Cellular, Molecular and Functional Characterization of the Tumor Suppressor Candidate MYO1C

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Cover illustration: Schematic diagram showing functions of MYO1C protein in GLUT-4 transportation and maintaining adherens junction by Woranop Sukparangsi

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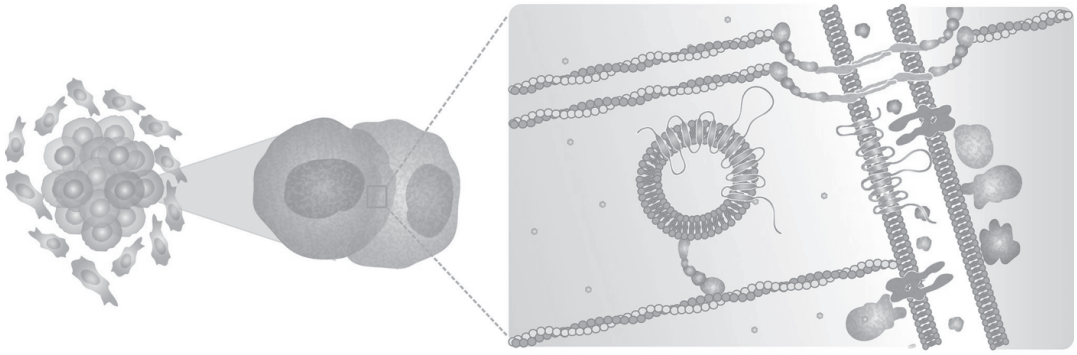
. . . dedicated to my dearest late grandmother Chinda and my family . . .



"*Knowing yourself is the beginning of all wisdom*."
— Aristotle

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ABSTRACT

Tumor suppressor genes play a role as a growth regulator and a gatekeeper of a cell. Their inactivation is often detected in malignant tumors. Identification of novel tumor suppressor gene candidates may help to further understand tumorigenesis and aid in the discovery of a new treatment leading toward cure of cancer.

This PhD research project aimed to understand functional significance of a novel tumor suppressor gene candidate, myosin IC (*MYO1C*) and to identify potential interaction(s) of the MYO1C protein with key components of the signaling pathways involving in cancer development.

In an experimental rat model for endometrial carcinoma (EC), detailed molecular genetic analysis of a candidate tumor suppressor region located distal to the tumor protein 53 (*Tp53*) suggested the myosin IC gene (*Myo1c*) as the best potential target for deletion of the genetic material. The question arising was whether and how *MYO1C* could function as a tumor suppressor gene. By using qPCR, Western blot or immunohistochemistry analyses, we examined MYO1C protein level in panels of well-stratified human colorectal cancer (CRC) and EC respectively. We found that MYO1C was significantly down-regulated in these cancer materials and that for the EC panel, the observed down-regulation of MYO1C correlated with tumor stage, where tumors at more advanced stages had less expression of MYO1C. In cell transfection experiments, we found that over-expression of MYO1C significantly decreased cell proliferation, and silencing *MYO1C* with siRNA increased cell viability. Additionally, knockdown of *MYO1C* impaired the ability of cells to migrate, spread and adhere to the surface. Recent published studies suggested a potential interplay between MYO1C and the phosphoinositide 3-kinase (PI3K)/AKT pathway. To examine this hypothesis, we analyzed the expression and/or activation of components of the PI3K/AKT and RAS/ERK signaling pathways *in vivo* in CRC samples, and *in vitro* in cells transfected with the *MYO1C* gene expression construct or *MYO1C*-targeted siRNA. To identify other potential pathways/ mechanisms through which MYO1C may exert its tumor suppressor activity, we additionally performed new sets of *MYO1C*-siRNA knockdown experiments. At different time points post transfection, we performed microarray global gene expression experiments followed by bioinformatics analysis of the data. Altogether, the results suggested an early PI3K/AKT response to altered *MYO1C* expression. We additionally identified several cancer-related genes/pathways with late response to *MYO1C* knockdown. All things considered, the identification of MYO1C-expression impact on cell proliferation, migration, and adhesion in combination with its interplay between several cancer-related genes and signaling pathways provide further evidence for the initial hypothesis of a tumor suppressor activity of MYO1C.

Keywords: MYO1C, myosin IC, tumor suppressor gene, cancer, tumor, PI3K/AKT signaling

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Cellulär, molekylär och funktionell karaktärisering av tumörsuppressor kandidatgenen MYO1C

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SAMMANFATTNING

Tumörsuppressorgener har en viktig roll i kontroll av celltillväxt och i att förhindra uppkomst av mutationer i arvsmassan. Inaktivering av dessa är ett viktigt steg i utveckling av maligna tumörer och därför kan identifiering av nya möjliga tumörsuppressorgener öka förståelsen för hur cancer uppstår och därmed ge ledtrådar till nya metoder för att bota cancer.

Projektet i denna avhandling är fokuserat på att undersöka den funktionella relevansen av myosin IC (*MYO1C),* som potentiell tumörsuppressorgen, samt att identifiera vilka interaktioner MYO1C har med andra protein i signalvägar med betydelse i utveckling av cancer.

Studier av en experimentell råttmodell av endometriecancer (EC) påvisade en genomisk region lokaliserad distalt om *TP53* (p53) vilken kunde innehålla en eller flera möjliga tumörsuppressorgener. Detaljerade molekylärgenetiska studier av denna region indikerade att genen för myosin IC (Myo1c) skulle kunna vara en kandidatgen och mål för deletioner av genetiskt material i dessa modeller. Frågan är då om och hur Myo1c kan fungera som en tumörsuppressorgen? Genom kvantitativ PCR (qPCR) och immunohistokemi undersöktes uttrycksnivåerna av MYO1C i välstratifierade paneler av humana kolorektalcancer (CRC)- och EC-tumörer. Vi såg att uttrycket av MYO1C var nedreglerat i dessa cancergrupper och att iEC-tumörer så korrelerade proteinnivåerna med tumörstadium, där tumörer med mer avancerat stadium hade lägre nivåer av MYO1C.

Genom cellbaserade försök visar vi att överuttryck av *MYO1C* signifikant minskar celltillväxten medan nedreglering av *MYO1C* med hjälp av siRNA ökar celltillväxten. Nedreglering av *MYO1C* leder också till försämrad kapacitet hos cellerna för migration och adhesion. Tidigare publicerade arbeten indikerar att det finns en koppling mellan MYO1C och phosphoinositide 3-kinase (PI3K)/Akt-signalering. För att undersöka denna hypotes analyserade vi uttryck och/eller aktivering av komponenter i PI3K/Akt eller MAPK signalering *in vivo* i kliniska CRC prover och *in vitro* i celler där *MYO1C* överuttryckts alternativt nedreglerats. För att identifiera andra möjliga mekanismer/signaleringsvägar genom vilka MYO1C kan utöva en tumörsupprimerande funktion undersökte vi genom microarray hur det globala genuttrycket förändrades över tid efter att *MYO1C* nedreglerats genom siRNA. Denna studie visade att nedreglering av *MYO1C* hade en tidig effekt på gener involverade i PI3K/Akt signalering.

För att summera, effekten av ett modulerat MYO1C-uttryck på celltillväxt, migration och adhesion tillsammans med dess interaktion med andra cancerrelaterade gener och signaleringsvägar bidrar tillsammans att stärka den ursprungliga hypotesen att MYO1C har tumörsupprimerande funktioner.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Hedberg Oldfors C, Dios DG, Linder A, **Visuttijai K**, Samuelson E, Karlsson S, Nilsson S, Behboudi A: Analysis of an independent tumor suppressor locus telomeric to *Tp53* suggested *Inpp5k* and *Myo1c* as novel tumor suppressor gene candidates in this region. *BMC genetics* 2015, 16:80

II. **Visuttijai K**, Pettersson J, Mehrbani Azar Y, van den Bout I, Örndal C, Marcickiewicz J, Nilsson S, Hörnquist M, Olsson B, Ejeskär K, Behboudi A: Lowered expression of tumor suppressor candidate *MYO1C* stimulates cell proliferation, activates AKT and suppresses cell adhesion. *Submitted*

III. **Visuttijai K**, Faura Tellez G, Wettergren Y, Pettersson J, Hedberg Oldfors C, Behboudi A, and Ejeskär K. Expression of *MYO1C* is down-regulated in primary colorectal tumors. *Submitted*

IV. Pfister A, **Visuttijai K**, Sjöback R, Purvén M, Behboudi A, and Olsson B: Knockdown of MYO1C resulted in early activation of the PI3K/AKT pathway and disruption of key genes and pathways involved in apoptosis and cell cycle progression. *Submitted*

