The role of neural cell adhesion molecule in melanoma progression and mesenchymal stromal cells migration

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**THE ROLE OF NEURAL CELL ADHESION MOLECULE
IN MELANOMA PROGRESSION AND MESENCHYMAL
STROMAL CELLS MIGRATION**

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SCHOOL OF BIOLOGICAL SCIENCES

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STROMAL CELLS MIGRATION**

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ABBREVIATIONS

AA	arachidonic acid
AD	Alzheimer's disease
2-AG	2-arachidonylglycerol
ALCAM	Activated leukocyte adhesion molecule
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATF-1	Activating transcription factor-1
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
bp	Base pair
BSA	Bovine serum albumin
CAMs	Cell adhesion molecules
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
CDK4	Cyclin-D-dependent protein kinase
CFU-F	Colony-forming unit fibroblasts
CMVE	Cardiac microvascular endothelial
CNS	Central nervous system
CREB	cAMP response element binding protein
CSF	Cerebrospinal fluid
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
ECM	Extracellular matrix

EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
F3	Fibronectin type-III homology module
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGFR	Fibroblast growth factor receptor
FGL	FG loop
FRS2	Fibroblast growth factor receptor substrate 2
Gab1	GRB2-associated-binding protein 1
GDNF	Glial cell line derived neurotrophic factor
GPI	Glycosylphosphoinositol
Grb2	Growth factor receptor-bound protein 2
GvHD	Graft-versus-host disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H&E	Hematoxylin and eosin
HGF	Hepatocyte growth factor
HG	High glucose
HDM2	Human double minute 2
HRP	Horseradish peroxidase
IACUC	Institutional animal care and use committee
IBMX	3-isobutyl-1-methylxanthine
ICAM-1	Intercellular adhesion molecule-1
IDO	Indoleamine 2,3-dioxygenase
Ig	Immunoglobulin
IFN- γ	Interferon- γ
IP ₃	Inositol 1,4,5-triphosphate
ISCT	International Society for Cellular Therapy

KD	Knock down
KID	Kinase-induce domain
KO	Knock out
LB	Luria-Bertani
LG	Low glucose
LTP	Long term potentiation
MAP-1A	Microtubule-associated protein 1A
MAPK	Mitogen-activated protein kinase
MCAM	Melanoma cell adhesion kinase
MEK	Mitogen activated protein (MAP)/extracellular signal regulated kinase (ERK) kinase
MET	Mesenchymal-epithelial transition
MITF	Microphthalmia-associated transcription factor
MMP-2	Matrix metalloproteinase-2
MSCs	Mesenchymal stromal cells
NCAM	Neural cell adhesion molecule
ORF	Open reading frame
PDGF	Platelet derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule-1
PKC	Protein kinase C
PLC γ	Phospholipase C γ
PNPP	P-nitrophenyl phosphate
PIB	Phosphatase inhibitor buffer
PI3K	Phosphatidylinositol 3-kinase
PIP ₂	Phosphoinositol 4,5-bisphosphate
PPI	Prepulse inhibition
PrP	Prion proteins
PSA-NCAM	Polysialyated-NCAM
PTEN	Phosphatase and tensin homolog
QRT-PCR	Quantitative reverse transcription-PCR

Rb	Retinoblastoma protein
RGP	Radial growth phase
ROCK	RhoA-binding kinase
s.d.	Standard deviation
SDF-1	Stromal cell derived factor-1
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
S1P	Sphingosine-1-phosphate
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
TAE	Tris-Acetic-EDTA
TNF- α	Tumor necrosis factor- α
TGF β -1	Transforming growth factor- β 1
VASE	Variable alternative splice exon
VCAM-1	Vascular cell adhesion molecule-1
VGP	Vertical growth phase
VLA-4	Very late antigen-4

SUMMARY

Neural cell adhesion molecule (NCAM), initially characterized in cells from nervous system, has also been found to express in a number of non-neuronal cell types including epithelial cells of various organs and muscle cells. A plethora of evidence have suggested the correlation between deregulated NCAM expression with increased cancer malignancy, however, its implication in melanoma remains elusive. The first part of the thesis aims to unravel the role of NCAM in melanoma progression. To this end, siRNA mediated approach was employed to stably silence NCAM expression in mouse melanoma B16F0 cells. We found that both cellular invasion and metastatic dissemination in vivo were significantly perturbed in the absence of NCAM. We further delineated the underlying molecular mechanism and showed that the pro-invasive function of NCAM was exerted via activation of both cAMP-dependent protein kinase (PKA) and phosphatidylinositol 3-kinase (PI3K) pathways. These two pathways converged at transcription factor cAMP response element binding protein (CREB), which in turn down-regulated mRNA expression of pro-invasive gene matrix metalloproteinase-2 (MMP-2). Together, these findings demonstrated the pro-metastatic function of NCAM thus suggesting the potential implication of anti-NCAM strategy in melanoma treatment.

The second part of the thesis consists of studies exploring the role of NCAM in MSCs migration and differentiation. MSCs represent promising tools for cellular therapy

owing to their multipotency and ability to localize to injured, inflamed sites and tumor. Various approaches to manipulate expression of MSC surface markers, including adhesion molecules and chemokine receptors, have been explored to enhance homing of MSCs. Recently, NCAM has been found to be expressed in MSCs yet its function remains largely elusive. Herein, we showed that bone marrow-derived MSCs from NCAM deficient mice exhibited defective migratory ability and significantly impaired adipogenic and osteogenic differentiation potential. We further explored the mechanism governing NCAM-mediated migration of MSCs and showed that the interplay between NCAM and fibroblast growth factor receptor (FGFR) induced activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling, thereby the migration of MSCs. In addition, re-expression of NCAM180, but not NCAM140, could restore the defective MAPK/ERK signaling thereby the migration of NCAM-deficient MSCs. Finally, we demonstrated that NCAM180 expression level could be manipulated by pro-inflammatory cytokine Tumor Necrosis Factor (TNF)- α treatment. Overall, our data revealed the vital function of NCAM in MSCs migration and differentiation thus raising the possibility of manipulating NCAM expression to enhance homing and therapeutic potential of transplanted MSCs in cell therapy.

CHAPTER 1. INTRODUCTION

1.1 Neural cell adhesion molecule: structure and isoforms

NCAM, also known as CD56, is a cell surface glycoprotein belonging to immunoglobulin superfamily mediating calcium-independent intercellular adhesion [1, 2]. Encoded by a single gene consisting 26 exons, *NCAM* locates on chromosome 11 in humans and on chromosome 9 in mice [3-5]. Alternative splicing of mRNA transcribed from this single gene generated numerous isoforms and the three major isoforms are termed as NCAM120, NCAM140 and NCAM180 on the basis of their apparent molecular weight [6]. As depicted in Figure 1, NCAM120 attaches to cell membrane via a glycosylphosphoinositol (GPI) anchor which is encoded by exon 15, whereas NCAM140 and NCAM180 are transmembrane proteins with their cytoplasmic domains encoded by exons 16, 17, 19 and 16-19, respectively[7]. All three major isoforms possess identical extracellular parts which are encoded by exons 1-14. Like other adhesion molecules from immunoglobulin superfamily, the extracellular parts of the three major isoforms share homology with the modules of immunoglobulin consisting five Ig-like domains and two fibronectin type-III repeats (F3) [8] .

Each of the three NCAM isoforms displays cell type related and temporal differences in expression. During embryonic development, NCAM140 and NCAM180 are the predominant isoforms present in many tissues, including epithelia. In adult tissue, however, NCAM changes from the two transmembrane isoforms to NCAM120 with

its expression mainly localizes in nervous system [9], skeletal muscle [10] and some neuroendocrine organs [11, 12]. In nervous system, NCAM140 is found transiently expressed on migratory growth cones and axon shaft of developing neurons, whereas NCAM180 appears late in development and is enriched at sites of contact between axonal growth cones as well as postsynaptic membranes of hippocampal neurons. NCAM120, however, is predominantly expressed in glia cells [13].

It has been reported that NCAM also exists in a secreted form (soluble NCAM), attributing to the expression of the NCAM-SEC-exon, which contains an in frame stop codon and locates between exons 12 and 13. The truncated form of 115 kDa NCAM lacks the hydrophobic domains and is expressed in brain postnatally [9, 14]. Enzymatic removal of any of the three major NCAM isoforms [15-17] could also contribute to the pool of soluble NCAM. Moreover, transmembrane isoforms of NCAM have also been found in soluble form, e.g. in cerebrospinal fluid (CSF) [18].

Additional variations of the extracellular parts of NCAM further diversify the pool of NCAM isoforms. The difference between these subtypes is achieved by optional insertion of the variable alternative splice exon (VASE) a, b, c, in the fourth Ig domain and AAG between the two F3 modules [19]. Alternatively, the structure and expression of NCAM could be complicated by post-translational modifications, including glycosylation and palmitoylation. Glycosylation of NCAM could be achieved by addition of α -2,8-linked sialic acid residues to the fifth Ig domain. Such polysialylated-NCAM (PSA-NCAM) is predominantly expressed during neuronal

development and its expression markedly decreased during embryonic ages into adulthood [20]. In adult brain, PSA-NCAM only presents in area retaining high degree of plasticity where neurogenesis is active, such as the hippocampus and the olfactory bulb [21]. In contrast, VASE exon expression increases during development. Together, PSA and VASE changes seem to act in concert to switch the function of NCAM from a plasticity-promoting to stability-promoting molecule [22].

Other than polysialylation, the two transmembrane isoforms of NCAM could also undergo palmitoylation at their C-terminus. This additional post-translational modification affects NCAM distribution within the plasma membrane and the downstream signaling and neurite outgrowth [23].

Figure 1. Schematic presentation of the three major NCAM isoforms.

There are three major isoforms of NCAM. Two of them are transmembrane protein with either long (NCAM180) or short (NCAM140) cytoplasmic domains. The other isoform, NCAM120, lacks a cytoplasmic domain and attaches to cell membrane via a GPI anchor. This figure depicts the exon constitution of NCAM gene and how each exon corresponds to various domains of NCAM isoforms. IgI-IgV indicates immunoglobulin homology modules. F3-I and F3-II represent fibronectin type III homology modules [7].

1.2 Mechanisms of NCAM homophilic and heterophilic interactions

1.2.1 Homophilic NCAM interactions

NCAM has long been known to mediate cell-cell interactions by means of homophilic binding. Regarding homophilic binding between NCAM molecules on two adjacent cells, several seemingly conflicting models have been proposed [24]. One model implicates an anti-parallel binding between the two N-terminal extracellular modules (IgI and IgII) of two opposing NCAM molecules [25, 26], whereas another one predicts an anti-parallel binding of all five NCAM-Ig modules [27, 28]. The

discrepancies between these models were later partially resolved when the structure of the IgI–IgII–IgIII triple modules of rat NCAM was resolved by X-ray crystallography [29]. Based on the structure, a new model involving three steps has been proposed: First, two NCAM molecules arrange in parallel forming *cis*-dimers, and bind to each other in a crosslink formation with IgI and IgII modules. Second, when NCAM *cis*-dimers locate on opposing cells, they bind to each other in an antiparallel manner with the second and third IgG of the two opposing NCAM, a mechanism called “flat zipper”; or the two NCAM *cis*-dimers form a “compact zipper” conformation, in which the binding sites locate at IgII modules and between IgI and IgIII modules. Last, combining both types of zippers leads to formation of a two dimensional “double zipper” NCAM adhesion complex involving numerous NCAM molecules.

1.2.2 Heterophilic NCAM interactions

NCAM also engages heterophilic interactions with various cell surface and extracellular matrix molecules. The following table lists some heterophilic ligands of NCAM that have been reported so far.

Table 1. Heterophilic ligands of NCAM

	Ligand	References
Co-receptors and cis interactors	FGF receptor	[30, 31]
	Glial cell line derived neurotrophic factor (GDNF)	[32]
	Platelet derived growth factor (PDGF)	[33]
	Brain derived neurotrophic factor (BDNF)	[34]
	Prion proteins (PrP)	[35]
	Adenosine triphosphate (ATP)	[30, 36, 37]
	L1	[38]
Extracellular matrix	Glycosaminoglycan heparin	[39]
	Chondroitin sulfate proteoglycan (Neuron can/Phosphacan/Protein-tyrosine phosphatase ζ / β)	[40, 41]
	Heparin sulfate proteoglycans (Agrin)	[26, 42]
	Collagen	[26]

FGFR is one of the most important extracellular interaction partners of NCAM. Fc-fusion protein of the extracellular part of NCAM, or an NCAM derived peptide (FG loop (FGL) peptide) has been shown to interact with FGFR. Such interaction is mediated by the second NCAM-F3 module and the second proximal membrane Ig domain of FGFR-1, thus resulting in FGFR phosphorylation thereby neurite outgrowth as demonstrated in PC12 cells and primary neurons of the hippocampus [30, 31].

Apart from FGFR, NCAM has also been shown to interact with a variety of co-receptors and *cis*-interactors. For example, GDNF is another heterophilic ligand of

NCAM and their interaction in the absence of the conventional GDNF receptor, the Ret receptor tyrosine kinase, results in neurite outgrowth in cultured primary hippocampal neurons [32]. In addition, ATP has been shown to directly bind to the extracellular domain of all NCAM isoforms which could serve as an ecto-ATPase [36, 43]. Interestingly, the binding of ATP to NCAM inhibits homophilic NCAM-NCAM interaction thereby growth of neurites. Hydrolysis of ATP on NCAM, on the other hand, promotes NCAM-FGFR interaction thus their corresponding signaling [37, 44]. Other related adhesion molecules could also serve as direct interaction partners of NCAM. For instance, NCAM has been shown to interact with the closely related adhesion molecule L1 to modulate neuronal function [38].

Several components of extracellular matrix (ECM), including glycosaminoglycan, heparan sulfate proteoglycans, chondroitin sulfate proteoglycans and collagen have also been reported as NCAM interaction partners [39-42]. Neurocan, for instance, is a member of the chondroitin sulfate proteoglycans. Binding of neurocan with NCAM has been shown to interfere with homophilic NCAM interactions thereby inhibiting neuronal adhesion and neurite outgrowth [45]. Collagens I-IC and IX have also been documented to bind with NCAM. Nevertheless, such interaction is considered as indirect and dependent on the presence of heparan sulfate [26].

1.3 NCAM-mediated intracellular signaling

Besides its role in cell adhesion, NCAM also functions as a signal transducer initiating intracellular signaling via direct or indirect interaction with intracellular signaling molecules and extracellular ligands listed above. The signaling events presented below are pathways involved in neuronal differentiation and neurite outgrowth, in which NCAM intracellular signaling has been extensively studied. Also, these signaling cascades would be employed by NCAM regulating other cellular attributes such as cell migration and survival [46].

1.3.1 NCAM signaling mediated via FGFR

Early indications that FGFR was involved in NCAM-mediated signaling came from studies showing that NCAM-mediated neurite outgrowth was impaired in neurons from transgenic mice expressing dominant negative FGFR and in PC12 cells treated with tyrosine kinase inhibitor [47-49]. Thereafter, several studies with various pharmacological inhibitors against enzymes [50] and second messenger molecules [51] revealed the sequence of events accompanying the binding of NCAM to FGFR. Upon binding to NCAM, FGFR undergoes dimerization and activation via auto-phosphorylation. Subsequently, several proteins dock to the phosphorylated residue in the cytoplasmic domain of FGFR molecule, one of them being the enzyme phospholipase C γ (PLC γ), which in turn catalyze the hydrolysis of phosphoinositol 4,5-bisphosphate (PIP₂) to generate inositol 1,4,5 triphosphate (IP₃) and diacylglycerol

(DAG). IP3 specifically binds to Ca^{2+} channel, leading to an increase in intracellular Ca^{2+} concentration. DAG, however, remains at the membrane and can either activate protein kinase C (PKC) or being converted to 2-arachidonylglycerol (2-AG) and arachidonic acid (AA), inducing various downstream signaling events [7].

1.3.2 NCAM signaling mediated via non-receptor tyrosine kinase

In addition to signaling through FGFR, NCAM has also been shown to mediate signal and induce neurite outgrowth via non-receptor tyrosine kinases. Fyn and focal adhesion kinase (FAK) are the two non-receptor tyrosine kinases that interact with NCAM and convey its signals. The involvement of Fyn in NCAM-mediated signaling was initially reported by Beggs, et al showing that neurite extension exhibited selective inhibition in sensory neurons from Fyn knockout ($\text{Fyn}^{-/-}$) mice grown on NCAM-expressing fibroblast [52]. Furthermore, clustering of NCAM molecules on neuroblastoma cell surface by means of antibodies leads to a transient increase in Fyn phosphorylation [52]. Activated Fyn in turn recruits FAK via its SH2 domain to form Fyn/FAK complex, and FAK was subsequently phosphorylated to mediate downstream signaling.

The involvement of Fyn and FAK in NCAM-mediated signaling and neurite outgrowth was later confirmed in studies where Fyn inhibitor PP2 and expression of dominant negative FAK inhibited the NCAM-mediated neuritogenic response [50, 53]. Moreover, it is worth noting that only the NCAM140 isoform, but not NCAM120 and

NCAM180, co-immunoprecipitates with Fyn and was implicated in activation of Fyn and FAK [54].

1.3.3 Cyclic adenosine monophosphate (cAMP) and PKA signaling

The role of PKA and the upstream activator cAMP in NCAM-mediated neurite outgrowth was initially being excluded [55]. Nevertheless, the same cAMP antagonist with different concentration was later shown to inhibit NCAM-mediated neurite outgrowth in PC12 cells [56]. Likewise, treatment with PKA inhibitor leads to attenuated NCAM dependent neuritogenic response [56]. Together, these lines of evidence confirm the involvement of cAMP/PKA pathway in NCAM mediated neurite outgrowth.

Downstream of PKA locates transcription factors CREB and c-Fos which are known to be activated and increasingly expressed, respectively, upon activation of cAMP-PKA pathway [56]. It has been shown that cAMP analogue dBcAMP could induce neurite outgrowth in PC12 to the same extent resembling the effect of NCAM stimulation. Moreover, expression of dominant negative CREB or c-Fos led to inhibition of cAMP analogue dBcAMP-induced neurite outgrowth. Therefore, it appears that cAMP/PKA induced activation of transcription factor CREB and c-Fos is involved in NCAM stimulated neuritogenic effect in PC12 cells [56]. Indeed, stimulation with NCAM mimetic ligand C3d induced phosphorylation of CREB confirming CREB as a necessary signaling component in NCAM-mediated neurite outgrowth.

1.3.4 PI3K /Akt pathway

PI3K and its downstream effector Akt, also known as protein kinase B, also contribute to NCAM-mediated neurite outgrowth [57]. Neuritogenic response in PC12 cells stimulated with NCAM mimetic peptide C3d was significantly inhibited by blocking PI3K pathway. Moreover, phosphorylation of Akt was significantly increased upon stimulation with C3d and inhibited by PI3K inhibitors [57] further consolidating the involvement of PI3K/Akt pathway in NCAM-mediated neuritogenesis.

There are several routes by which NCAM mediates activation of PI3K/Akt pathway. For instance, PI3K can be activated by FGFR via a FGFR-Grb2-Gab1 complex or by binding to phosphorylated FAK, or via Ras and G-proteins [58, 59]. In addition, transcription factors CREB and NF κ B, whose activities have been shown to be regulated by Akt might serve as the downstream targets of PI3K/Akt pathway contributing NCAM-mediated neurite outgrowth [60, 61].

1.3.5 MAPK/ERK pathway

Other than above mentioned FGFR-PLC γ -DAG-AA signaling cascade, NCAM could also induce activation of MAPK pathway via FGFR. Upon stimulation by C3d, increased phosphorylation and activation of the terminal MAPKs, ERK1 and ERK2 have been observed in primary hippocampal neurons and PC12 cells [50]. Likewise in NCAM140-expressing neuroblastoma B35 cells stimulated with NCAM-Fc fusion

proteins and in primary cerebella neurons stimulated with NCAM antibodies or NCAM–Fc fusion proteins [53], elevated phosphorylation level of ERK1/2 is noted. Pharmacological inhibition of MEK, which is upstream of ERK, has been shown to inhibit NCAM-mediated neurite outgrowth [50, 53]. Moreover, expression of dominant negative forms of Ras and Raf, which are upstream activators of MEK, leads to impaired NCAM-mediated neurite outgrowth in PC12 cells and B35 cells [50]. In addition, expression of dominant negative form of Ras in NCAM140-expressing B35 cells and COS cells led to decreased phosphorylation of ERK, further confirming Ras being upstream of MEK and ERK [53].

In addition to signaling through FGFR, NCAM could also signal through non-receptor tyrosine kinases Fyn/FAK to induce activation of MAPK pathway. Upon activation, phosphorylated FAK recruits adaptor protein Grb2 and guanine nucleotide exchange factor Sos to form Grb2/Sos complex which in turn activates Ras resulting in phosphorylation of MAPKs and downstream transcription factor CREB [53, 56, 62, 63].

As stated above, both Fyn/FAK and FGFR mediated signaling induced by NCAM could contribute to activation of MAPK/ERK pathway. However, whether the Ras/MAPK pathway is activated via the Fyn/FAK complex or FGFR depends on the compartmentalization of NCAM in cell membrane. Only lipid-raft associated NCAM can induce signaling via Fyn/FAK complex, whereas FGFR-mediated signaling is provoked by non-raft fraction of NCAM [64].

Figure 2. Signaling pathways activated by NCAM140 and NCAM180 isoforms in neuronal cells.

Co-signaling from NCAM inside and outside lipid raft activates cytoskeletal and transcriptional events which in turn contribute to neurite outgrowth [13].

1.4 NCAM in central nervous system (CNS)

NCAM was initially identified and abundantly expressed in cells from nervous system where its function has been studied extensively and implicated in various cellular

attributes including intercellular adhesion, cell migration, proliferation and survival. These events are triggered by the homophilic binding of NCAM on adjacent cells or heterophilic binding with other ligands contributing to diverse aspects of neuronal development [65], synaptogenesis [66], neuroprotection [67], learning and memory consolidation [68]. In humans, dysregulation of NCAM has also been reported to contribute to the development of neuropsychiatric and neurodegenerative disorders such as schizophrenia, bipolar disorder, depression and anxiety [13, 69-71]. Elevated levels of soluble NCAM fragment was found in the postmortem brain and in CSF of patients correlating with the severity and duration of schizophrenia [72-74]. Moreover, reduced NCAM level has been observed in frontal and temporal cortex of patients with Alzheimer's disease (AD) suggesting the correlation between NCAM and the cognitive deficits in AD patients [75]. The functional importance of NCAM in nervous system could be further exemplified by deficits observed in NCAM knockout (NCAM^{-/-}) mice. Though NCAM^{-/-} mice appear healthy and fertile, ablation of NCAM gene leads to many functional and behavioral abnormalities. For instance, impaired synaptic plasticity for learning and memory is observed in NCAM^{-/-} mice, specifically, hippocampal long term potentiation (LTP) is reduced and long term depression in CA1 region is abolished [66, 76, 77]. Moreover, abnormal synaptic vesicle release at neuromuscular junction [78, 79], corticospinal tract hypoplasia [80], impaired migration of olfactory neurons and reduced size of the olfactory bulb, body weight and brain weight [81, 82] have also been noted in NCAM^{-/-} mice. In a recent study, it was demonstrated that NCAM loss led to hippocampal dysplasia and loss of cholinergic neurons in forebrain of adult mice might contribute to the impaired cognition [83]. At

behavioral level, NCAM^{-/-} mice exhibit compromised spatial learning [81], increased anxiety[84], elevated inter-male aggression [85], impaired contextual and cued fear conditioning [86] and abnormal circadian clock [87]. Moreover, depression like behaviors accompanied with impaired hippocampal neurogenesis have been demonstrated in NCAM^{-/-} mice [88] and these phenotypes could be ameliorated by systemic administration of NCAM mimetic peptide, FGL [88]. Also, exaggerated dopamine related-locomotion was shown in NCAM^{-/-} mice owing to disruption of NCAM-mediated dopamine D2 receptor internalization [89]. In mice with NCAM180 isoform specifically being knocked out, increased lateral ventricle size and reduced prepulse inhibition (PPI) of startle response were observed [90].

1.5 NCAM in cancer

1.5.1 Cancer metastasis

Though surgery and radiation could effectively control many localized tumors, the development of metastatic lesion signals a poor prognosis and causes 90% cancer death [91]. Hence, a mechanistic understanding of metastasis is imperative and would lead to better therapies and improved patient outcome.

Metastasis, or the spread of cancer cells from its primary site to a distant organ, is an extraordinarily complex process. The initial step requires the proliferation of the primary tumor, diminishing of intercellular adhesion, degradation of extracellular matrix, and invasion into surrounding tissue and basement membrane [92]. This process continues until tumor cells invade into lymphatics or directly into tumor

associated vasculature. Once in the bloodstream, tumor cells must survive several stresses, including physical damage from velocity induced shear forces, lack of substratum and attack from immune cells. Having invaded and endured in circulation, metastatic tumor cells will then manage to arrest in circulatory system and extravasate into a target tissue by inducing endothelial retraction. Colonization at the secondary sites is an inefficient business/ rate-limiting step of metastatic progression. Stephen Paget proposed that disseminated cancer cells or “seeds”, would only colonize organ microenvironments or “soil” that are compatible with their growth. Indeed, clinical observation of cancer patients supports this notion that different cancers displayed specific metastatic tropisms. For instance, systemic breast cancer frequently metastasizes to the lung, bone, liver and brain, whereas advanced gastrointestinal malignancy and uveal melanomas metastasize specifically to the liver [93]. Recent advances have enriched our understanding of the word “microenvironment” and unraveled the determinants affecting such site-specific metastatic growth. First, a “premetastatic niche” may be formed by bone marrow–derived hematopoietic progenitor cells within the target organ to support the initial survival of extravasated tumor cells [94, 95]. Subsequently, the invading metastatic cells must display appropriate functions to effectively colonize the target site [95].

