into and transferring through the lymphatic vessels and blood stream, arresting in the parenchyma of distant tissues, formation of small nodules (micrometastasis), and finally, growth of micrometastatic lesions into macroscopic tumors (colonization). The whole process is then repeated to produce secondary metastasis. Despite the prevalence of tumor metastasis in cancer patients, metastasis is an extremely inefficient process and only a few cells in a primary tumor are considerate to be able to give rise to a metastasis. To successfully metastasize to a distant location, tumor cells must undergo all of the stems of the process. A failure or an insufficiency at any of the steps can result in failure to colonize and proliferate in the distant organ [236, 238-239].

Figure 14. The main steps in the development of metastasis.

a: cellular transformation and tumor growth; b: angiogenesis; c: detachment and local invasion; d: embolism, and circulation; e: extravasation; f: proliferation [236].

5.3 pDisrup 8 vector

The pDisrup 8 vector we used was specially designed by Dr. Han Jiahuai and colleagues based on retroviral insertion-mediated mutagenesis [240-242]. The pDisrup 8 vector which is able to randomly induce insertions into the genomic DNA contains two functional domains and the 5' and 3' long terminal repeat (LTR) from retrovirus (Fig. 15A). The first functional domain of the vector contains a splice acceptor (SA), a ribozyme sequence (Rib) (Fig. 15B) and a polyA signal sequence (pA). The second

domain contains the cytomegalovirus (CMV) promoter, a blasticidin resistant gene (*Blasticidin⁺*) and a splice donor (SD). Retroviral insertion can produce a truncated gene product or create a single mutation to partially abolish gene expression depending upon the site of insertion [243]. Truncation of the gene product might yield unpredictable results, since some truncated proteins might display dominant properties, causing either a gain- or loss-of-function. In order to produce gene deletion and avoid gene truncation, the pDisrup 8 vector was incorporate with a Rib sequence which is a RNA molecule that possesses enzymatic, self-cleaving activities. Any transcript containing this ribozyme will be destroyed because the mRNA will be completely self-cleaved and subsequently degraded. By adopting a poly A trapping strategy, only cells harboring the pDisrup 8 vector in a gene area (either in an intron or exon) could be selected by blasticidin. This is because the transcription of *blasticidin⁺* gene is dependent on the endogenous polyA, when the functional insertion occurs in a non-gene region, or in a gene but in a wrong direction, the transcript of *blasticidin⁺* gene driven by the CMV promoter will be degraded due to lacks a poly A signal and no *blasticidin⁺* gene product is produced.

Figure 15. Representation of pDisrup 8 vector-mediated insertional mutagenesis.

(A) Diagram of the pDisrup 8 vector used for gene knock down. (B) Sequence of the ribozyme in the vector [242].

5.4 Dph3 and diphthamide

5.4.1 Dph3

Dph3 is a small acidic protein with 82 amino acids, encoding a CSL zinc finger containing protein [244-245]. It is evolutionally conserved and ubiquitously expressed in various mouse and human adult tissues. Dph3 is originally identified as a gene involved in the first step in the biosynthesis of diphthamide, a unique posttranslationally modified histidine residue occurs only in translation elongation factor 2 (eEF-2) [246]. Except for its role in synthesis of diphthamide, Dph3 also plays an important role in embryonic development, as its genetic ablation causes embryonic lethality in mice. In addition, down regulation of Dph3 in HeLa cells induces increased extracellular secretion of proteoglycans by association with deafness locus associated

putative guanine nucleotide exchange factor (DelGEF), a homologue of Ran guanine nucleotide exchange factor (RanGEF) [247-248]. The analogue of Dph3 in yeast, Kti11 (*Kluyveromyces lactis* killer toxin insensitive 11), has been found to be involved in regulating the sensitivity of yeast to zymocin [249-251]. Furthermore, Kti11 has been reported to interact with various proteins, such as core-Elongator (Elp1-Elp3), ribosome proteins, eEF2 and the diphthamide synthesis factors (Dph1 and Dph2), indicating that Dph3 may play roles in various biological processes [246, 252].

5.4.2 Diphthamide

Diphthamide is conserved from archaeobacteria to eukaryotes [244, 253]. Diphthamide biosynthesis is carried out by stepwise modifications to the His⁷¹⁵ residues (His⁶⁹⁹ in yeast) of eEF-2 that requires the action of five proteins including Dph1-Dph5 [246]. Although the physiological roles of diphthamide are still not clear, the presentation of Dph1 to Dph5 in all eukaryotic organisms indicated that this modified histidine residue may have important biological functions. This notion is well supported by the fact that Dph1, also known as ovarian cancer-associated gene 1 protein (Ovca1), is a tumor suppressor gene that is frequently lost its heterozygosity in breast and ovarian tumors [254-255]. Further studies with Ovca1 heterozygous mice ascertained the significant roles of Ovca1 in cell proliferation, embryonic development and tumorigenesis [256-257].