CONTENT

ABBREVIATIONS

AI	Allelic imbalance
a.k.a.	Also known as
BDII	BDII/Han (inbred rat strain)
BN	Brown Norway (inbred rat strain)
bp	Base pair
BSA	Bovine serum albumin
°C	Degree Celsius
CaM	Calmodulin
cDNA	Complementary deoxyribonucleic acid
CRC	Colorectal cancer
DNA	Deoxyribonucleic acid
EC	Endometrial carcinoma
e.g.	Exempli gratia
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
F1	First generation of a cross, first filial
F2	Second generation inter-cross (F1xF1)
GLUT4	Glucose transporter type 4
hr	Hour
IGF-1	Insulin growth factor 1
IHC	Immunohistochemistry
$InsP_3$	Inositol 1,4,5-trisphosphate
IRS-1	Insulin receptor substrate 1
kb	Kilobase (1000bp)
kDa	Kilodalton
mg	Milligram
min	Minute
mRNA	Messenger ribonucleic acid
MYO18B	Myosin XVIIIB
MYO1C	Myosin IC

n, N	Sample size
N1	Backcross generation
ng	Nanogram
nM	Nanomolar
NUT	Rat uterine tumor developed in the backcross (N1) progeny
pAKT	Phosphorylated AKT
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
pERK	Phosphorylated ERK
PEST	Penicillin/streptomycin
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PCR	Polymerase chain reaction
PH	Pleckstrin homology
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative polymerase chain reaction
REF	Rat embryo fibroblast
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RUT	Rat uterine tumor developed in first generation and intercross progeny
S473	Serine residue 473
SD	Standard deviation
siRNA	Small interference ribonucleic acid
T308	Threonine residue 308
TP53	Tumor protein p53
TSG	Tumor suppressor gene
VEGF	Vascular endothelial growth factor
μg	Microgram
μM	Micromolar

1 INTRODUCTION

1.1 Cancer

Cancer is a group of genetic diseases characterized by uncontrolled cell growth and the invasion and spread of cells from the site of origin (primary site) to other sites of the body.

Cancer can arise from all different cell types and tissues in the human body. The most common cancers in adults are the cancers that occur in epithelial cells and are called carcinomas while cancers of glandular tissues such as breast are called adenocarcinomas. Cancers derived from dividing cells in the pigment-containing cells known as melanocytes (and rarely in ocular retina), neurons and neural glia are called melanomas, retinoblastomas, neuroblastomas and glioblastomas respectively. Lymphomas and leukemias, sometimes referred to as the blood cell tumor or liquid tumors, develop from the tissues that give rise to lymphoid and blood cells. All of these diseases, from different cell types and in various locations are collectively group members of 'cancer'.

Environmental factors play a significant role in carcinogenesis and DNA is the critical target for environmental carcinogens, such as viruses, xenobiotic chemicals and radiation. It is believed that malignant transformation and tumor growth is a multi-stage process during which accumulation of several mutations that lead to deregulation of cell signaling pathways central to the control of cell growth and cell fate occurs [1]. Two major groups of genes responsible for cancer development are oncogenes and tumor suppressor genes. Tumor initiation and progression are results of the conversion of normal proto-oncogenes to activated oncogenes and/or inactivation of tumor suppressor genes.

1.2 Genetic basis of cancer

1.2.1 Tumor heterogeneity

Cancer research has been trying to describe the precise mechanism that transforms normal cells in the human body into malignant tumor and to identify mutations in a subset of genes that enhance growth advantage [2].

The current studies show that the mutational profile and genomic landscape varies widely both within and between classes of cancer. Among all cancer cells, the subclones that pass through the selection pressure with the best evolutionary fitness will influence the cancer cell population, and each will be marked by the presence of mutations that provide a direct competitive advantage (driver mutations) and by others acquired during clonal evolution that contribute nothing to the subclone's oncogenic potential (passenger mutations) [2]. The alteration in these key drivers, once acquired by somatic mutation, triggers the development of cancer [3].

The significance of tumor heterogeneity is that tumors display substantial difference in terms of morphological and phenotypic profiles, including cellular morphology, gene expression profile, metabolism, motility, and angiogenic, proliferative, immunogenic, and metastatic potential. These distinctive events, occurring both between tumors (inter-tumor heterogeneity) and within tumors (intra-tumor heterogeneity), are to be stratified to the cancer subtypes in order to understand the development and design of cancer- and patient-specific treatment plans [4].

1.2.2 Tumor suppressor genes

Tumor suppressor genes are also called gatekeepers of the cell due to their role in controlling cell proliferation and their loss/deletion is often found during the development of tumors. According to Knudson's "two-hit" model, for inactivation of a tumor suppressor gene, loss or inactivation of both alleles of the gene is required. For hereditary tumors, this model proposes that tumorigenesis requires a second somatic mutation while predisposed inherited germline mutation has already existed in the cells [5]. Inactivation of tumor suppressor genes can occur through deletion, mutation, epigenetic silencing, such as promoter methylation, or other mechanisms leading to a phenomenon called loss of heterozygosity as a consequence. Among different tumors, occurring of the repeated loss of heterozygosity in a given chromosome region suggests the presence of tumor suppressor gene in that region. There is a group of tumor suppressor genes identified as a haploinsufficient tumor suppressor. These genes are exceptions to Knudson's "two-hit" model. In this small group of tumor suppressor genes, mutation of a single allele causes an increase in carcinogen susceptibility.

1.3 Cell signaling pathway

Over 45 years since the discovery of the first cancer-causing gene or 'oncogene' to the coming forth of the new era for cancer treatment – those

targeted at specific signaling molecules, cancer research has been focusing on signal transduction related to the multistep development of human cancers. Those signaling pathways are invariably altered in cancer leading to an uncontrollable change in particular '*Hallmarks of Cancer*' such as evading growth suppressors, sustaining proliferation, and resisting cell death [6].

These interconnected pathways are being deciphered and depicted, but the most substantial challenge for all researchers across the world is set to understand the alterations that lead to cancer and how to correct them as defining the signaling mechanisms will define druggable targets and facilitate the development of effective therapies.

Among the crucial pathways are those controlling cell growth, proliferation, and apoptosis, which coordinate a response to the cellular environment, with the phosphatidylinositol-3 kinase (PI3K) and the mammalian target of rapamycin (mTOR) as critical nodes. Both signaling pathways demonstrate the regulation of cell proliferation, survival as well as angiogenesis. Both non-malignant tumor cells and malignant tumor cells depend on these two pathways, which have an important role in the cellular response to hypoxia and energy depletion. Moreover, it is shown that aberrant activation of these pathways has been linked to the development of cancer [7].

1.3.1 Activation of PI3K /AKT signaling

PI3K/AKT signaling is involved in diverse cell activities, including cell growth, survival, motility, and metabolism. Changes in this pathway are suggested as an essential step towards the initiation and maintenance of cancer development as somatic mutation and amplification of genes encoding key components of PI3K/AKT pathway has been reported in different cancer types [8, 9]. Therefore, there are several approaches to identify targets in this pathway for potential therapeutic approaches [10].

Phosphatidylinositol 3 kinase (PI3K) is an intracellular lipid kinase enzyme that is involved in the addition of a phosphate to 3' hydroxyl group of the inositol at the hydrophilic head of the membrane-associated phosphatidylinositols and phosphoinositides. The family of the PI3Ks, was discovered in the 1980s. They are classified into three classes; class I (Ia and Ib), II, and III. The eight members of the PI3K family are classified into these three groups based on their primary sequence homology, domain structure, *in vitro* substrate preference, and mode of regulation [8, 11] and play a role as key regulators in various cell activities including cell growth, proliferation, differentiation, and survival [12]. With an exception of the p53 and

retinoblastoma (Rb) pathways, many components of this signaling pathway are affected by genomic alterations like mutation (germline mutation or somatic mutation), rearrangement, and amplification more often than any other pathways in human cancer [13] especially class Ia PI3K [14].

Research on PI3K signal transduction is mainly focused on PI3K class Ia. This class of PI3K enzymes includes heterodimers comprised of a p110 catalytic subunit (with three isoforms: p110α, p110β, and p110δ) and a smaller p85 regulatory subunit (with five isoforms: p85α, p85β, p50α, p55γ, and p55α). The p85 regulatory subunit has two Src homology 2 (SH2) domain [15, 16]. The role for the p85 subunit is to maintain the p110 catalytic subunit in a low-activity state in inactive cells. Through direct interaction with activated growth factor receptor tyrosine kinases (RTK) or adaptor molecules like insulin receptor substrate 1 (IRS-1) at its SH2 domains, p85 activates p110 [15-17].

The PI3Ks transduce signals from various growth factors and regulators such as cytokines as major effectors downstream of receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs). Transduction of the signal leads to generation of phospholipids that further activate serine/threonine protein kinase AKT, also known as protein kinase B (PKB), and its downstream components in the pathway [18]. The signaling pathway can also be activated by genetic hits that affect different components of the pathway [19].