1.5.2 Cell adhesion molecules (CAMs) in cancer

On progression to advanced metastatic stage, cancer cells undergo multiple molecular alterations to get equipped to invade neighboring tissues, infiltrate into vascular and lymphatic vessels, extravasate and eventually colonize at distant organs [95]. At

molecular level, such changes mainly involve cell adhesion molecules, proteases and proteins regulating actin cytoskeleton reorganization. CAMs are cell surface molecules widely implicated in cell growth, migration and differentiation. In addition to its adhesive functions modulating intercellular and cell-matrix interactions, cell adhesion molecules participate in signal transduction, cellular communication and recognition, embryogenesis, inflammatory response and apoptosis [96]. In adult organism, CAMs are also particularly important in maintenance of tissue integrity and guidance of leukocyte migration [97]. Perturbances in expression and function of CAMs in tumor cells can lead to altered cellular adhesive repertoire and intercellular signaling, both of which will contribute to tumor progression and metastasis formation.

On the basis of molecular structure and function, cell adhesion molecules can be further grouped into three main classes which are the integrins, the cadherin and immunoglobulin superfamily [96]. Ample evidence demonstrated the pivotal regulatory role of each group of CAMs in metastatic cascade. For example, loss of E-cadherin has been demonstrated to coincide with the progression of most human epithelial cancers including carcinomas of the breast, colon, stomach, etc [98]. Furthermore, loss of E-cadherin is frequently accompanied with gain of mesenchymal cadherins, such as N-cadherin or cadherin 11 [99], thus raising the possibility that a ‘cadherin switch’ from pro-adhesive, epithelial cadherins (e.g. E-cadherin) to mesenchymal migratory cadherins (e.g. N-cadherin) often accompanies with tumor progression to malignancy [100].

Integrins, the prime mediator of cell-matrix adhesion and one of the key regulators controlling signaling pathways contributing to actin dynamics and cell movement, has also been found to be involved in tumor invasion and metastasis [101]. One of the most intensively studied integrin $\alpha\beta3$, for instance, serves as currently the best molecular marker correlating with the change from radial growth phase (RGP) to the metastatically competent vertical growth phase (VGP) of melanoma. Upon introduction of $\beta3$ integrin, it has been shown that RGP melanoma cell lines were converted into VGP-like melanoma cells. Other than melanoma, elevated expression of $\alpha\beta3$ has also been shown to be associated with increased bone metastasis in breast and prostate cancers [102, 103]. Besides $\alpha\beta3$, other members of the integrin family expressed on epithelial cells, such as $\alpha6\beta4$, $\alpha6\beta1$, $\alpha3\beta1$, are generally retained in epithelial tumors with altered expression level. Moreover, it has been shown that integrin $\alpha6\beta4$ cooperates with the function of hepatocyte growth factor (HGF) to regulate the invasive properties of carcinoma cells [104].

CAMs of the immunoglobulin superfamily (Ig-CAM) have also been found implicated in cancer progression by facilitating information exchange between cells and support neoplastic progression thereby leading to the development of metastases [105]. For instance, intercellular adhesion molecule-1(ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), platelet endothelial cell adhesion molecule-1(PECAM-1) have been demonstrated to be involved in the progression of colorectal carcinoma [106]. In addition, expression of L1, which is also known as CD171, was shown to be up-regulated and contributed to the metastatic dissemination of breast cancer, prostate

cancer and melanoma cells [96, 107]. Over-expression of MUC18/CD146 (Melanoma cell adhesion molecule) has been reported to associate with advanced primary and metastatic melanoma [108, 109]. However, the role of MCAM seems controversial and tumor context dependent. In breast carcinoma, MCAM expression is frequently lost and smaller metastases were generated from breast cancer cells over-expressing MCAM in nude mice [110].

1.5.3 NCAM in cancer

Besides cells from CNS, NCAM is also expressed in non-neuronal cell types. Studies in various human cancer biopsies as well as mouse tumor models reveal the importance of NCAM in the progression to tumor malignancy [111]. In many cancers, the expression of NCAM shifts from the adult NCAM120 isoform to the embryonic NCAM140 and NCAM180 isoforms. Moreover, NCAM expression level is deregulated exerting either positive or negative regulation in tumor progression. In neuroblastoma and tumors of neuroendocrine origin, elevated expression level and extensive polysialylation of NCAM has been observed [112-114]. Likewise, poor prognosis correlates with up-regulated NCAM level in myeloma, acute myeloid leukemia and lung cancer [115-117]. Also, it has been demonstrated recently that NCAM promotes ovarian cancer progression via coordination with FGFR signaling [118]. In contrast, an overall reduction of NCAM expression level has been found in colon carcinoma, pancreatic cancer and astrocytoma in association with poor prognosis [119-122]. Moreover, ablation of NCAM function in Rip1Tag2 transgenic mice, the only mouse model of spontaneous tumorigenesis for functional study of

NCAM in cancer progression, results in increased tissue disaggregation of primary tumor [123], lymphangiogenesis and formation of metastases at local lymph nodes [124, 125]. In melanoma, constitutive expression of NCAM has been found in cell lines derived from CNS metastatic lesions [126]. In addition, significantly higher expression level of NCAM was documented in aggressive, rapidly metastasizing primary and metastatic uveal melanoma [127]. However, a recent study reported controversy results showing that only a small proportion of tumor samples were NCAM positive in patients who underwent enucleation for primary uveal melanoma [128]. Moreover, only 14 out of 34 tumor samples were NCAM positive in another study with conjunctival melanoma patients [129]. Therefore, it seems that the correlation between NCAM expression and melanoma prognosis is still obscure and how NCAM modulates the progression of melanoma is yet to be answered.

1.5.4 Melanoma

Melanoma arises from the malignant transformation of neural-crest derived pigment-producing cells, melanocytes [130]. Recognized as the most fatal form of skin cancer, melanoma accounts for 75% of mortality associated with skin cancer [131] owing to its rapid growth and highly metastatic proclivity. The incidence of melanoma has been increasing at an alarming rate, with the number of cases worldwide doubled in the past 20 years [130, 132]. Currently, the risk of developing melanoma is 1/58 for males in the United States, and the increase in mortality rate is the second highest among all cancers [132].

As depicted in the Clark model (Fig.3), melanoma progresses from the benign naevi, to RGP melanoma, an intra-epidermal lesion lacking the capacity to invade the dermis and metastasize, and subsequently to VGP, a more dangerous stage in which cells are metastatic competent, with nodules invading the dermis and deeper structure [130]. Though early stage thin lesion of melanoma can be cured by surgical resection, treatment options, cure rate and survival rate decrease dramatically as melanoma progresses. Once diagnosed as metastatic phase, it is always fatal, largely refractory to existing therapies with 5-year survival rate of less than 5% [130]. Therefore, in-depth understanding of the mechanism underlying the complex metastatic process of melanoma and the molecular changes occurring in highly malignant cells are imperative and would be of great help for novel regime development.

As a complex genetic disease, melanoma arises from accumulation of mutations in genes residing within critical developmental pathways that are central to proliferation, differentiation and survival of melanocytic lineage [91]. In the past decades, the rate at which these oncogenic molecular ‘drivers’ being identified and catalogued in melanoma has accelerated greatly, with several of the oncogenes already being targeted with small molecules. However, no case of melanoma has been identified in which deregulation of only one oncogene or tumor suppressor gene is responsible, although some oncogenic mutations are mutually exclusive [133]. The table below summarizes the key genetic alterations in melanoma.

Table 2. Selected genetic alterations in malignant melanomas

Gene Type	Gene	Alteration frequency/types in melanoma (%)	Pathways affected by alteration	References
Oncogene	BRAF	50%-70%, mutation	point MAPK	[134, 135],
	NRAS	15%-30%, mutation	point MAPK, PI3K	[135]
	KIT	1% overall, mutation	point MAPK,PI3K	[136]
	CDK4	5%, point mutation or amplification	Cell cycle	[137]
	AKT1,AKT2 and AKT3	<1% point mutation; 25% amplification (AKT3)	PI3K	[138, 139]
Tumor suppressors	CDKN2A	30-70%, point mutation or deletion	Cell cycle	[140]
	p53	10%, point mutation	Cell cycle	[141]
	APAF-1	40%, point mutation	Cell cycle	[142]
	PTEN	50-60% point mutation or hemizygous deletion, 10% homozygous deletion	PI3K	[143, 144]
Transcription factors	MITF	20%, Amplification	Melanocyte lineage and cell cycle	[145]
	Cyclin D1	6-44%, Amplification	Cell cycle	[146]
	Myc	20%, Amplification	Cell cycle	[147]

The most commonly observed recurrent mutations reside within the Ras/Raf/MEK/ERK pathway which regulates cell fate decision downstream of receptor tyrosine kinases, cytokines and heterotrimeric G-protein-coupled receptors [148]. In melanoma, this pathway acts as a key regulator of cell proliferation, with ERK hyper-activation being observed in around 90% human melanomas [149]. ERK phosphorylation and activation could be achieved through autocrine growth factors [150]: mutational activation of autocrine growth factor receptor KIT [151], or most commonly through gain of function mutation in NRAS, one of the three Ras genes in humans (the other being HRAS and KRAS) which has been reported in 15% to 30% of human melanoma [130]. NRAS mutations are thought to be mutually exclusive to activating mutations in BRAF. BRAF mutations have been identified in 50% to 70% of melanoma cases, with the vast majority found in melanomas arising from intermittently sun-exposed skin [134, 135]. The most common mutation is a glutamic acid for valine substitution at position 600 (V600E), resulting in constitutive activation of mitogen activated protein (MAP)/extracellular signal regulated kinase (ERK) kinase (MEK) [134].

Besides its canonical effector pathway RAF/MAPK/ERK, NRAS mutation could simultaneously activate the PI3K pathway. The first indication regarding the involvement of PI3K/Akt pathway in melanoma progression came from the observation that phosphatase and tensin homolog (PTEN), the negative regulator of PI3K pathway inhibiting the phosphorylation of Akt by PI3K, was frequently lost in melanocytic lesions. In melanoma, allelic loss of PTEN on chromosome 10q has been

described in 30-50% of malignant melanoma, and 5-15% of uncultured melanoma specimen and metastases were shown to have PTEN mutations [152, 153]. More recently, constitutive activation of Akt has been reported in up to 60% of melanomas, with higher frequencies of activation at malignant stage of the disease [154, 155]. Furthermore, gene amplification of AKT3 isoform is another mechanism contributing to the constitutive activation of ERK and progression of malignant melanoma [155].

Other than above mentioned mutations, melanoma cells have been demonstrated to override senescence signals by inactivating tumor suppressor pathways such as p16^{INK4a} cyclin dependent kinases Cdk4 and 6/retinoblastoma protein (p16^{INK4a}/CDK4,6/pRb) and p14^{ARF}/human double minute 2/p53 (p14^{ARF}/HDM2/p53) pathways [156, 157]. p16^{INK4a} functions by binding to the cyclin-D-dependent protein kinase, CDK4 and 6, thereby blocking phosphorylation of Rb and cell cycle progression. In familial and most sporadic melanomas, mutation of *CDKN2A*, the gene encoding p16^{INK4a} and p14^{ARF} have been described and generally affect only p16^{INK4a} or p16^{INK4a} and p14^{ARF} together [157], rendering these kinases constitutively active.

Another concept that has been advanced in melanoma is that lineage-specific pathways critical in melanocyte development might also play pivotal roles in melanomagenesis. Microphthalmia-associated transcription factor (MITF) is considered as the master regulator of melanocyte development, differentiation and pigmentation [158]. Amplification of MITF has been identified in 16% of metastatic

melanoma in which BRAF is mutated, and such constitutive expression is essential for melanoma cell proliferation and survival [145].

Figure 3. Progression of melanocyte transformation.

A, Normal skin with evenly distributed dendritic melanocytes in the basal layer of the epidermis. B, Naevus. In early stages, benign melanocytic naevi occur with increased number of melanocyte. Some naevi are dysplastic with structural and architectural atypia. C, RGP melanoma which is considered as the primary malignant stage. D, VGP melanoma. Melanoma cells show pagetoid spread and have penetrated the dermal-epidermal junction [130].

1.6 NCAM in MSCs

1.6.1 MSCs

Mesenchymal stromal cells, also referred as connective tissue progenitor cells are non-hematopoietic stem cells that are capable of self-renewal and can give rise to multiple lineages [159]. They were originally identified by Friedenstein et al as fibroblast-like cells from bone marrow aspirate that can adhere to tissue culture plastic. Friedenstein defined them as colony-forming unit fibroblasts (CFU-F) and showed that they were able to differentiate into cells of mesodermal lineage such as adipocytes, chondrocytes and osteocytes [160].

1.6.1.1 Characterization and phenotypes of MSCs

One of the most elusive problems in MSC biology has been the identification of a single unique specific cell marker that distinguishes the population of MSCs with a defined set of functional properties. Numerous studies have shown that MSCs can be recovered from a variety of adult tissue, exhibiting different proliferative and differentiation potential. Moreover, different protocols for isolation and in vitro culture methods adopted by different laboratories further complicate the picture by contributing resulting cell population with variable phenotypes and function. Given the lack of universally accepted criteria for defining an MSC, the International Society for Cellular Therapy (ISCT) provided the following minimum criteria for defining multi-potent human mesenchymal stromal cells used for clinical studies [159]:

- 1) MSCs must be plastic adherent under standard culture conditions;

- 2) MSCs express a specific set of membrane molecules including CD105,CD73 and CD90 but not hematopoietic cell surface markers CD34, CD45 ,CD11a,CD79a ,CD19 and HLA-DR;
- 3) MSCs must be able to differentiate into osteocytes, adipocytes and chondrocytes under standard in vitro differentiating conditions.

The immunophenotypic profile used for MSC characterization is summarized in the table below and this list would be further complicated by the differences between species and between strains of species.

Table 3. Surface antigen profile of MSCs

	Positive selection	Negative selection
Minimal immunophenotypic criteria recommended by ISCT	CD73,CD105,CD90	CD34,CD45,CD14,CD11b,CD79 α ,CD19,HLA-DR
	CD9,CD10,CD13,CD29,CD44,CD49a,CD56,CD106,CD140b,CD146,CD166,	CD86,CD80/CD40,CD15,CD18,CD25,CD31,CD49d,CD50,CD117
Other antigens used for MSC isolation	CD271, CD340,CD349 CCR1,CCR4,CCR7,CXCR5,CCR10,VCAM-1,ALCAM, ICAM-1, STRO-1	
Data compiled from references: [159, 161-163]		

Other than specific immunophenotypic surface markers, the phenotype of MSCs is also defined by the multipotency of these cells in culture and in vivo. As far back as Friedenstein, MSCs have been shown to be able to differentiate into adipocytes, osteocytes and chondrocytes cells. Moreover, ectopically transplanted of MSCs grown ex vivo have been demonstrated to maintain their differentiation potential and are capable of inducing bone and marrow formation [160]. Several other studies have indicated the potential of MSCs differentiate into unrelated tissues. In particular, MSCs are capable of differentiating into myoblasts [164], cardiomyocytes [165], neurons and glial cells [166-168] in vitro and in vivo.

1.6.1.2 MSCs localization and homing

MSCs are a minor population, accounting 0.001%-0.01% of the total nucleated cells in human bone marrow [169]. Besides this primary source, MSCs can also be isolated from almost every postnatal organ, including periosteum [170], adipose tissue [171], brain [172], liver [163], skeletal muscle [172], hair follicles [173], peripheral blood [174], umbilical cord blood [175], and placenta [163].

Recently, it has been reported that only a minute quantity of MSCs consistently circulates in the peripheral blood under stationary conditions, and this circulating pool increases significantly under hypoxic conditions [176], in case of major injury such as myocardial infarction [177, 178], or acute skin burns [179]. However, not all attempts to isolate MSCs from peripheral blood have been successful and there are several

studies reported contrasting results on the presence of circulating MSCs in peripheral blood [180].

MSC homing defines a process in which MSCs arrest within the vasculature of a tissue followed by transmigration across the endothelium and engraftment into tissues in which they will exert their functional and protective effects [181]. In an animal model of cerebella ischemia, MSCs have been shown to home to site of injury and ameliorate neurological deficits in the animals [182]. Moreover, Chapel et al. showed in a model of multi-organ failure that green fluorescent protein tagged MSCs homed to numerous tissues with localization correlating to the severity of sites of injury [183]. This inherent migratory ability thus makes MSCs a promising candidate for cell therapy, which can be delivered systemically, avoiding the presumed complications associated with intra-muscular or site-specific injection [184].

However, little is known about the precise mechanism by which MSCs are recruited and home to sites of injury. The process of leukocyte homing to peripheral sites of inflammation was proposed as a paradigm [185]. During inflammation, the inflammatory cells were recruited following a complex sequence of adhesive events, including tethering, selectin mediated rolling on activated vasculature where a chemokine gradient is established, activation of integrin mediating firm adhesion on endothelium, transendothelial migration and finally migration into extracellular matrix [186, 187]. Unlike leukocytes, MSCs employed P-selectin instead of L- and E-selectin in the initial rolling. Using P-selectin knockout mice, Ruster et al showed via intra-

vital microscopy that much less MSCs rolling along the walls of post-capillary venules compared to wild type mice [188].

It is well known that chemokine gradient serves as environmental cues guiding the migration of inflammatory cells. In response to injury, chemokines, cytokines and growth factors are released to induce expression of selectins, integrins and several other chemokine /growth factor receptors on cell surface, enabling cells to interact with endothelium [189]. For instance, in vitro migration study has demonstrated that stromal cell derived factor-1 α (SDF-1 α) can serve as chemotactic stimulus for MSCs [190]. Early passage MSCs retaining higher migratory potential have been shown to express the specific SDF-1 chemokine receptor CXCR4 [191]. Blocking of SDF-1/CXCR4 axis was shown to partially inhibit homing of CXCR4 expressing cells to sites of injury, whereas transfection of MSCs with CXCR4 resulted in increased migration toward SDF-1 α in vitro and toward infarcted myocardium in vivo [192, 193]. Other than SDF-1/CXCR4 axis, there are several other ligand receptor interactions involved in MSC homing. As demonstrated by Sasaki et al, interaction between CCR7 expressed on MSCs with CCL21 expressed on keratinocytes within sites of wounded skin has been shown to contribute to the recruitment of MSCs to the wound site in vivo[194].

Besides chemokines and their receptors, a plethora of signaling pathways have also been demonstrated to regulate MSC migration. This could be exemplified by canonical Wnt signaling which has been shown to regulate the invasive capacity of human MSCs.

Stimulation of Wnt signaling with recombinant Wnt3a or Wnt signaling activator, LiCl resulted in increased migration of cells through transwell filters coated with human ECM whereas inhibition of Wnt signaling led to attenuated expression of Wnt target genes, thereby diminishing the invasive capacity of MSCs [195]. PI3k/Akt signaling was also employed by MSC mediating TNF- α , SDF-1 or basic fibroblast growth factor (bFGF) induced cell migration [196-198]. To add on, MAPK/ERK signaling also plays critical role in migration of MSCs. Both SDF-1 and TNF- α induced migration of MSCs employs this pathway and the induced migration could be abrogated upon pharmacological inhibition of ERK [199-201].

1.6.1.3 Clinical implication of MSCs

Emerging evidence suggesting the regenerative potential and immune-suppressive capacity of MSCs has prompted a new age for MSCs as a promising candidate for tissue-sparing and immune-mediated diseases treatment. Transplanted MSCs have been applied for treatment of many devastating diseases, including osteogenesis imperfecta [202], large bone defects [203], myocardial infarction [178, 204, 205], renal disease [206], Crohn's disease [207], etc. Cardiovascular disease is one of the foci of interest for MSC-based therapeutics. In preclinical studies using experimental models of cardiac injury, such as myocardial infarction, transplantation of MSCs led to significant reduction in infarct size, improved cardiac function, as well as reduction in myocyte apoptosis [208-210]. MSCs could also be employed for renal disease treatment. When administered to animals undergoing acute kidney injury, MSC engrafted within the damaged kidney, prevented apoptosis and elicited proliferation of

tubular epithelial cells [206, 211-214]. Interestingly, a more recent study demonstrated that rather than the initially proposed strategy in which MSCs trans-differentiate into tubular epithelial cells resulting in restoration of renal structure and function, it is more probably that the paracrine actions, including reduction in expression of pro-inflammatory cytokines and the up-regulation of anti-inflammatory cytokines are responsible for accelerated recovery of renal function in MSC-treated kidneys [215].

Therefore, in addition to contributing to tissue repair directly, MSCs could also modulate the immune system to ameliorate excessive inflammation induced tissue damage. Initial indication regarding the immunomodulatory aspects of MSCs was from transplantation studies in animals and clinical trials showing that autologous and allogeneic MSCs could be transplanted without immune rejection [202, 216]. Further studies demonstrated that MSCs expressed functionally active indoleamine 2,3-dioxygenase (IDO) to inhibit allogeneic T-cell response [217]. Alternatively, MSCs could reduce T-cell activation through inhibiting the maturation of dendritic cells [218, 219]. The immunosuppressive ability of MSCs was then exploited clinically for acute graft-versus-host disease (GvHD) treatment. The 8-year old boy with refractory steroid immunosuppression was successfully cured by MSCs transplantation [220]. To add on, Gonzalo-Daganzo et al reported that MSCs were better employed prophylactically when used to treat acute GvHD in umbilical cord blood transplantation [221].

During the past 10 years, MSC transplantation has been considered safe and no adverse side effects have been shown affecting their safety profile. However, recent preclinical studies have highlighted the potential long term risks associated with MSC therapeutics including maldifferentiation [222], immunosuppression [223] and instigation of malignant tumor growth [224-227]. Also, unresolved issues such as lack of conformity with respect to cell isolation and culture remain as obstacles for MSCs research. Therefore, it is clear that more work is needed to tackle those potential risks and long term studies will be needed to further validate the nature of MSC-based therapy.

1.6.2 NCAM in MSCs

1.6.2.1 Adhesion molecules in MSC

Besides above mentioned P-selectin, which involves in adhesion of MSCs to the endothelium, various integrin subunits including $\alpha 1$, $\alpha 2$, $\alpha 4$, $\alpha 6$, αv , $\beta 1$, $\beta 3$, and $\beta 4$ are known to expressed in MSCs [228]. Very late antigen-4 (VLA-4), also known as $\alpha 4\beta 1$ integrin has been demonstrated to be involved in firm adhesion of hMSCs to endothelial cells under conditions of shear flow. Treatment of MSCs with neutralizing antibody against VLA-4 or treatment of endothelial cells with antibody to its counterpart adhesion molecule, namely VCAM-1, induced a significant decrease in MSCs adherence to endothelial cells [188]. Moreover, Ip et al identified integrin $\beta 1$ instead of CXCR4 as a novel pathway for trafficking and engraftment of MSCs to the ischemic myocardium in a model of acute myocardial infarction [229].

Other adhesion molecules including VCAM-1, ICAM-1, ICAM-2 and ALCAM (Activated leukocyte adhesion molecule) are also present on the surface of MSCs [230]. In Segers and colleagues' study, treatment with TNF- α leads to an increased expression of VCAM-1 on rat MSCs, and the MSCs adherence to cardiac microvascular endothelial cells (CMVE) was abolished after treatment with anti-VCAM-1 antibody [231].

Based on evidence reported so far, adhesion molecules are functionally important in the MSCs adhesion to endothelial cells, one of the critical steps of homing. Given the inherent homing capacity of MSCs, it would thus be interesting to make a comparison regarding the cell adhesion molecule expression profile between these mobilized, circulating MSCs and tissue-derived MSCs thereby providing further insight into the potential mechanisms of MSC homing, as well as expanding the functional importance of adhesion molecules in MSCs.

1.6.2.2 NCAM in MSCs

Recently, NCAM was found to express in MSCs [162, 232], yet its function in MSCs remains largely elusive. In a previous study, we identified the novel regulatory role of NCAM in MSCs differentiation into adipocytes [233]. In addition, NCAM has been shown to contribute to hematopoietic supporting function of MSCs [234, 235]. In MSCs of monkey origin, elevated NCAM expression level was noted with increasing passages. Taking into consideration the regulatory role of NCAM in cancer [236, 237]

and neuronal cells [238] migration, it would thus be interesting to explore the possible function of NCAM in MSCs mobilization as well as the underlying mechanism.

1.7 Aims of Study

As already mentioned, NCAM is present in a wide variety of non-neuronal cell types including skeletal muscle [10] and pancreatic β -cells [11], where its function remains largely elusive. In many cancers, the deregulated NCAM expression pattern correlates with cancer progression as demonstrated in various studies involving human cancer biopsies as well as mouse tumor models [111]. However, the function of NCAM in cancer progression remains controversial and tumor context dependent. Previous studies have identified the constitutive expression of NCAM in cell lines derived from malignant melanoma patients with CNS metastatic lesions [126], as well as in aggressive metastasizing uveal melanoma [127], implying the potential implication of NCAM in melanoma development. However, the concrete role of NCAM in melanoma progression remains elusive. To this end, the first aim of this study is to characterize the function of NCAM in melanoma metastasis and this will be covered in Chapter 3. Moreover, the molecular mechanism underlies will be investigated and the data obtained will be presented in Chapter 3.