5.5 Aim of the study

There is currently no effective treatment for melanoma once it is progressed to metastatic stage. Therefore, further study to elucidate the molecular mechanism underlying the metastasis of melanoma cells is urgently required. In the present study, we aimed to identify novel genes involved in the metastasis of melanoma cells and investigated the underlying mechanism of the novel genes in the melanoma metastasis, with the goal of finding new targets for treatment of metastatic melanoma. The first

aim of our study is using retroviral-mediated mutagenesis to randomly disrupt genes in B16F10 cells, followed by screening for changes in metastatic properties and subsequently the disrupted genes were identified. The second aim is to confirm the role of a disrupted gene, Dph3, in melanoma metastasis with up- and down-regulated expression of Dph3. The third aim is to investigate the underlying mechanism in Dph3-mediated melanoma metastasis. It is hoped that these results would eventually produce a useful clue for effective cure for melanoma, especially in the metastatic stage of melanoma.

CHAPTER 6 MATERIALS AND METHODS

6.1 Cell culture and reagents

Marine melanoma B16F10, B16F0 cells, human colon cancer HCT116 cells, human ovary cancer A2780 cells, human skin cancer A431 cells and NIH 3T3 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium with 10% fetal calf serum (FCS) (Gibco, Invitrogen, USA), and 1% Penicilin/Streptomycin mix (Gibco, Invitrogen, USA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Specific inhibitor for AKT (LY294002) was obtained from Calbiochem (USA). Lipofectamine 2000 was purchased from Invitrogen (USA).

6.2 Medium

Luria Bertani (LB) medium	10 g/L bacto-tryptone, 5 g/L bacto-yease extract, 8.56 mM NaCl, pH7.4
LB agar	LB medium with 15g/L Bacto-agar

6.3 Plasmids and DNA constructs

The pDisrup 8 vector was a gift from Dr. Han Jiahuai (Xiamen University, China). The short small interfering RNA (siRNA) was constructed with a sequence specifically targeted to the mRNA of mouse Dph3 gene: (5'-C AAG GAG UUA GUU AAA UGC -3'). Target and scrambled control oligonucleotides duplexes were cloned into pSilencer4.1-CMV vector (Ambion, USA) according to the manufacturer's instructions. The Dph3 and its truncated clones were cloned into the sites of EcoRI

and XhoI of pIRES2-EGFP vector (Clontech, USA) containing a Myc-tag with gene specific primers. The primers used were as follows: Dph3 (5'- CCG CTCGAG GCC ACC ATG GCG GTG TTT CAC GAC -3' and 5'- G GAATTC GCA TTT AAC TAA CTC CTTG-3'), Dph3 1-40aa (5'- CCG CTCGAG GCC ACC ATG GCG GTG TTT CAC GAC -3' and 5'-G GAATTC TTCC AAA TCT TCC TTG GTG ATG-3'), Dph3 21-60aa (5'- CCG CTCGAG GCC ACC ATG ACA TAT TTC TAC CCT TGCC-3' and 5'-G GAATTC GTC ATAAA TCA CTTTT ATAATG-3'), Dph3 41-82aa (5'- CCG CTCGAG GCC ACC ATG AAT GGA GAA GAT GTG GCC ACG-3' and 5'- G GAATTC GCA TTT AAC TAA CTC CTTG-3'), Dph3 α -helix deletion (5'- CCG CTCGAG GCC ACC ATG GCG GTG TTT CAC GAC -3', 5'- G GAATTC GCA TTT AAC TAA CTC CTTG-3', 5'-GGA TAA CTT TGC CAT CAC CGG AGA AGA TGT GGC CACG-3', 5'-CGT GGC CAC ATC TTC TCC GGT GAT GGC AAA GTT ATC C-3'). A constitutively active mutant D2AKT (T308D/S473D) plasmid was kindly provided by Dr. Takashi Tsuruo [258]. Transfection was performed with lipofectamine 2000 following the manufacture's manual. For pDisrup 8 clone selections, cells were selected with Blasticidin S.HCl at 25 μ g/ml (Invitrogen, USA).

6.4 Western blot

Western blot was performed as described in Part I, Chapter 2. The antibodies used were summarized in table 4.

Table 4. Antibodies used for Western Blot

Antibody	Company	Dilution factor
anti FAK	BD Bioscience	1000
phospho-FAK	Upstate	1000
Anti Flag-tag	Sigma-Aldrich, USA	1000
Anti Myc-Tag	Invitrogen	1000

Anti β -Catenin	Cell signaling technology, USA	1000
Anti phospho- β -catenin	Cell signaling technology, USA	1000

6.5 Cell attachment assay

96-well tissue culture plates were coated with Collagen Type IV (Sigma Aldrich, USA) followed by washing with PBS before blocking with 0.5% BSA. 1×10^5 melanoma cells were seeded onto the pre-coated 96-well plates and incubated for 15, 30, 60 and 120 min. After incubation, the unattached cells were removed and the plate was stained with 0.5% crystal violet in 20% methanol for 20 min at room temperature and then washed with tap water. Cell attachment was evaluated spectrophotometrically by dissolving the stain with 20% acetic acid and measured at a wavelength of 570 nm with Tecan (Männedorf, Switzerland).