Prior to signal transduction via PI3K, the inositols are in their inactive form as phosphatidylinositol-4,5-biphosphate (PtdIns(4,5)P_2 or PIP_2). Upon activation of PI3K, PIP_2 becomes phosphorylated and PtdIns(3,4,5)P_3 or PIP_3 is generated. PIP_3 in turn targets those cytosolic proteins that have a pleckstrin homology (PH) domain, including the serine/threonine kinase AKT (protein kinase B, PKB). The interaction between PIP_3 (in the membrane) and the PH domain of the AKT (at the vicinity of the membrane) causes this molecule to undergo structural conformational changes. These conformational changes result in exposure of two phosphorylation sites in AKT protein, threonine residue 308 (Thr-308) and serine residue 473 (Ser-473) residues that subsequently become phosphorylated by PDK1 (3-phosphoinositide-dependant kinase-1) and mTORC2 (mammalian target of rapamycin-rictor kinase complex) protein kinases, respectively and thus AKT becomes fully activated [16].

Activated AKT in turn acts as a kinase enzyme that phosphorylates series of downstream signaling proteins, controlling three main biological effects

attributed to AKT activation: (1) facilitating cell survival by anti-apoptosis; (2) stimulating cell proliferation, and (3) promoting cell growth [16].

On the other hand, dephosphorylation of PIP_3 at D-3 or D-5 positions of inositol by enzyme phosphatase family members, e.g. PTEN (5'-phosphatase and tensin homolog) and/or INPP5K (inositol polyphosphate-5'-phosphatase K) leads to regulated termination of PI3K/AKT signaling [15-17, 20]. The haploinsufficient tumor suppressor *PTEN* is an important regulator to the PI3K/AKT signaling pathway. Inactivating mutations or deletion of *PTEN* results in constitutive activation of PI3K/AKT/mTOR axis [21].

The consequence of persistent activation of the PI3K signaling pathway leads to a disturbance of crucial cell activities as well as contributes to competitive growth advantage, metastatic competence, and resistance to treatment. Taking all the facts into account, the PI3K becomes an obvious target of choice for cancer treatment in contrast with other tumor-suppressor pathways such as p53 because amendment of activation by pharmacological intervention is easier than recovering the tumor-suppressor function [13].

1.3.2 The PI3K /AKT pathway as a target of treatment

A variety of signals is integrated inside the cells to determine cell fate in term of survival and proliferation as well as to maintain the functions. It is rarely that a single checkpoint is for signaling convergence [22], but lots of research to date indicates that PI3K/AKT signaling axis is a gatekeeper for tumor growth. Being a central regulation point and therefore activated as a result of loss of function of tumor suppressor gene *PTEN* or gain of function mutations, this axis is a target of many attempts to develop therapeutic targets for a variety of cancers.

Certain drug candidates, including pan-PI3K inhibitors, PI3K isoform specific inhibitors, and mTOR inhibitors, are being tested as monotherapy or as combinations in Phase II-III clinical trials in an array of cancer indications [19, 23]. Moreover, new candidates as dual PI3K/mTOR inhibitors (e.g. bay80-6946, GDC0980, BGT226 and PF-04691502) seem to be new development trend that is lined-up entering into Phase I clinical trials according to public data extracted from NIH Clinical Trials Registry (available at http://clinicaltrials.gov).

1.4 The MYO1C gene and protein

The myosin IC (*MYO1C*) gene, also known as myosin I-beta (*MMIb*, *MMIβ*, *MMI-beta*), nuclear myosin I (*NMI*), and *Myr2*, is located adjacent to *INPP5K* on the human chromosome 17p13.3 (HSA17p13.3). This gene encodes a member of the unconventional myosin gene family, which is single-headed myosin molecule that dynamically links membrane to the actin cytoskeleton (Figure 1). There are three isoforms of the MYO1C protein, two of which are found in the cytoplasm and nucleus, whereas the third with a unique N-terminus containing additional 16 amino acids is found exclusively in the cell nucleus [24-26] (Figure 2).

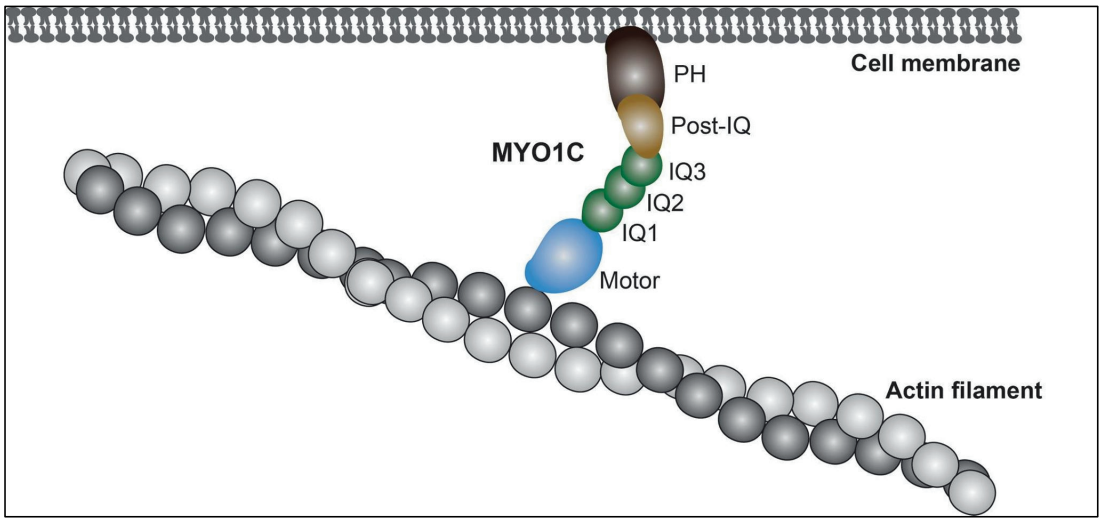

Figure 1. A schematic model of the full-length Myo1c protein (modified from Lu et al. [27]). Each Myo1c contains, a motor domain at N-terminus (blue), a regulatory domain (neck region; green) and a tail region (brown) at C-terminus. Illustration: Woranop Sukparangsi.

From N-terminus to C-terminus, MYO1C contains a motor domain, a regulatory domain (neck region) consisting of several calmodulin (CaM)-binding IQ motifs [28] and a tail region. The binding site at the regulatory domain appears to exert a non-specific binding, whereas the second binding site in the tail domain is more active in binding to membrane phosphoinositides. It has been shown that point mutations in the binding site at the tail domain of the protein inhibit the binding between MYO1C and PIP_2 *in vitro* as well as disrupt binding to membrane and its cellular localization *in vivo* [29, 30]. This specific binding site has later been identified as an embedded pleckstrin homology (PH) domain, which allows MYO1C to tightly bind with soluble inositol phosphates e.g. $InsP_3$ as well as PIP_2 [31].

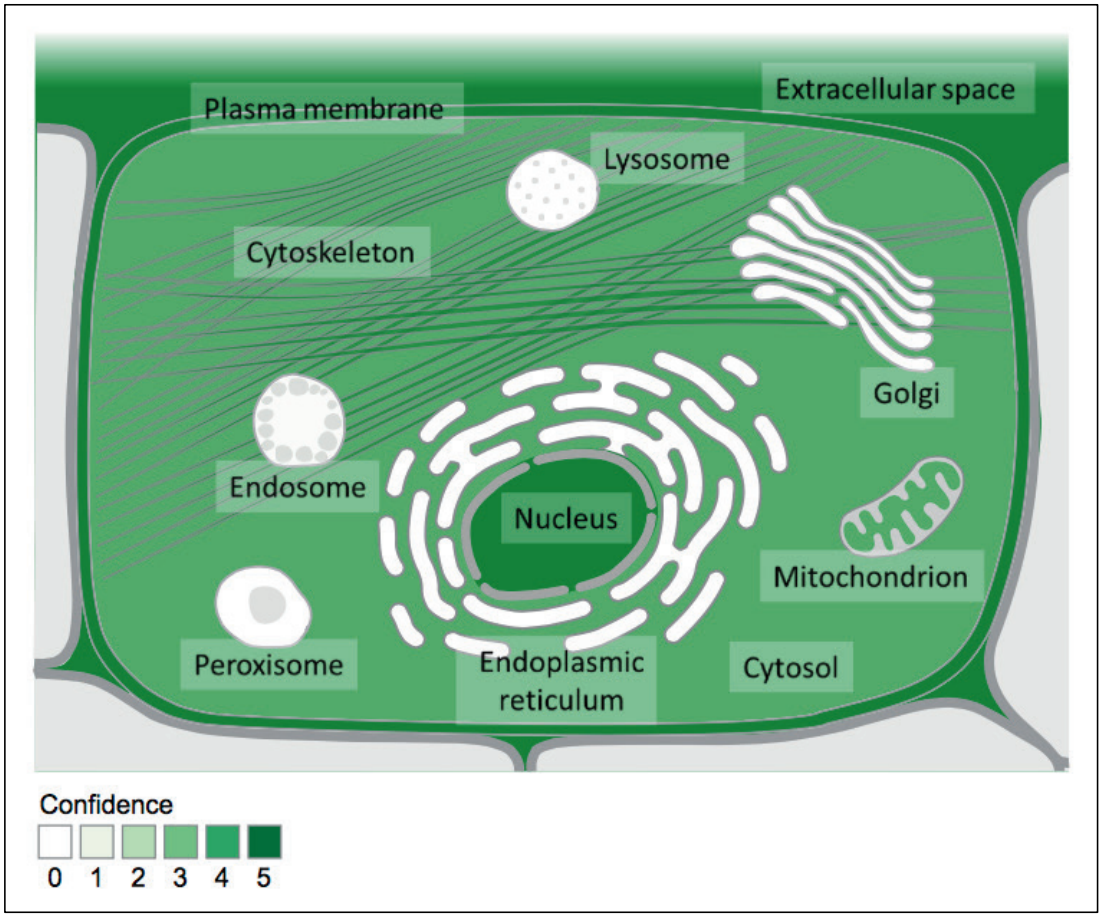

Figure 2. Endogenous MYO1C is localized in both the nucleus and cytoplasm. Subcellular locations of MYO1C protein derived from COMPARTMENTS (available at http://compartments.jensenlab.org). Unified confidence scores of the localization evidence are assigned based on evidence type and source. Color-coded confidence scale is ranging from light green (1) for lowest confidence to dark green (5) for highest confidence. White (0) indicates an absence of localization evidence.

