Impact of tululin binding cofactor C (TBCC) on microtubule mass and dynamics, cell cycle, tumor growth and response to chemotherapy in breast cancer

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TITRE:

IMPACT OF TUBULIN BINDING COFACTOR C (TBCC) ON MICROTUBULE MASS AND DYNAMICS, CELL CYCLE, TUMOR GROWTH AND RESPONSE TO CHEMOTHERAPY IN BREAST CANCER

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The difference between perseverance and obstinacy is that one comes from a strong will, and the other from a strong won't.

Henry Ward Beecher

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Thank you for strengthening my faith in God…
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I dedicate my thesis

To my lovely parents Hiba and Saleh

I hope that this achievement will complete the dream that you had for me all those many years.....

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Abbreviations

AJC: apical junctional complex

ARF: ADP ribosylation factor

Arl2: ADP ribosylation factor like 2

Arl3: ADP ribosylation factor like 3

ATCC: American type culture collection

ATP: adenosine-5'-triphosphate

BRCA1: breast cancer type 1

BRCA2: breast cancer type 2

BrdU: 5-bromo-2-deoxyuridine

CAK: CDK-activating kinase

CCT: cytosolic chaperonin

cDNA: complementary DNA

CdK1: cyclin dependent kinase 1

CdK2: cyclin dependent kinase 2

CLASP: CLIP-associated proteins

CLIP: cytoplasm linker protein

DAPI: Di-aminido-phenyl-indol

DMEM: Dulbecco's Modified Eagle medium

DNA: Deoxyribonucleic acid

dNTP: deoxynucleotide triphosphate

DTT: Dithiothreitol

EGTA: ethylene glycol tetraacetic acid

ER: estrogen receptor

FITC: fluorescein isothiocyanate

GDP: guanosine-5'-diphosphate

Gem: gemcitabine

GTP: guanosine-5'-triphosphate

GTPase: guanosine-5'-triphosphatase

HDAC6: Histone deacetylase 6

HRD: syndrome of congenital hypoparathyroidism-mental retardation-facial dysmorphism

hTert: human telomerase reverse transcriptase

HUGO: human genome organization

IκB: inhibitor of NF-κB

JNK: C-jun N-terminal

MAP: microtubule associated proteins

MAPK: mitogen activated protein kinase

MCAK: mitotic centromere associated kinesin

MC+: MCF7 cells with the sens of *TBCC*

MC-: MCF7 cells with the antisense of *TBCC*

Mdm2: murin double minute 2

MDR: multidrug resistance phenotype

MP6: MCF7 cells with the empty vector

mRNA: messenger RNA

MT: microtubules (or microtubule fraction tubulins)

MTOC: microtubule organizing center

MTT: methylthiazoletetrazolium

NEB: nuclear envelope breakdown

NF-κB: nuclear factor kappa B

NPT: nonpolymerizable tubulins

NSCLC: Non-small cell lung carcinoma

OD: optical density

PAK: Serine/threonine-protein kinase

PARP: Poly ADP ribose polymerase

PCR: polymerase chain reaction

PP2A: protein phosphatase 2A

PT: polymerizable tubulins

RNA: Ribonucleic acid

RP2: retinitis pigmentosa 2 protein

RT-PCR: reverse transcription PCR

S-phase: phase of synthesis

SERM: selective estrogen receptor co-activator-1

siRNA: small interference RNA

SIRT2: silent mating type information regulation 2

SLC: small lung carcinoma

STOP: stable tubule-only polypeptide

TBC: Tubulin binding cofactor

TBCC: tubulin binding cofactor C

TTL: tubulin tyrosine ligase

TUBA: alpha tubulin

TUBB: beta tubulin

γ-TURC: γ-tubulin ring complex

γ-TuSC: γ-tubulin small complex

UICC: union internationale contre le cancer

α tubulin: total alpha tubulin

β tubulin: total beta tubulin

INTRODUCTION

TUBULINS AND MICROTUBULES

Cell behavior and function depend to a large extent on the cytoskeleton that consists of three interconnected filament systems: intermediate filaments (IFs), actin-containing microfilaments (MFs) and microtubules (MTs). Microtubules are composed of αβ tubulin heterodimers arranged to form polar hollow cylinders. The folding pathway of α and β tubulins involves many tubulin binding cofactors (A to E). Tubulin binding cofactor C (TBCC) is crucial to obtain the αβ tubulin heterodimers that polymerize into microtubules. Microtubules are involved in many cellular processes including cellular motility, cytoplasmic transport and mitosis which make them major targets of anticancer drugs, especially in breast cancer chemotherapy. Many studies have been performed and others are in progress in order to understand the mechanism of action of antimicrotubule agents in order to enhance response to these treatments and overcome resistance phenotypes observed in patients.