6.6 Wound healing assay

Wound healing assay was performed as previously described [259]. Briefly, B16F10 and B16F0 murine melanoma cells were seeded in 60 mm dishes and cultured at 37 °C overnight to produce a confluent monolayer. After starvation in serum-free medium for 24 hours, a wound was created by scratching the monolayer with a 200 μ l yellow sterile pipette tip. The wounded monolayer was then washed twice to remove cell debris and incubated with fresh normal medium. The area of cell-free scratch was photographed at 0 h, 12 h and 24 h after scratching respectively. The wound healing effect was determined by measuring the percentage of the remaining cell-free area compared with the area of the initial wound.

6.7 In vitro invasion and migration assay

Invasion of melanoma cells was determined by BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences, USA) assay in vitro according to the manufacturer's instructions. In brief, 1×10^5 cells with 500 μ l in serum-free medium were added into the upper chamber and 750 μ l of NIH-3T3 fibroblast conditioned medium was added into the lower chamber, serving as chemo-attractant. After incubation in humidified tissue culture incubator, 37 °C, 5% CO₂ atmosphere for 24 h, the non-invasive cells in the upper surface of the membrane were removed by “scrubbing” with cotton tipped swab and the invasive cells migrating to the lower surface of the membrane were fixed and stained with 0.5% crystal violet for 30 minutes. Cell counting was then carried out by photographing the membrane through the microscope. 20 random fields under microscope at 20X magnification are taken. The migration assay was performed with the same strategy, just that the chamber membrane was not coated with matrigel while assessing cell motility.

6.8 RNA isolation and reverse transcription

Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the product instruction. Briefly, B16F10 and B16F0 murine melanoma cells were seeded on 60mm dishes and allowed to reach 80-90% confluent. Cells were lysed directly by adding 1ml of TRIzol reagent, and pipetting the cells up and down several times. The homogenized samples were incubated for 5 min at room temperature and then 0.2ml of chloroform was added into the samples, mixed by shaking vigorously for 15 s and incubated at room temperature for 3 min, followed by centrifugation at 12,000 g for 15 min at 4°C. The colorless supernatant was transferred to a fresh tube and then mixed with 500 μ l of isopropanol to precipitate the RNA. The samples were incubated for 10 min at room temperature and centrifuge at 12,000 g for 10 min at 4°C. Supernatant was aspirated and the RNA pellet was washed with 75% ethanol, followed by centrifugation at 7,500 g for 5 min at 4°C again. RNA pellet was air-dried briefly and

subsequently dissolved in 20µl of DEPC-treated water. The concentration of total RNA was detected by spectrophotometry at OD260. The extracted RNAs were stored at -80°C for later use.

Reverse transcription (RT) was carried out using superscript III reverse transcriptase (Invitrogen, USA) as described in the manufacturer's manual. In brief, 1 µg of total RNAs was mixed with 1 µl oligo-d(T)18 primer (50 µM), 1 µl dNTP (10mM each), and DEPC-treated water to a final volume of 13 µl. This mixture was incubated at 65 °C for 5 min, and cooled on ice for at least 1 min. The sample was briefly centrifuged to collect content, followed by adding of a reaction mixture of 4 µl 5X First-Strand Buffer, 1 µl DTT (0.1 M), 1 µl RNaseOUT™ Recombinant RNase Inhibitor (40 units/µl), and 1 µl of SuperScript™ III RT (200 units/µl). The sample was mixed by pipetting gently up and down and incubated at 50 °C for 1 h. Finally, the reaction was stopped by heating at 70 °C for 15 min. The cDNAs were stored at -20 °C until further use.

6.9 Real-time PCR

The real-time PCR was performed on ABI Prism 7500 Sequence detection system (Applied Biosystems, CA) with the KAPA SYBR® qPCR Kit (KAPA Biosystems, USA) according to the manufacturer's instructions. The conditions for real-time PCR amplification were as follows: 2 min at 50 °C, 1 min at 95 °C, 40 cycles at 95 °C for 3 s and 60 °C for 1 min, followed by melting curve analysis at the end of each run from 60 °C to 95 °C. The primers used were as follows: Dph3 (Forward: 5'- TTG CCA TCA CCA AGG AAG ATTT-3', Reverse: 5'-GTG CTG GGA CTG TTT CTC CAC - 3'), MMP-9 (Forward: 5'-CTG GAC AGC CAG ACA CTA AAG -3', Reverse: 5'-CTC GCG GCA AGT CTT CAG AG -3'), β-actin (Forward: 5'-GCT CTT TTC CAG CCT TCCTT-3', Reverse: 5'-TGA TCC ACA TCT GCT GGAAG-3'). The target

mRNA level of control cells normalized to the level of β -actin mRNA, was defined as 1. Results were obtained from three independent experiments.