There is no solid evidence suggesting a direct association of MYO1C to the PI3K/AKT pathway. However, there are some indications: during the glucose metabolism process, it has been shown that MYO1C is involved in insulin-stimulated fusion of vesicles containing glucose transporter-4 (GLUT-4) with the plasma membrane by controlling the movement of these vesicles to the membrane [32]. Insulin-dependent phosphorylation of MYO1C through PI3K/AKT pathway was additionally shown to be mandatory for GLUT-4 translocation [33]. In these studies, it was shown that enhanced polymerization of actin filaments by insulin stimulation through a PI3K-independent mechanism could recruit MYO1C to the membrane through its actin-binding sites. This then resulted in the accumulation of GLUT-4-containing vesicles at the cell cortex along with MYO1C-mediated membrane ruffling before they underwent fusion to the membrane. These events occurred in parallel with GLUT-4 translocation to the membrane for

which PI3K activation was required. Thus, taken together, in response to insulin stimulation, activated MYO1C (through PI3K/AKT) is required for the mobilization of GLUT-4-containing vesicles to the cell membrane as well as for their anchoring to the actin cytoskeleton prior to fusion of vesicles to the membrane [32, 34]. In summary, through PI3K/AKT pathway, MYO1C is involved in glucose metabolism.

There has been no previous report on potential tumor suppressor activity of *MYO1C*. However, there are reports on a cancer-related profile among other members of the myosin I gene family, such as *MYO18B* as a tumor suppressor gene identified in lung, ovarian and colorectal cancer [35-37] and *MYO1F* involved in gene fusion (chromosomal translocation) in infant acute monocytic leukemia [38].

2 AIM

The ultimate goal of this PhD research project was to characterize functional significance of a recently identified tumor suppressor candidate, MYO1C, in tumorigenesis processes. In order to gain insight, various molecular and functional analyses using cell-based methods were carried out and classified clinical samples were analyzed. The specific aims of the studies included in this thesis were:

Paper I: In this work, started before my PhD enrolment, we aimed to identify the most prominent tumor suppressor candidate(s) among a panel of 19 genes located in a candidate tumor suppressor region distal to *Tp53* using expression analysis in a panel of rat endometrial carcinoma compared to non-malignant rat endometrium cells. *Myo1c* was one of the two identified targets in this region.

Paper II: In this work, we aimed to investigate if MYO1C could influence expression and/or activation of a number of key components of the PI3K/AKT and RAS/ERK signaling pathways. Additionally, we aimed to examine functional significance of MYO1C over-expression or silencing on a number of cancer-related phenotypes e.g. cell proliferation, migration, and adhesion. Finally, we examined expression of MYO1C in a well-stratified panel of endometrial carcinoma to investigate for potential correlation between tumor stage/grade and MYO1C expression.

Paper III: This work was set to investigate expression of the tumor suppressor candidate MYO1C in colorectal cancer clinical samples to assess potential correlation between MYO1C mRNA expression and protein level with the tumor stage. We further investigated the possible correlation between level of MYO1C and protein level and/or activation of a number of key components of the PI3K/AKT and RAS/ERK signaling pathways in the clinical samples.

Paper IV: The potential interplay between MYO1C and the PI3K/AKT signaling pathway might not singularly explain cancer-related phenotypes exerted by MYO1C. Therefore, the present work was designed to explore other potentially involved genes and pathways in MYO1C exerted cancer-related phenotypes. The goal was to identify a number of novel genes and pathways expression of which was significantly altered in response to *MYO1C* knockdown, suggesting an interplay between these genes/pathways and MYO1C.

3 MATERIALS AND METHODS

The present PhD research project used both clinical samples and genetic materials derived from cell culture to investigate alterations in the central dogma of biology at different levels from DNA to translated protein by performing various molecular techniques (Figure 3).

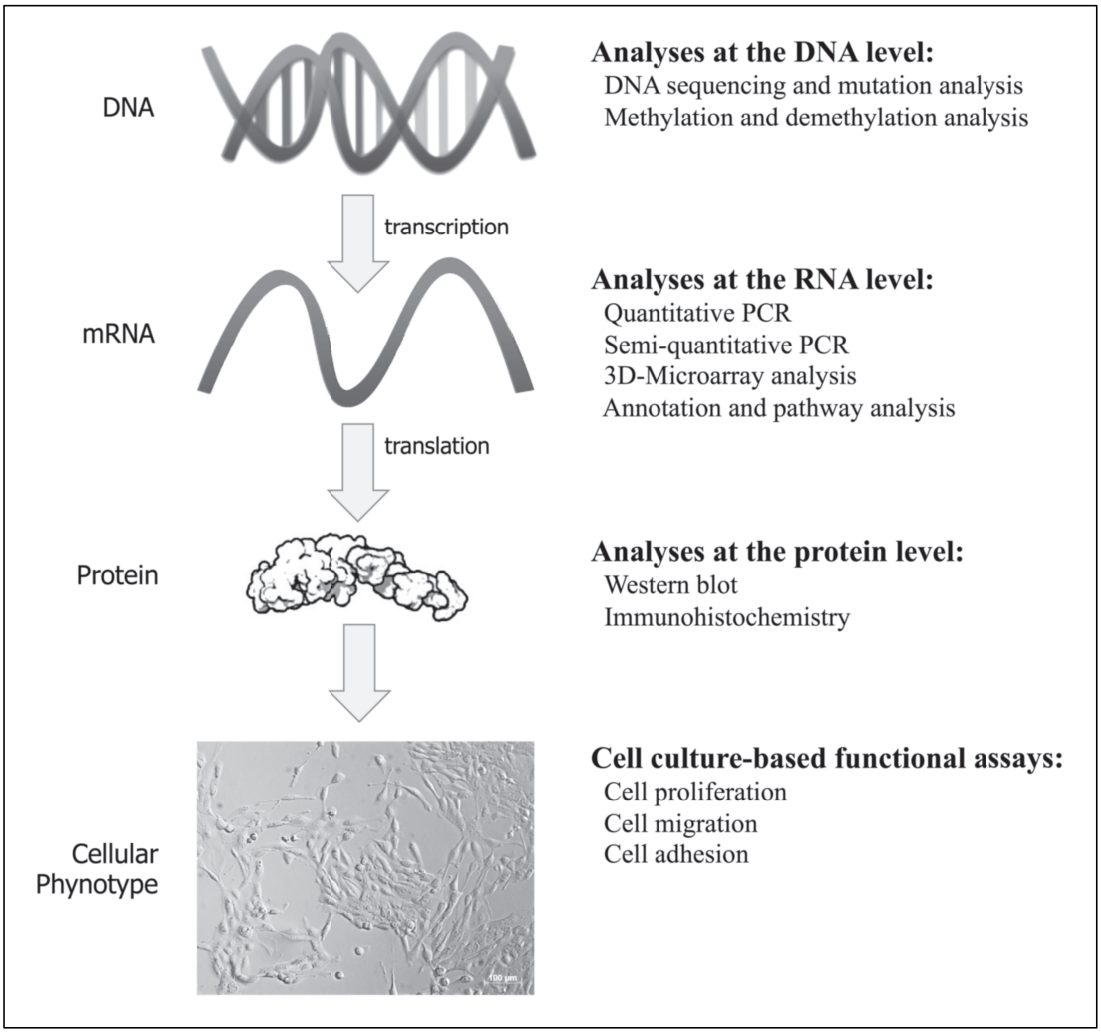

Figure 3. Schematic summary of different methods and assays performed in the present PhD research project. A crystal structure of protein reproduced and modified courtesy of the RCSB Protein Data Bank [39]

3.1 Clinical samples and cell lines

Human tumor samples

In Paper II, a total of 62 human endometrial carcinomas (EC) were analyzed. The tumor samples were randomly selected based on their pathology in the tissue bank of paraffin blocks at Sahlgrenska University Hospital and classified according to FIGO staging for endometrial carcinoma (Table 1).