Recently, NCAM was found to be expressed in MSCs, which are promising tools in tissue engineering as well as multiple cellular and gene therapies owing to their multipotency[161] and ability to mobilize and specifically localize to tumor [239, 240] and injury sites [241, 242]. However, little is known regarding the function of NCAM

in MSCs. Our previous study demonstrated that NCAM potentiated adipogenesis of MSCs via activation of insulin signaling [233]. Given the tri-lineage differentiation potential of MSCs, we thus hypothesize that NCAM may also affect osteogenesis and chondrogenesis of MSCs. Moreover, it has been noted that NCAM expression level in MSCs of monkey origin increases with passages [243]; together with the fact that NCAM regulates cell migration in other cell types [238], we wonder the possible link between NCAM and MSCs mobilization as well as the underlying mechanisms. These questions will be addressed and discussed in Chapter 4.

CHAPTER 2 MATERIALS AND METHODS

2.1 Reagents

PKA inhibitor (H89), Wnt agonist (LiCl), TNF- α human buffered aqueous solution, ROCK inhibitor (Y27632) and Collagen I solution were purchased from Sigma-Aldrich. PI3K inhibitor (LY294002) was from Cell Signaling Technology. MEK inhibitor (U0126) and FGFR specific inhibitor (SU5402) were from Calbiochem. Lipofectamine 2000, G418 and puromycin were from Invitrogen. For adipogenic, oestrogenic and chondrogenic differentiation, the following reagents were obtained from Sigma-Aldrich: Insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), ascorbic acid-2-phosphate, β -glycerophosphate, transforming growth factor β 1 (TGF β -1). Phalloidin-Alexa 546 was purchased from Sigma-Aldrich for F-actin staining.

2.2 Antibodies

Table 4. Primary Antibodies

Antibody	Species	Source
Anti-NCAM	Rabbit	Chemicon
Anti-phospho-Akt	Mouse	Cell Signaling Technology
Anti-Akt	Rabbit	Cell Signaling Technology
Anti-phospho-CREB	Rabbit	Cell Signaling Technology
Anti-CREB	Rabbit	Cell Signaling Technology
Anti-phospho-c-Raf	Rabbit	Cell Signaling Technology
Anti-phospho-MEK	Rabbit	Cell Signaling Technology
Anti-phospho-ERK	Rabbit	Cell Signaling Technology

Anti-ERK	Rabbit	Cell Signaling Technology
Anti-phospho- β -catenin	Rabbit	Cell Signaling Technology
Anti- β -catenin	Rabbit	Cell Signaling Technology
Anti-phospho-GSK3 β	Rabbit	Cell Signaling Technology
Anti-GSK3 β	Rabbit	Cell Signaling Technology
Anti-c-myc-tag	Mouse	Invitrogen
Anti- β -actin	Mouse	Chemicon
Anti-CD73 (PE)	Mouse	BD Biosciences
Anti-CD90 (FITC)	Mouse/Rat	BD Biosciences
Anti-CD105 (PE)	Mouse	BD Biosciences
Anti-CD29 (PE)	Mouse	BD Biosciences
Anti-CD34 (PE)	Mouse	BD Biosciences
Anti-CD45 (PerCP-cy5.5)	Mouse	BD Biosciences
Anti-CD31 (PE)	Mouse	BD Biosciences
Anti-CD44 (PE)	Mouse	BD Biosciences

Table 5. Secondary antibodies

Antibody	Source
Goat anti-mouse IgG, HRP conjugated	Sigma-Aldrich
Goat anti-rabbit IgG, HRP conjugated	Sigma-Aldrich
Goat anti-rabbit IgG, Alexa Fluora 488	BD Biosciences

2.3 Media

All media were sterilized by autoclaving or filtered unless otherwise stated.

Table 6. Media

Luria Bertani (LB) medium	10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 8.56 mM NaCl, pH 7.4
LB agar	LB medium plus 15 g/l Bacto-agar
Culture medium	Dulbecco's modified Eagle's medium (DMEM)-low glucose (LG)/high glucose (HG), supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 10% heat-inactivated fetal bovine serum (FBS) for DMEM-LG or 10% heat-inactivated fetal calf serum (FCS) for DMEM-HG (Invitrogen)
Adipogenic differentiation medium	DMEM-LG supplemented with Insulin (1 µg/ml), IBMX (50 µM) ,dexamethasone (1 µM) and 1% FBS
Osteogenic differentiation medium	50 µM ascorbic acid-2-phosphate, 10 mM β-glycerophosphate and 100nM dexamethasone in basal culture medium
Chondrogenic differentiation medium	DMEM-LG supplemented with 1% FBS, insulin (6.25 µg/ml), TGF-β1(10 ng/ml), and ascorbic acid-2-phosphate (50 nM)

2.4 General buffers and solutions

Table 7. General buffers and solutions

10× PBS (phosphate buffered saline)	80 g NaCl, 2 g KCl, 14.4 g Na ₂ HPO ₄ , 2.4 g KH ₂ PO ₄ in 1 L ddH ₂ O (distilled, deionized water), pH 7.4
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Lysis buffer	20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor (Rhoche Applied Sciences)
4 $\times$ Resolution gel buffer	1.5 M Tris-HCl, pH 8.8, 0.4% SDS
4 $\times$ Stacking gel buffer	0.5 M Tris-HCl, pH 6.8, 0.4% SDS
10 $\times$ SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) Running Buffer	0.25 M Tris, 1.9 M glycine, 1% SDS
5 $\times$ Sample loading buffer	250 mM Tris HCl, pH6.8, 10% SDS, 30% glycerol, 5% β -mercaptoethanol (or 0.5 M DTT (Dithiothreitol)), 0.02% bromophenol blue
Transfer buffer (Immunoblotting)	25 mM Tris, 191.8 mM glycine
Blocking buffer (Immunoblotting)	PBS-T (PBS with 0.1% Tween-20) containing 5% non-fat milk
50 $\times$ TAE (Tris-Acetic-EDTA) buffer	2 M Tris base, 5.71% acetic acid, 0.05 M EDTA, pH 8.0
PKA dilution buffer	350 mM K_3PO_4 , pH 6.8, 0.1 mM DTT
PKA extraction buffer	25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β -mercaptoethanol, 1 mg/ml leupeptin, 1 mg/ml aprotinin
Phosphatase Inhibitor Buffer (PIB)	125 mM NaF, 250 mM β -glycerophosphate, 250 mM p-nitrophenyl phosphate (PNPP), 25 mM NaVO_3

Hypertonic Buffer (HB)	20	mM	HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5, 5 mM NaF, 0.1 mM EDTA
PBS/PIB	0.5 ml PIB in 10 ml 1×PBS			

2.5 Cell culture and Animals

2.5.1 Culture of murine melanoma cells

The murine melanoma B16F0 and F10 cells were obtained from American Type Culture Collection (ATCC) and routinely maintained in DMEM-HG supplemented with 10% FCS and 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). For routine sub-culturing, cells were washed once with PBS and incubated with 0.5 ml of 0.25% trypsin until cells are detached from substratum. Culture medium was then added to stop trypsinization. The cells were gently flushed off the culture dish and pelleted by centrifugation at 800 rpm for 5 minutes and resuspended in fresh culture medium.

2.5.2 Isolation and culture of MSCs

MSCs were isolated from NCAM wild-type and knockout mice. NCAM wild-type and knockout mice were bred on a C57BL/6J background [244]. Male and female heterozygous NCAM^{+/-} mice were used for breeding homozygous NCAM^{+/+} (wild-type) and NCAM^{-/-} mice. Genotyping was performed as previously described to confirm the ablation of all NCAM isoforms in NCAM knockout mice [81]. Animal husbandry and procedures for sacrificing the animals were approved by the

institutional animal care and use committee (IACUC) from Nanyang Technological University. MSCs were isolated from wild type and NCAM^{-/-} mice bone marrow and cultured as described previously [233]. Briefly, whole bone marrow was harvested from the femurs and tibias of 8-week male C57BL/6J mice. Cells were cultured in DMEM-LG supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). After 5-7 days of incubation in a 37°C humidified incubator containing 5% CO₂, non-adherent hematopoietic cells were discarded. The adherent cells were further purified through repetitive passaging and reached homogeneous at passage 6. All the MSCs used in current study were around passage 10 with fibroblast like morphology.

2.5.3 Experimental metastasis assay

C57BL/6J Mice (6–8 weeks) were obtained from the laboratory Animal Center of NUS (Singapore). For experimental metastasis analysis, single cell suspensions (1×10⁶ B16F0 cells) in 100 µl PBS were injected into the lateral tail vein of the mice. On day 14 post injection, mice were euthanized and all organs were inspected for the presence of macroscopic metastases. Lungs and livers with metastatic nodules were removed and fixed in 4% paraformaldehyde for histological analysis. Lung and liver metastatic nodules were counted under a dissecting microscope.

2.6 Plasmid and DNA constructs

The dominant negative CREB construct named ACREB and its corresponding control construct were kindly provided by Dr. Charles Vinson [245]. A constitutively active

mutant D2-Akt (T308D/S473D) construct was a gift from Dr. Takashi Tsuruo [246] and the insert encoding mutant Akt was subcloned into *Xho* I/ *Eco*R I sites of pIRES2-EGFP (Clontech). The dominant negative and constitutive active forms of MEK were gifts from Dr Sheng-Cai Lin (Xiamen University, China).

cDNAs containing full-length mouse NCAM140 or NCAM180 open reading frames (ORFs) were individually cloned into *Hind*III/*Xho*I sites of pcDNA4/Myc. Transfection was conducted using Lipofectamine 2000 following manufacturer's instructions. For transient transfection of plasmid, cells were fed with fresh medium containing 10% FCS/FBS 6 hours post transfection and harvested 36 hours later for further experiments.

2.7 Small interfering RNA (siRNA) and Transfection

The siRNA template oligonucleotides targeting two different 19 nucleotides of mouse NCAM transcript were generated as follows: NCAM-3.1F siRNA, nucleotides 83-102, 5'- GGAGAAATCAGCGTTGGAG-3' NCAM-3.1R, 5'- CTCCAACGCTGATTCTCC-3'; NCAM-4.1F siRNA 109-128: 5'- ATTCTTCCTGTGTCAAGTG -3', NCAM-4.1R, 5'- CACTTGACACAGGAAGAAT-3'. Restriction sites were designed at the 5' and 3' ends to facilitate directional cloning into *Bam*HI and *Hind*III digested pSilencer 3.1-U6 and pSilencer 4.1-CMV vectors (Ambion). The oligonucleotides were annealed in annealing buffer (100 mM NaCl and 50 mM HEPES, pH 7.4) and incubated at 95°C for 5 min, followed by chilling on ice for 1 min. The annealed oligonucleotides were

then cloned into two separate siRNA expression vectors, pSilencer 3.1-U6 and pSilencer 4.1-CMV, respectively. The same vectors containing hairpin siRNAs with a scrambled sequence not found in the mouse database was constructed and used as siRNA control. Transfection was conducted using Lipofectamine 2000 following manufacturer's instructions. Briefly, B16F0/F10 cells plated at 80% confluence in 60-mm dishes were co-transfected with pSilencer 3.1- and pSilencer 4.1-based recombinant plasmids, 4 µg each. To generate stable cell lines harboring the desired plasmids, transfectants were selected with 1 µg/ml puromycin and 800 µg/ml G418 for two weeks. Knockdown efficiency was determined by immunoblotting.

2.8 Cell adhesion assay

For cell adhesion assay, the Nunc 96-well plate was coated with 40 µg/ml Collagen I solution in PBS at 4°C overnight. After 12 hours coating, collagen solution was removed and the plate was air-dried at room temperature in tissue culture hood. Prior plating cells, the coated wells were washed with ddH₂O for three times. To seed the cells, 100 µl cell suspension (2×10^5 cells/ml) was added to each well of the 96-well plates and incubated for 10, 30, 60 and 120 minutes. Each time point was stopped by decanting the floating cells, followed by staining those attached cells with 0.5% crystal violet in 20% methanol. Cell density was determined spectrophotometrically by dissolving the stain with 20% acetic acid and followed by measuring the absorbance at OD570 with using a TECAN Genios plate reader and Magellan Version 4.0 (TECAN).

2.9 Wound healing assay

Cells were seeded in 60-mm dishes and allowed to reach complete confluence. A wound was inflicted by making a scratch through the confluent layer of cells with a 200 μ l pipette tip. The wounded cell layer was subsequently washed once with fresh medium to remove loosely attached cells and refed with fresh culture medium. Microphotographs of the scratch were taken at 0, 12 and 24 hours post scratching, respectively. The distance migrated was determined by measuring the width of the wound, dividing by two and subtracting this value from the initial half-width of the wound.

2.10 In vitro migration and invasion assay

2.10.1 Invasion assay

For melanoma cells, *in vitro* invasion assay were performed as previously described with some modification [247]. Briefly, a suspension of 1×10^5 cells in 500 μ l serum free DMEM was added to the upper chamber of BioCoat Matrigel invasion chambers (BD Biosciences) whereas the lower chamber was filled with chemo-attractant. NIH-3T3 fibroblast-conditioned medium served as chemo-attractant here. To prepare NIH-3T3 conditioned medium, mouse NIH-3T3 fibroblast of 80% confluent was washed once with PBS and cultured for 18 hours in serum free DMEM. Thereafter, the cultured medium was collected, centrifuged at 4,000 rpm for 5 min, filtered through 0.22 μ m filters and stored at -80°C until use. After 24 hours incubation at 37°C , the cells on the upper surface of the filter were carefully removed with a cotton swab and those that had traversed the membrane were stained with 0.5% crystal violet. To quantify

the migrated cells microscopically, twenty random fields/filter (magnification $\times 200$) were selected followed by cell count. The migration assay was performed in a similar fashion with transwell (Corning) without Matrigel coated.

2.10.2 Migration assay

MSCs migration was evaluated with a two-chamber Transwell system (8 μm pore size and 6.5 mm diameter; Costar) as previously described with some modification [247]. Briefly, a suspension of 1.5×10^4 cells in 100 μl serum free DMEM was seeded in the top chamber of Transwell in the presence of inhibitor when indicated. 500 μl of culture medium with serum was placed in the bottom chamber as chemo-attractant. After 24 hours incubation at 37°C , cells remaining on the top surface of the filter were removed with a cotton swab and the cells that had traversed the membrane to the bottom side were stained with 0.5% crystal violet. As stated above, twenty random fields/filter (magnification $\times 200$) were selected followed by cell count to quantify the migrated cells microscopically.

2.11 Protein extraction and Immunoblotting

Subconfluent cells were washed twice with cold PBS, harvested by scraping, lysed in lysis buffer followed by centrifugation at $14,000 \times g$ for 20 min at 4°C . The supernatant was collected and protein concentration was determined using Bradford assay (BioRad). For immunoblotting, samples were heated at 100°C for 5 min and resolved by 8%~10% SDS-PAGE and transferred onto a polyvinylidene difluoride

membrane (Millipore). After blocking with 5% skim milk in PBST (0.1% Tween-20 in PBS) for 1 hour, membranes were incubated with specific primary antibodies at 4°C overnight. Thereafter, membrane was washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit (Sigma) secondary antibody for 1 hour at room temperature with gentle agitation. The secondary antibodies were prepared in 5% skim milk in PBST and the washing steps were performed with PBST, for three times of 10 minutes each at room temperature. After thrice wash in PBST, membranes were subjected to chemiluminescence detection using Immobilon Western Chemiluminescent HRP Substrate system (Millipore) following manufacture's protocol and exposed to an X-ray film (Kodak) and developed using Kodak X-OMAT ME processor.

2.12 PepTag[®] Non-Radioactive cAMP-dependent protein kinase assay

The PepTag[®] Non-Radioactive cAMP-dependent Protein Kinase Assay (Promega) was performed to examine melanoma cell PKA activity following manufacturer's protocol (Fig. 4). Briefly, 1×10^7 cells were trypsinized and washed twice with PBS. Thereafter, cells were suspended in 0.5 ml cold PKA extraction buffer and then homogenized by sonication for 1 min. Cell lysates were centrifuged at $14,000 \times g$ for 5 minutes at 4°C; the supernatant was collected and the concentration for each sample was determined. Thereafter, each sample was mixed with PepTag[®] PKA Reaction 5× buffer, PepTag[®] A1 peptide, PKA Activator 5× solution and water and incubated at room temperature for 30 minutes. The reaction was stopped by placing the tube on a 95°C heating block for 10 minutes followed by running the samples on 0.8% agarose

gel at 100 V for 20 minutes until the separation of the bands was apparent. The phosphorylated and non-phosphorylated forms of PepTag® peptide bands were pink in color and visible to the naked eye. To attain higher sensitivity, the gel was photographed under UV light. To further quantify the kinase activity, the negatively charged phosphorylated bands were excised from the gel and heated at 95°C until the gel splice was melted. The hot agarose of 125 µl was mixed with 75 µl Gel Solubilization Solution and 50 µl of glacial acetic acid and transferred to a 96 well plate. To detect the signal, absorbance at 570 nm was measured and quantified.

Figure 4. Schematic diagram of the PepTag® Non-Radioactive Protein Kinase Assay procedure.

2.13 Transcription factor CREB reporter assay

2.13.1 Preparation of nuclear extract

5×10^6 cells were washed once with and suspended in ice-cold PBS/PIB followed by centrifugation at $300 \times g$ for 5 minutes at 4°C . The cell pellet was then resuspended in 0.5 ml ice-cold HB buffer and incubated on ice for 15 minutes to allow the cells to swell. Thereafter, 25 μl 10% Nonidet P-40 was added and mixed with the cell

suspension by vigorously vortexing for 10 seconds until the cell membrane was completely lysed while the nuclear membrane remained intact. The homogenate was then subjected to centrifugation for 30 seconds at 4°C. The nuclear pellet was resuspended in 25µl Complete Lysis Buffer and incubated for 30 minutes at 4°C with constant agitation. After that, the nuclear suspension was centrifuged for 10 minutes at $14,000 \times g$ at 4°C. The supernatant was saved as the nuclear extract and the protein concentration of the extract was determined using Bradford-based assay.

2.13.2 CREB reporter assay

The DNA binding activity of CREB was determined using the TransAM™ Assay kit (Active Motif) based on manufacturer's recommendations. Briefly, 2-10 µg of nuclear extracts was incubated in oligonucleotide-coated 96 well plates for 3 hours at room temperature with mild agitation (100 rpm on a rocking platform). Thereafter, the bond complex was washed 3 times with 200 µl 1× Wash buffer and probed with specific primary antibody against phospho-CREB suspended in 1× Antibody Binding Buffer. After 1 h incubation, each well was washed 3 times with 1× Wash Buffer and HRP-conjugated anti-rabbit IgG antibody in 1× Antibody Binding Buffer was added. Incubation with secondary antibody for 1 hour was then followed by 4 times thoroughly washes with 1× Wash Buffer. To detect the signal, the samples were incubated with Developing Solution for 15 minutes at room temperature protected from direct light followed by stopping the reaction with Stop Solution and measurement of absorbance at 450 nm with reference wavelength of 655 nm.

2.14 Immunohistochemistry

The animals were euthanized 2 weeks post inoculation of melanoma cells to examine formation of metastasis. The dissected lung and tumor tissues were further cut into smaller blocks of about 2 mm × 2 mm, fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4°C for 18 hours and transferred to 30% sucrose in PBS. To allow fully impregnation, the tissue was immersed in 30% sucrose for 2 days until it sunk. The tissue blocks with metastases were sectioned in cryostat at 10 µm and mounted onto poly-L-lysine-coated glass slides. Hematoxylin and eosin (H&E) staining was performed on at least one slide for each animal. In detail, mounted slides were first rinsed in distilled water for 1 minute before transferring into Hematoxylin solution (Sigma-Aldrich). After staining with Hematoxylin, the slides were rinsed in distilled water twice followed by incubation in differentiating solution (1% HCl in 100% ethanol). Thereafter, the slides were again rinsed, soaked in Scotts tap water substitute (Magnesium sulfate buffered with sodium bicarbonate) (Electron Microscopy Sciences) for 5 minutes, in 95% ethanol for 30 seconds then transferred to Eosin Y solution (Sigma-Aldrich) and stain for 20 seconds. After that, excess Eosin was removed by emerging the slides in 70% ethanol followed by PBS for 1 minute. The slides were then covered with mounting media and investigated under an Olympus IX71 fluorescent microscope (Nikon). For immunostaining against NCAM, the sections were rinsed, blocked with 4% bovine serum albumin (BSA) and incubated with rabbit anti-NCAM antibody (1:1000 in PBS/ Triton X-100) overnight at 4°C. Following thrice wash with PBS-TX, they were then incubated with anti-rabbit FITC antibody,

counterstained with DAPI for visualization of nuclei, washed and mounted with FluorSave Reagent (Calbiochem). The staining was visualized and images were acquired by a Zeiss Axiovert LSM-510-META-inverted microscope (Carl Zeiss).

2.15 Quantitative reverse transcription-PCR (QRT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen), and reverse transcribed to generate cDNA using SuperScript II reverse transcriptase and anchored oligo (dT) primers (Invitrogen) according to manufacturer's recommendations. QRT-PCR was performed with KAPA SYBR green QRT-PCR master mix (KAPA Biosystems). The primers used for SYBR Green quantitative reverse transcription PCR were as follows: MMP-2, 5'-CAAGTTCCCCGGCGATGTC-3' and 5'-TTCTGGTCAAGGTCACCTGTC-3' ; β -actin, 5'-GCTCTTTTCCAGCCTTCCTT - 3' and 5'-TGATCCACATCTGCTGGAAG-3'. β -actin served as an endogenous control. QRT-PCR was performed on an ABI Prism 7500 Sequence detection system (Applied Biosystems) with 20 ng cDNA in KAPA SYBR fast QRT-PCR master mix per 20 μ l reaction. Reaction conditions were as follows: 50°C for 2 minutes, 95°C for 1 minute, followed by 40 cycles of 95°C for 3 seconds and 60°C for 1 minute. Melting curve analysis was performed at the end of each run from 60°C to 95°C. At the end of QRT-PCR, the relative expression of mRNA was calculated and analyzed using the comparative *Ct* method according to manufacturer's instructions (Applied Biosystems). The target mRNA level of control cells normalized to that of β -actin, was set to 1. The *Ct* value for each gene was determined in the linear phase of amplification, and the fold change for each gene was obtained using $2^{-(\text{mean } \Delta C_t(\text{gene1}) - \text{mean } \Delta C_t(\text{gene2}))}$.

2.16 Flow cytometry analysis

For cell surface marker immunophenotyping, MSCs were detached and washed once with 0.1% sodium azide and 0.5% BSA in PBS, and incubated with the following primary antibodies including antibodies against NCAM, CD73, CD90, CD105, CD34, CD45, CD117, CD31. For indirect immunofluorescence, cells were washed and incubated with corresponding fluorescein conjugated secondary antibody for 30 minutes. Cells were subsequently analyzed with a FACSCalibur flow cytometer (BD Biosciences). Background fluorescence was subtracted after analyzing cells stained with relevant isotype control.

2.17 In vitro differentiation of MSCs

2.17.1 Adipogenic differentiation

To induce adipogenic differentiation, MSCs were incubated for 3 days in adipogenic differentiation medium. The medium was replaced with culture medium containing insulin alone on day 4, and changed every 2 days thereafter for 2 weeks. For visualization of lipid vacuole accumulation, the cells were fixed with 2% paraformaldehyde and stained with Sudan Black for 20 minutes at room temperature.

2.17.2 Osteogenic differentiation

To induce osteogenic differentiation, 1×10^4 cells per well were plated onto a 24 well-plate and the medium was changed to osteogenic differentiation medium 24 hours later. Thereafter, the medium was changed every 3 days for 3 weeks. Alizarin Red S

staining was performed to assess osteogenic differentiation. In brief, cells were fixed in ice cold 70% ethanol for an hour at -20°C followed by staining with 40 mM Alizarin Red S (pH 4.1) for 20 minutes. Secretion of calcified extracellular matrix was shown as red stains.

2.17.3 Chondrogenic differentiation

To induce chondrogenic differentiation, 1×10^4 culturing cells were centrifuged at 1,000 rpm for 5 minutes to form a pelleted micromass (10 μ l) and cultured in chondrogenic differentiation medium for 3 weeks. Medium changes were carried out every 2 days and chondrogenesis was assessed by the end of 3 weeks post induction. The pellets were fixed with 2% paraformaldehyde for 15 minutes followed by staining with 1% Alcian Blue for 20 minutes. The highly sulfated proteoglycans of cartilage matrices were stained blue as control.

2.18 Indirect immunofluorescence staining

MSCs cultured on glass coverslips were washed twice and fixed with 4% paraformaldehyde in PBS for 5 minutes and then permeabilized with 0.1% Triton X-100/PBS (PBS-TX) for 30 min. Thereafter, cells were incubated with fluorescent Phalloidin conjugate solution in PBS for 40 minutes at room temperature. All washings were performed with 0.1% Triton X-100 in PBS. The coverslip were dried briefly and mounted on glass slides with FlourSave™ Reagent (Calbiochem). Finally, the slides were scrutinized field by field with Zeiss Axiovert LSM-710-META-

inverted microscope LSM710 and images were processed using the Zeiss LSM Image Examiner (Carl Zeiss).

2.19 Statistical analysis

Data were expressed as means $\pm$ s.d.. 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. For the rest, statistical analysis was done using student's t-test or one-way ANOVA for groupwise comparisons. A *p* value of < 0.05 was considered statistically significant.