6.10 MMP-9 activity assay

The activity of MMP-9 was determined by QuickZyme Mouse MMP-9 activity assay (QuickZyme BioSciences, Netherlands) according to the manufacturer's instructions. Briefly, after transfection for 48 h, cells were washed with fresh medium and replaced with serum-free medium. After additional 24 h, the medium was collected and centrifuged at 10000 g for 10 min. Respective supernatant was added to the 96-well strip coated with MMP-9 antibody and incubated at 4 °C overnight. After washing with wash buffer for 4 times, 50 μ l assay buffer was added into the well, followed by adding 50 μ l detection reagent. After incubation at 37 °C for 1h, OD405 was measured with Tecan (Männedorf, Switzerland).

6.11 Animals and experimental metastasis assay

Female C57BL/6J mice at 6–8 weeks old (15-20g) were purchased from the laboratory Animal Center of NUS (Singapore). Mice were maintained at dark/light cycles of 12 h duration with food and water available *ad libitum*. 12 animals were randomly divided into two experiment groups. For experimental metastasis analysis, the mice were injected at the lateral tail vein with (5×10^5) B16F10 cells carrying control or Dph3 siRNA plasmids. Mice were sacrificed 2 weeks after inoculation and all organs were examined for the presence of macroscopic metastases. Lung and liver metastatic nodules were determined under a dissecting microscope. Animal handling and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University (ARF SBS/NIE-A0075).

6.12 Statistical Analysis

For quantitative PCR analyses, results were obtained from triplicate experiments on all the samples and data from all trials were averaged. Numerical results were analyzed using independent mean T-test and expressed in mean $\pm$ standard error (SE). Statistical analysis was performed using post hoc testing using Bonferroni's method. To analyze the data from experimental lung metastasis assay, we used the nonparametric Mann-Whitney U test to compare the mean values between two groups. Differences were considered statistically significant at $p < 0.05$.

CHAPTER 7 RESULTS

7.1 Identification of a novel role of Dph3 in melanoma metastasis

The murine melanoma B16F10 cell line is a widely used model to study the metastasis of melanoma for its high metastatic potential after intravenous injections [260-261]. To identify genes involved in melanoma metastasis, we transfected melanoma B16F10 cells with pDisrup 8 vector to randomly produce insertions into the genomic DNA, followed by selection with blasticidin (25 µg/ml) to obtain mutated cell clones [240]. The migratory ability of the selected mutant cell clones was then determined by wound healing assay. Finally, cell clones with increased or decreased migration potential were further analyzed by the RT-PCR and 3' RACE to identify the genes disrupted by pDisrup 8 vector. With this strategy, several candidate genes were identified, including a gene named Dph3 and this candidate was designated as Dph3^{mut} which exhibited decreased motility potential.

To verify whether the gene identified by this method was indeed disrupted in melanoma B16F10 cells, real-time PCR was carried out to determine the gene expression of Dph3, as shown in Fig. 16A, the expression of Dph3 was greatly reduced in this cell clone compared to the control cells. Cellular invasion, a characteristic of metastatic tumors, involves cell attachment to extracellular matrix (ECM), ECM degradation and cell migration [262-263]. To determine if loss function of Dph3 affects cell attachment and migration, cell attachment experiment was performed. Compared to control cells, disruption of Dph3 significantly reduced cell adhesion to substratum at different attaching period of time (Fig. 16B). Then we performed wound healing and transwell assays to evaluate the cell motility. As shown in Fig. 16C, 12 h after scratching, the area of wound recovered by the migration of Dph3^{mut} cells was not significant and only less than half of that for control ones. 24

hours later, wild type cells had almost closed up the wound, but not Dph3^{mut} cells. Consistently, there were less Dph3^{mut} cells that migrated across the membrane of the Boyden chamber compared to the wild type cells (Fig. 16D). In summary, disruption of Dph3 led to reduced cell adhesion and significantly impaired the migration of B16F10 cells.

Figure 16. Identification of a novel role of Dph3 in the metastasis of melanoma B16F10 cells.

A, Dph3 expression in Dph3^{mut} cells was analyzed by real-time PCR. B, a total of 1×10^5 cells were seeded into pre-treated 96-well plates for the indicated times. Attached cells were stained with crystal violet and measured spectrophotometrically. Results are expressed as absorbance values. C, wound healing of control and Dph3^{mut} cells was performed and representative pictures of the wound distance were taken at each time point as indicated. Scale bars: 1mm. D, the cell motility was evaluated by transwell assays. Representative pictures were taken after staining with crystal violet. Scale bars: 200 μ m. Data are collected from three independent experiments and are average $\pm$ S.E. values. * $p < 0.05$, ** $p < 0.01$, compared to wild type cells.

7.2 Confirmation of the role of Dph3 in melanoma migration by gene silencing and overexpression

To ascertain the gene identified was indeed responsible for reduced migration in B16F10 cells, we investigated whether reduced migration of melanoma cells could be reproduced by gene silencing with siRNA. To perform this experiment, we first silenced the expression of Dph3 in melanoma B16F10 and B16F0 cells with a siRNA-incorporated plasmid targeting a specific site of mouse *Dph3* mRNA. As shown in Fig. 17A, the expression of Dph3 in cells transfected with siRNA plasmid (siDph3) was significantly decreased compared with the cells transfected with scrambled siRNA (siCTL). Transfected cells were then subjected to wound healing and transwell assays to evaluate their migratory potential. Both B16F10 and B16F0 cells transfected with a control siRNA were able to close a wound by 24 h. However, the wound inflicted on cells transfected with Dph3 siRNA had not yet closed up at this time (Fig. 17B). Consistently, results from transwell assay also showed that the cell number of siDph3 cells moved across the membrane was much fewer than the siCTL cells (Fig. 17C). Taken together, these results indicated that silencing of Dph3 could reproduce the effect of Dph3 disruption by pDisrup 8 plasmid and drastically reduced the cell motility of melanoma cells.