Table 1. EC tumor samples used in Paper II and their stages according to FIGO staging for carcinoma of the endometrium [40, 41].

Stage (Description)	Number of samples
Hyperplasia	10
Stage I (highly differentiated)	19
Stage II (intermediately differentiated)	24
Stage III (poor differentiated)	19
Total	**62**

In Paper III, we used a panel of 24 surgically removed CRC samples, 12 from colon and 12 from rectum, obtained from Sahlgrenska University Hospital, paired with their corresponding normal-appearing colon or rectum mucosa located 10 cm away from the location of the tumor from each patient. All specimens were pathologically staged according to Duke's classification [42, 43] (Table 2). Total RNA was extracted from tissue samples using RNAeasy mini kit (Qiagen) according to manufacturer's protocol and protein extraction was made from frozen homogenized tissue samples with RIPA lysis buffer (Thermo Scientific).

Table 2. CRC tumor samples and stages according to Duke's classification used in Paper III.

Stage	Colon cancer	Rectal cancer	Total
Stage II	5	3	8
Stage III	5	6	11
Stage IV	2	3	5
Total	**12**	**12**	**24**

All experiments on human tumor samples were approved by the local ethical committee (Sahlgrenska Academy, University of Gothenburg).

Experimental tumor materials, cell lines and culture

In Paper I, ECs developed in animals of inbred rat strain BDII/Han were used as the experimental model for EC. The virgin females of this rat strain spontaneously develop endometrial cancer at a high frequency (more than 90%) before the age of 24 months [44, 45]. To obtain the tumor material used in this study, rats from the BDII strain were crossed with rats from the two inbred rat strains BN and SPRD that have a very low frequency of EC, and a F1 progeny was developed. A backcross was performed by mating heterozygote males (BDII/BN or BDII/SPRD) with female BDII rats and in this backcross about 25 percent of the progeny developed EC [46] (Figure 4). After the pathological assessment, in some cases, no malignant cells could be detected. In cytogenetic analysis, these samples showed only minor numerical chromosomal aberrations; these samples were referred as non-malignant endometrium (NME) and used as control samples. At necropsy, the tumor specimens were collected for DNA extraction with Genepure 341 Nucleic Acid Purification System (PE Applied Biosystems). Small pieces of fresh tumor as well as NME were transferred to set up primary cell cultures. Later on, DNA and total RNA were extracted from the cultures using GenElute kit (Mammalian Total RNA Kit, Sigma).

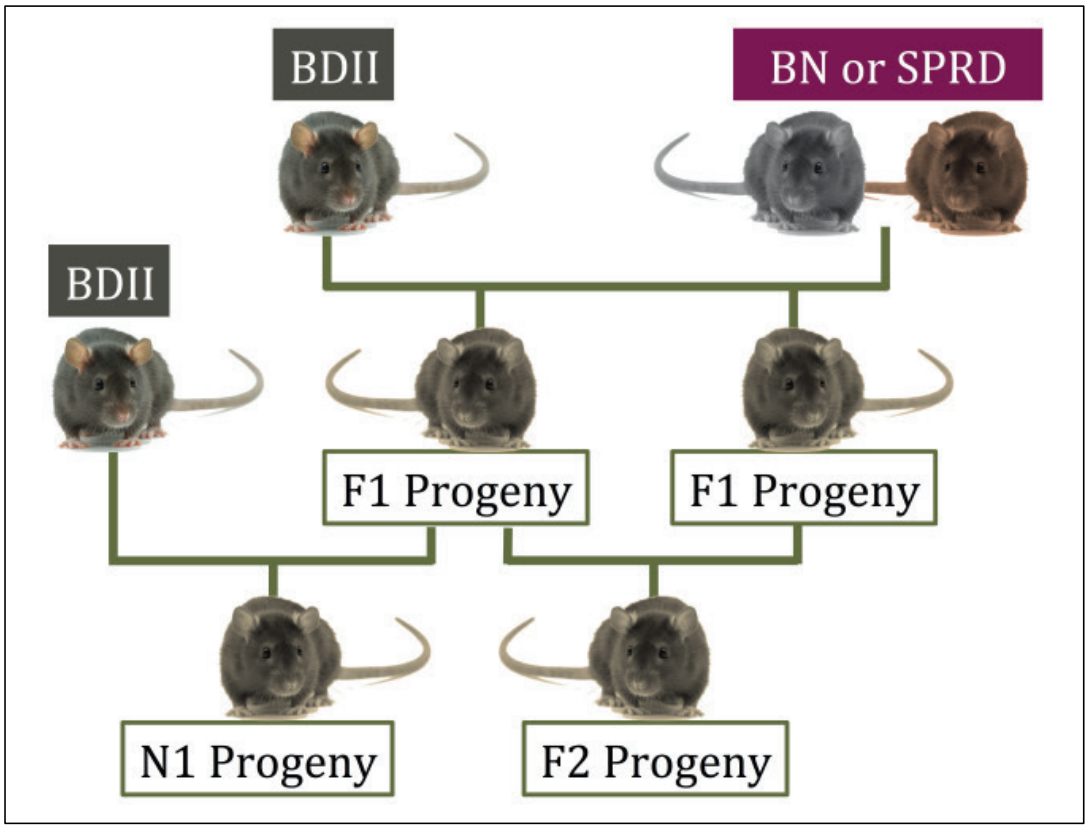

Figure 3. Crossing schedule for the BDII rats. The N1 (backcross) progeny is used in this study.

All animal works were performed under supervision of Professor Hans J. Hedrich and approved by the local ethical committee (Institute of Laboratory Animal Science and Central Animal Facility, Hannover Medical School, Germany). All cell lines used in Paper I were established from fresh tissues at CMB-Genetics, University of Gothenburg, Gothenburg, Sweden.

In Paper II, HEK-293 (or 293) cells (human embryonic kidney cell line, American Type Culture Collection, ATCC) and HeLa cells (Sigma-Aldrich/The European Collection of Cell Cultures, ECACC) with limited *de novo* protein expression of MYO1C were used for *MYO1C* gene expression transfection studies. The cell lines were cultured in DMEM medium, supplemented with 10% bovine growth serum (BGS), 1% penicillin and streptomycin (PEST).

In paper II and IV and for siRNA gene silencing experiments the immortalized normal breast epithelium MCF10A cells (Michigan Cancer Foundation, ATCC) with medium to high *de novo* protein expression of MYO1C were used. The cells were cultured, as described earlier [47], in DMEM/F12 medium, supplemented with 5% horse serum, 20 ng/ml epidermal growth factor (EGF), 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin, 1% PEST.

All cells were cultured in the atmosphere of 5% carbon dioxide at 37°C.

3.2 Analyses at DNA level

DNA sequencing for mutation analysis

DNA sequencing is a method to determine precise order of nucleotides within a DNA molecule. It is a classic method to identify mutation in DNA sequence or change in nucleic acid by different mechanisms e.g. substitution, insertion or deletion. The change in DNA sequence that alters protein-coding sequence is called frameshift mutation. The original sequencing method was firstly introduced by Sanger *et al.* in 1977 [48] after a report of DNA polymerase usage for primed synthesis in 1975 [49]. The sequencing technique makes use of the 2',3'- dideoxynucleotide (ddNTP) analogs. Once the ddNTP is incorporated, it results in chain termination as ddNTPs lacks the hydroxyl group at 3' carbon atom that allows chain elongation. In Paper I, based on the availability of material and the genetic background, a panel of 32 EC was selected for mutation sequencing of *Myo1c*. Primer pairs were designed by a commercial supplier, SIGMA-Genosystem. PCR primers set corresponding to the coding sequence were amplified and

screened for mutations. We also sequenced the promoter region of *Myo1c*. Sequencing products were separated on a denaturing polyacrylamide gel on a 3130xl Genetic Analyzer (Applied Biosystems) and analyzed using the software's Sequencing Analysis v5.2 and SeqScape v2.5 (Applied Biosystems). DNA sequencing was also performed to verify the sequence of a full-length insert of *MYO1C* in a construct for down-stream overexpression experiment (Paper II).

DNA methylation and analysis

Hypermethylation of a gene promoter is an important process to negatively regulate gene transcription and inactivation of a gene. In paper I, promoter methylation analysis was performed using a panel of DNA samples, 14 experimental ECs and three NMEs (control). The DNA samples were subjected to bisulfite modification and purified using Epitech Bisulfite Kit (Qiagen) according to the protocol from manufacturer. In theory, treatment with bisulfite causes deamination and turn unmethylated cytosines to uracils. During the amplification by PCR, the deaminated cytosines that converted to uracils are recognized as thymines in the sequencing analysis. In this experiment, we used web server CpG Island Searcher (URL: http://www.uscnorris.com/cpgislands2/cpg.aspx) to predict the location of CpG islands that associated with the promoter regions. Primers suitable for bisulfite sequencing were designed using the MethPrimer software (URL: http://urogene.org/methprimer/) and BiSearch web server (URL: http://bisearch.enzim.hu/) and synthesized by a commercial supplier (SIGMA-Genosystem). Promoter regions were amplified using three different overlapping sets of primers for *Myo1c* using treated DNA as a template. The methylation status was then analyzed using bisulfite sequencing as described in Carén *et al.* [50].