CHAPTER 3 Neural cell adhesion molecule potentiates invasion and metastasis of melanoma cells through cAMP-dependent protein kinase and phosphatidylinositol 3-kinase pathways

3.1 Results

3.1.1 Down-regulation of NCAM alters the morphology of mouse melanoma B16F0 cells.

To explore the function of NCAM in melanoma progression, suppression of its expression by siRNA-mediated approach in the mouse melanoma B16F0 cells was performed. In detail, B16F0 cells were transfected with pSilencer 3.1 and pSilencer 4.1 based plasmids expressing siRNAs targeting different regions of NCAM coding sequence or scrambled siRNAs with no homology to any known mouse gene serving as control. Stable cell line with silenced NCAM expression was obtained and the knock down efficiency was examined by immunoblotting. As shown in Fig. 5A, more than 70% NCAM expression level (quantified in Fig .5B) was successfully suppressed compared to Control cells and these cells were used for subsequent experiments.

It is worth noting that B16F0 cells lost their mesenchymal cell morphology upon NCAM ablation (Fig. 5C). In contrast to Control cells which are of spindle shape and sparsely distributed, NCAM knock down (KD) cells assumed an enlarged, flattened

appearance and clustered together which may indicate the mesenchymal-epithelial transition (MET) [248] of melanoma cells upon NCAM silencing.

Figure 5. Down-regulation of NCAM alters the morphology of mouse melanoma B16F0 cells.

A, Expression level of NCAM in melanoma B16F0 cell stably co-transfected with pSilencer-3.1 and pSilencer-4.1 based plasmids containing scrambled sequence (Control) or 19-bp insert targeting NCAM was determined by immunoblotting. **B**, morphology of Control and NCAM KD cells is shown. Scale bar, 25 μ m. *, $p < 0.05$. **, $p < 0.01$.

3.1.2 NCAM silencing retards melanoma cell migration and invasion.

Cellular invasion, a characteristic of metastatic tumors, involves cell attachment, ECM degradation and cell migration [95]. To determine if NCAM loss affects above aspects,

cell attachment experiment was performed first [249]. Compared to Control cells, silencing of NCAM significantly enhanced B16F0 cell adhesion to substratum at different attaching period of time (Fig. 6A). Thereafter, cell motility and invasiveness representing the characteristics of metastatic tumors [95] were gauged to examine the impact of NCAM silencing. As shown in Fig. 6B, silencing of NCAM significantly impaired the migration of B16F0 cells in a wound healing assay. Twelve hours after scratching, the area of wound recovered by the migration of NCAM KD cells was not significant and only less than half of that for control ones. Moreover, the wound inflicted on control cell monolayer was completely healed in 24 hours. Nevertheless, 24 hours post wounding, the migration distance for NCAM KD cells was even less than that for Control cells 12 hour after scratching (Migration distance: 12 h, Control, 0.42 mm, NCAM KD, 0.13 mm, $p < 0.01$; 24 h, Control, 0.64 mm, NCAM KD, 0.3 mm, $p < 0.01$). Such inhibitory effect of NCAM silencing on cell motility was further validated by transwell assay (Fig. 6C, *left*). Likewise in matrigel invasion assay (Fig. 6C, *right*) where matrigel was used to coat the porous membrane serving as the surrogate of ECM, the invasiveness of NCAM KD cells was less than 50% of Control cells ($p < 0.01$). In summary, silencing of NCAM led to reduced cell motility and invasiveness.

Figure 6. NCAM gene silencing affects adhesion, migration and invasion of melanoma cells.

A, A total of 1.5×10^4 cells were seeded into 96 well plates for the indicated times. Adherent cells were stained with crystal violet and quantified spectrophotometrically. **B**, Wound healing of Control and NCAM KD cells was performed and pictures of the wound distance were taken at each time point as indicated. **C**, the cell motility (*left*) and invasiveness (*right*) were evaluated by transwell migration and matrigel invasion assays. Data are given as mean of three independent experiments. *Columns*, mean of three independent experiments, *bars*, s.d.; *, $p < 0.05$; **, $p < 0.01$.

3.1.3 Down-regulation of NCAM perturbs the metastatic competence of mouse melanoma B16F0 cells.

Cancer metastasis is the end product of a micro-evolutionary process in which tumor cells eventually populate and flourish at new tissue habitats. However, the secondary organ to which metastatic cancer cells colonize is dependent on tumor context. In this regard, metastatic melanoma preferentially metastasizes into lymph nodes, lung, liver and brain [250-252]. To investigate whether NCAM down-regulation affects the metastatic dissemination of melanoma cells, we injected B16F0 Control and NCAM KD cells into the lateral tail vein of C57BL/6J mice. Fourteen days post inoculation, animals were sacrificed and major organs including brain were examined for the presence of tumor metastases. In such experimental metastasis assay, we found that melanoma cells directly introduced into the bloodstream mainly formed metastatic deposits in lungs. As shown in Fig. 7A, inoculation of B16F0 Control cells resulted in the formation of numerous lung metastases (median, 437; range, 397-517) whereas silencing of NCAM reduced the number of metastases to one third (median 162; range, 87-163; $p < 0.01$). B16F0 Control cells generated bigger lesions occupying higher percentage of total lung area. In stark contrast, metastatic nodules of NCAM KD cells exhibited as small, discrete black foci. Furthermore, we identified the presence of hepatic metastases in mice inoculated with B16F0 Control cells but not for NCAM KD cells (Fig. 7A). Collectively, these observations suggested that silencing of NCAM expression suppressed the metastatic potential of B16F0 cells.

To analyze if NCAM down-regulation affects the tumor histological structure, immunohistochemical experiments were carried out. Immunostaining revealed high expression level of NCAM in metastases generated by Control cells but no in the NCAM KD nodules (Fig. 7B, *upper*). However, no drastic histological difference was observed between metastases formed by control and NCAM KD cells as revealed by H&E staining (Fig. 7B, *bottom*).

Figure 7. Loss of NCAM perturbs the metastatic competence of mouse melanoma B16F0 cells.

Experimental metastasis assay was performed to evaluate the metastatic potential of Control and NCAM KD melanoma cells. *A*, Upper panel shows representative pictures of lungs and livers from mice injected with B16F0 Control cells or with NCAM KD cells. Numbers of lung and liver metastatic foci were quantified (*bottom*) as described in Materials and Method and represented by each data point. *B*, Lung nodules were sectioned and subjected to immunostaining with NCAM antibody (*upper panel*) or staining with H&E (*bottom*). The dotted lines partition the metastases lesion from normal lung tissue and the asterisks denotes metastases. Scale bar, 50 µm for immunostaining, 200 µm for H&E staining.

3.1.4 NCAM knock down leads to attenuated CREB activity and MMP2 expression.

CREB, which binds to the consensus motif 5'-TGACGTCA-3', is a well characterized transcription factor belonging to the basic leucine zipper family [253]. In response to various stimuli such as growth factor, neurotransmitter, etc, CREB is activated through phosphorylation at Ser133 located in the KID (kinase-induce domain) by many serine/threonine (Ser/Thr) protein kinases. Phosphorylated CREB will in turn bind to the transcriptional co-activator, CBP (CREB-binding protein) thus enabling the recruitment of other transcriptional machinery to initiate CREB-dependent gene transcription [253, 254]. Previously, it has been shown that CREB plays an important role in melanoma progression [255-257]. Moreover, quenching its activity with dominant-negative form of CREB (KCREB) led to decreased melanoma tumorigenicity and metastatic potential [258]. As mentioned in the Introduction, clustering of NCAM could stimulate phosphorylation of CREB in neuronal cells thereby modulating expression of genes responsible for axonal growth and synaptic plasticity [53]. In addition, remarkable defects in CREB activity and signaling pathways have been observed in NCAM knockout mice [259]. Therefore, we speculate that CREB activation is required for NCAM mediated melanoma cellular invasion. To test this hypothesis, immunoblotting was performed using antibody against phospho-CREB. As shown in Fig. 8A, a drastic reduction of phosphorylated CREB level was identified as the effect of NCAM silencing. Moreover, reporter assay monitoring DNA binding activity of CREB was conducted. In consonance, silencing of NCAM led to reduced levels of DNA binding activity of CREB (Fig. 8B). To determine whether

attenuated CREB activity affects cellular behavior of melanoma cells, B16F0 cells were transiently transfected with a dominant-negative form of CREB (ACREB). As expected, expression of ACREB clearly decreased the migration of melanoma cells (Fig. 8C).

Previous study has demonstrated that CREB could regulate the transcription, expression and activity of type IV collagenase MMP2, which in turn accounts for the invasiveness and metastatic potential of melanoma cells [258]. We therefore examined the effect of NCAM down-regulation on MMP-2 mRNA expression. Results from QRT-PCR showed that MMP-2 mRNA expression level of NCAM KD cells was only 30% of that of Control cells. Consistently, similar reduction of MMP-2 expression level was observed in melanoma cells with CREB activity quenched by ACREB (Fig. 8D). Thus, these results may suggest that down-regulation of MMP2 contributed to the compromised CREB activity thereby reduction in invasiveness and metastatic potential of melanoma B16F0cells.

Figure 8. NCAM knock down leads to attenuated CREB activity and MMP2 expression.

A, Lysate derived from NCAM KD and Control cells were analyzed by immunoblotting with anti-phospho CREB antibody. *B*, CREB reporter assay was performed to evaluate the CREB activation status in Control and NCAM KD cells. *C*, Expression of dominant negative form of CREB (ACREB) impeded cell migration. Scale bar, 1 mm. The percentage of wound closure was quantified (*right*). *D*, Quantitative reverse transcription-PCR (QRT-PCR) was employed to evaluate the mRNA expression of MMP-2 in NCAM KD, Control cells as well as F0 cells transfected with ACREB or empty vector (Veh). *Columns*, mean of three independent experiments; *bars*, s.d.; *, $p < 0.05$; **, $p < 0.01$.

3.1.5 NCAM potentiates invasion and phosphorylation of CREB in melanoma cells via activation of PKA pathway

To determine the protein kinases participating in relaying NCAM-mediated signal and in turn inducing the phosphorylation of CREB, we examined the activation status of a panel of intracellular signaling effectors likely regulated by NCAM. As discussed earlier, NCAM induced activation of a variety of signaling cascades such as PI3K, MAPK and PKC pathways via binding to FGFR. Though MAPK/ERK pathway is highly activated in melanoma cells [130], we did not detect any significant difference between NCAM KD cells and their Control counterparts in terms of ERK phosphorylation level therefore excluding the possibility of its involvement in NCAM-potentiated invasion of melanoma cells (Fig. 15A).

Other than above mentioned pathways, NCAM could also elicit activation of cAMP/PKA signaling putatively via Ca^{2+} or G-proteins [13, 260]. To examine whether PKA activity was compromised as the effect of NCAM silencing, PepTag[®] cAMP-dependent protein kinase assay was performed. PKA catalyzed phosphorylation of its substrate Kemptide, which was subsequently separated on the agarose gel moving towards the anode. The intensity of the band, therefore, is indicative of PKA activity. As shown in Fig. 9A, we found that down-regulation of NCAM led to drastic reduction in PKA activity. Based on manufacture's protocol, PKA kinase activity were subsequently quantified and shown in the right panel of Fig. 9A. To verify the role of PKA pathway in melanoma cellular invasion, B16F0 cells were treated with PKA

specific inhibitor, H89, demonstrating that both migration and invasion were significantly suppressed by H89 (Fig. 9B). Moreover, immunoblotting results using H89 treated F0 cell lysates revealed that phosphorylation level of CREB was reduced significantly upon inactivation of PKA (Fig. 9C), thereby suggesting that NCAM-mediated phosphorylation of CREB was achieved via activation of PKA pathway. To further prove the role of PKA downstream of NCAM-mediated signaling, we treated NCAM KD cells with PKA inhibitor and analyzed cellular invasive properties. Indeed, H89 treatment could not yield any further reduction in NCAM KD cells in contrast to NCAM-expressing cells (Fig. 9D).

Figure 9. NCAM potentiates invasion and phosphorylation of CREB in melanoma cells via activation of PKA.

A, PepTag[®] cAMP-dependent protein kinase assay was carried out. The intensity of band representing phosphorylated kemptide separated from non-phosphorylated one reflects the activity of PKA. PKA activity was further quantified based on manufacturer's instruction (*right*). B, Attenuated cell migration and invasion in the presence of PKA inhibitor H89. C, Treatment with H89 reduced phosphorylation level of CREB. Immunoblotting probed against CREB serves as loading control. D, Treatment with H89 did not lead to further reduction in migration and invasion of NCAM KD cells. Columns, mean of three independent experiments, bars, s.d.; *, $p < 0.05$; **, $p < 0.01$.

3.1.6 Involvement of PI3K, other than PKA, in NCAM-mediated cellular invasion and phosphorylation of CREB in melanoma cells

Other than PKA and MAPK, the downstream effector of PI3K could also induce phosphorylation thus activation of CREB. Moreover, aberrant PI3K pathway has been reported in up to 60% of melanoma and deregulated Akt3 has been shown to promote the development of malignant melanoma [138]. Therefore, we speculated that PI3K/Akt pathway may also contribute to NCAM-potentiated invasion of melanoma cells. As what has been demonstrated for the PKA pathway, results from immunoblotting revealed significant reduction in the phosphorylation of Akt owing to NCAM silencing (Fig. 10A) thus suggesting impaired PI3K/Akt signaling. To verify the contribution of PI3K/Akt signaling in melanoma cellular invasion, B16F0 cells were treated with PI3K specific inhibitor, LY294002. In the presence of LY294002, the activity of PI3K downstream effector, Akt, and melanoma cellular invasion were significantly impaired. In addition, CREB activity was compromised owing to deregulated PI3K/Akt pathway (Fig. 10B). Together, we could conclude that PI3K/Akt, other than PKA pathway, participated in transmitting NCAM-mediated signal to CREB thereby regulating cellular invasion of melanoma cells.

To verify this notion, forced expression of constitutively active form of Akt (D2-Akt) in NCAM KD cells was conducted. As shown in Fig. 10C, the motility and invasiveness of NCAM KD cells were restored to approximately 90% of Control cells. Moreover, the phosphorylation level of CREB was elevated as demonstrated by

immunoblotting further bolstering the role of PI3K/Akt axis in phosphorylation of CREB by NCAM in melanoma cells.

Figure 10. Involvement of PI3K, other than PKA, in NCAM-mediated cellular invasion and phosphorylation of CREB in melanoma cells.

A, Lysate from NCAM KD and Control cells were analyzed by immunoblotting for phospho-Akt content. B, PI3K inhibitor, LY294002, treatment led to compromised cell motility, invasiveness and CREB phosphorylation level. C, Expression of constitutively activated Akt (D2-Akt) restored cellular invasion and CREB activation in NCAM KD cells. Columns, mean of three independent experiments; bars, s.d.; *, $p < 0.05$; **, $p < 0.01$.

3.1.7 Concurrent commission of PKA and PI3K pathways is required for NCAM-mediated cellular invasion of melanoma cells.

As shown in above results, NCAM-activated PKA and PI3K/Akt axes converged at CREB. Though both cellular invasion as well as phosphorylation of CREB were significantly compromised in the presence of either PKA or PI3K inhibitor, neither H89 nor LY294002 alone could abolish NCAM-mediated cellular invasion of melanoma cells, likewise the activation of CREB. Thus, one can exclude the possibility of pathway redundancy mediating signals from NCAM.

However, we still wondered whether there is any crosstalk between these two pathways at kinase level. To address this issue, immunoblotting was performed to examine phospho-Akt level following H-89 treatment. As shown in Fig. 11A, phosphorylation of Akt remained comparable level after H-89 treatment indicating PI3K/Akt signaling could not be regulated by PKA. We also examined whether PI3K inhibitor has any inhibitory effect on PKA kinase activity. In consistence, no significant change of PKA activity was observed in the presence of LY294002 (Fig. 11B). Therefore, these results suggest that there is no crosstalk at kinase level between PKA and PI3K/Akt pathways in melanoma cells under our experimental conditions.

We also examined melanoma cell migration and invasion in the presence of both H89 and LY294002. By combining the effect of both inhibitors, further reduction in migration was observed compared to those under the effects of either inhibitor alone

(Fig. 11C). Similar result was observed in invasion assay (Fig. 11D). In all, these results further corroborating the idea that NCAM induced migration and invasion of melanoma cells requires cooperated action of PKA and PI3K signaling.

Figure 11. Concurrent commission of PKA and PI3K pathways is required for NCAM-mediated cellular invasion of melanoma cells.

A, PKA inhibitor, H89, did not affect the phosphorylation level of Akt. B, Similarly, treatment with LY294002 did not affect PKA activity. C&D, Combining H89 and LY294002 followed by examination of cell migration and invasion. Columns, mean of three independent experiments; bars, s.d.; *, $p < 0.05$; **, $p < 0.01$.

3.1.8 Over-expression of NCAM180 enhances melanoma cellular invasion in vitro.

As mentioned in the Introduction, each NCAM isoforms serves distinct role and the expression of NCAM undergoes an isoforms switch from NCAM120 to the transmembrane NCAM140 and 180 isoform during melanoma progression. Both NCAM140 and NCAM180 have been reported serving regulatory role in cancer cell motility [238] yet their individual roles in melanoma cellular invasion remain elusive. To address this issue, B16F0 cells were transiently transfected with constructs expressing either NCAM140 or NCAM180. We found that NCAM180 transfectants exhibited around 20% increment ($p < 0.05$) in cell motility and invasiveness as compared with Control cells transfected with an empty vector (Veh) (Fig. 12A&B). Conversely, NCAM140 when expressed at comparable level had no significant effect on cellular invasion (Fig. 12 A&B). Thus, these data suggested that NCAM180 may play a predominant role regulating cellular invasion of melanoma cells presumably through activation the pathways aforementioned.

Figure 12. Over-expression of NCAM180 enhances melanoma cellular invasion in vitro.

B16F0 cells over-expressing either NCAM140 or NCAM180 were subjected to migration (A) and invasion assay (B) to assess their motility and invasiveness. Columns, mean of three independent experiments; bars, s.d.; *, $p < 0.05$; **, $p < 0.01$.

3.1.9 The promoting role of NCAM in melanoma cellular invasion is confirmed in highly metastatic B16F10 cells.

The role of NCAM in melanoma cellular invasion and metastasis was further confirmed in a more invasive cell line F10 cells. Like in F0 cells, robust NCAM silencing efficiency was achieved via co-transfection of B16F10 cells with pSilencer 3.1 and pSilencer 4.1 based plasmids expressing siRNAs targeting different regions of NCAM coding sequence. As control, B16F10 cells were transfected with scrambled siRNA with no sequence homology to any known mouse gene. The reduction of NCAM expression level was determined by immunoblotting (Fig. 13A, left). Cells with greater than 50% reduction of NCAM protein level (Fig. 13A, right) compared with transfectants expressing non-targeting siRNA (Control) were subjected to

transwell migration and matrigel invasion assay. Resembling what has been shown in F0 cells, NCAM silencing significantly impeded the migration and invasion of the more invasive line B16F10 cells (Fig. 13B). Moreover, we noted that NCAM expression level was higher in F10 cells comparing that in F0 cells (Fig. 13C). Taking into consideration the higher metastatic potential of F10 cell line, this result further confirmed the promoting role of NCAM in melanoma cellular invasion.

Figure 13. The promoting role of NCAM in melanoma cellular invasion is confirmed in highly metastatic B16F10 cells.

A, Expression level of NCAM in melanoma B16F10 cell stably co-transfected with pSilencer-3.1 and pSilencer-4.1 based plasmid containing scrambled sequence (Control) or 19-bp insert targeting NCAM was determined by immunoblotting. *B*, Cell motility and invasiveness of F10 NCAM KD cells were examined. *C*, NCAM expression level were compared between B16F0 and F10 cells. *Columns*, mean of three independent experiments; *bars*, s.d.; *, $p < 0.05$; **, $p < 0.01$.

3.2 Discussion

There is ample evidence demonstrating that deregulated NCAM expression level in different types of cancers, to be a marker of more aggressive tumors, and to promote the growth and cellular invasion of cancer cells. The role of NCAM in melanoma progression, however, has not been previously elucidated. In present study, we adopted siRNA-based approach to investigate the function of NCAM in murine melanoma cellular invasion and metastasis. Our result demonstrated that stable silencing of NCAM with siRNA induced MET-like morphological transition and significantly perturbed both melanoma cellular invasion and metastatic dissemination. Mechanistically, such metastatic inhibitory effect of NCAM down-regulation was mediated through deregulated PKA and PI3K pathways converging at transcription factor CREB, which in turn led to decreased pro-invasive gene MMP-2 expression .

3.2.1 NCAM in melanoma cell adhesion, migration and invasion

Cellular invasion is an integral process involving attachment, matrix degradation and invasion. Therefore, we examined first the effect of NCAM ablation on cell adhesion of B16F0 cell. Loss of cell adhesion molecule, such as MCAM, a critical marker of melanoma metastasis [109], has been shown to lead to diminished interaction with endothelial cells thereby retarded melanoma cellular invasion through ECM, entry into vasculature and metastasize at distant organ. Intriguingly, it was shown in our study that NCAM KD cells exhibited elevated adhesiveness against substratum. Though such inverse correlation between the expression level of NCAM and attachment ability

of melanoma cells is consistent with what happened in neuroblastoma cells where low NCAM level are necessary for cancer cells to leave tumor aggregate and adhere to endothelial cells [261], further study examining the adhesion of melanoma cells to endothelial cells may be needed to fully clarify the influence of NCAM on melanoma cell attachment as the regulation of NCAM expression during the melanoma progression might be highly dynamic.

Besides enhanced adhesion to substratum, ablation of NCAM also led to formation of compact colonies of cells in contrast to the scattered cell distribution as seen in control ones. Scattering of compact colonies of cells correlates with tumor cell invasiveness [262]. Indeed, we found that both migration and invasion of melanoma cells was reduced owing to NCAM loss. Such pro-invasive role of NCAM is similar to what has been reported under various other tumor contexts, such as in neuroblastoma and lung cancer. Moreover, not only restricted to only one cell line of melanoma, the highly invasive line F10 cells with NCAM knocked down also displayed impaired cellular invasion. If NCAM-negative melanoma cell line is available, ectopic expression of NCAM followed by assessing cell migration and invasion will be done to further consolidate the idea that NCAM plays a pro-invasive role in melanoma. However, there is no murine NCAM-negative melanoma cell line as far as we know. To generate NCAM-negative melanoma cells, transgenic mouse may need to be generated by crossing NCAM knock out- C57BL/6J mice with mouse lines expressing oncogenes such as Ret or mutant form of Ras and Raf which could develop spontaneous melanoma [263, 264]. Taking into consideration the availability of MCAM-negative

human primary cutaneous melanoma SB-2 cells which are non-metastatic [109], we would also speculate the existence of NCAM-negative human melanoma cells which might reside at early stage non-metastatic melanoma. Screening human melanoma specimen may help to identify NCAM-negative human melanoma cells. Isolation of these cells followed by expressing NCAM would not only verify the role of NCAM in melanoma metastasis but also expand the generality of our observations. To add on, B16F0 cells could also be treated with NCAM mimetic peptide FGL [265] or C3 [266] to verify the function of NCAM in melanoma cellular invasion.

3.2.2 Experimental VS Spontaneous metastasis model

To investigate the role of NCAM ablation in melanoma metastatic dissemination, experimental metastasis assay was performed. Direct transplantation of melanoma cells into the systemic circulation generated significant amount of lung metastatic deposits as well as liver metastases. It has been documented that tail vein injection generally results in pulmonary metastases whereas liver metastases are primarily developed from intrasplenic or portal vein injection of tumor cells [267]. Here in current study, the liver metastases observed may indicate the high metastatic burden thus further consolidating the pro-metastatic function of NCAM.

Though experimental metastasis model has been generally adopted to assess the metastatic potential of cancer cells, the fact that early steps in the metastatic cascades are eliminated from this model is of potential limitation. To address this issue and to further confirm the pro-metastatic role of NCAM, we could also perform spontaneous

metastasis assay. This could be done by orthotopic transplantation of melanoma cells into the flank region of the mice followed by excision of the primary tumor burden allowing for the development of distant metastases [267]. In this way, not only the transplanted tumor model may more closely resemble human cancer including tumor histology and metastatic biology [268], but also many aspects of the metastatic cascades that are bypassed using experimental metastasis model such as shedding of tumor cells from primary site and host-tumor interactions could be further studied.

3.2.3 NCAM-mediated signaling in melanoma cellular invasion and metastasis

3.2.3.1 PKA axis contributes to NCAM-mediated melanoma cellular invasion

While extensive investigations have been done to delineate the complex signaling network controlled by NCAM in neural cells [260], little information is available regarding how NCAM modulates tumor progression. Using the transgenic Rip1Tag2 mouse model with spontaneous pancreatic β cell carcinoma, a first glimpse regarding NCAM elicited cellular and molecular mechanism came from a previous study showing that NCAM modulates tumor cell-matrix adhesion by cross-talking with FGFR signaling machinery [269]. Further study expanded these observations by showing that NCAM-mediated signal was transmitted either through activated PLC γ and MAPK pathway downstream of FGFR or Fyn/ FAK pathway thereby contributing to increased cell spreading and migration in insulinoma and lobular breast carcinoma cells [247, 270]. Other than these signaling cascades, NCAM induced activation of PKA and PI3K/Akt pathways could also contribute to cancer progression as demonstrated by our results in melanoma. PKA, as a crucial effector transmitting

NCAM-mediated signal to regulate neurite outgrowth, has long been shown controlling cell motility and cytoskeleton dynamics. The regulatory role of PKA was exerted through either increasing cell-substrate adhesion [271] or induction of MMP-2 secretion to its active form as demonstrated in endometrial cancer [272]. Moreover, activation of PKA axis was shown accompanied with enhanced invasion and metastasis of Lewis lung carcinoma cells [273]. Here, we showed that PKA clearly promoted melanoma cellular invasion which contributed to NCAM-mediated metastatic dissemination. Up to our knowledge, this is the first time revealing PKA as a promoting factor in melanoma metastasis. Our results not only supported the positive effect of PKA in regulation of cell migration but also proposed a novel potential target pathway for melanoma treatment.