Figure 17. Dph3 silencing decreases the migration of melanoma B16F10 and B16F0 cells.

A, pSilence4.1-CMV vectors carrying siDph3 or siCTL were transfected into melanoma B16F10 and B16F0 cells separately. mRNA expression level of Dph3 was determined by real-time PCR. B, both siDph3 and siCTL transfected cells were plated in 6cm dishes. A wound healing assay was performed to determine the metastatic potential of cells and representative pictures of the wound distance were taken at 0 and 24 h post scratching as indicated (left). The percentage of wound closure was quantified (right). C, the cell motility was evaluated by transwell assays. Data are from three repeated experiments and are average $\pm$ S.E. values. * $p < 0.05$, ** $p < 0.01$, compared to control cells.

To determine whether the effect of Dph3 in cell metastasis is general to cancer cells or cell type specific, we transfected human colon cancer cell HCT116, human ovary cancer cell A2780 and human skin cancer cell A431 with scramble siRNA (siRNA) and Dph3 specific siRNA (siDph3) and examined the migration of these cell lines by transwell assay or wound healing. As shown in Fig. 18, siDph3 only affects the migration of A431 cells (Fig. 18A) and has no effect on the migration of A2780 (Fig. 18B) and HCT116 cells (Fig. 18C), indicating that the role of Dph3 in metastasis may be cell type specific and only has effect on the metastasis of skin cancer cells.

Figure 18. Dph3 silencing decreases the migration of human skin cancer A431 cells, not human ovary cancer A2780 cells and human colon cancer HCT116.

pSilence4.1 vectors carrying siDph3 or siCTL were transfected into human skin cancer A431 cells, human ovary cancer A2780 cells and human colon cancer HCT116 separately. A, the cell motility of A431 cells was determined by wound healing assay and representative pictures of the wound distance were taken at 0 and 18 h post scratching as indicated (upper panel). The percentage of wound closure was quantified (lower panel). The cell motility of A2780 (B) or HCT116 (C) was evaluated by transwell assay. Data are from three repeated experiments and are average $\pm$ S.E. values. * $p < 0.05$, ** $p < 0.01$, compared to control cells.

To further confirm the role of Dph3 in melanoma motility, we cloned Dph3 into pIRES-EGFP vector and transfected it into melanoma B16F10 and F0 cells. The transfection efficiency was confirmed by the expression of green fluorescence protein (GFP) (Fig 19A). To further determine the overexpression of Dph3, we examined the expression of Dph3 by western blot with a Myc-tag antibody (Fig 19B). The migration of Dph3 overexpressed cells was then examined by wound healing assay and migration assay. As shown in Fig. 19C, the wound gaps of cells which were stably

expressing Dph3 gene healed faster than control cells. In accordance with this result, there were more Dph3 overexpressed cells migrated across the membrane (Fig 19D). Matrix metalloproteinases (MMP), which are capable of degrading the various structural components of the ECM, play a critical role in tumor invasion and metastasis and the up-regulation of matrix metalloproteinase (MMP)-9 has been considered as one of the markers for the metastasis of melanoma cells [264-265]. To determine if Dph3 overexpression affects the transcription level of MMP-9, we then tested the effect of Dph3 overexpression on the expression of MMP-9 by real-time PCR. As expected, the mRNA expression of MMP-9 was significantly up-regulated after Dph3 overexpression (Fig. 19E). Consistent with this result, the activity of MMP-9 was also markedly increased with the overexpression of Dph3 (Fig. 19F). Collectively, these results suggest that Dph3 promotes motility of melanoma cells.

Figure 19. Dph3 overexpression promotes the migration of melanoma B16F10 and B16F0 cells.

Dph3 was cloned into pIRES2-EGFP vector and transfected into melanoma B16F10 and B16F0 cells. The cells transfected with an empty vector were used as control. The transfection efficiency was evaluated by the expression of green fluorescence protein (GFP) (A) and the expression of Dph3 was determined western blot with Myc-tag antibody (B). Scale bars: 200 μ m. β -actin was used as a loading control. The cell motility was determined by wound healing assay 18h post scratching and the percentage of wound closure was quantified (C). D, the cell motility was evaluated by transwell assays. Scale bars: 200 μ m. E, real-time PCR was carried out to evaluate the mRNA expression level of MMP-9 in control and Dph3 overexpression cells. F, activity of MMP-9 in control and Dph3 overexpression cells was determined by MMP-9 activity assay. Data are from three independent experiments and are average $\pm$ S.E. values. * $p < 0.05$, ** $p < 0.01$, compared to control cells.