DNA demethylation and restoration of gene expression

In Paper I, four experimental EC cell lines, NUT12 and NUT50 with- and NUT51 and NUT98 without deletion/AI (allelic imbalance) at the candidate tumor suppressor region and all with low expression of *Myo1c*, as well as rat embryo fibroblasts (REF) as control, were selected for the analysis. All cell cultures were treated with the demethylating agent 5-aza-2′-deoxycytidine (5-Aza-dC, Sigma-Aldrich) and/or with the histone deacetylase inhibitor trichostatin A (TSA; Sigma-Aldrich). Total RNA was isolated from using RNeasy Protect Mini kit (Qiagen) according to the manufacturer's protocol. Protein extraction was performed according to standard protocol. This analysis assisted us to understand if epigenetic regulation was the rationale of low expression of *Myo1c* in tumor samples tested.

3.3 Analyses at RNA level

Polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR)

The polymerase chain reaction (PCR) is an *in vitro* molecular technique to amplify small amounts of DNA fragments of interest in an exponential pattern. For each cycle, the DNA made in the previous cycles also serves as template. This technique was invented in the 1980s and later patented by Kary Mullis [51, 52]. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA denaturation, hybridization, and enzymatic replication (Figure 5). These steps are usually repeated 20-35 rounds before depletion of the starting components occurs. Primers containing specific sequences complementary to the target region along with a thermostable DNA polymerase, which the method is named after, are key features to enable amplification using PCR technique [53, 54].

Figure 4. Step in PCR amplification of DNA consisting of 3 steps; denaturation, annealing, and elongation.

The products of PCR are usually examined by running on 0.5-2% agarose gel electrophoresis after staining with fluorescent dye such as ethidium bromide or non-toxic Gel Red (Biotium) to enable observation under ultraviolet light. By observation, it allows size estimation of PCR product run in parallel with the DNA ladder and amount estimation by measuring the band intensity. This method was used in Paper II to verify the genomic insert in a DNA construct for *MYO1C* during the cloning process. In addition, a modified variant of the PCR technique for the analysis of gene expression at RNA level (reverse transcription PCR or RT-PCR) was used in Paper I and IV to observe gene expression. In RT-PCR, the starting material is a complementary DNA (cDNA) that is produced from RNA through a reverse transcription process.

Real-time reverse transcription PCR (real-time RT-PCR)

Real-time RT-PCR, also known as quantitative PCR (qPCR), is a variant of PCR, in which amplification and detection (quantification) of cDNA is performed at the same time. This method allows real time quantification of the starting material based on signals from non-specific fluorescent DNA-binding dyes or sequence-specific DNA probes labeled with a fluorescent reporter during each cycle of PCR. This method was performed for quantitative examination of the gene expression in Paper I. A set of housekeeping genes, including *Gapdh*, *Actb,* and *Rps9*, was used for normalization. The quality control and data analysis were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [55].

Microarray analysis

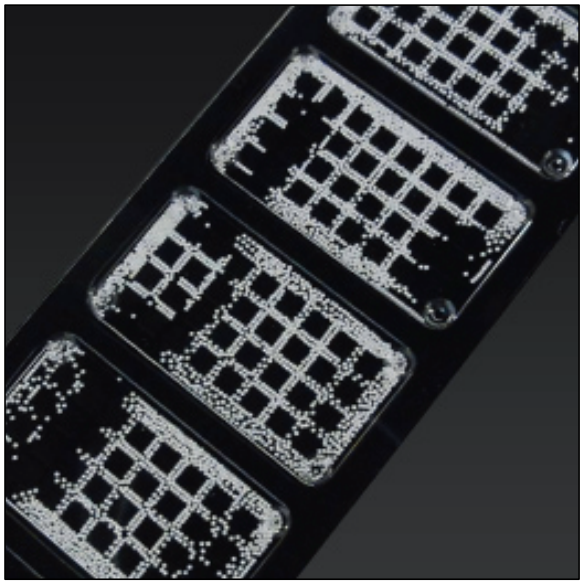

Figure 5. 3D-microarray chip filled with white beads in the black resin slide. Courtesy of Toray Industries.

DNA microarray analysis is a technique used to determine gene expression from the starting material mRNA. In Paper IV, we used a 3D-microarray system (3D-Gene, Toray Industries) that is a black colored chip (Figure 6) to analyze potential time-dependent alterations in expression of all of the 25000 annotated and predicted genes in response to *MYO1C*

depletion. The 3D-microarray system is a variant of the conventional 2D-microarray technique with special features that allow effective elimination of the background noise and more specific binding of target sequences to the probes. These features include 1) use of black resin substrate with dye absorbing autologous fluorescence to reduce the background noise; 2) the DNA probe is immobilized on a special unique surface to enable dense and uniform immobilization of probe; 3) the surface that probes are immobilized to are located at the upper end of an uneven columnar structure to enable the stabilization of spot morphology and a uniform signal detection; 4) and constantly active bead agitation enhancing DNA hybridization, thus increases signal intensity.

Clustering and identification of regulatory events

In Paper IV a data set from 3D-microarray analyses was produced and subjected to bioinformatics analysis. The microarray data was clustered using the Short Time-series Expression Miner (STEM) [56] v1.3.8 according to their expression pattern across all time points. For this analysis, standard settings were used except that the minimum absolute expression change was measured between the maximum and minimum (rather than the difference from $t = 0$) and that the option "No normalization/add 0" was chosen. To normalize for differences in absolute expression values, the gene expression vectors used in this analysis contained the ratio of expression between *MYO1C*-siRNA treated and scrambled-siRNA treated for each of the time points.

The Dynamic Regulatory Events Miner (DREM) [57] v2.0 was used to derive a regulatory map indicating bifurcation events and identifying candidate transcription factors causing the major changes in the expression. Standard settings were used except that expression differences were measured between the maximum and minimum, and that path merges were allowed. The maximum number of paths out of a split was set to four.

Annotation and pathway analysis

Annotation enrichment analysis was performed in Paper IV using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [58]. Pathways discovered by clustering and found in the KEGG database (Kyoto Encyclopedia of Genes and Genomes) were analyzed for enrichment using the desktop version of the Gene Set Expression Analysis (GSEA) software v2.1.0 [59].

3.4 Analyses at protein level

Western blotting

The Western blot or immunoblot is a semi-quantitative method to detect specific protein using the interaction between an antibody and target antigen that is usually a protein or peptide. This technique was firstly introduced by Towbin *et al.* in 1979 [60, 61]. The specificity of the interaction is determined by an epitope (small fragment of the antigen) and recognition sites on the molecule of an antibody. To increase the specificity, two types of antibodies are used; a primary antibody that is specific to the protein of interest and a secondary antibody that is a counterpart to the primary antibody which now serves as an 'antigen'. This method was used in Paper I, II and III to examine the protein expression.

The detection is achieved by using a conjugated secondary antibody. In this PhD research work, the chemiluminescent system, in which peroxidase enzyme tagged to secondary antibody cleaves the substrate allowing light emission to be detected was used. The signals were recorded with a LAS1000 camera (Luminescent Image Analyzer, LAS1000 Plus, Fuji-Film, Japan) using Image Reader LAS1000 V2.6 program. Western blots were performed at least independent triplicates for each protein extraction experiment. Immunoblotting signals were quantified by ImageJ (http://rsbweb.nih.gov/ij) and protein levels were calculated and presented as relative protein level after normalized against the protein expression of GAPDH.

Immunohistochemistry analysis

Immunohistochemistry (IHC) is a technique to detect the protein of interest *in situ* using an antibody against the target protein in cells of a tissue section by exploiting the principle of immunological specificity, binding between antibodies and antigens in biological samples. The assay was firstly implemented by Albert Coons in 1941 [62]. IHC is widely used in clinical research to determine distribution and localization of differentially expressed protein of interest and cellular biomarkers within cells and in the tissue section. Visualization is achieved by using secondary antibodies conjugated with an enzyme, e.g. horseradish peroxidase (HRP) or alkaline phosphatase (AP), which can produce either chromogenic insoluble precipitates or fluorescent signals.

In paper II, this method was used to evaluate MYO1C protein expression in a panel of 62 paraffin-embedded EC samples. The signals were evaluated using conventional light microscopy, following parameters were recorded: i)

localization of the signal in cells with positive staining, ii) the intensity of the signal (0 to 4) in the tumor cell population, and iii) fraction of positive cells in the tumor cell population. Corresponding sections stained with haematoxylin-eosin were used to determine areas of tumor in each section.