1.1. Tubulins

1.1.1. α and β tubulins

The synthesis of α and β tubulins is coordinated and the amount of the folded and functional monomers is controlled and does not exceed the cellular need under normal conditions (Burke et al. 1989; Weinstein and Solomon 1990). This is achieved by an autoregulatory mechanism which leads to degradation of tubulin mRNA if the intracellular concentration of tubulin subunits increases (Cleveland et al. 1981). At the end of the 1980s, it was shown that for the RNA to be a substrate for autoregulation it must carry the 13-nucleotide segment encoding the first four β tubulin amino acids methionine-arginine-glutamic acid-isoleucine (MREI), must be ribosome bound and must already been translated beyond the codon 41 (Yen et al. 1988). The α and β tubulins are 55 kDa proteins of about 450 amino acids. They have different protein sequences with 40% amino-acid identity encoded by

different genes and conserved across species to a degree of 60 % (Wade 2009). α tubulin binds to β tubulin and they form the dimer subunits that assemble into microtubules. An atomic model of the α and β tubulins obtained by electron crystallography showed that both tubulins are basically identical; each monomer is formed by a core of two β-sheets of 6 and 4 strands surrounded by 12 α-helices (Nogales et al. 1998b). The monomer structure is very compact, but can be divided into three functional domains: the amino-terminal domain (residues 1–205) containing the guanine nucleotide-binding site, the intermediate domain (residues 206–381) containing the paclitaxel-binding site, and the carboxy-terminal domain (residues 382-450), which probably constitutes the binding surface for motor proteins **(Figure 1)**. The N-terminal domain is formed of 5 α-helices (H1 to H5) and 6 parallel stands of β-sheet (B1 to B6) that alternate with helices. Helices H1 and H2 are on one side of the sheet, whereas helices H3, H4 and H5 are on the other. Strand B6 leads to the intermediate domain formed of 4 stands of β-sheet (B7 to B10) and 5 α-helices (H6 to H10). The loop connecting B7 and H9 is more ordered in α-tubulin, where it is involved in strong lateral contacts. The loop between B9 and B10 where the paclitaxel binds includes an 8-residue insertion in the α subunit. The C-terminal domain is formed by antipararrel helices H11 and H12. These helices are probably involved in the binding of MAPs and motor proteins. The loop connecting H11 and H12 is important for the interaction with the next monomer along the protofilament (Nogales et al. 1998b). The C-terminal tails of α and β tubulins can either project outwards from the tubulin and the microtubule surface or lye on the surface. Projecting ones play role in signaling pathways and are more accessible for enzymes of post-translational modifications (Tuszynski et al. 2006).