To determine the functional motif of Dph3 required for the metastasis of melanoma cells, a mutant with a predicted 6 amino acids helix deletion and 3 truncated mutants were constructed and transfected into melanoma B16F10 cells (Fig. 20A). The migration of cells transfected with truncated Dph3 clones was then examined by transwell migration assay. As shown in Fig. 20B, all mutants had no effects on the migration of melanoma B16F10 cells, indicating that the full length of Dph3 is required for its role in melanoma migration.

Figure 20. Full length of Dph3 was required for the metastasis of murine melanoma B16F10 cells.

A, Schematic presentation of a mutant with a predicted 6 amino acids helix deletion between 36-41 of the whole protein sequence and 3 truncated mutants of Dph3. B, the cell motility was evaluated by transwell assays after transfection with different mutants.

7.3 Dph3 knock down reduces the metastasis of mouse melanoma B16F10 in vivo

To further investigate the role of Dph3 in the metastasis of melanoma cells in vivo, an experimental metastasis assay was performed. Control and Dph3 knock down cells were injected into the lateral tail vein of C57BL/6J mice. 2 weeks post inoculation, animals were sacrificed and all the major organs were checked for the generation of tumor metastasis. The tumor metastasis was mainly observed in the lungs as previously reported [266]. We found that injection of B16F10 control cells resulted in the formation of numerous lung colonies (median, 280; range, 201-427) whereas silencing of Dph3 significantly suppressed pulmonary metastasis and only generated

one third of lung colonies (median, 90; range, 48-160, $p < 0.01$) (Fig. 21B). In addition, B16F10 control cells produced nodules that occupied a higher percentage of the total lung area, while metastatic nodules of siDph3 cells generated discrete black foci (Fig. 21A). These results implied that Dph3 silencing indeed perturbed the metastasis of melanoma cells not only in vitro but also in vivo.

Figure 21. The metastasis of mouse melanoma B16 F10 was impaired by Dph3 silencing in vivo.

siCTL and siDph3 cells were injected into the lateral vein of C57BL/6J mice respectively. A, representative pictures of lungs from mice were taken after 2 weeks of injection with B16F10 control cells or with Dph3 KD cells. B, numbers of lung metastasis were quantified and showed by each data point, $**p < 0.01$, compared to control cells.

7.4 AKT pathway is involved in Dph3-mediated B16F10 migration and invasion

To determine the signaling pathways which are involved in Dph3-mediated melanoma cell migration and invasion, multiple potential signaling pathways related to migration and invasion of cancer cells were screened. We first examined the phosphorylation of focal adhesion kinase (FAK) which is a crucial signaling component that is activated by numerous stimuli and functions as a biosensor or integrator to control cell motility by performing western blot as FAK had been demonstrated to be involved in the migration of melanoma cells [267-268]. Western blot result showed no significant change for FAK activation in Dph3 overexpressing cells compared to control cells (Fig. 22A). Besides FAK signaling, JNK and ERK signalings had been reported as key regulators of melanoma migration [269-270]. To check whether Dph3-promoted metastasis of melanoma was dependent on JNK and ERK signaling pathways, the activation of JNK and ERK was assessed by western blot. As shown in Fig. 22A, comparable level of phospho-JNK and phospho-ERK was detected in Dph3-overexpressing melanoma cells. In addition, Wnt/ β -catenin signaling pathway which is involved in various cellular activities, including determination, proliferation, differentiation and migration, was also recorded as a key regulator governing the migration of melanoma, we then compared the activation status of β -catenin and GSK-3 β which can directly phosphorylate β -catenin and promote its degradation by western blot to determine the involvement of Wnt/ β -catenin pathway in Dph3 mediated migration of melanoma cells [271-272]. Western blot results demonstrated no drastic difference in the phosphorylation of GSK-3 β and the expression of β -catenin between cells overexpressing Dph3 and control ones (Fig. 22A). Lastly, we had recently identified that PI3K/AKT pathway is involved in NCAM-mediated migration of melanoma cells [259]. To examine whether PI3K/AKT signaling also contributes to Dph3 mediated mobilization of melanoma B16F10 cells, the activation of AKT was analyzed by western blot. As shown in Fig. 22A, phosphorylation of AKT was

strongly upregulated in cells overexpressing Dph3. Above data suggest that overexpression of Dph3 upregulates the activation of AKT. Consistently, when Dph3 was silenced by siRNA in melanoma cells, AKT activation was also down regulated (Fig. 22B). In combination, these results strongly suggest that Dph3 facilitates the activation of AKT signaling pathway in B16F19 murine melanoma cells. To confirm the role of AKT in Dph3-mediated cell migration, constitutively active form of AKT (D2AKT) was introduced into Dph3-silenced cells and the expression of D2AKT was confirmed by western blot with anti-Flag and anti-AKT antibody (Fig. 22C). As expected, active AKT largely restored the impaired migration in Dph3-silenced cells (Fig. 22D). Furthermore, LY292004, a PI3K/AKT specific inhibitor was also employed to dissect the role of PI3K/AKT signaling in cell migration and invasion. As shown in Fig. 22E, Dph3-mediated phosphorylation of AKT was completely blocked by LY294002. As a result, Dph3-promoted migration and invasion in B16F10 cells were also abolished by LY294002, as shown by the transwell migration and invasion assays (Fig. 22F). To conclude, these data indicate that AKT signaling is involved in Dph3 promoted metastasis of melanoma cells.