3.5 Cell-based functional assays

Cell transfections

The transient transfections used in the present PhD research work was a liposome-mediated transfection technique, in which liposomes were used to deliver either DNA construct or siRNA into the target mammalian cells [63]. In Paper II and IV, we performed transfection to manipulate gene expression either to over-express or knockdown the target gene, *MYO1C*, using gene expression construct carrying full-length *MYO1C* or siRNA targeting MYO1C, respectively.

The vectors or plasmid used in gene overexpression transfection experiments are autonomously replicating DNA molecules that are used to carry foreign DNA fragments into the transfected cells [64]. The vector is constructed to contain regulatory sequences that act as enhancer and promoter regions and lead to efficient transcription of the gene inserted in the expression vector. It also contains antibiotic-resistance genes to facilitate identification of the positive clones (clones with correct insert and correct orientation) during the screening process. Use of expression vector for overexpression experiments is a basic tool for understanding the effect of gene expression and its translated products in mammalian cells [65].

The siRNA (short interfering RNA or silencing RNA) is a synthetic RNA duplex of 20-25 base pairs length that specifically targets a mRNA molecule. The effect of siRNA in RNA interference (RNAi) depends on its complementary binding to the target mRNA; it can lead to either inhibiting translation or directly causing degradation of the transcript. The first report of siRNA utilization in mammalian cells was introduced in the early 2000s by Thomas Tuschl *et al.* by the name of the RNA interference process [66].

Cell proliferation assay

The assay was performed in Paper II to examine cell proliferation, which is the cellular process to increase the number of viable cells in balance or compensation with the cell loss through cell death or differentiation.

The assay used in this work is based on the use of tetrazolium compound [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron-coupling reagent (phenazine ethosulfate; PES) [67, 68]. In brief, the viable cells metabolize the tetrazolium compound in to an insoluble colored product, formazan. The quantity of metabolized formazan, directly proportional to the number of living cells in culture, is measured at 490 nm-absorbance and recorded using Mithras LB940 (Berthold Technologies) with the program MikroWin v.4.31 (Mikrotek Laborsystems).

Cell migration assay

Cell migration is a basic cellular process in the development and maintenance of multicellular organisms. Tissue formation during embryonic development, wound healing and immune responses all require the orchestrated movement of cells in particular directions and to specific locations.

In cancer, this process may imply to invasion to adjacent tissue, penetration into vascular system or metastasis to distant location of the body. In paper II, we performed migration assay using OrisTM Cell Migration Assay plates (Platypus Technologies), containing a cell-seeding stopper. First, cells were allowed to attach and spread overnight at 37°C before the stoppers were removed and the medium was refreshed. Migration was measured in real time using the POLARstar Omega plate reader (BMG Labtech). Migration speed was measured by calculating the area under the curve for each well and pictures were taken before and after the migration assays.

Continuous real-time cell analysis (RTCA) assay

In paper II, we additionally performed an assay to examine cell adhesion based on continuous measurement of electrode impedance. The presence of cells on electrodes' surface affects the local ionic environment at the electrode/solution interface, leading to an increase in electrode impedance (Figure 7). The more cells attached to the electrodes, the more electrode impedance increases. The electrode impedance, which is displayed as Cell Index (CI) values, can hence be used to monitor and quantify the ability of cells to adhere to the electrode.

In this study we used the xCELLigence RTCA system (Roche Applied Science) to examine cell adhesion and spreading after knockdown of *MYO1C* compared to the control samples.

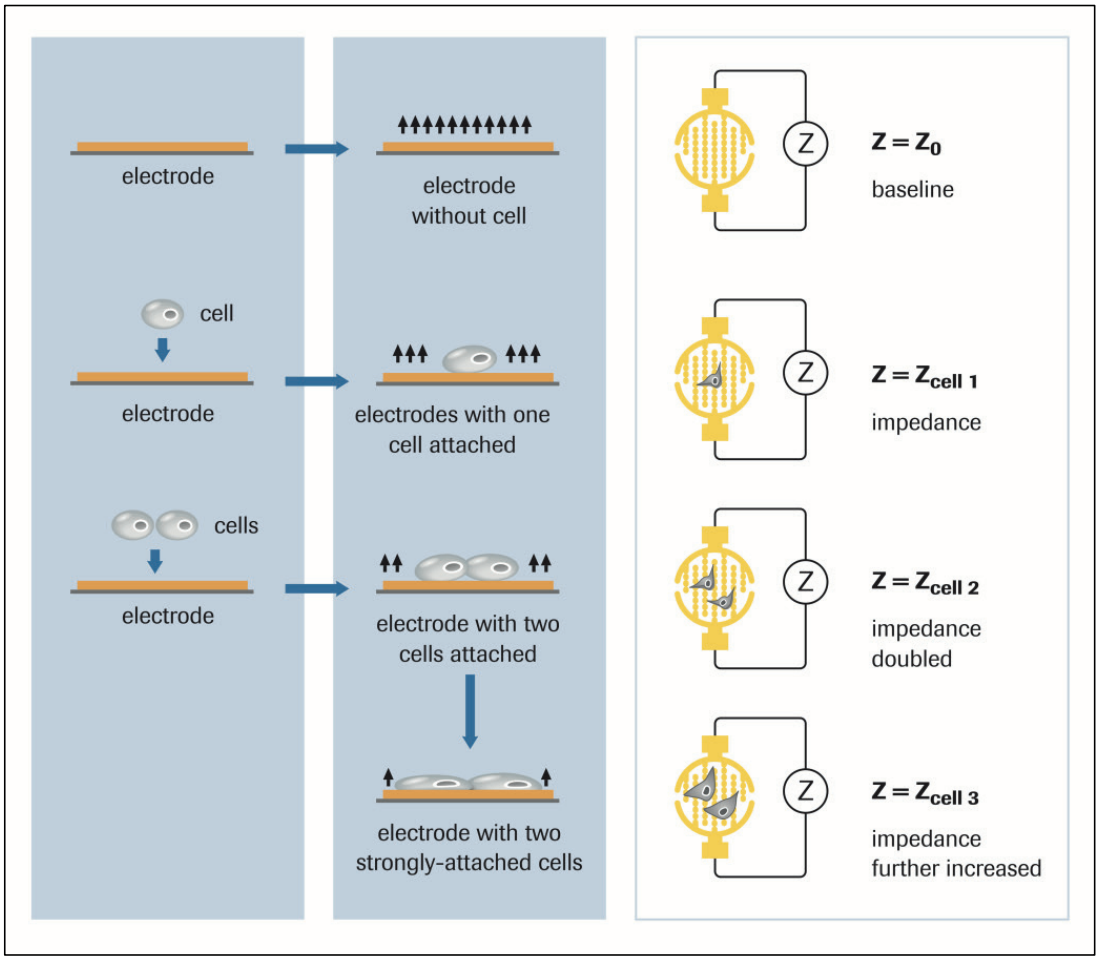

Figure 6. A schematic drawing of measuring the signal in of RTCA system to gain information corresponding to cell adhesion. Courtesy of ACEA Biosciences.

Serum activation assay

Growth factor can stimulate signaling pathways through binding to their respective receptors and activating the downstream signal transduction cascade. In paper II, we were interested to examine whether MYO1C is involved in activation of PI3K/AKT and ERK/RAS signaling pathways. To address this, we performed serum activation assay. Cells treated with MYO1C-targeted siRNA, hence with depleted MYO1C expression, were serum-starved for 24 hrs and subjected to serum stimulation. At 1, 5 and 20 minutes post serum stimulation we examined expression of AKT and ERK and their activated forms using Western blot analysis.

3.6 Statistical analysis

The statistical analysis software SPSS (Statistical Package for the Social Sciences; IBM SPSS Data Collection) was used to compare two populations by applying two-tailed Student's *t*-test in Paper I and II. In addition, normalized values were arranged and plotted as bar diagram using Prism6 (GraphPad Software) that once again tested for statistically significant difference in a non-pairwise fashion. Paired analysis based on Student's *t*-test was only employed to assess differential protein expression between CRC samples and its corresponding mucosa in Paper III. The data were presented as mean ± standard deviation (SD). A probability value (*p*-value) less than 0.05 were regarded as a level of statistical significance in all tests.

4 RESULTS AND DISCUSSION

In the previous work, a minimal region of recurrent deletion/allelic loss distal to the *Tp53* gene was identified in an experimental rat model for endometrial carcinoma (EC) [69]. This finding was intriguing, since similar observation of deletion at the homologous position on human chromosome 17, not associated with *TP53* mutation, has been reported in several types of human tumors [70-73]. An important tumor suppressor gene located close to, but distinct of Tp53 was thus suggested. In a comparative genomic analysis, the candidate region was narrowed down to a segment harboring 16 known and three predicted genes. Using quantitative real-time PCR for all 19 genes in a panel of experimental ECs and control samples, *Myo1c* (myosin IC) and *Inpp5*k (inositol polyphosphate 5-phosphatase K) were singled out as the best candidates [74]. In the present PhD research work, we focused on the potential tumor suppressor activity of *MYO1C*.