3.2.3.2 NCAM-mediated melanoma cellular invasion requires concurrent commitment of PI3K pathway

Other than PKA pathway, our results demonstrated the co-commitment of another critical signaling cascade in melanoma progression, the PI3K/Akt axis. Blocking PI3K activity thereby its downstream signaling significantly impaired melanoma cellular invasion, a defect resemble the effect of NCAM silencing. Restoring phospho-Akt level, however, could rescue the retarded cellular invasion hence confirming the commitment of PI3K axis in NCAM-mediated melanoma cellular invasion. The pro-invasive role of NCAM activated PI3K pathway can be supported by previous study showing that up-regulated phospho-Akt level associated with severely dysplastic nevi and metastatic melanomas compared with normal or mildly dysplastic nevi [274].

Moreover, not only in melanoma, human breast cancer cells and ovarian cancer cells also required activated PI3K pathway to promote cellular invasion thereby enhancing metastasis [275]. Together, the involvement of both PI3K and PKA pathways indicated that multiple signaling cascades have been employed by NCAM to promote melanoma cellular invasion and metastasis.

3.2.4 Putative mediators bridging NCAM-mediated signal to PKA and PI3K pathway

As mentioned in Introduction, homophilic binding of NCAM as well as heterophilic binding with FGFR could elicit a complex network of signaling cascades. Here in current study, however, we did not go into detail to elaborate the mediators between NCAM and its downstream PKA and PI3K pathways.

The exact mechanisms by which NCAM activates PKA or PI3K pathway remain unknown [13]. However, these two pathways share common putative effectors mediating signals from NCAM. In PKA pathway, PKA is dependent on cAMP which is generated by adenylyl cyclases for activation. Earlier studies have shown that adenylyl cyclases can be activated by α -subunit of heterometric G-proteins or Ca^{2+} /calmodulin [260]. G proteins have been demonstrated to be involved in NCAM-mediated neurite outgrowth, as pertussis toxin which inhibits heterotrimeric Gi and G0 proteins blocks NCAM-mediated neuritogenesis. Together with the fact that cholera toxin, a stimulator of adenylyl cyclases [276] induces neurite extension in PC12 cells [55], it is thus plausible to speculate that G protein might serve as an effector mediating signals from NCAM to PKA pathway. As NCAM could also induce

activation of PI3K via G protein, we therefore hypothesize that G protein might serve as a common mediator for these two pathways. However, the possible role of G-protein in NCAM-signaling has not been fully confirmed. Thus, NCAM induced activation of PKA and PI3K pathways could be mediated through other routes as well.

Ca²⁺ influx has also been shown to induce the generation of cAMP thereby activation of PKA in PC12 cells [277]. In addition, Ca²⁺ is an important signaling molecule in NCAM-mediated growth cone guidance and neurite outgrowth. As NCAM activation leads to an elevation of intracellular Ca²⁺ level through activation of both Src-family kinases and the FGFR/PLC/DAG/arachidonic acid pathway [260], Fyn/FAK complex and FGFR might serve as effectors mediating signals from NCAM to PKA. For PI3K pathway, FAK has been shown to bind to PI3K [278]. Also, Ong *et al* has demonstrated that NCAM induced activation of PI3K pathway could be achieved by coordination with FGFR, with the signal mediated by a FRS2 (Fibroblast growth factor receptor substrate 2)-Grb2 (Growth factor receptor-bound protein 2)-Gab1 (GRB2-associated-binding protein 1) complex [279]. Therefore, both Fyn/FAK complex and FGFR might serve as common mediators conducting signals from NCAM to PKA and PI3K pathways.

Based on our results, reduction in terms of FAK phosphorylation level upon NCAM ablation might suggest the possible role of Fyn/FAK complex (Fig. 14) in NCAM mediated signaling thereby melanoma cellular invasion. However, expression of NCAM140 which directly binds to Fyn/FAK complex could barely induce any increment in melanoma cellular invasion. Therefore, further experiments, such as

treating F0 WT cells with Fyn specific inhibitor PP2 and assessing the activation status of PKA and PI3K pathways thereafter, would be needed to verify the role of Fyn/FAK complex in NCAM-mediated signaling thereby cellular invasion of melanoma cells.

Figure 14. NCAM suppression leads to compromised FAK phosphorylation.

Immunoblotting was performed to examine the effect of NCAM loss on activation of Fyn/FAK pathway the band intensity was quantified (right). β -actin served as a loading control. Representative blots of at least three independent experiments were shown. *, $p < 0.05$.

As compared to NCAM140, NCAM180 seems to play a predominant role in melanoma cellular invasion thus suggesting the involvement of FGFR in NCAM-mediated melanoma cellular invasion as NCAM180 could only signal through FGFR [54, 280]. However, further experiment such as treatment of WT F0 cells with FGFR inhibitor followed by evaluating the activation status of PKA and PI3K pathways is still needed. If FGFR inhibitor could impede the activation of PKA and PI3K signaling thereby cellular invasion of melanoma cells, NCAM might directly associate with FGFR as postulated by Cavallaro et al. [269] or indirectly modulates the activity of FGFR [281], which in turn contributes to melanoma cellular invasion. However, if activation of PKA and PI3K signaling could not be completely blocked by the FGFR

inhibitor in F0 cells, provided that Fyn/FAK complex and G-protein do not involve in activation of NCAM downstream signaling, this might imply that other than FGFR, there might be some other yet unidentified mediators participated in this process.

3.2.5 Transcription factors involved in NCAM-mediated melanoma cellular invasion

3.2.5.1 CREB and its downstream pro-invasive gene MMP-2 mediate NCAM-potentiated cellular invasion of melanoma cells

Based on our results, PKA and PI3K axes converged on transcription factor CREB, which plays critical role in cell differentiation, proliferation and survival in the nervous system [282]. Recently, mounting evidences has revealed the critical role of CREB in tumor initiation, progression and metastasis. Up-regulated CREB expression and activity was detected in a panel of human cancers such as acute myeloid leukemia, breast cancer, ovarian cancer and lung cancer [283, 284]. In addition, positive staining of p-CREB and elevated mRNA level of CREB was only found in poorly-differentiated prostate cancers and bone metastatic tissue samples suggesting the critical role of CREB in tumor progression and metastasis [285]. Also, increased CREB expression level and activity has been shown to contribute to tumorigenesis and metastasis of human melanoma (MeWo) cells via up-regulating expression of MMP-2 and cell adhesion molecule, MUC18, as aforementioned [258]. Here in B16F0 melanoma cells, we confirmed the critical role of CREB in cellular invasion by showing compromised cellular invasion after transfection with ACREB. In line with this, ACREB rendered the mRNA level of MMP-2 which is consistent with previous evidence in human melanoma cell line [258].

Given this critical regulatory function in tumor malignancy, the function of CREB is tightly regulated through phosphorylation at serine 133. In the present study, CREB activity was governed by NCAM-mediated signals. In NCAM-silenced melanoma cells, the phosphorylation of CREB at Ser133 was markedly reduced, which was further proved to be important for melanoma cellular invasion. This result may explain why NCAM potentiates cellular invasion and metastasis of melanoma cells in vitro and in vivo. Actually, there are several lines of evidence to support the speculation that NCAM can induce the activation of CREB, though they are mainly from studies on neural cells. In mouse brain regions, NCAM deficiency results in a significant reduction of the phosphorylation of CREB at Ser133, which is related to the formation of memory [259]. Moreover, previous studies suggest that NCAM-mediated CREB activation during neurite outgrowth is dependent on MAPK/MEK [53, 286] or PKA signaling pathways [56]. Similarly, we also demonstrated that NCAM induced CREB activation is through PKA signaling. However, in our study, no relationship between CREB and MAPK/MEK signaling was found in NCAM potentiated cell migration and invasion, as evidenced by that MEK-specific inhibitor (U0126) did not affect the CREB activity in melanoma cells (Fig. 15B). In fact, we even did not observe a difference in ERK phosphorylation level between Control and NCAM KD B16F0 cells (Fig. 15A). Instead, we revealed a link between CREB and NCAM-induced PI3K/Akt signaling. Taken together, these results suggest that NCAM-mediated CREB phosphorylation could be achieved via activation of different signaling pathways with respect to different cell types or processes.

Figure 15. MAPK/ERK signaling is not involved in NCAM-mediated cellular invasion of melanoma cells.

A, Activation status of MAPK/ERK pathway in NCAM KD cells was examined by probing the content of phospho-ERK. B, Treatment with MEK specific inhibitor U0126 did not affect the activation of CREB. β -actin served as a loading control. Representative blots of at least three independent experiments were shown.

3.2.5.2 Other transcription factors that may be involved in NCAM-mediated melanoma cellular invasion

It is worth noting that other than CREB, activity of Activating Transcription Factor-1 (ATF-1) (Fig. 8) and c-jun (Fig. 17) were compromised in NCAM knocked down cells. ATF-1 belongs to the leucine zipper class of transcription factor and its overexpression contributed the same way as CREB to the metastatic potential of melanoma cells [258]. Moreover, previous study has demonstrated the promoting role of c-jun in cellular invasion [287] thus posing the possibility of its involvement in NCAM mediated invasion of melanoma cells. Further studies could be done to address this question and to explore whether there is any interaction between these three transcription factors.

Figure 16. C-jun activation in B16F0 cells was attenuated after NCAM knocked down.

Immunoblotting was performed to examine the effect of NCAM loss on activation of c-jun and the band intensity was quantified (right). β -actin served as a loading control. Representative blots of at least three independent experiments were shown. *, $p < 0.05$.

3.2.6 The role of specific NCAM isoform in melanoma cellular invasion

Besides expression level changes, the isoform switch of NCAM was also documented in tumor progression [92]. But the significance of such switch and the concrete role of individual NCAM members in melanoma cellular invasion are still obscure. To this end, over-expression of each major isoforms of NCAM in melanoma cells was performed followed by evaluation of their cellular invasion. However, it seems that only the NCAM180 isoform could led to significant increase in cell migration and invasion and such pro-invasive role is probably mediated via FGFR as NCAM180 could only signal through FGFR but not Fyn/FAK complex [54, 280]. In consistent with our observation, in neuroblastoma, NCAM180 was shown to increase cell motility due to its contact of the long intracellular domain with the cytoskeleton [288]. Interestingly, the function of NCAM180 varies across different cancers. Loss of NCAM180 expression resulted in increased invasion and aggressiveness of the colon cancer suggesting its role as tumor suppressor [289]. Unlike NCAM180, the 140 kDa

isoform barely had increasing effect on melanoma cellular invasion. It has been noted that NCAM140 expression level is relatively higher as opposed to that of NCAM180 in both B16F0 and F10 cells. Therefore, we cannot exclude the possibility that the pathway controlled by NCAM140 was saturated by the endogenous protein thus leading to no additive effect upon forced expression of NCAM140. Further study would be needed to elucidate the role of this specific isoform in melanoma cell invasion. Moreover, differential polysialylation in various isoforms of NCAM has been observed to modulate the adhesive function and cell migration [290], whether it contributes to NCAM180 induced melanoma invasion requires further study.

In summary, the study presented here revealed that NCAM, an important adhesion molecule, contributes to the acquisition of a highly motile and invasive phenotype which is consistent with the aggressive melanoma behavior. NCAM exerts its effect by inducing phosphorylation and transcriptional activity of CREB thereby expression of MMP-2 via PKA and PI3K pathways (Summarized in Fig. 17). These findings not only provide further insight into the mechanism of melanoma metastasis but also pave the way for novel therapeutic approaches targeting NCAM or concomitant inhibition of both signaling cascades to combat melanoma progression.

Figure 17. Schematic model summarizing how NCAM modulates melanoma cellular invasion and metastasis.

CHAPTER 4 Neural cell adhesion molecule modulates mesenchymal stromal cells migration via activation of MAPK/ERK signaling

4.1 Results

4.1.1 Isolation and characterization of MSCs

To further elucidate the role of NCAM in MSCs, we isolated MSCs from bone marrow stroma of wild-type (WT) and NCAM^{-/-} (KO) mice and separated them from hematopoietic cells based on their adhesive nature on the plastic culture plate. After 6 passages, a homogeneous population with fibroblast-like morphology was obtained and cells at around the 10th passage were used for subsequent experiments. Flow cytometry analysis was performed to ensure complete ablation of NCAM expression in MSCs. As shown in Fig. 18A, more than 95% of the WT MSC cells expressed high level of NCAM and almost all (99.35%) NCAM^{-/-} cells were NCAM deficient. Immunoblotting analysis further confirmed the expression of NCAM in WT but not in NCAM^{-/-} MSCs (Fig. 18B).

Subsequently, immunophenotype of cultured MSCs were examined. Flow cytometry analysis revealed that WT and NCAM^{-/-} MSCs expressed MSCs characteristic cell surface antigens, CD73, CD105 and CD90, but not hematopoietic markers CD31, CD34 and CD45 (Fig. 18C) thus confirmed the identity of cultured MSCs.

To verify their multipotency, cultured MSCs were exposed to adequate differentiating conditions for adipocyte, osteocyte and chondrocyte induction. As reported in our previous study, NCAM deficiency leads to impaired adipogenic differentiation [233]. When cultured in adipogenic medium, intercellular lipid vacuoles accumulation indicates successful differentiation into adipocyte and this was detected by Sudan Black staining. As shown in Fig. 18D (a-b), WT MSCs were readily differentiated into adipocytes. However, NCAM deficient MSCs generated much less and smaller lipid droplets compared to their WT counterparts. In accordance with this phenomenon, NCAM^{-/-} MSCs differentiation into osteocytes was also found to be impaired significantly. As described previously [291], MSCs cultured in osteo-inductive medium underwent a dramatic morphological change from spindle shaped cell to cuboidal, accompanying with an increase in alkaline phosphatase activity. Moreover, WT MSCs formed aggregates or nodules with islands of ECMs secreted three weeks after osteogenic induction as shown in Fig. 18D (c). In contrast, staining with Alizarin red revealed much less calcium deposition in NCAM deficient MSCs Fig. 18D (d). To induce chondrogenesis, MSCs were centrifuged to form pelleted micromass and cultured in chondrogenic medium for three weeks. Both WT and NCAM^{-/-} MSCs micromass bodies developed into multilayered cartilaginous nodules associated with a well-organized ECM rich in sulfated proteoglycans [161, 292]. These sulfated proteoglycans were specifically detected using Alcian blue staining under acidic conditions. However, we did not observe any significant difference in terms of cartilaginous nodule formation between WT and NCAM^{-/-} MSCs (Fig. 18D (e-f)).

Collectively, these results suggested that NCAM participated in MSCs differentiation into the adipocytes and osteocytes.

Figure 18. Isolation and characterization of MSCs.

A, Flow cytometry analysis was performed to examine NCAM expression on bone-marrow-derived wild-type (WT; right panel) and NCAM deficient (NCAM^{-/-}; left panel) MSCs (passage 10). Histograms with black outline show data from negative controls and those with red histograms show data obtained with rabbit polyclonal antibody against NCAM. B, Immunoblotting was performed to further confirm the expression of NCAM in WT and NCAM^{-/-} MSCs. β-actin was detected as a loading control. Data are representative of three independent experiments. C, Immunophenotype of WT and NCAM^{-/-} MSCs was examined. Flow cytometry analysis was performed to check the expression of MSCs surface marker CD73, 90, 105 while excluding the expression of hematopoietic marker CD31, CD34 and CD45. D, WT and NCAM^{-/-} MSCs were induced to differentiation into adipocyte, osteocyte, or chondrocyte. Formation of lipid droplets, the key feature indicating successful differentiation of adipocyte was visualized by Sudan Black staining at day 14 after treatment with adipogenic differentiation medium (a-b), Original magnification, × 200. Scale bars indicate 100 μm; For osteogenic differentiation, MSCs were cultured for 3 weeks in osteogenic induction medium, and calcium deposition was visualized by Alizarin Red S staining (c-d). For chondrogenic differentiation, cartilage specific metachromasia generated was viewed with Alcine Blue staining (e-f), Original magnification, × 100. Scale bars indicate 200 μm.

4.1.2 NCAM deficiency impairs MSCs motility

As an adhesion molecule, NCAM has been shown to be implicated in neuronal outgrowth, migration of neural cells as well as many cancer cells [236-238]. To explore the role of NCAM in MSCs migration, wound healing assay, which is generally considered as a simple and reliable method to quantitatively evaluate cell motility, was performed. A scratch was inflicted on a confluent cell monolayer followed by 24 hours incubation. As shown in Fig. 19A, NCAM deficient MSCs barely exhibited any healing 24 hours post scratching. In contrast, migrated WT MSCs almost filled the wound and the distance migrated was approximately 3 times more than that of NCAM^{-/-} MSCs.

During cell migration, an early event following the stimulation of rounded cells by chemo-attractant is dynamic reorganization of filamentous F-actin and formation of stress fibers [293]. To determine if NCAM deficiency affects cytoskeleton rearrangement during MSCs migration, cultured MSCs were stained with rhodamine-labeled phalloidin to monitor F-actin polymerization. Immunocytochemical analysis revealed formation of actin longitudinal stress fiber organized in tight cortical bundles in cell cortex of WT MSCs. However, such redistribution of actin fibers in cell cortex was not evident in NCAM^{-/-} MSCs, implying that NCAM deficiency leads to impaired stress fiber formation thereby resulting in reduced cell motility of MSCs (Fig. 19B).

We also tested the effect of NCAM loss on MSCs in a directional migration assay using transwell. In response to chemo-attractant at the bottom chamber, cells seeded on top of the porous filter will traverse through the random distributed pores and migrate to the bottom surface of the filter. In consistent with the wound healing result, migrated NCAM^{-/-} MSCs through the porous filter was around one third of that for WT MSCs (Fig. 19C). Together, these results indicated the positive regulatory role of NCAM in migration of MSCs.

Figure 19. NCAM deficiency impairs MSCs motility and invasiveness.

A, WT and NCAM^{-/-} MSCs were subjected to wound healing assay and representative pictures of the wound distances were taken at each time point as indicated. Original magnification $\times 40$, scale bar indicates 1 mm. B, Morphology of WT and NCAM^{-/-} MSCs stained with rhodamine-conjugated phalloidin antibody to visualize the actin cytoskeleton, scale bar indicates 10 μ m. C, Transwell migration assay was performed to quantitatively gauge the migratory behavior. Left panel, WT MSCs, right, NCAM^{-/-} MSCs. Original magnification $\times 100$, scale bar indicates 200 μ m. Columns, means from three independent experiments, bars, s.d.; *, $p < 0.05$; **, $p < 0.01$.

4.1.3 Neither PI3K nor Wnt signaling contributes to NCAM-mediated migration of MSCs

As demonstrated earlier, activation of PI3K pathway contributes to NCAM-mediated cellular invasion of melanoma cells [294]; also in MSCs, the PI3K signaling is shown

to be adopted in HGF potentiated migration [196]. To examine whether PI3K signaling contributes to NCAM-mediated mobilization of MSCs, the activation status of Akt, the serine/threonine kinase downstream of PI3K, was examined. Immunoblotting revealed only a slight reduction in phospho-Akt level upon loss of NCAM (Fig. 20A). However, such defect did not seem to contribute to the impaired migration of NCAM^{-/-} MSCs as expression of constitutively active form of Akt (D2-Akt) could not restore the defective migration of NCAM^{-/-} cells (Fig. 20B).

Besides PI3K signaling, canonical Wnt signaling regulating various stem cell attributes has also been reported as a crucial regulator governing the transmigration of hMSCs through ECM [195]. We previously identified the association of β -catenin and GSK-3 β with NCAM in melanoma cells [294]. NCAM in turn induces activation of β -catenin signaling contributing to the growth of melanoma. To examine whether NCAM convey its signal via activation of β -catenin signaling in MSCs, we assessed the activation status of β -catenin and GSK-3 β . Immunoblotting result revealed no significant difference in terms of β -catenin expression level in NCAM^{-/-} MSCs. In consonance, phosphorylation level of GSK-3 β , which sequesters and phosphorylates β -catenin in the absence of Wnt ligands, remains the same despite the absence of NCAM (Fig. 20C).

To further assess the attributes of Wnt/ β -catenin signaling in NCAM-mediated migration, NCAM^{-/-} MSCs were exposed to Wnt pathway activator LiCl (25 mM) for 1 hour and their migration was assessed. Indeed, impaired migration of NCAM^{-/-}

MSCs could not be restored (Fig. 20D). Collectively, neither PI3K nor Wnt signaling contributed to NCAM mediated migration of MSCs.

Figure 20. Neither PI3K nor Wnt signaling contributes to NCAM-mediated migration of MSCs.

A, Akt and phospho-Akt level in WT and NCAM^{-/-} MSCs were examined by immunoblotting. β-actin served as a loading control. B, Effect of constitutively active form of Akt (D2-Akt) on migration of NCAM^{-/-} MSCs was assessed and compared with that of WT MSCs. C, Lysates from WT and NCAM^{-/-} MSCs were analyzed by immunoblotting with antibody against β-catenin, phospho-β catenin, GSK-3β and its phosphorylation form. β-actin served as a loading control. D, NCAM deficient MSCs were pre-treated with Wnt agonist LiCl (25mM) for 1 hour followed by transwell migration assay. Columns, means from three independent experiments, bars, s.d.; *, $p < 0.05$; **, $p < 0.01$.

4.1.4 NCAM employs MAPK/ERK signaling to regulate MSCs migration.

In bone marrow or umbilical cord blood derived MSCs, MAPK/ERK signaling has been shown to contribute to sphingosine-1-phosphate (S1P) or stromal cell-derived factor-1(SDF-1) induced cell migration [199-201]. Together with the fact that MAPK/ERK is one of the major signaling components downstream of NCAM, it is thus plausible to speculate the involvement of MAPK/ERK signaling in NCAM-mediated migration of MSCs. To test this notion, we proceeded to examine the effect of NCAM deficiency on MAPK/ERK signaling. As expected, the phosphorylation level of ERK and that of upstream kinases, MEK and Raf, are much lower in NCAM deficient cells compared to WT counterparts under our experimental conditions thus suggesting the impaired MAPK/ERK signaling in NCAM^{-/-} MSCs (Fig. 21A). To elucidate the contribution of MAPK/ERK signaling in NCAM-mediated MSC migration, WT MSCs were subjected to treatment with MEK specific inhibitor U0126 followed by migration assay. As shown in Fig. 21B, inhibition of MEK significantly impeded the migration of MSCs and the number of cells crossing the filters was around 40% less than the untreated control when incubated with 10 μ M of U0126. Likewise, cell motility was drastically reduced upon transfection of WT MSCs with dominant negative MEK (AMEK) (Fig. 21C). Expression of the constitutively active form of MEK (EMEK), on the contrary, could successfully restore the impaired migration of NCAM^{-/-} MSCs (Fig. 21D). Together, MAPK/ERK pathway, rather than PI3K or Wnt signaling, contributed to NCAM mediated migration of MSCs.

Figure 21. NCAM employs MAPK/ERK signaling to regulate MSCs migration.

A, Lysates from WT and NCAM^{-/-} MSCs were subjected to immunoblotting analysis examining the expression levels of phospho-c-Raf, phospho-MEK, phospho-ERK and ERK with corresponding antibodies. β-actin served as a loading control. Representative blots of at least three independent experiments were shown. **B**, WT MSCs were treated with MEK inhibitor U0126 (10 μM or 20 μM) for 1 hour followed by migration assay. **C**, Expression of the dominant negative form of MEK (AMEK) in WT cells followed by examination of cell motility. **D**, NCAM^{-/-} MSCs transfected with the constitutively active form of MEK (EMEK) was subjected to transwell migration assay. *Columns*, means from three independent experiments, *bars*, s.d.; *, $p < 0.05$; **, $p < 0.01$.

4.1.5 NCAM 180 reconstitutes MAPK/ERK signaling and migration of NCAM^{-/-} MSCs

In neural cells, distinct functions were served by the three major isoforms of NCAM. To elucidate the regulatory attributes of each NCAM isoform in MSCs migration, NCAM^{-/-} MSCs were transfected with plasmids expressing full length NCAM120,

NCAM140 and NCAM180, independently. As shown in Fig. 22A, the expression levels of specific isoforms of NCAM in NCAM^{-/-} MSCs transfected cells are comparable to those in WT cells. Thereafter, we gauged the activation status of ERK in transfected NCAM^{-/-} MSCs. Intriguingly, impaired MAPK/ERK signaling in NCAM deficient MSCs could be restored efficiently by re-expression of NCAM180. Re-expression of NCAM140, however, barely induced any significant increment in terms of ERK phosphorylation.

When subjected to migration assay, transfected NCAM^{-/-} MSCs expressing NCAM180 exhibited more than four-fold increase in cell migration compared to NCAM^{-/-} MSCs transfected with control vector. In contrast, expression of NCAM140 only induced a slight increment in cell migration, and NCAM120 did not contribute to MSCs migration at all (Fig. 22B). Collectively, these results implied the predominant role of NCAM180 in activation of MAPK/ERK signaling thereby migration of MSCs.