Figure 22. Dph3 facilitates the invasion and migration of melanoma cells via AKT pathway.

A, western blot shows that the phosphorylation of AKT at Ser-473 was elevated in cells transfected with pIRES-EGFP-Dph3. AKT was used as a loading control. B, the phosphorylation of AKT at Ser-473 was perturbed in cells transfected with Dph3 siRNA plasmid. AKT was used as a loading control. C, expression of D2AKT was confirmed by western blot with antibody against Flag-tag and AKT, and β-actin was used as loading control. D, transwell assays were performed to determine the motility of cells cotransfected with Dph3 silencing plasmid and D2AKT plasmid. Columns are data collected from three independent experiments. E, in the presence of LY294002 (20 μM), cells were incubated for 1 h, protein extracts were analyzed by western blot with antibodies against phosphorylated AKT (S473) or AKT. F, in the presence of LY294002 (20 μM), transwell assays (left panel) and Matrigel invasion assays (right panel) were conducted to evaluate the cell motility and invasiveness after transfection. Representative pictures were taken after staining with crystal violet. Columns are data

collected from three independent experiments and are average $\pm$ S.E. values. * $p < 0.05$, ** $p < 0.01$, compared to control cells.

CHAPTER 8 DISCUSSION AND CONCLUSION

8.1 Discussion

Malignant melanoma is the skin cancer with the highest risk of death for its highly metastatic potential [273]. Both incidence and mortality rate of malignant melanoma continue to climb in recent years [274]. However, there is currently no effective treatment for metastatic melanoma partly due to the complicated mechanism underlying its metastasis. In the present study, we identified a novel role for Dph3 in the metastasis of murine melanoma cells. We found that Dph3 promotes the metastasis of murine melanoma cells in vitro and in vivo through the AKT signaling pathway. Our results may provide a new target for intervention in the melanoma treatment and may improve the future treatment of melanoma.

8.1.1 Insertional mutagenesis as a functional screen for genes involved in murine melanoma metastasis

Insertional mutagenesis is an effective method to induce high-throughput genetic mutations in targeted cells [275]. Compared to chemical mutagenesis, it is relatively easier to identify mutated genes by determine the insertion site of the vector. However, insertion of the vector may abolish gene expression or generate a truncated gene product depending on the insertion site. Truncation of gene product might cause unpredictable effects, because some truncated proteins might show dominant potentials, inducing either gain- or loss- of-function. In order to generate gene deletion and avoid gene truncation, the pDisrup 8 vector was incorporated with a self-cleavage Rib sequence (Fig 15) [242]. The other limitation of this method is that it is more suitable to identity genes conveying a selective growth or survival advantage, because this advantage can significantly reduce the number of cell clones after selection with antibiotics or chemicals. In our experiment, we have to pick up more clones to screen

genes related to metastasis because we use wound healing to isolate genes conferring enhanced or reduced metastatic potential.

8.1.2 Dph3 is involved in the metastasis of murine melanoma cells.

Dph3 is originally identified as a gene involved in the biosynthesis of diphthamide. Diphthamide is conserved in all eukaryotes and archaea and is important for ribosomal protein synthesis, especially for translation fidelity in preventing -1 frameshift mutation [276]. Dph1-4 and Dph5 catalyzed the first and second step of diphthamide biosynthesis, respectively [277]. Although diphthamide is essential for cellular translation, abolishing its synthesis is not embryonic lethal [244]. However, it has been recently reported that Dph3 knock out in mice leads to embryonic lethality [250]. In addition, recent studies had shown that loss of Kti11, the homologue of Dph3 in yeast, can also cause growth defects [251]. Hence, it was proposed that Dph3 may play additional roles, other than diphthamide synthesis in the cells. However, no investigation has been carried out on the involvement of Dph3 in cancer.

In this work, we firstly reported a novel role for Dph3 in the metastasis of melanoma cells. We found that the metastasis of B16F10 murine melanoma cells was significantly inhibited, when the Dph3 gene was disrupted by insertional mutagenesis. Further investigation with gene silencing of Dph3 showed that the metastasis of melanoma cells was significantly decreased as revealed by the wound healing assay and migration assay. Interestingly, this effect is revealed to be specific to skin cancer cells. In contrast, the overexpression of Dph3 in murine B16F10 and B16F0 melanoma cells greatly enhanced the migration of melanoma cells. More convincingly, Dph3 silencing markedly impaired the lung metastasis of murine melanoma B16F10 cells in vivo. All these data presented that Dph3 is involved in the metastasis of melanoma cells. Interestingly, compared to the pro-metastatic effect of Dph3, Dph1 was reported as a tumor suppressor gene for breast and ovarian cancer [278-279]. Dph1 was originally identified to be deleted from chromosome 17p13.3 which is a hot spot for genetic alterations in breast and ovarian cancer. Deletion of Dph1 in mouse leads to

lethality during embryonic development or shortly after birth with delay and defects in the development of multiple organ systems. In Dph1 heterozygous mice, the frequency of tumor formation was increased and cancer can be spontaneously developed at 80-102 weeks of age [257]. Moreover, forced overexpression of Dph1 retarded the proliferation of epithelial ovarian cancer cells by stopping cell cycle in the G0-G1 phase and accelerating the degradation of cyclin D1 [280-281]. The reason why Dph1 and Dph3 function so differently in tumor cells needs to be further investigated.