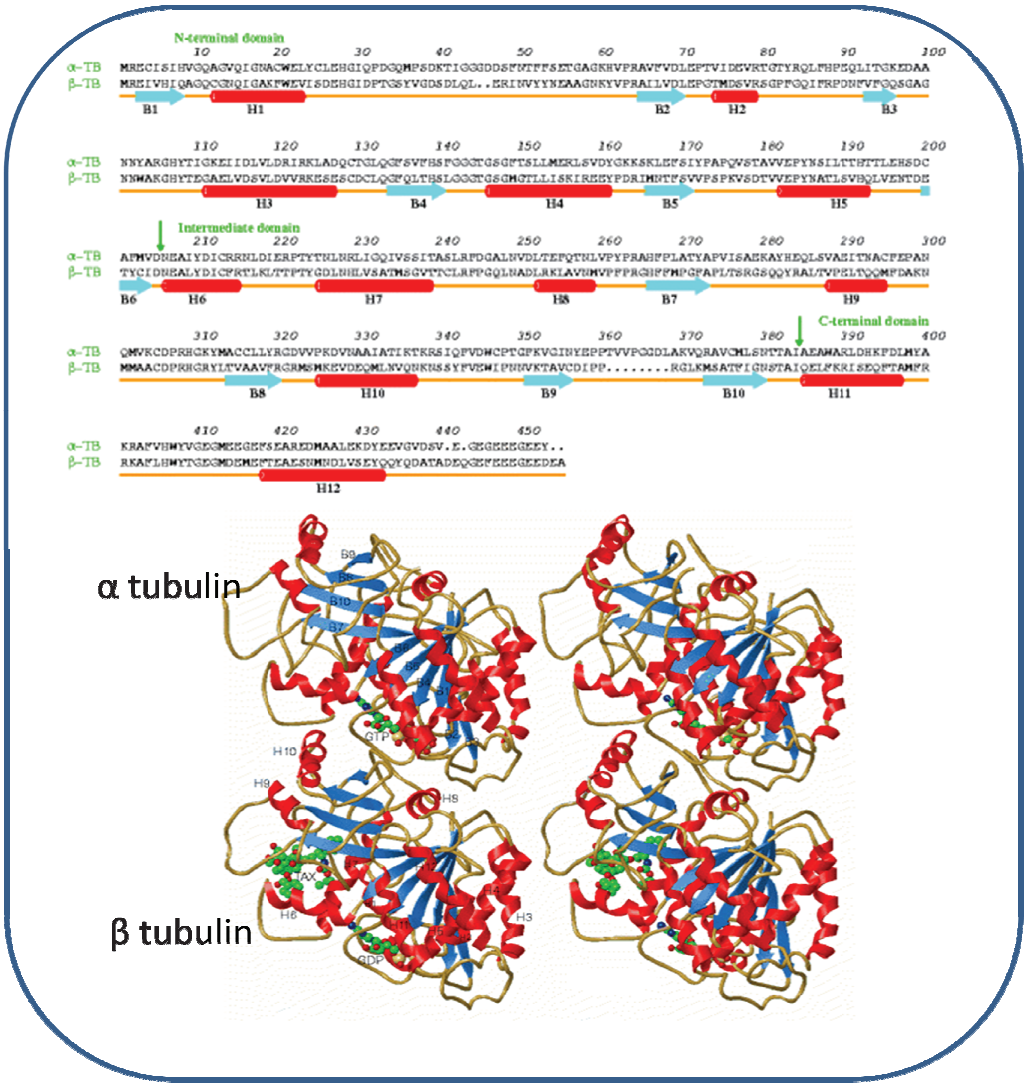


Figure 1. Sequences of α and β tubulins used in the atomic models of Nogales,1998 with the secondary structures (red : α helices, blue : β sheets) and representative 3D structures of α tubulin bound to GTP (upper) et β tubulin bound to GDP or paclitaxel (down). Adapted from (Nogales et al. 1998b).

1.1.2. Diversity of tubulins

1.1.2.1 α and β tubulin isotypes

Both α and β tubulins exist in several isotypic forms encoded by different genes. Unicellular organisms tend to have only one or two genes for the α and β tubulins whereas vertebrates have six or more genes for each tubulin as well as many pseudogenes that are non-functional sequences closely similar to functional genes (Dutcher 2003). In humans, there are 8 identified genes coding for α tubulins (TUBA1A NM_006009, TUBA1B NM_006082, TUBA1C NM_032704, TUBA3C NM_006001, TUBA3D NM_080386, TUBA3E NM_207312, TUBA 4A NM_006000 and TUBA8 NM_018943) and 3 pseudogenes (TUBA4B NR_003063, TUBAP and TUBAP2). Some of the α tubulin isotypes and their tissue distribution are shown in **table 1**. In humans, 9 genes coding for the β tubulins (TUBB NM_178014, TUBB1 NM_030773, TUBB2A NM_001069, TUBB2B NM_178012, TUBB2C NM_006088, TUBB3 NM_006086, TUBB4 NM_006087, TUBB4Q NM_020040, TUBB6 NM_032525) and 7 pseudogenes (TUBB1P1 NG_002948, TUBB1P2 NG_002947, TUBBP1 NG_001206, TUBBP2, TUBBP3, TUBBP4 and TUBBP5) have been described (Sullivan and Cleveland 1986; Berrieman et al. 2004; Khodiyar et al. 2007) (HUGO Gene Nomenclature Committee at http://www.genenames.org/). β tubulin isotypes and their tissue distribution are shown in **table 1**.