It is known that NCAM-mediated activation of MAPK/ERK signaling could be achieved through the interplay with FGFR or activation of non-receptor tyrosine kinase Fyn/FAK complex. NCAM180, however, could not associate with Fyn/FAK complex but only signal through FGFR [54]. To confirm the involvement of FGFR in NCAM180-mediated ERK activation thereby migration of MSCs, MSCs re-expressing NCAM180 were pretreated with FGFR specific inhibitor SU5402 (10 μ M) for 1 hour followed by migration assay. As shown in Fig. 22C, the increased cell migration by

expression of NCAM180 was successfully abrogated by SU5402 treatment thus suggesting NCAM induced MAPK/ERK signaling is FGFR-dependent.

Figure 22. NCAM180 reconstitutes MAPK/ERK signaling and migration of NCAM^{-/-} MSCs.

A, NCAM^{-/-} MSCs were transfected with pcDNA4/Myc-NCAM180 (180), pcDNA4/Myc-NCAM140 (140), pcDNA/Myc-NCAM120 (120) or pcDNA4/Myc (Veh). The expression of NCAM was analyzed by immunoblotting with antibodies against NCAM or c-Myc tag. Wild type counterpart transfected with pcDNA4/Myc was used as a control. Also, activation status of ERK was examined under the above five conditions. β-actin served as a loading control. Representative blots of at least three independent experiments are shown. B, NCAM^{-/-} MSCs transfected with plasmids expressing NCAM120, 140, or 180 were subjected to transwell migration assay. C, NCAM180 expressing cells were treated with FGFR inhibitor SU5402 (10 μM) followed by assessing their migration. The expression level of phospho-ERK was examined in NCAM180 expressing cells with or without SU5402 treatment. Columns, means from three independent experiments, bars, s.d.; *, $p < 0.05$; **, $p < 0.01$.

4.1.6 TNF- α up-regulates NCAM180 expression in MSCs

TNF- α , one of the major pro-inflammatory cytokine released at sites of injury, has been demonstrated as a potent regulator of MSCs migration. In response to its cues, MSCs would mobilize from bone marrow and subsequently home to injured tissue contributing to injured cell substitution and tissue architecture repair [295, 296].

To examine whether TNF could regulate the expression of NCAM thereby NCAM-mediated MSC migration, MSCs were exposed to 50 ng/ μ l TNF- α for different times as indicated in Fig. 23A followed by RNA extraction. As shown by QRT-PCR analysis, a maximum increment in NCAM180 expression of 2.7 folds was observed at 6 hours upon stimulation with TNF- α as compared to untreated control cells. mRNA expression of NCAM180, however, declined to comparable level as opposed to control cells 24 hours post treatment with TNF- α (Fig. 23A). Thereafter, we performed immunoblotting to further confirm whether elevated NCAM180 mRNA expression in response to TNF- α stimulation correlated with an increase in protein level of NCAM180. As showed in Fig. 23B, NCAM180 expression level increased significantly and peaked at around 4 to 6 hours post TNF- α exposure, compared to that of untreated control cells. No significant increment in NCAM140 expression, however, was observed thus further corroborating the predominant function of NCAM180.

Given the strong chemotactic potential of TNF- α in hMSCs migration [296] , together with its regulatory role in NCAM expression as demonstrated above, one would expect

a differential response in terms of cell migration between WT and NCAM deficient MSCs in response to TNF- α . We treated WT and NCAM deficient MSCs with TNF- α followed by evaluation of their migratory potential. As expected, TNF- α treatment revealed a profound increment in the basal rate of cell migration in WT MSCs. However, TNF- α was unable to induce an increase of cell motility in NCAM deficient MSCs thus confirming the critical role of NCAM in MSCs migration (Fig. 23C)

Figure 23. TNF- α up-regulates NCAM180 expression in MSCs.

A rapid induction of NCAM180 was observed after TNF- α (50 ng/ μ l) stimulation for 1 hour and persisted for more than 6 hours. *A*, QRT-PCR was performed to monitor the induction of NCAM180 at mRNA level. *B*, Immunoblotting further confirmed such observation by showing an up-regulated NCAM180 at protein level with anti-NCAM antibody. Representative blots of at least three independent experiments are shown. *C*, Migration of WT and NCAM^{-/-} MSCs was evaluated before and after treatment with TNF- α . *Columns*, means from three independent experiments, *bars*, s.d.; *, $p < 0.05$; **, $p < 0.01$.

4.2 Discussion

Most research concerning NCAM has been focusing on nervous system owing to its ubiquitous expression on almost all neural cell types. The critical function of NCAM ranges from early brain development, neurite outgrowth, synaptic plasticity to memory consolidation [13]. Recent studies show that NCAM is also expressed in adult stem cells supporting hematopoiesis [234, 235]. Moreover, we have previously identified the novel function of NCAM in mediating adipogenesis of MSCs in vitro [233]. In this study, we further demonstrated that NCAM potentiated migration of MSCs by activating the MAPK/ERK signaling cascade through crosstalk with FGFR .

4.2.1 The role of NCAM in MSCs differentiation

MSCs are characterized by their ability to differentiate into various mesodermal cell types such as adipocytes, osteocytes, and chondrocytes. Moreover, accumulating evidences have also demonstrated their capacity of differentiating into cells beyond the germ layers with visceral mesoderm, neuroectoderm and endoderm characteristics [165, 297, 298]. These observations supporting multipotential of MSCs prompt the expectation of their implication in cell therapy and thus formed the basis for most current studies of MSCs. For instance, preclinical studies have demonstrated that MSCs facilitate myocardial repair in models of cardiac injury through cardiomyocytes differentiation [165]. In the current study, we have verified the multipotency of culturing MSCs with differentiation assays. Interestingly, the efficacy of adipogenesis and osteogenesis for NCAM^{-/-} MSCs were significantly impaired. In previous study, we reported the novel function of NCAM in adipogenic differentiation and elaborated

the molecular mechanism underlying. Either NCAM deficiency in MSCs or NCAM down-regulation in pre-adipocytes disrupts adipogenesis owing to impaired insulin signaling [233]. In line with this result, current study extends the vital function of NCAM from adipogenic to osteogenic differentiation of MSCs. However, further studies with pre-osteocytes are necessary to confirm the decisive role of NCAM in osteogenesis. As for the chondrogenic differentiation of MSCs, quantitative assessment by measuring DNA contents or glycosaminoglycan content within the aggregates would be needed to compare the chondrogenesis capacity between WT and NCAM^{-/-} cells. Taken together, the defective adipogenesis and osteogenesis owing to NCAM deficiency suggest the critical role of NCAM in fat and cartilage tissue development from MSCs. In addition, impaired differentiation capacity might result in reduced therapeutic potential of NCAM^{-/-} MSCs in treatment of related diseases such as osteogenic imperfecta. Thus, it would also be interesting to explore the possible role of NCAM in MSCs differentiation into other cell types such as neurons or cardiomyocytes which might provide insight into the implication of cell adhesion molecule in MSCs-based cell therapy.

4.2.2 Modulation of NCAM could be implicated in MSCs mobilization

As stated in Introduction, several studies have reported the presence of a very small number of circulating MSCs in the peripheral blood [176-178]. Though the presence of this circulating pool of MSCs is still debatable, studies have consistently shown that MSCs are capable of migrating to inflamed or traumatized tissue to contribute to tissue repair [180]. In animal models of cerebral ischemia and multiple organs failure [183,

299], for example, MSCs have been shown to migrate to site of inflammation and injury and correlates with the severity of injury. However, migration of infused MSCs to intended correction and their homing efficiency remains a great challenge in therapeutic MSCs transplantation. In the past decade, various approaches have been explored to enhance migration of MSCs. Since the chemotactic response of MSCs decreases along with expression of adhesion molecules, chemokine receptors during culture expansion [190, 191], modulating the expression of these receptors would thus be a feasible strategy to enhance MSCs migration. Previous studies have shown that retroviral overexpression of chemokine receptor CXCR4 led to increased migration of MSCs towards SDF-1 α [300]. In the current work, we reported the correlation between NCAM and MSCs migration thereby exemplifying the implication of adhesion molecules in MSCs migration. Therefore, NCAM expression modulation could be explored to enhance MSCs migration.

Cell migration is an integrated process including polarization of cells towards a chemo-attractant and cytoskeletal rearrangement. NCAM has been demonstrated to associate with cytoskeletons and the reorganization of the submembrane F-actin network has been found to be defective in NCAM deficient mice [244]. Indeed, we noted that F-actin polymerization/ actin stress fiber formation is impaired in NCAM-deficient cells, thus implying defective motility of NCAM^{-/-} MSCs. The impaired migration of MSCs as the effect of NCAM loss corresponds well to what has been observed in NCAM-deficient tumor cells of neuroendocrine origin [237] as well as melanoma cell lines as what we have identified earlier [236], thus further consolidates

the promoting role of NCAM in cell migration. Moreover, NCAM has been shown to facilitate adhesion of solid tumor lines to endothelial cells [301]. It is thus tempting to speculate that NCAM, through binding with its heterophilic ligands such as heparan sulfate on endothelial cells [301], facilitates the transmigration of MSCs thereby contributing to the homing of MSCs. Further experiment will be required to examine this notion in detail.

4.2.3 Signaling cascade contributes to NCAM-mediated MSCs migration

In the past few decades, a plethora of evidence have been reported stressing the importance of signaling pathways regulating migration of MSCs. Wnt/ β -catenin and PI3K/Akt pathways are examples of those pathways shown to govern the mobilization of MSCs under various contexts. However, in present study, neither one was exploited by NCAM to regulate MSCs migration. Instead, we found that activation of MAPK/ERK signaling played a pivotal role in NCAM-mediated migration of MSCs. Several studies have already highlighted the importance of MAPK/ERK signaling in MSCs. For instance, MAPK/ERK couples with signal transducer and activator of transcription (STAT)-signaling to contribute to SDF-1 induced migration of human MSCs [200, 201]. Besides chemokines and cytokines, growth factor such as epidermal growth factor (EGF) also employs MAPK/ERK signaling to regulate chemotactic activities of rat bone marrow-derived MSCs [196, 302]. In current work, our observation added that other than chemokines, cytokines and growth factors, adhesion molecules such as NCAM also exploited MAPK/ERK signaling to relay its signal thereby governing migration of MSCs. We showed that treatment with MEK specific

inhibitor (U0126) or dominant negative form of MEK (AMEK) led to drastic attenuation of MSCs migration. In contrast, the constitutively active form of MEK (EMEK) could rescue the defective migration of NCAM-deficient MSCs.

NCAM induced activation of MAPK/ERK signaling could be achieved via activation of Fyn/FAK complex or FGFR signaling [260]. However, the contribution of Fyn/FAK to NCAM mediated MSCs migration seems minimal as treatment with Fyn inhibitor PP2 failed to impede migration of WT MSCs (Fig. 24). In consistent, re-expression of NCAM180, which could only signal through FGFR restored not only the MAPK/ERK signaling but also the migration of MSCs. Together with our observation that FGFR specific inhibitor SU5402 significantly abolished the increase in MSCs migration resulted from forced expression of NCAM180, we concluded that crosstalk of receptor tyrosine kinase FGFR with NCAM was crucial in MAPK/ERK activation thereby NCAM mediated migration of MSCs.

Figure 24. Fyn/FAK is not involved in NCAM-mediated signaling thereby migration of MSCs.

Migration of WT MSCs was assessed after treatment with Fyn specific inhibitor PP2. *Columns*, means from three independent experiments, *bars*, s.d..

4.2.4 NCAM180 could reconstitute the defective MAPK/ERK signaling in NCAM-deficient MSC

It is generally known that different functions are served by specific isoforms of NCAM. Interestingly, expression of NCAM180 alone could efficiently reconstituted the defective MAPK/ERK signaling thereby restoring the impaired migration of NCAM^{-/-} MSCs. By contrast, NCAM140 did not seem to contribute to either the signal reconstitution or migration of MSCs. Together these results suggest that NCAM180 but not NCAM140 plays a predominant role regulating NCAM-mediated MAPK/ERK signaling thereby MSCs migration. It is generally known that the cytoplasmic domain of NCAM140 associates with many signaling molecules including Fyn/FAK complex. NCAM180, however, interacts with membrane-cytoskeleton linker proteins instead [303]. This might endow NCAM180 a more

efficient association with cytoskeleton or related signaling molecules thereby facilitating migration of MSCs.

Based on previous study summarizing binding partners for NCAM140 and NCAM180, β -actin, tropomyosin, microtubule-associated protein (MAP)-1A, and RhoA-binding kinase (ROCK) are molecules which specifically bind to NCAM180 [303]. As ROCK has been implicated in cell migration [304], we wondered whether binding of NCAM180 with ROCK would contribute to ERK activation thereby MSCs migration. However, though inhibition of ROCK could lead to dramatic increment of MSC cell motility, such effect on MSCs migration seems NCAM-independent (Fig. 25), resembling what has been observed in neurite outgrowth experiments reported before [303].

Figure 25. ROCK signaling is not involved in NCAM-mediated migration of MSCs.

Migration of WT and NCAM^{-/-} MSCs was assessed after treatment with ROCK specific inhibitor Y27632. *Columns*, means from three independent experiments, *bars*, s.d.; *, $p < 0.05$; **, $p < 0.01$.

In addition, it is worth noting that NCAM180 plays a predominant role regulating both MSCs as well as melanoma cell migration. Together, our results not only highlight the novel regulatory function of NCAM180 in cell migration but also enrich our understanding towards the role of specific NCAM isoform.

4.2.5 Pro-inflammatory cytokine TNF- α could induce up-regulation of NCAM180

The increase of inflammatory cytokine concentration is the key mediator of recruiting MSCs to the site of injury. In the early course of tissue injury, TNF- α induces MSCs homing and increases adhesion of hMSCs to endothelial cells during extravasation [188]. Moreover, previous studies reported that incubation of MSCs with TNF- α resulted in increased chemotaxis activity and up-regulated expression of adhesion molecules, such as ICAM-1 [305]. Considering the promoting role of NCAM on MSC migration, as well as the fact that NCAM expression increases in response to INF- γ and TNF- α in thyroid follicular cells [306], it would thus be plausible to manipulate NCAM expression thereby MSCs migration by cytokine treatment. Indeed, we observed an elevated NCAM180 expression level in MSCs in response to TNF- α treatment thus suggesting a novel linkage between pro-inflammatory cytokine and NCAM in MSCs.

In summary, the study presented here revealed the regulatory role of NCAM in differentiation and mobilization of MSCs. NCAM promotes MSC homing via activation of MAPK/ERK signaling. In addition, its expression level could be modulated by TNF- α treatment. Therefore, modulation of NCAM expression in MSCs

would be a feasible strategy to enhance homing efficiency and therapeutic potential of transplanted MSCs in cell therapy (Summarized in Fig. 26).

Figure 26 .Schematic model summarizing how NCAM modulates the migration of MSCs.

CHAPTER 5 CONCLUSION

As the most intensively studied member of immunoglobulin superfamily, NCAM has been widely implicated in many cellular attributes, including cell adhesion, migration and differentiation, etc. In current study, we identified the novel regulatory role of NCAM in melanoma metastasis and in MSCs migration. Emerging evidence has reported the deregulated NCAM expression level in many cancers, however it seems that the function of NCAM in cancer progression remains controversial and tumor context development. Herein, we demonstrated the promoting role of NCAM in melanoma cellular invasion and metastatic dissemination. Such pro-invasive role was subsequently implicated in MSCs, a promising avenue in cell therapy which could mobilize to site of injury and subsequently contribute to tissue repair. Our study uncovered the regulatory role of adhesion molecule NCAM in MSC mobilization and detailed the concrete role of individual members of NCAM in this process. In addition, we found that NCAM also participated in adipogenic and osteogenic differentiation of MSCs.

Like other adhesion molecules, NCAM could serve as a signal transducer eliciting a complex network of signaling cascades. In melanoma cells, the pro-metastatic role of NCAM is mediated through concurrent activation of PKA and PI3K pathways. These two pathways converge at transcription factor CREB, which in turn induces elevated expression of pro-invasive gene MMP-2. In MSCs, NCAM-mediated MSCs

mobilization requires activation of MAPK/ERK pathway, which seems not involved in NCAM induced melanoma cellular invasion. PI3K pathway, however, was not employed by MSCs in cell migration suggesting that different signaling pathways are adopted by NCAM under different cell context. The secondary messengers, growth factors, cytokines and heterophilic ligands of NCAM vary under different cellular context. Moreover, the expression level, isoforms and polysialylation of NCAM might be highly dynamic across different cell types and different developmental stages. Together, these factors would contribute to the distinct signaling elicited by NCAM under different cellular context thereby its diverse functions.

In all, our studies not only provide further insight into NCAM implication but also pave the way for anti-NCAM therapy in melanoma treatment as well as modulation of NCAM in MSC mediated cell therapy. To modulate the expression of NCAM, retroviral overexpression or mRNA transfection of NCAM could be performed in MSCs. Regarding anti-NCAM therapy for melanoma treatment in future, we could start from a spectrum of existing anti-NCAM compounds, antibodies, radioimmunoconjugates and immunotoxins which representing the clinically most advanced and successful strategies. For instance, the fully chimeric antibody Ch.MK1 against NCAM which was shown effective in a neuroblastoma SCID mice model [307] could be explored for the treatment of melanoma. In vivo experiment could be done first by either locoregionally introduction of the antibodies to mice with melanoma xenograft or systemically to mice after the primary melanoma was removed. Only when strong and specific effects of these antibodies are shown, could we proceed to

clinical trials for melanoma treatment in patients. Besides antibodies alone, we could also utilize antibodies coupled with radioisotope, or cytotoxic drugs to enhance the efficacy of the treatment. For instance, the novel anti-NCAM-mayatansine immunotoxin huN901-DM1 which is currently under clinical trial [308, 309] could be evaluated in vitro followed by in vivo model of melanoma. Other than immuno-conjugates, we could also introduce the genetically modified T cells with chimeric anti-NCAM T cell receptors developed by Guest *et al* [310] for melanoma treatment. Back in 2006, Morgan *et al* have already generated genetically engineered T cells with melanoma-specific T cell receptor and significant cancer regression was observed in patients with malignant melanoma after adoptive transferred of these transduced cells [311]. Thus, for anti-NCAM therapy, T cells could be retrovirally transduced with genes encoding with the anti-NCAM T cell receptors followed by adoptively transferring to mice with malignant melanoma. Thereafter, these transduced cells might secrete interferon- γ (IFN- γ) and kill NCAM-expressing melanoma cells upon activation after antigen contact like what has been observed in neuroblastoma in Guest *et al*'s study [310]. Last but not least, therapeutic Abs targeting NCAM downstream signaling to inhibit cell proliferation and invasion of melanoma cells could also be developed. In that case, NCAM elicited PKA and PI3K signaling could be taken into consideration.

CHAPTER 6 REFERENCES

1. Crossin, K.L. and L.A. Krushel, *Cellular signaling by neural cell adhesion molecules of the immunoglobulin superfamily*. Developmental dynamics : an official publication of the American Association of Anatomists, 2000. **218**(2): p. 260-79.
2. Cunningham, B.A., et al., *Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing*. Science, 1987. **236**(4803): p. 799-806.
3. Nguyen, C., et al., *Localization of the human NCAM gene to band q23 of chromosome 11: the third gene coding for a cell interaction molecule mapped to the distal portion of the long arm of chromosome 11*. The Journal of cell biology, 1986. **102**(3): p. 711-5.
4. Walsh, F.S., et al., *Human N-CAM gene: mapping to chromosome 11 by analysis of somatic cell hybrids with mouse and human cDNA probes*. Brain research, 1986. **387**(2): p. 197-200.
5. D'Eustachio, P., et al., *Chromosomal location of the gene encoding the neural cell adhesion molecule (N-CAM) in the mouse*. Proceedings of the National Academy of Sciences of the United States of America, 1985. **82**(22): p. 7631-5.
6. Owens, G.C., G.M. Edelman, and B.A. Cunningham, *Organization of the neural cell adhesion molecule (N-CAM) gene: alternative exon usage as the basis for different membrane-associated domains*. Proceedings of the National Academy of Sciences of the United States of America, 1987. **84**(1): p. 294-8.
7. Walmod, P.S., et al., *Zipper make signals: NCAM-mediated molecular interactions and signal transduction*. Neurochem Res, 2004. **29**(11): p. 2015-35.
8. Cunningham, B.A., *Cell adhesion molecules as morphoregulators*. Curr Opin Cell Biol, 1995. **7**(5): p. 628-33.
9. Gower, H.J., et al., *Alternative splicing generates a secreted form of N-CAM in muscle and brain*. Cell, 1988. **55**(6): p. 955-64.
10. Dickson, G., et al., *Human muscle neural cell adhesion molecule (N-CAM): identification of a muscle-specific sequence in the extracellular domain*. Cell, 1987. **50**(7): p. 1119-30.
11. Cirulli, V., et al., *Expression of neural cell adhesion molecule (N-CAM) in rat islets and its role in islet cell type segregation*. J Cell Sci, 1994. **107** (Pt 6): p. 1429-36.
12. Rouiller, D.G., V. Cirulli, and P.A. Halban, *Differences in aggregation properties and levels of the neural cell adhesion molecule (NCAM) between islet cell types*. Exp Cell Res, 1990. **191**(2): p. 305-12.

13. Maness, P.F. and M. Schachner, *Neural recognition molecules of the immunoglobulin superfamily: signaling transducers of axon guidance and neuronal migration*. Nat Neurosci, 2007. **10**(1): p. 19-26.
14. Bock, E., et al., *Characterization of soluble forms of NCAM*. FEBS letters, 1987. **225**(1-2): p. 33-6.
15. He, H.T., et al., *Phosphatidylinositol is involved in the membrane attachment of NCAM-120, the smallest component of the neural cell adhesion molecule*. The EMBO journal, 1986. **5**(10): p. 2489-94.
16. Diestel, S., et al., *NCAM140 stimulates integrin-dependent cell migration by ectodomain shedding*. Journal of neurochemistry, 2005. **95**(6): p. 1777-84.
17. Hinkle, C.L., et al., *Metalloprotease-induced ectodomain shedding of neural cell adhesion molecule (NCAM)*. Journal of neurobiology, 2006. **66**(12): p. 1378-95.
18. Olsen, M., et al., *Intact transmembrane isoforms of the neural cell adhesion molecule are released from the plasma membrane*. The Biochemical journal, 1993. **295** (Pt 3): p. 833-40.
19. Doherty, P., G. Williams, and E.J. Williams, *CAMs and axonal growth: a critical evaluation of the role of calcium and the MAPK cascade*. Molecular and cellular neurosciences, 2000. **16**(4): p. 283-95.
20. Ni Dhuill, C.M., et al., *Polysialylated neural cell adhesion molecule expression in the dentate gyrus of the human hippocampal formation from infancy to old age*. J Neurosci Res, 1999. **55**(1): p. 99-106.
21. Seki, T. and Y. Arai, *Distribution and possible roles of the highly polysialylated neural cell adhesion molecule (NCAM-H) in the developing and adult central nervous system*. Neuroscience research, 1993. **17**(4): p. 265-90.
22. Ronn, L.C., V. Berezin, and E. Bock, *The neural cell adhesion molecule in synaptic plasticity and ageing*. Int J Dev Neurosci, 2000. **18**(2-3): p. 193-9.
23. Little, E.B., G.M. Edelman, and B.A. Cunningham, *Palmitoylation of the cytoplasmic domain of the neural cell adhesion molecule N-CAM serves as an anchor to cellular membranes*. Cell adhesion and communication, 1998. **6**(5): p. 415-30.
24. Kiselyov, V.V., et al., *Structural biology of NCAM homophilic binding and activation of FGFR*. J Neurochem, 2005. **94**(5): p. 1169-79.
25. Kasper, C., et al., *Structural basis of cell-cell adhesion by NCAM*. Nat Struct Biol, 2000. **7**(5): p. 389-93.
26. Kiselyov, V.V., et al., *The first immunoglobulin-like neural cell adhesion molecule (NCAM) domain is involved in double-reciprocal interaction with the second immunoglobulin-like NCAM domain and in heparin binding*. The Journal of biological chemistry, 1997. **272**(15): p. 10125-34.
27. Ranheim, T.S., G.M. Edelman, and B.A. Cunningham, *Homophilic adhesion mediated by the neural cell adhesion molecule involves multiple immunoglobulin domains*. Proc Natl Acad Sci U S A, 1996. **93**(9): p. 4071-5.
28. Rao, Y., et al., *Identification of a peptide sequence involved in homophilic binding in the neural cell adhesion molecule NCAM*. J Cell Biol, 1992. **118**(4): p. 937-49.