4.1 Paper I

Several reports indicate a commonly deleted chromosomal region independent from, and distal to the *TP53* locus in a variety of human tumors. In a previous study from our group, we reported a similar finding in a rat tumor model for endometrial carcinoma. Through developing a deletion map, we narrowed the candidate region to 700 kb, harboring 19 genes.

The real-time qPCR analysis suggested *Hic1* (hypermethylated in cancer 1), *Inpp5k* (inositol polyphosphate-5-phosphatase K; a.k.a. *Skip*, skeletal muscle and kidney-enriched inositol phosphatase) and *Myo1c* (myosin IC) as the best targets for the observed deletions. No mutation in coding sequences of these genes was detected; hence the observed low expression suggested a haploinsufficient mode of function for these potential tumor suppressor genes. Both Inpp5k and Myo1c were down regulated at mRNA and/or protein levels, which could be rescued in gene expression restoration assays. This could not be shown for Hic1. *Innp5k* and *Myo1c* were thus suggested as the best targets for the deletions in the candidate tumor suppressor region.

Then, *Innp5k* and *Myo1c* were identified as the best targets for the deletions in the candidate tumor suppressor region. *INPP5K* and *MYO1C* are located adjacent to each other within the reported independent region of tumor suppressor activity located on chromosome arm 17p distal to *TP53* in human tumors. There was no previous report on the tumor suppressor activity of *MYO1C* and *INPP5K*; however, overlapping roles in PI3K/AKT signaling, known to be vital for the cell growth and survival, were reported for both

genes. Moreover, there were reports on tumor suppressor activity of other members of the gene families that *INPP5K* and *MYO1C* belong. Functional significance of the *MYO1C* candidate tumor suppressor gene in cancer pathways was selected for further investigation in the continued research work.

4.2 Paper II

In this work, by using clinical samples along with cell-based functional assays, we aimed to examine the original hypothesis that *MYO1C* acts as a tumor suppressor gene in relation to a number of the Hanahan and Weinberg's proposed '*Hallmarks of Cancer*' [75, 76].

Our analysis showed a significant negative association between MYO1C protein level and the endometrial carcinoma tumor stage. Over-expression of MYO1C led to a significant decrease in cell proliferation and reduction of MYO1C protein resulted in an opposite effect. Furthermore, we found that reduced MYO1C expression impaired cell migration, cell spreading and cell adhesion. Overexpression of MYO1C protein resulted in an increase of p110α protein, decrease of PTEN and AKT levels as well as a decrease of activated AKT. Lastly, we showed that the decrease of MYO1C protein sensitized fast serum-induced activation of AKT.

Taken altogether, our analysis revealed a negative correlation between high MYO1C level and activation of PI3K/AKT signaling as well as the ability of cells to proliferate. We additionally showed that lowered expression of MYO1C resulted in impaired cell migration and cell adhesion that could support of cells escaping from cell contact inhibition in favor of cancer development.

4.3 Paper III

Alterations resulting in activation of the PI3K/AKT pathway are commonly found in many types of human cancers, including in colorectal cancer (CRC). There is evidence suggesting that deregulated activation of the PI3K/AKT signaling pathway can function both as early and late event during CRC carcinogenesis. Against this background, we aimed to investigate expression of MYO1C, which was suggested to be involved in the PI3K/AKT signaling pathway, in CRC samples as well as to assess a potential correlation between MYO1C mRNA expression and protein level with tumor stage.

Our analysis revealed that MYO1C was significantly down-regulated in CRC samples in comparison with their corresponding normal-appearing mucosa. However, down-regulation of MYO1C was not correlated with tumor stage,

suggesting that depletion of MYO1C was important for CRC development in general. We additionally found that levels of PTEN, ERK and pERK were lower in CRC in comparison with their corresponding tumor-adjacent normal-appearing mucosa sample, and the total RAS protein was significantly higher in tumor-adjacent mucosa compared to both tumor tissue and normal healthy mucosa. However, we could not link low levels of myosin IC to activation of neither the PI3K/AKT nor RAS pathways in the CRC samples. Accordingly, our data suggested either depletion of MYO1C affected these pathways in early steps of CRC tumorigenesis, which could not be detected in fully blown CRC samples, or MYO1C exerted its tumor suppressor function through a different mechanism(s).

4.4 Paper IV

The previous work suggested that PI3K/AKT and RAS/ERK are most likely not the only pathways through which *MYO1C* may exert its tumor suppressor activity. Accordingly, we designed a high-throughput genome-wide assay to understand new interactions of *MYO1C* with other gene candidates/pathways.

We identified an early and significant response to *MYO1C* knockdown on PI3K/AKT signaling pathway of which six genes were identified as having expression patterns highly correlated to that observed for *MYO1C*. The analysis additionally identified several late response genes/pathways with known cancer-related function, providing additional supporting evidence for the initial hypothesis of tumor suppressor activity of *MYO1C*. Identification of PI3K/AKT signaling as an early response to *MYO1C* knockdown could explain the lack of correlation between *MYO1C* expression and expression of components of PI3K/AKT in the fully developed CRC samples, as identified in Paper III.

As a purely exploratory study, the presented data have some limitations. The lack of replicates in the experimental design is a definite limitation, but when designing the study, we prioritized more time points over duplicates, to ensure capturing the dynamics of gene regulation and provide sufficient data for correlation analyses. Another limitation is the inaccuracy in measuring low concentration gene expressions when using microarray analysis in comparison with qPCR. To minimize this limitation, we chose to use the 3D-Gene platform from Toray, which has special features, such as a black resin substrate which produces a lower background signal and a columnar structure upon which the probers are mounted giving more uniform results [77].

5 CONCLUSION AND FUTURE PERSPECTIVE

Discovery of a novel cancer-related gene may not only help to understand cancer pathogenesis, but it also gives a new hint of hope for new treatments leading toward the cure of cancer. Moreover, identification of new cancer biomarkers will serve as efficient tools for early detection, better diagnosis and prognosis, and improved classification of cancers.

This PhD project is a very first step to allow a better understanding of the potential role(s) of a novel tumor suppressor candidate, *MYO1C*, in different cell phenotypes such as cell proliferation, adhesion, and migration together with investigating the interplay of this protein with various components of the signaling pathways involved in cancer development.

In this study, we found the expression of MYO1C was strongly and significantly lowered in at least two cancer types: endometrial carcinoma and colorectal cancer. We additionally found that low expression of MYO1C in endometrial carcinoma was correlated with tumor stage; however, these initial findings need to be verified and confirmed in bigger panels of tumors as well as in other cancer types.

Through our analyses, we could additionally produce some initial data on the potential tumor suppressor functions of *MYO1C* that can be directly related to at least three of the Hanahan and Weinberg's proposed '*Hallmarks of Cancer*':

I. Sustaining proliferative signaling through negative correlation between MYO1C expression and ability of cells to proliferate
II. Evading growth suppressors through evading cell contact inhibition, as depletion of MYO1C resulted in reduced ability of cells to adhere to the surface
III. Resisting cell death as indicated by the data from 3D-miroarray analysis that suggested apoptosis pathway was down-regulated in response to MYO1C depletion

Data from the 3D-miroarray experiments additionally suggested that depletion of MYO1C resulted in up-regulation of cell metabolism, which is an emerging '*Hallmarks of Cancer*' [76] that features in the context of cancer development.

Future perspective

Evasion from growth inhibitory signals and acquiring unlimited replicating potential are two hallmarks of cancer that contribute to tumorigenesis in the favor of net cell growth. The work presented here is just the beginning of the whole story. The very next step for this project will be to verify data from high throughput 3D-microarray by performing real-time RT-PCR, and in doing so, it is interesting to explore the expression of genes related to apoptosis pathway and shelterin complex, vesicle transport, metabolism, cell cycle pathway, to name a few. These investigations are in process.

Our research group is additionally planning to investigate the potential role of MYO1C in other cancer types, namely prostate cancer and neuroblastoma. Another dimension of the ongoing research is to identify protein(s) to which MYO1C can physically interact through performing pull-down experiment, using a yeast two-hybrid system. These approaches together will certainly produce further evidence for the functional significance of myosin IC in cancer.

"As the ending of the thesis, I would like to repeat myself how we began with this PhD research project. The main aim of the project was to investigate the role and function of myosin IC as a potential tumor suppressor gene. At the end of my PhD study I could produce some evidences to support this original assumption as well as could propose a number of genes and pathways that MYO1C could possibly exert its tumor suppressor function by the interplay of them. However, there is still much work left for identification of the precise mechanism(s) through which MYO1C may exert its role in tumor development. It is also good to notify that INPP5K, the second tumor suppressor candidate identified in paper I, is waiting for its turn to be attended."

"Cancer affects all of us, whether you're a daughter, mother, sister, friend, coworker, doctor, or patient."
– Jennifer Aniston

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All PhD roads lead to one destination called 'Dissertation', and it is the one in your hands now. At the end of this PhD work, I would like to express my sincere gratitude and acknowledge to all the supporters who help me finalize this thesis even during the last-minute call for help.

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