8.1.3 The role of AKT signaling pathway in Dph3-mediated melanoma metastasis

The underlying molecular mechanism for melanoma metastasis is quite complicated and several signaling pathways had been reported to be involved in the metastasis of melanoma cells. For example, FAK, a cytoplasmic tyrosine kinase, had been demonstrated to promote the migration and invasion of melanoma cells through increasing the activities of urokinase, matrix metalloproteinase-2 (MMP-2), and membrane type 1-matrix metalloproteinase (MT1-MMP) by MAPK/ERK pathway. Inhibition of FAK-mediated signaling pathway with FAK-related nonkinase (FRNK), a dominant negative protein of FAK, leads to a decrease in ERK activity and metastatic ability of aggressive melanoma cells [282]. Besides FAK signaling, CD151, a tetraspanin membrane protein, had been suggested to promote the metastasis of human melanoma cells through phosphorylation of c-Jun by JNK signaling [283]. In addition, imidazole, a heterocyclic aromatic organic compound, inhibited migration of B16 melanoma cell through activation of GSK-3 β and subsequent phosphorylation and degradation of β -catenin [284]. Lastly, we had recently identified that NCAM enhanced the metastatic potential of B16F0 melanoma cells through PI3K/AKT pathway [285]. To determine the underlying mechanism of Dph-3-mediated melanoma metastasis, we checked the activation of these pathways and found that only the activation of AKT pathway was affected in cells with Dph3 overexpression. Our results showed that overexpression of Dph3 in B16F10 murine melanoma cells induces upregulation of AKT phosphorylation and Dph3 silencing leads to reduced

AKT phosphorylation. Furthermore, restored AKT activity by an active form AKT plasmid could rescue the impaired migration of B16F10 cells induced by Dph3 silencing. In the presence of LY294002, a specific inhibitor of AKT signaling pathway, Dph3 overexpression-promoted migration and invasion were significantly inhibited as revealed by the transwell migration and invasion assay. The pro-metastatic potential of AKT pathway can be supported by the involvement of upregulation of phosphorylated AKT in severely dysplastic nevi and metastatic melanomas compared with normal or mildly dysplastic nevi [286]. Recently, AKT was reported to be a downstream effector of UCH-L1 in regulating the tumor-cell invasion [287]. Moreover, activation of AKT pathway is also found to promote cellular invasion and metastasis of human breast cancer cells and ovarian cancer cells [288]. However, the detail mechanisms underlying Dph3 regulating the activation of AKT is still not clear. We speculated that the mechanism leading to this specificity may involve the association of Dph3 with DelGEF, a homologue of RanGEF, the guanine nucleotide exchange factor (GEF) for the small Ran GTPase [289]. GEFs regulate the transition of small GTPases from inactive GDP-bound state to active GTP-bound state [290]. Dph3 directly interacts with DelGEF, which has been previously reported to regulate extracellular secretion of proteoglycans in HeLa cells by association with Sec 5, a constitutive component of Sec 6/8 complex [289, 291]. Dph3, possibly in combination with DelGEF, regulates the activity of Ran GTPase. Ran GTPase has been reported to activate the PI3K/AKT pathway to promote the metastasis of Rama 37 rat epithelial mammary cancer cells [292]. It is possible that Dph3 might regulate the activation of AKT by the small Ran GTPase through association with DelGEFs. This notion will be explored in detail in the future.

Due to the important roles of Dph3 in the metastasis of melanoma B16F10 cells, it may serve as an attractive target for molecular targeting cancer therapy. In the further work, it is worthwhile to elucidate the precise roles of Dph3 in regulating the AKT signaling thus mediating the metastasis in melanoma cells.

8.2 Conclusion

Metastatic melanoma is the most fatal skin cancer and there is currently no effective treatment. Hence, it is urgently required to elucidate the mechanism underlying melanoma metastasis. In the current study, we uncovered a novel role of Dph3 in promoting the metastasis of melanoma cells. Such pro-metastatic role was further confirmed by up- and down- regulation of the Dph3 expression in vitro. Consistent with these results, silencing Dph3 expression strikingly inhibits their cellular invasion and metastasis in vivo. Finally, we found that the pro-metastatic role of Dph3 is mediated through the AKT signaling pathway. In conclusion, our findings provide a novel mechanism underlying the metastasis of melanoma cells which might help develop novel treatment for melanoma.

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