29. Soroka, V., et al., *Structure and interactions of NCAM Ig1-2-3 suggest a novel zipper mechanism for homophilic adhesion*. Structure, 2003. **11**(10): p. 1291-301.
30. Kiselyov, V.V., et al., *Structural basis for a direct interaction between FGFR1 and NCAM and evidence for a regulatory role of ATP*. Structure, 2003. **11**(6): p. 691-701.
31. Saffell, J.L., et al., *Expression of a dominant negative FGF receptor inhibits axonal growth and FGF receptor phosphorylation stimulated by CAMs*. Neuron, 1997. **18**(2): p. 231-42.
32. Paratcha, G., F. Ledda, and C.F. Ibanez, *The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands*. Cell, 2003. **113**(7): p. 867-79.
33. Zhang, H., et al., *A role for the polysialic acid-neural cell adhesion molecule in PDGF-induced chemotaxis of oligodendrocyte precursor cells*. Journal of cell science, 2004. **117**(Pt 1): p. 93-103.
34. Vutskits, L., et al., *PSA-NCAM modulates BDNF-dependent survival and differentiation of cortical neurons*. The European journal of neuroscience, 2001. **13**(7): p. 1391-402.
35. Santuccione, A., et al., *Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth*. The Journal of cell biology, 2005. **169**(2): p. 341-54.
36. Dzhandzhugazyan, K. and E. Bock, *Demonstration of an extracellular ATP-binding site in NCAM: functional implications of nucleotide binding*. Biochemistry, 1997. **36**(49): p. 15381-95.
37. Skladchikova, G., et al., *Extracellular adenosine triphosphate affects neural cell adhesion molecule (NCAM)-mediated cell adhesion and neurite outgrowth*. Journal of neuroscience research, 1999. **57**(2): p. 207-18.
38. Kristiansen, L.V., et al., *Homophilic NCAM interactions interfere with L1 stimulated neurite outgrowth*. FEBS letters, 1999. **464**(1-2): p. 30-4.
39. Herndon, M.E., C.S. Stipp, and A.D. Lander, *Interactions of neural glycosaminoglycans and proteoglycans with protein ligands: assessment of selectivity, heterogeneity and the participation of core proteins in binding*. Glycobiology, 1999. **9**(2): p. 143-55.
40. Grumet, M., A. Flaccus, and R.U. Margolis, *Functional characterization of chondroitin sulfate proteoglycans of brain: interactions with neurons and neural cell adhesion molecules*. The Journal of cell biology, 1993. **120**(3): p. 815-24.
41. Retzler, C., W. Gohring, and U. Rauch, *Analysis of neurocan structures interacting with the neural cell adhesion molecule N-CAM*. The Journal of biological chemistry, 1996. **271**(44): p. 27304-10.
42. Storms, S.D. and U. Rutishauser, *A role for polysialic acid in neural cell adhesion molecule heterophilic binding to proteoglycans*. The Journal of biological chemistry, 1998. **273**(42): p. 27124-9.

43. Dzhandzhugazyan, K. and E. Bock, *Demonstration of (Ca²⁺)-Mg²⁺)-ATPase activity of the neural cell adhesion molecule*. FEBS letters, 1993. **336**(2): p. 279-83.
44. Hubschmann, M.V., et al., *Neural cell adhesion molecule function is regulated by metalloproteinase-mediated ectodomain release*. J Neurosci Res, 2005. **80**(6): p. 826-37.
45. Friedlander, D.R., et al., *The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth*. The Journal of cell biology, 1994. **125**(3): p. 669-80.
46. Povlsen, G.K., et al., *Intracellular signaling by the neural cell adhesion molecule*. Neurochem Res, 2003. **28**(1): p. 127-41.
47. Ronn, L.C., et al., *Neurite outgrowth induced by a synthetic peptide ligand of neural cell adhesion molecule requires fibroblast growth factor receptor activation*. J Neurochem, 2000. **75**(2): p. 665-71.
48. Ronn, L.C., et al., *Identification of a neuritogenic ligand of the neural cell adhesion molecule using a combinatorial library of synthetic peptides*. Nat Biotechnol, 1999. **17**(10): p. 1000-5.
49. Doherty, P. and F.S. Walsh, *CAM-FGF receptor interactions: a model for axonal growth*. Mol Cell Neurosci, 1996. **8**(2-3): p. 99-111.
50. Kolkova, K., et al., *Neural cell adhesion molecule-stimulated neurite outgrowth depends on activation of protein kinase C and the Ras-mitogen-activated protein kinase pathway*. J Neurosci, 2000. **20**(6): p. 2238-46.
51. Williams, E.J., et al., *Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin*. Neuron, 1994. **13**(3): p. 583-94.
52. Beggs, H.E., P. Soriano, and P.F. Maness, *NCAM-dependent neurite outgrowth is inhibited in neurons from Fyn-minus mice*. J Cell Biol, 1994. **127**(3): p. 825-33.
53. Schmid, R.S., et al., *NCAM stimulates the Ras-MAPK pathway and CREB phosphorylation in neuronal cells*. J Neurobiol, 1999. **38**(4): p. 542-58.
54. Beggs, H.E., et al., *NCAM140 interacts with the focal adhesion kinase p125(fak) and the SRC-related tyrosine kinase p59(fyn)*. J Biol Chem, 1997. **272**(13): p. 8310-9.
55. Doherty, P., et al., *Morphoregulatory activities of NCAM and N-cadherin can be accounted for by G protein-dependent activation of L- and N-type neuronal Ca²⁺ channels*. Cell, 1991. **67**(1): p. 21-33.
56. Jessen, U., et al., *The transcription factors CREB and c-Fos play key roles in NCAM-mediated neuritogenesis in PC12-E2 cells*. J Neurochem, 2001. **79**(6): p. 1149-60.
57. Ditlevsen, D.K., et al., *The role of phosphatidylinositol 3-kinase in neural cell adhesion molecule-mediated neuronal differentiation and survival*. J Neurochem, 2003. **84**(3): p. 546-56.

58. Ong, S.H., et al., *Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins*. Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6074-9.
59. Steelman, L.S., et al., *JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis*. Leukemia, 2004. **18**(2): p. 189-218.
60. Du, K. and M. Montminy, *CREB is a regulatory target for the protein kinase Akt/PKB*. J Biol Chem, 1998. **273**(49): p. 32377-9.
61. Kane, L.P., et al., *Induction of NF-kappaB by the Akt/PKB kinase*. Curr Biol, 1999. **9**(11): p. 601-4.
62. Schaller, M.D., *Biochemical signals and biological responses elicited by the focal adhesion kinase*. Biochim Biophys Acta, 2001. **1540**(1): p. 1-21.
63. Barberis, L., et al., *Distinct roles of the adaptor protein Shc and focal adhesion kinase in integrin signaling to ERK*. J Biol Chem, 2000. **275**(47): p. 36532-40.
64. Leshchyn'ska, I., et al., *Neural cell adhesion molecule (NCAM) association with PKCbeta2 via beta1 spectrin is implicated in NCAM-mediated neurite outgrowth*. J Cell Biol, 2003. **161**(3): p. 625-39.
65. Cremer, H., et al., *NCAM is essential for axonal growth and fasciculation in the hippocampus*. Molecular and cellular neurosciences, 1997. **8**(5): p. 323-35.
66. Cremer, H., et al., *Long-term but not short-term plasticity at mossy fiber synapses is impaired in neural cell adhesion molecule-deficient mice*. Proc Natl Acad Sci U S A, 1998. **95**(22): p. 13242-7.
67. Skibo, G.G., et al., *A synthetic NCAM-derived peptide, FGL, protects hippocampal neurons from ischemic insult both in vitro and in vivo*. Eur J Neurosci, 2005. **22**(7): p. 1589-96.
68. Cambon, K., et al., *A synthetic neural cell adhesion molecule mimetic peptide promotes synaptogenesis, enhances presynaptic function, and facilitates memory consolidation*. J Neurosci, 2004. **24**(17): p. 4197-204.
69. Vawter, M.P., *Dysregulation of the neural cell adhesion molecule and neuropsychiatric disorders*. European journal of pharmacology, 2000. **405**(1-3): p. 385-95.
70. Tanaka, Y., et al., *Alteration in serum neural cell adhesion molecule in patients of schizophrenia*. Human psychopharmacology, 2007. **22**(2): p. 97-102.
71. Brennaman, L.H. and P.F. Maness, *NCAM in neuropsychiatric and neurodegenerative disorders*. Advances in experimental medicine and biology, 2010. **663**: p. 299-317.
72. Poltorak, M., et al., *Disturbances in cell recognition molecules (N-CAM and LI antigen) in the CSF of patients with schizophrenia*. Exp Neurol, 1995. **131**(2): p. 266-72.
73. Vawter, M.P., et al., *Abnormal expression of cell recognition molecules in schizophrenia*. Exp Neurol, 1998. **149**(2): p. 424-32.
74. Vawter, M.P., et al., *CSF N-CAM in neuroleptic-naive first-episode patients with schizophrenia*. Schizophr Res, 1998. **34**(3): p. 123-31.
75. Aisa, B., et al., *Altered NCAM expression associated with the cholinergic system in Alzheimer's disease*. Journal of Alzheimer's disease : JAD, 2010. **20**(2): p. 659-68.

76. Bukalo, O., et al., *Conditional ablation of the neural cell adhesion molecule reduces precision of spatial learning, long-term potentiation, and depression in the CA1 subfield of mouse hippocampus*. J Neurosci, 2004. **24**(7): p. 1565-77.
77. Stoenica, L., et al., *In vivo synaptic plasticity in the dentate gyrus of mice deficient in the neural cell adhesion molecule NCAM or its polysialic acid*. The European journal of neuroscience, 2006. **23**(9): p. 2255-64.
78. Polo-Parada, L., C.M. Bose, and L.T. Landmesser, *Alterations in transmission, vesicle dynamics, and transmitter release machinery at NCAM-deficient neuromuscular junctions*. Neuron, 2001. **32**(5): p. 815-28.
79. Rafuse, V.F., L. Polo-Parada, and L.T. Landmesser, *Structural and functional alterations of neuromuscular junctions in NCAM-deficient mice*. J Neurosci, 2000. **20**(17): p. 6529-39.
80. Rolf, B., et al., *Pathfinding errors of corticospinal axons in neural cell adhesion molecule-deficient mice*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2002. **22**(19): p. 8357-62.
81. Cremer, H., et al., *Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning*. Nature, 1994. **367**(6462): p. 455-9.
82. Tomasiewicz, H., et al., *Genetic deletion of a neural cell adhesion molecule variant (N-CAM-180) produces distinct defects in the central nervous system*. Neuron, 1993. **11**(6): p. 1163-74.
83. Tereshchenko, Y., et al., *Neural cell adhesion molecule ablation in mice causes hippocampal dysplasia and loss of septal cholinergic neurons*. J Comp Neurol, 2011. **519**(12): p. 2475-92.
84. Stork, O., et al., *Anxiety and increased 5-HT1A receptor response in NCAM null mutant mice*. Journal of neurobiology, 1999. **40**(3): p. 343-55.
85. Stork, O., et al., *Increased intermale aggression and neuroendocrine response in mice deficient for the neural cell adhesion molecule (NCAM)*. The European journal of neuroscience, 1997. **9**(6): p. 1117-25.
86. Pillai-Nair, N., et al., *Neural cell adhesion molecule-secreting transgenic mice display abnormalities in GABAergic interneurons and alterations in behavior*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2005. **25**(18): p. 4659-71.
87. Glass, J.D., et al., *Polysialylated neural cell adhesion molecule modulates photic signaling in the mouse suprachiasmatic nucleus*. Neurosci Lett, 2000. **280**(3): p. 207-10.
88. Aonurm-Helm, A., et al., *Depression-like behaviour in neural cell adhesion molecule (NCAM)-deficient mice and its reversal by an NCAM-derived peptide, FGL*. The European journal of neuroscience, 2008. **28**(8): p. 1618-28.
89. Xiao, M.F., et al., *Neural cell adhesion molecule modulates dopaminergic signaling and behavior by regulating dopamine D2 receptor internalization*. J Neurosci, 2009. **29**(47): p. 14752-63.
90. Wood, G.K., et al., *NCAM-180 knockout mice display increased lateral ventricle size and reduced prepulse inhibition of startle*. Neuroreport, 1998. **9**(3): p. 461-6.

91. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer*. Cell, 2000. **100**(1): p. 57-70.
92. Cavallaro, U. and G. Christofori, *Cell adhesion and signalling by cadherins and Ig-CAMs in cancer*. Nature reviews. Cancer, 2004. **4**(2): p. 118-32.
93. Fidler, I.J., *The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited*. Nature reviews. Cancer, 2003. **3**(6): p. 453-8.
94. Kaplan, R.N., et al., *VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche*. Nature, 2005. **438**(7069): p. 820-7.
95. Gupta, G.P. and J. Massague, *Cancer metastasis: building a framework*. Cell, 2006. **127**(4): p. 679-95.
96. Cavallaro, U. and G. Christofori, *Cell adhesion and signalling by cadherins and Ig-CAMs in cancer*. Nat Rev Cancer, 2004. **4**(2): p. 118-32.
97. Springer, T.A., *Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration*. Annu Rev Physiol, 1995. **57**: p. 827-72.
98. Christofori, G. and H. Semb, *The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene*. Trends Biochem Sci, 1999. **24**(2): p. 73-6.
99. Cavallaro, U., B. Schaffhauser, and G. Christofori, *Cadherins and the tumour progression: is it all in a switch?* Cancer Lett, 2002. **176**(2): p. 123-8.
100. Hazan, R.B., et al., *Cadherin switch in tumor progression*. Ann N Y Acad Sci, 2004. **1014**: p. 155-63.
101. Hynes, R.O., *Integrins: bidirectional, allosteric signaling machines*. Cell, 2002. **110**(6): p. 673-87.
102. Desgrosellier, J.S. and D.A. Cheresh, *Integrins in cancer: biological implications and therapeutic opportunities*. Nat Rev Cancer, 2010. **10**(1): p. 9-22.
103. Hsu, M.Y., et al., *Adenoviral gene transfer of beta3 integrin subunit induces conversion from radial to vertical growth phase in primary human melanoma*. Am J Pathol, 1998. **153**(5): p. 1435-42.
104. Trusolino, L., A. Bertotti, and P.M. Comoglio, *A signaling adapter function for alpha6beta4 integrin in the control of HGF-dependent invasive growth*. Cell, 2001. **107**(5): p. 643-54.
105. Johnson, J.P., *Cell adhesion molecules in the development and progression of malignant melanoma*. Cancer metastasis reviews, 1999. **18**(3): p. 345-57.
106. Paschos, K.A., D. Canovas, and N.C. Bird, *The role of cell adhesion molecules in the progression of colorectal cancer and the development of liver metastasis*. Cell Signal, 2009. **21**(5): p. 665-74.
107. Thies, A., et al., *Overexpression of the cell adhesion molecule L1 is associated with metastasis in cutaneous malignant melanoma*. Eur J Cancer, 2002. **38**(13): p. 1708-16.
108. Hsu, M.Y., F. Meier, and M. Herlyn, *Melanoma development and progression: a conspiracy between tumor and host*. Differentiation, 2002. **70**(9-10): p. 522-36.

109. Xie, S., et al., *Expression of MCAM/MUC18 by human melanoma cells leads to increased tumor growth and metastasis*. Cancer Res, 1997. **57**(11): p. 2295-303.
110. Shih, I.M., *The role of CD146 (Mel-CAM) in biology and pathology*. The Journal of pathology, 1999. **189**(1): p. 4-11.
111. Zecchini, S. and U. Cavallaro, *Neural Cell Adhesion Molecule in Cancer: Expression and Mechanisms*. Neurochem Res, 2008.
112. Lantuejoul, S., et al., *Neural cell adhesion molecules (NCAM) and NCAM-PSA expression in neuroendocrine lung tumors*. Am J Surg Pathol, 1998. **22**(10): p. 1267-76.
113. Trouillas, J., et al., *Polysialylated neural cell adhesion molecules expressed in human pituitary tumors and related to extrasellar invasion*. J Neurosurg, 2003. **98**(5): p. 1084-93.
114. Lantuejoul, S., et al., *NCAM (neural cell adhesion molecules) expression in malignant mesotheliomas*. Hum Pathol, 2000. **31**(4): p. 415-21.
115. Campodonico, P.B., et al., *The neural cell adhesion molecule is involved in the metastatic capacity in a murine model of lung cancer*. Mol Carcinog. **49**(4): p. 386-97.
116. Ely, S.A. and D.M. Knowles, *Expression of CD56/neural cell adhesion molecule correlates with the presence of lytic bone lesions in multiple myeloma and distinguishes myeloma from monoclonal gammopathy of undetermined significance and lymphomas with plasmacytoid differentiation*. Am J Pathol, 2002. **160**(4): p. 1293-9.
117. Novotny, J.R., H. Nuckel, and U. Dührsen, *Correlation between expression of CD56/NCAM and severe leukostasis in hyperleukocytic acute myelomonocytic leukaemia*. Eur J Haematol, 2006. **76**(4): p. 299-308.
118. Zecchini, S., et al., *The adhesion molecule NCAM promotes ovarian cancer progression via FGFR signalling*. EMBO molecular medicine, 2011. **3**(8): p. 480-94.
119. Fogar, P., et al., *Neural cell adhesion molecule (N-CAM) in gastrointestinal neoplasias*. Anticancer Res, 1997. **17**(2B): p. 1227-30.
120. Kameda, K., et al., *Expression of highly polysialylated neural cell adhesion molecule in pancreatic cancer neural invasive lesion*. Cancer Lett, 1999. **137**(2): p. 201-7.
121. Sasaki, H., et al., *Expression of the neural cell adhesion molecule in astrocytic tumors: an inverse correlation with malignancy*. Cancer, 1998. **82**(10): p. 1921-31.
122. Tezel, E., et al., *Expression of neural cell adhesion molecule in pancreatic cancer*. Pancreas, 2001. **22**(2): p. 122-5.
123. Cavallaro, U., et al., *N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling*. Nature cell biology, 2001. **3**(7): p. 650-7.
124. Crnic, I., et al., *Loss of neural cell adhesion molecule induces tumor metastasis by up-regulating lymphangiogenesis*. Cancer Res, 2004. **64**(23): p. 8630-8.

125. Perl, A.K., et al., *Reduced expression of neural cell adhesion molecule induces metastatic dissemination of pancreatic beta tumor cells*. Nat Med, 1999. **5**(3): p. 286-91.
126. Geertsens, R., et al., *Inverse regulation of neuronal cellular adhesion molecule (NCAM) by IFN-gamma in melanoma cell cultures established from CNS lesions*. Int J Cancer, 1999. **83**(1): p. 135-40.
127. Mooy, C.M., et al., *Neural cell adhesion molecule distribution in primary and metastatic uveal melanoma*. Hum Pathol, 1995. **26**(11): p. 1185-90.
128. Anastassiou, G., et al., *Expression of the cell adhesion molecules ICAM-1, VCAM-1 and NCAM in uveal melanoma: a clinicopathological study*. Oncology, 2000. **58**(1): p. 83-8.
129. Anastassiou, G., et al., *Expression of cell adhesion molecules and tumour infiltrating leucocytes in conjunctival melanoma*. Melanoma research, 2004. **14**(5): p. 381-5.
130. Gray-Schopfer, V., C. Wellbrock, and R. Marais, *Melanoma biology and new targeted therapy*. Nature, 2007. **445**(7130): p. 851-7.
131. Jerant, A.F., et al., *Early detection and treatment of skin cancer*. Am Fam Physician, 2000. **62**(2): p. 357-68, 375-6, 381-2.
132. Hocker, T.L., M.K. Singh, and H. Tsao, *Melanoma genetics and therapeutic approaches in the 21st century: moving from the benchside to the bedside*. J Invest Dermatol, 2008. **128**(11): p. 2575-95.
133. Flaherty, K.T., F.S. Hodi, and D.E. Fisher, *From genes to drugs: targeted strategies for melanoma*. Nat Rev Cancer, 2012. **12**(5): p. 349-61.
134. Davies, H., et al., *Mutations of the BRAF gene in human cancer*. Nature, 2002. **417**(6892): p. 949-54.
135. Curtin, J.A., et al., *Distinct sets of genetic alterations in melanoma*. N Engl J Med, 2005. **353**(20): p. 2135-47.
136. Curtin, J.A., et al., *Somatic activation of KIT in distinct subtypes of melanoma*. J Clin Oncol, 2006. **24**(26): p. 4340-6.
137. Muthusamy, V., et al., *Amplification of CDK4 and MDM2 in malignant melanoma*. Genes Chromosomes Cancer, 2006. **45**(5): p. 447-54.
138. Stahl, J.M., et al., *Deregulated Akt3 activity promotes development of malignant melanoma*. Cancer Res, 2004. **64**(19): p. 7002-10.
139. Bastian, B.C., et al., *Chromosomal gains and losses in primary cutaneous melanomas detected by comparative genomic hybridization*. Cancer Res, 1998. **58**(10): p. 2170-5.
140. Sharpless, E. and L. Chin, *The INK4a/ARF locus and melanoma*. Oncogene, 2003. **22**(20): p. 3092-8.
141. Straume, O. and L.A. Akslen, *Alterations and prognostic significance of p16 and p53 protein expression in subgroups of cutaneous melanoma*. Int J Cancer, 1997. **74**(5): p. 535-9.
142. Soengas, M.S., et al., *Inactivation of the apoptosis effector Apaf-1 in malignant melanoma*. Nature, 2001. **409**(6817): p. 207-11.
143. Wu, H., V. Goel, and F.G. Haluska, *PTEN signaling pathways in melanoma*. Oncogene, 2003. **22**(20): p. 3113-22.

144. Tsao, H., et al., *Genetic interaction between NRAS and BRAF mutations and PTEN/MMAC1 inactivation in melanoma*. J Invest Dermatol, 2004. **122**(2): p. 337-41.
145. Garraway, L.A., et al., *Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma*. Nature, 2005. **436**(7047): p. 117-22.
146. Sauter, E.R., et al., *Cyclin D1 is a candidate oncogene in cutaneous melanoma*. Cancer Res, 2002. **62**(11): p. 3200-6.
147. Kraehn, G.M., et al., *Extra c-myc oncogene copies in high risk cutaneous malignant melanoma and melanoma metastases*. Br J Cancer, 2001. **84**(1): p. 72-9.
148. Wellbrock, C., M. Karasarides, and R. Marais, *The RAF proteins take centre stage*. Nat Rev Mol Cell Biol, 2004. **5**(11): p. 875-85.
149. Cohen, C., et al., *Mitogen-activated protein kinase activation is an early event in melanoma progression*. Clin Cancer Res, 2002. **8**(12): p. 3728-33.
150. Satyamoorthy, K., et al., *Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation*. Cancer Res, 2003. **63**(4): p. 756-9.
151. Carvajal, R.D., et al., *KIT as a therapeutic target in metastatic melanoma*. JAMA, 2011. **305**(22): p. 2327-34.
152. Guldberg, P., et al., *Disruption of the MMAC1/PTEN gene by deletion or mutation is a frequent event in malignant melanoma*. Cancer research, 1997. **57**(17): p. 3660-3.
153. Teng, D.H., et al., *MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines*. Cancer research, 1997. **57**(23): p. 5221-5.
154. Dhawan, P., et al., *Constitutive activation of Akt/protein kinase B in melanoma leads to up-regulation of nuclear factor-kappaB and tumor progression*. Cancer research, 2002. **62**(24): p. 7335-42.
155. Stahl, J.M., et al., *Deregulated Akt3 activity promotes development of malignant melanoma*. Cancer research, 2004. **64**(19): p. 7002-10.
156. Serrano, M., G.J. Hannon, and D. Beach, *A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4*. Nature, 1993. **366**(6456): p. 704-7.
157. Hayward, N.K., *Genetics of melanoma predisposition*. Oncogene, 2003. **22**(20): p. 3053-62.
158. Levy, C., M. Khaled, and D.E. Fisher, *MITF: master regulator of melanocyte development and melanoma oncogene*. Trends in molecular medicine, 2006. **12**(9): p. 406-14.
159. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement*. Cytotherapy, 2006. **8**(4): p. 315-7.
160. Friedenstein, A.J., et al., *Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues*. Transplantation, 1968. **6**(2): p. 230-47.

161. Pittenger, M.F., et al., *Multilineage potential of adult human mesenchymal stem cells*. Science, 1999. **284**(5411): p. 143-7.
162. Buhring, H.J., et al., *Novel markers for the prospective isolation of human MSC*. Ann N Y Acad Sci, 2007. **1106**: p. 262-71.
163. da Silva Meirelles, L., P.C. Chagastelles, and N.B. Nardi, *Mesenchymal stem cells reside in virtually all post-natal organs and tissues*. J Cell Sci, 2006. **119**(Pt 11): p. 2204-13.
164. Ferrari, G., et al., *Muscle regeneration by bone marrow-derived myogenic progenitors*. Science, 1998. **279**(5356): p. 1528-30.
165. Toma, C., et al., *Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart*. Circulation, 2002. **105**(1): p. 93-8.
166. Jiang, Y., et al., *Neuroectodermal differentiation from mouse multipotent adult progenitor cells*. Proc Natl Acad Sci U S A, 2003. **100 Suppl 1**: p. 11854-60.
167. Brazelton, T.R., et al., *From marrow to brain: expression of neuronal phenotypes in adult mice*. Science, 2000. **290**(5497): p. 1775-9.
168. Hermann, A., et al., *Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells*. J Cell Sci, 2004. **117**(Pt 19): p. 4411-22.
169. Caplan, A.I., *The mesengenic process*. Clin Plast Surg, 1994. **21**(3): p. 429-35.
170. Nakahara, H., et al., *In vitro differentiation of bone and hypertrophic cartilage from periosteal-derived cells*. Exp Cell Res, 1991. **195**(2): p. 492-503.
171. Zuk, P.A., et al., *Human adipose tissue is a source of multipotent stem cells*. Mol Biol Cell, 2002. **13**(12): p. 4279-95.
172. Jiang, Y., et al., *Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain*. Exp Hematol, 2002. **30**(8): p. 896-904.
173. Sellheyer, K. and D. Krahel, *Cutaneous mesenchymal stem cells: status of current knowledge, implications for dermatopathology*. J Cutan Pathol, 2010. **37**(6): p. 624-34.
174. Roufosse, C.A., et al., *Circulating mesenchymal stem cells*. Int J Biochem Cell Biol, 2004. **36**(4): p. 585-97.
175. Erices, A., P. Conget, and J.J. Minguell, *Mesenchymal progenitor cells in human umbilical cord blood*. Br J Haematol, 2000. **109**(1): p. 235-42.
176. Rochefort, G.Y., et al., *Multipotential mesenchymal stem cells are mobilized into peripheral blood by hypoxia*. Stem Cells, 2006. **24**(10): p. 2202-8.
177. Devine, S.M., et al., *Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates*. Blood, 2003. **101**(8): p. 2999-3001.
178. Kawada, H., et al., *Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction*. Blood, 2004. **104**(12): p. 3581-7.
179. Mansilla, E., et al., *Bloodstream cells phenotypically identical to human mesenchymal bone marrow stem cells circulate in large amounts under the*

- influence of acute large skin damage: new evidence for their use in regenerative medicine.* Transplant Proc, 2006. **38**(3): p. 967-9.
180. He, Q., C. Wan, and G. Li, *Concise review: multipotent mesenchymal stromal cells in blood.* Stem Cells, 2007. **25**(1): p. 69-77.
 181. Karp, J.M. and G.S. Leng Teo, *Mesenchymal stem cell homing: the devil is in the details.* Cell Stem Cell, 2009. **4**(3): p. 206-16.
 182. Chen, J., et al., *Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats.* J Neurol Sci, 2001. **189**(1-2): p. 49-57.
 183. Chapel, A., et al., *Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome.* J Gene Med, 2003. **5**(12): p. 1028-38.
 184. Breitbach, M., et al., *Potential risks of bone marrow cell transplantation into infarcted hearts.* Blood, 2007. **110**(4): p. 1362-9.
 185. Ley, K., et al., *Getting to the site of inflammation: the leukocyte adhesion cascade updated.* Nat Rev Immunol, 2007. **7**(9): p. 678-89.
 186. Imhof, B.A. and M. Aurrand-Lions, *Adhesion mechanisms regulating the migration of monocytes.* Nature reviews. Immunology, 2004. **4**(6): p. 432-44.
 187. Luster, A.D., R. Alon, and U.H. von Andrian, *Immune cell migration in inflammation: present and future therapeutic targets.* Nature immunology, 2005. **6**(12): p. 1182-90.
 188. Ruster, B., et al., *Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells.* Blood, 2006. **108**(12): p. 3938-44.
 189. Spring, H., et al., *Chemokines direct endothelial progenitors into tumor neovessels.* Proc Natl Acad Sci U S A, 2005. **102**(50): p. 18111-6.
 190. Son, B.R., et al., *Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases.* Stem Cells, 2006. **24**(5): p. 1254-64.
 191. Honczarenko, M., et al., *Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors.* Stem Cells, 2006. **24**(4): p. 1030-41.
 192. Bhakta, S., P. Hong, and O. Koc, *The surface adhesion molecule CXCR4 stimulates mesenchymal stem cell migration to stromal cell-derived factor-1 in vitro but does not decrease apoptosis under serum deprivation.* Cardiovascular revascularization medicine : including molecular interventions, 2006. **7**(1): p. 19-24.
 193. Cheng, Z., et al., *Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance.* Molecular therapy : the journal of the American Society of Gene Therapy, 2008. **16**(3): p. 571-9.
 194. Sasaki, M., et al., *Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type.* J Immunol, 2008. **180**(4): p. 2581-7.

195. Neth, P., et al., *Wnt signaling regulates the invasion capacity of human mesenchymal stem cells*. Stem Cells, 2006. **24**(8): p. 1892-903.
196. Forte, G., et al., *Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation*. Stem Cells, 2006. **24**(1): p. 23-33.
197. Schmidt, A., et al., *Basic fibroblast growth factor controls migration in human mesenchymal stem cells*. Stem Cells, 2006. **24**(7): p. 1750-8.
198. Li, Y., et al., *Insulin-like growth factor 1 enhances the migratory capacity of mesenchymal stem cells*. Biochem Biophys Res Commun, 2007. **356**(3): p. 780-4.
199. Meriane, M., et al., *Cooperation of matrix metalloproteinases with the RhoA/Rho kinase and mitogen-activated protein kinase kinase-1/extracellular signal-regulated kinase signaling pathways is required for the sphingosine-1-phosphate-induced mobilization of marrow-derived stromal cells*. Stem Cells, 2006. **24**(11): p. 2557-65.
200. Gao, H., et al., *Activation of signal transducers and activators of transcription 3 and focal adhesion kinase by stromal cell-derived factor 1 is required for migration of human mesenchymal stem cells in response to tumor cell-conditioned medium*. Stem Cells, 2009. **27**(4): p. 857-65.
201. Ryu, C.H., et al., *Migration of human umbilical cord blood mesenchymal stem cells mediated by stromal cell-derived factor-1/CXCR4 axis via Akt, ERK, and p38 signal transduction pathways*. Biochem Biophys Res Commun, 2010. **398**(1): p. 105-10.
202. Horwitz, E.M., et al., *Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta*. Nat Med, 1999. **5**(3): p. 309-13.
203. Quarto, R., et al., *Repair of large bone defects with the use of autologous bone marrow stromal cells*. N Engl J Med, 2001. **344**(5): p. 385-6.
204. Chen, S.L., et al., *Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction*. Am J Cardiol, 2004. **94**(1): p. 92-5.
205. Ripa, R.S., et al., *Bone marrow derived mesenchymal cell mobilization by granulocyte-colony stimulating factor after acute myocardial infarction: results from the Stem Cells in Myocardial Infarction (STEMMI) trial*. Circulation, 2007. **116**(11 Suppl): p. I24-30.
206. Morigi, M., et al., *Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure*. J Am Soc Nephrol, 2004. **15**(7): p. 1794-804.
207. Duijvestein, M., et al., *Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study*. Gut, 2010. **59**(12): p. 1662-9.
208. Shake, J.G., et al., *Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects*. Ann Thorac Surg, 2002. **73**(6): p. 1919-25; discussion 1926.
209. Nagaya, N., et al., *Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through*

- angiogenesis and myogenesis*. Am J Physiol Heart Circ Physiol, 2004. **287**(6): p. H2670-6.
210. Amado, L.C., et al., *Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction*. Proc Natl Acad Sci U S A, 2005. **102**(32): p. 11474-9.
 211. Lange, C., et al., *Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats*. Kidney Int, 2005. **68**(4): p. 1613-7.
 212. Togel, F., et al., *Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms*. Am J Physiol Renal Physiol, 2005. **289**(1): p. F31-42.
 213. Morigi, M., et al., *Human bone marrow mesenchymal stem cells accelerate recovery of acute renal injury and prolong survival in mice*. Stem Cells, 2008. **26**(8): p. 2075-82.
 214. Kale, S., et al., *Bone marrow stem cells contribute to repair of the ischemically injured renal tubule*. J Clin Invest, 2003. **112**(1): p. 42-9.
 215. Togel, F., et al., *Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury*. Am J Physiol Renal Physiol, 2007. **292**(5): p. F1626-35.
 216. Lazarus, H.M., et al., *Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use*. Bone Marrow Transplant, 1995. **16**(4): p. 557-64.
 217. Meisel, R., et al., *Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation*. Blood, 2004. **103**(12): p. 4619-21.
 218. Jiang, X.X., et al., *Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells*. Blood, 2005. **105**(10): p. 4120-6.
 219. Li, Y.P., et al., *Human mesenchymal stem cells license adult CD34⁺ hemopoietic progenitor cells to differentiate into regulatory dendritic cells through activation of the Notch pathway*. J Immunol, 2008. **180**(3): p. 1598-608.
 220. Le Blanc, K., et al., *Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells*. Lancet, 2004. **363**(9419): p. 1439-41.
 221. Gonzalo-Daganzo, R., et al., *Results of a pilot study on the use of third-party donor mesenchymal stromal cells in cord blood transplantation in adults*. Cytotherapy, 2009. **11**(3): p. 278-88.
 222. Kunter, U., et al., *Mesenchymal stem cells prevent progressive experimental renal failure but maldifferentiate into glomerular adipocytes*. J Am Soc Nephrol, 2007. **18**(6): p. 1754-64.
 223. Sundin, M., et al., *Mesenchymal stem cells are susceptible to human herpesviruses, but viral DNA cannot be detected in the healthy seropositive individual*. Bone Marrow Transplant, 2006. **37**(11): p. 1051-9.

224. Matushansky, I., et al., *Derivation of sarcomas from mesenchymal stem cells via inactivation of the Wnt pathway*. J Clin Invest, 2007. **117**(11): p. 3248-57.
225. Iwamoto, S., et al., *Mesenchymal cells regulate the response of acute lymphoblastic leukemia cells to asparaginase*. J Clin Invest, 2007. **117**(4): p. 1049-57.
226. Williams, D.A., *A new mechanism of leukemia drug resistance?* N Engl J Med, 2007. **357**(1): p. 77-8.
227. Dierks, C., et al., *Essential role of stromally induced hedgehog signaling in B-cell malignancies*. Nat Med, 2007. **13**(8): p. 944-51.
228. Docheva, D., et al., *Human mesenchymal stem cells in contact with their environment: surface characteristics and the integrin system*. J Cell Mol Med, 2007. **11**(1): p. 21-38.
229. Ip, J.E., et al., *Mesenchymal stem cells use integrin beta1 not CXC chemokine receptor 4 for myocardial migration and engraftment*. Molecular biology of the cell, 2007. **18**(8): p. 2873-82.
230. Majumdar, M.K., et al., *Characterization and functionality of cell surface molecules on human mesenchymal stem cells*. J Biomed Sci, 2003. **10**(2): p. 228-41.
231. Segers, V.F., et al., *Mesenchymal stem cell adhesion to cardiac microvascular endothelium: activators and mechanisms*. Am J Physiol Heart Circ Physiol, 2006. **290**(4): p. H1370-7.
232. Crigler, L., et al., *Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis*. Experimental neurology, 2006. **198**(1): p. 54-64.
233. Yang, H.J., et al., *A novel role for neural cell adhesion molecule in modulating insulin signaling and adipocyte differentiation of mouse mesenchymal stem cells*. J Cell Sci, 2011. **124**(Pt 15): p. 2552-60.
234. Wang, X., et al., *Neural cell adhesion molecule contributes to hemopoiesis-supporting capacity of stromal cell lines*. Stem Cells, 2005. **23**(9): p. 1389-99.
235. Wang, X., et al., *Mouse mesenchymal stem cells can support human hematopoiesis both in vitro and in vivo: the crucial role of neural cell adhesion molecule*. Haematologica, 2010. **95**(6): p. 884-91.
236. Shi, Y., et al., *Neural cell adhesion molecule potentiates invasion and metastasis of melanoma cells through CAMP-dependent protein kinase and phosphatidylinositol 3-kinase pathways*. Int J Biochem Cell Biol, 2011. **43**(4): p. 682-90.
237. Zecchini, S. and U. Cavallaro, *Neural cell adhesion molecule in cancer: expression and mechanisms*. Adv Exp Med Biol, 2010. **663**: p. 319-33.
238. Prag, S., et al., *NCAM regulates cell motility*. J Cell Sci, 2002. **115**(Pt 2): p. 283-92.
239. Studeny, M., et al., *Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors*. Cancer Res, 2002. **62**(13): p. 3603-8.
240. Menon, L.G., et al., *Differential gene expression associated with migration of mesenchymal stem cells to conditioned medium from tumor cells or bone marrow cells*. Stem Cells, 2007. **25**(2): p. 520-8.

241. Satake, K., J. Lou, and L.G. Lenke, *Migration of mesenchymal stem cells through cerebrospinal fluid into injured spinal cord tissue*. Spine (Phila Pa 1976), 2004. **29**(18): p. 1971-9.
242. Ji, J.F., et al., *Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury*. Stem Cells, 2004. **22**(3): p. 415-27.
243. Kato, J., et al., *Contribution of neural cell adhesion molecule (NCAM) to hemopoietic system in monkeys*. Ann Hematol, 2008. **87**(10): p. 797-807.
244. Olofsson, C.S., et al., *Impaired insulin exocytosis in neural cell adhesion molecule-/- mice due to defective reorganization of the submembrane F-actin network*. Endocrinology, 2009. **150**(7): p. 3067-75.
245. Ahn, S., et al., *A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-fos*. Mol Cell Biol, 1998. **18**(2): p. 967-77.
246. Sato, S., N. Fujita, and T. Tsuruo, *Modulation of Akt kinase activity by binding to Hsp90*. Proc Natl Acad Sci U S A, 2000. **97**(20): p. 10832-7.
247. Lehenbre, F., et al., *NCAM-induced focal adhesion assembly: a functional switch upon loss of E-cadherin*. EMBO J, 2008. **27**(19): p. 2603-15.
248. Thiery, J.P. and J.P. Sleeman, *Complex networks orchestrate epithelial-mesenchymal transitions*. Nat Rev Mol Cell Biol, 2006. **7**(2): p. 131-42.
249. Weeraratna, A.T., et al., *Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma*. Cancer Cell, 2002. **1**(3): p. 279-88.
250. Huang, F.J., et al., *Molecular basis for the critical role of suppressor of cytokine signaling-1 in melanoma brain metastasis*. Cancer Res, 2008. **68**(23): p. 9634-42.
251. Minn, A.J., et al., *Genes that mediate breast cancer metastasis to lung*. Nature, 2005. **436**(7050): p. 518-24.
252. Kang, Y., et al., *A multigenic program mediating breast cancer metastasis to bone*. Cancer Cell, 2003. **3**(6): p. 537-49.
253. Shaywitz, A.J. and M.E. Greenberg, *CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals*. Annual review of biochemistry, 1999. **68**: p. 821-61.
254. Radhakrishnan, I., et al., *Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:coactivator interactions*. Cell, 1997. **91**(6): p. 741-52.
255. Aucoin, R., et al., *Dominant-negative CREB inhibits heparanase functionality and melanoma cell invasion*. J Cell Biochem, 2004. **93**(2): p. 215-23.
256. Rutberg, S.E., et al., *Expression and transcriptional activity of AP-1, CRE, and URE binding proteins in B16 mouse melanoma subclones*. Mol Carcinog, 1994. **10**(2): p. 82-7.
257. Yang, Y.M., L.R. Dolan, and Z. Ronai, *Expression of dominant negative CREB reduces resistance to radiation of human melanoma cells*. Oncogene, 1996. **12**(10): p. 2223-33.
258. Xie, S., et al., *Dominant-negative CREB inhibits tumor growth and metastasis of human melanoma cells*. Oncogene, 1997. **15**(17): p. 2069-75.

259. Aonurm-Helm, A., et al., *Dysregulated CREB signaling pathway in the brain of neural cell adhesion molecule (NCAM)-deficient mice*. Brain Res, 2008. **1243**: p. 104-12.
260. Ditlevsen, D.K., et al., *NCAM-induced intracellular signaling revisited*. J Neurosci Res, 2008. **86**(4): p. 727-43.
261. Blaheta, R.A., et al., *Expression level of neural cell adhesion molecule (NCAM) inversely correlates with the ability of neuroblastoma cells to adhere to endothelium in vitro*. Cell Commun Adhes, 2002. **9**(3): p. 131-47.
262. Thiery, J.P. and D. Chopin, *Epithelial cell plasticity in development and tumor progression*. Cancer metastasis reviews, 1999. **18**(1): p. 31-42.
263. Noonan, F.P., et al., *Animal models of melanoma: an HGF/SF transgenic mouse model may facilitate experimental access to UV initiating events*. Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society, 2003. **16**(1): p. 16-25.
264. Zaidi, M.R., C.P. Day, and G. Merlino, *From UVs to metastases: modeling melanoma initiation and progression in the mouse*. The Journal of investigative dermatology, 2008. **128**(10): p. 2381-91.
265. Cambon, K., et al., *A synthetic neural cell adhesion molecule mimetic peptide promotes synaptogenesis, enhances presynaptic function, and facilitates memory consolidation*. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2004. **24**(17): p. 4197-204.
266. Soroka, V., et al., *Induction of neuronal differentiation by a peptide corresponding to the homophilic binding site of the second Ig module of the neural cell adhesion molecule*. The Journal of biological chemistry, 2002. **277**(27): p. 24676-83.
267. Khanna, C. and K. Hunter, *Modeling metastasis in vivo*. Carcinogenesis, 2005. **26**(3): p. 513-23.
268. Bibby, M.C., *Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages*. European journal of cancer, 2004. **40**(6): p. 852-7.
269. Cavallaro, U., et al., *N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling*. Nat Cell Biol, 2001. **3**(7): p. 650-7.
270. Francavilla, C., et al., *Neural cell adhesion molecule regulates the cellular response to fibroblast growth factor*. J Cell Sci, 2007. **120**(Pt 24): p. 4388-94.
271. Leve, F., W. de Souza, and J.A. Morgado-Diaz, *A cross-link between protein kinase A and Rho-family GTPases signaling mediates cell-cell adhesion and actin cytoskeleton organization in epithelial cancer cells*. J Pharmacol Exp Ther, 2008. **327**(3): p. 777-88.
272. Dabizzi, S., et al., *Luteinizing hormone increases human endometrial cancer cells invasiveness through activation of protein kinase A*. Cancer Res, 2003. **63**(14): p. 4281-6.
273. Young, M.R., et al., *Regulation of Lewis lung carcinoma invasion and metastasis by protein kinase A*. Int J Cancer, 1995. **61**(1): p. 104-9.

274. Dhawan, P., et al., *Constitutive activation of Akt/protein kinase B in melanoma leads to up-regulation of nuclear factor-kappaB and tumor progression.* Cancer Res, 2002. **62**(24): p. 7335-42.
275. Arboleda, M.J., et al., *Overexpression of AKT2/protein kinase Bbeta leads to up-regulation of beta1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells.* Cancer Res, 2003. **63**(1): p. 196-206.
276. Li, A.P., et al., *Correlation between changes in intracellular level of cyclic AMP, activation of cyclic AMP-dependent protein kinase, and the morphology of Chinese hamster ovary cells in culture.* Archives of biochemistry and biophysics, 1977. **182**(1): p. 181-7.
277. Impey, S., et al., *Cross talk between ERK and PKA is required for Ca²⁺ stimulation of CREB-dependent transcription and ERK nuclear translocation.* Neuron, 1998. **21**(4): p. 869-83.
278. Chen, H.C., et al., *Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase.* The Journal of biological chemistry, 1996. **271**(42): p. 26329-34.
279. Ong, S.H., et al., *Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(11): p. 6074-9.
280. Kolkova, K., et al., *Identification of an amino acid sequence motif in the cytoplasmic domain of the NCAM-140 kDa isoform essential for its neuritogenic activity.* Journal of neurochemistry, 2000. **75**(3): p. 1274-82.
281. Kamiguchi, H. and V. Lemmon, *IgCAMs: bidirectional signals underlying neurite growth.* Current opinion in cell biology, 2000. **12**(5): p. 598-605.
282. Mayr, B. and M. Montminy, *Transcriptional regulation by the phosphorylation-dependent factor CREB.* Nat Rev Mol Cell Biol, 2001. **2**(8): p. 599-609.
283. Linnerth, N.M., et al., *cAMP response element-binding protein is expressed at high levels in human ovarian adenocarcinoma and regulates ovarian tumor cell proliferation.* Int J Gynecol Cancer, 2008. **18**(6): p. 1248-57.
284. Xiao, X., et al., *Targeting CREB for cancer therapy: friend or foe.* Curr Cancer Drug Targets, 2010. **10**(4): p. 384-91.
285. Dai, Y., et al., *Skin epithelial cells in mice from umbilical cord blood mesenchymal stem cells.* Burns, 2007. **33**(4): p. 418-28.
286. Ditlevsen, D.K., et al., *Relative role of upstream regulators of Akt, ERK and CREB in NCAM- and FGF2-mediated signalling.* Neurochem Int, 2008. **53**(5): p. 137-47.
287. Jiao, X., et al., *c-Jun induces mammary epithelial cellular invasion and breast cancer stem cell expansion.* J Biol Chem, 2010. **285**(11): p. 8218-26.
288. Pollerberg, G.E., et al., *The 180-kD component of the neural cell adhesion molecule N-CAM is involved in cell-cell contacts and cytoskeleton-membrane interactions.* Cell Tissue Res, 1987. **250**(1): p. 227-36.
289. Huerta, S., et al., *Alternative mRNA splicing in colon cancer causes loss of expression of neural cell adhesion molecule.* Surgery, 2001. **130**(5): p. 834-43.

290. Franceschini, I., et al., *Migrating and myelinating potential of neural precursors engineered to overexpress PSA-NCAM*. Mol Cell Neurosci, 2004. **27**(2): p. 151-62.
291. Jaiswal, N., et al., *Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro*. J Cell Biochem, 1997. **64**(2): p. 295-312.
292. Mackay, A.M., et al., *Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow*. Tissue Eng, 1998. **4**(4): p. 415-28.
293. Rottner, K. and T.E. Stradal, *Actin dynamics and turnover in cell motility*. Curr Opin Cell Biol, 2011. **23**(5): p. 569-78.
294. Liu, R., et al., *Neural cell adhesion molecule potentiates the growth of murine melanoma via beta-catenin signaling by association with fibroblast growth factor receptor and glycogen synthase kinase-3beta*. J Biol Chem, 2011. **286**(29): p. 26127-37.
295. Ponte, A.L., et al., *The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities*. Stem Cells, 2007. **25**(7): p. 1737-45.
296. Hemeda, H., et al., *Interferon-gamma and tumor necrosis factor-alpha differentially affect cytokine expression and migration properties of mesenchymal stem cells*. Stem Cells Dev, 2010. **19**(5): p. 693-706.
297. Jiang, Y., et al., *Pluripotency of mesenchymal stem cells derived from adult marrow*. Nature, 2002. **418**(6893): p. 41-9.
298. Bianco, P., et al., *Bone marrow stromal stem cells: nature, biology, and potential applications*. Stem Cells, 2001. **19**(3): p. 180-92.
299. Wang, L., et al., *MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture*. Hematology, 2002. **7**(2): p. 113-7.
300. Bhakta, S., P. Hong, and O. Koc, *The surface adhesion molecule CXCR4 stimulates mesenchymal stem cell migration to stromal cell-derived factor-1 in vitro but does not decrease apoptosis under serum deprivation*. Cardiovasc Revasc Med, 2006. **7**(1): p. 19-24.
301. Zocchi, M.R., M. Vidal, and A. Poggi, *Involvement of CD56/N-CAM molecule in the adhesion of human solid tumor cell lines to endothelial cells*. Exp Cell Res, 1993. **204**(1): p. 130-5.
302. Tamama, K., et al., *Epidermal growth factor as a candidate for ex vivo expansion of bone marrow-derived mesenchymal stem cells*. Stem Cells, 2006. **24**(3): p. 686-95.
303. Buttner, B., et al., *The neural cell adhesion molecule is associated with major components of the cytoskeleton*. Biochemical and biophysical research communications, 2003. **310**(3): p. 967-71.
304. Narumiya, S., M. Tanji, and T. Ishizaki, *Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion*. Cancer Metastasis Rev, 2009. **28**(1-2): p. 65-76.

305. Fu, X., et al., *Migration of bone marrow-derived mesenchymal stem cells induced by tumor necrosis factor-alpha and its possible role in wound healing*. Wound Repair Regen, 2009. **17**(2): p. 185-91.
306. Vargas, F., et al., *Characterization of neural cell adhesion molecule (NCAM) expression in thyroid follicular cells: induction by cytokines and over-expression in autoimmune glands*. Clin Exp Immunol, 1994. **98**(3): p. 478-88.
307. Jensen, M. and F. Berthold, *Targeting the neural cell adhesion molecule in cancer*. Cancer letters, 2007. **258**(1): p. 9-21.
308. Tassone, P., et al., *In vitro and in vivo activity of the maytansinoid immunoconjugate huN901-N2'-deacetyl-N2'-(3-mercapto-1-oxopropyl)-maytansine against CD56+ multiple myeloma cells*. Cancer research, 2004. **64**(13): p. 4629-36.
309. Smith, S.V., *Technology evaluation: huN901-DM1*, ImmunoGen. Current opinion in molecular therapeutics, 2005. **7**(4): p. 394-401.
310. Guest, R.D., et al., *The role of extracellular spacer regions in the optimal design of chimeric immune receptors: evaluation of four different scFvs and antigens*. Journal of immunotherapy, 2005. **28**(3): p. 203-11.
311. Morgan, R.A., et al., *Cancer regression in patients after transfer of genetically engineered lymphocytes*. Science, 2006. **314**(5796): p. 126-9.

CHAPTER 7 PUBLICATIONS

1. Shi Y, Liu R, Zhang S, Xia YY, Yang HJ, Guo K, Zeng Q, Feng ZW. *Neural cell adhesion molecule potentiates invasion and metastasis of melanoma cells through CAMP-dependent protein kinase and phosphatidylinositol 3-kinase pathways*. *Int J Biochem Cell Biol*. 2011 Apr;43(4):682-90.
2. Shi Y, Wang L, Ju PJ, Xia YY, Feng ZW. *Neural cell adhesion molecule modulates migration of mesenchymal stromal cells via activation of MAPK/ERK signaling*. *Experimental Cell Research*, 2012 Jun 5
3. Liu R, Shi Y, Yang HJ, Wang L, Zhang S, Xia YY, Wong JL, Feng ZW. *Neural cell adhesion molecule potentiates the growth of murine melanoma via β -catenin signaling by association with fibroblast growth factor receptor and glycogen synthase kinase-3 β* . *J Biol Chem*. 2011 Jul 22; 286(29):26127-37.
4. Wang L, Shi Y, Ju PJ, Liu R, Yeo SP, Xia YY, Feng ZW. *Silencing of Diphthamide Synthesis 3 (Dph3) Reduces Metastasis of Murine Melanoma*. *PLoS One* 2012;7(11):e49988, Epub 2012 Nov 20
5. Liu R, Zhang S, Yang HJ, Ju PJ, Xia YY, Shi Y, Lim TH, Alvin St Lim, Liang FY, Feng ZW. *Characterization and therapeutic evaluation of a Nestin+ CNP+ NG2+ cell population on mouse spinal cord injury*. (Submission in progress)