The specificity of α and β tubulin isotypes resides in the carboxy-terminal residues in both α and β monomers. Each evolutionarily conserved isotype tends to have specific negatively charged stretches of 15 or more amino acid residues at their C-terminal tail. The N-terminal is less specific of the isotypes (Wade 2009). Many hypotheses explain the existence of many isotypes, such as the possibility that these isotypes improve the capacity of organisms to adapt and respond to environmental challenges such as temperature (Wade 2009). It is believed that the different isotypes are often distributed in different cell types or in different

sets of microtubules in a single cell (Luduena 1998). Other studies showed that the isotypes are partially but not completely interchangeable (Raff et al. 1997; Wilson and Borisy 1997). In humans, at the proteins levels, there are 6 isotypes of β tubulin classified as I, II, III, IVA, IVB, and VI (Berrieman et al. 2004) **(Table 1)**. The existence of the seventh isotype class VII (h4q) is probable in neurons (Dumontet 2000).

Class I β tubulin is the most commonly expressed isotype in human beings and the most common in cancer cells. At least nine pseudogenes have been identified with homology to the corresponding regions of the class I β tubulin gene **(Figure 2 and Table 2)**. The intronless ones might have arisen from cDNA incorporated into the genome (Hasegawa et al. 2002). The involvement of this isotype and its pseudogenes as well as other isotypes of β tubulin in resistance phenotypes is discussed later. A recent study from our group showed that Class III β tubulin is induced by estradiol in human breast cancer cells through an estrogen-receptor dependent manner, an observation which sheds light on the implication of tubulins in hormone dependent pathways (Saussede-Aim et al. 2009a).

Table 1. Human α and β tubulin Isotypes and distribution

Isotype **Protein, Gene (accession #)**	**C-terminus sequence**	**Tissue Expression**
α tubulins		
1, TUBA/k- α1 (I77403)	MAALEKDYEEVGVDSVEGEEEEGEEY	Constitutive
6, TUBA1C (Q9BQE3)	MAALEKDYEEVG**A**DS**AD**GE**D**E**G**E**EY**	Constitutive
1, TUBA3/b- α1 (CAA25855)	MAALEKDYEEVGV**H**SVEGEGEEEGEEY	Brain-specific
3, TUBA3C (Q13748)	**L**AALEKDYEEVGVDSVE**A**E**A**EE**GEEY**	Testis-specific
4, TUBA4 (A25873)	MAALEKDYEEVG**I**DS**Y**E**D**E**D**E**G**E**E**	Brain, muscle
8, TUBA8 (Q9NY65)	**L**AALEKDYEEVG**T**DS**F**E**E**EN**E**G**EEF**	Heart, muscle, testis
β tubulins		
Class I, hM40/TUBB (AAD33873)	YQDATAEEEEDFGEEAEEEA	Constitutive
Class II, hβ9/TUBB2 (AAH01352)	YQDATA**D**E**QGE**F**E**EEE**G**E**DEA**	Major neuronal
Class III, hβ4/TUBB4 (AAH00748)	YQDATAEEE**GEMY**E**DD**EEE**SEAQGPK**	Minor neuronal
Class IVA, hβ5/TUBB5 (P04350)	YQDATAE**QG**E**FEE**E**A**EEE**V**A	Brain-specific
Class IVB, hβ2 (P05217)	YQDATAEEE**GEF**E**E**EEAEEE**VA**	Constitutive
Class V, 5-beta/ Beta V not in humans (NP_115914)	DATANDGEEAFEDDEEEEIDG	Constitutive
Class VI, hβ1/TUBB1 (NP_110400)	**F**DQA**K**A**VL**EED**EEVTE**EA**E**MEPEDKGH**	Haemopoietic-specific

Table 2. Functional gene and pseudogenes of human class I β tubulin

Gene Type	Nomenclature (GenBank number)	Introns
Functional	hM40 (J00314)	3
Pseudogenes	1β (J00315)	1 short
	7β (K00842)	None
	14β (K00840)	None
	21β (K00841)	None
	11β (J00316)	None
	46β (J00317)	None
	β9 (M28484)	None

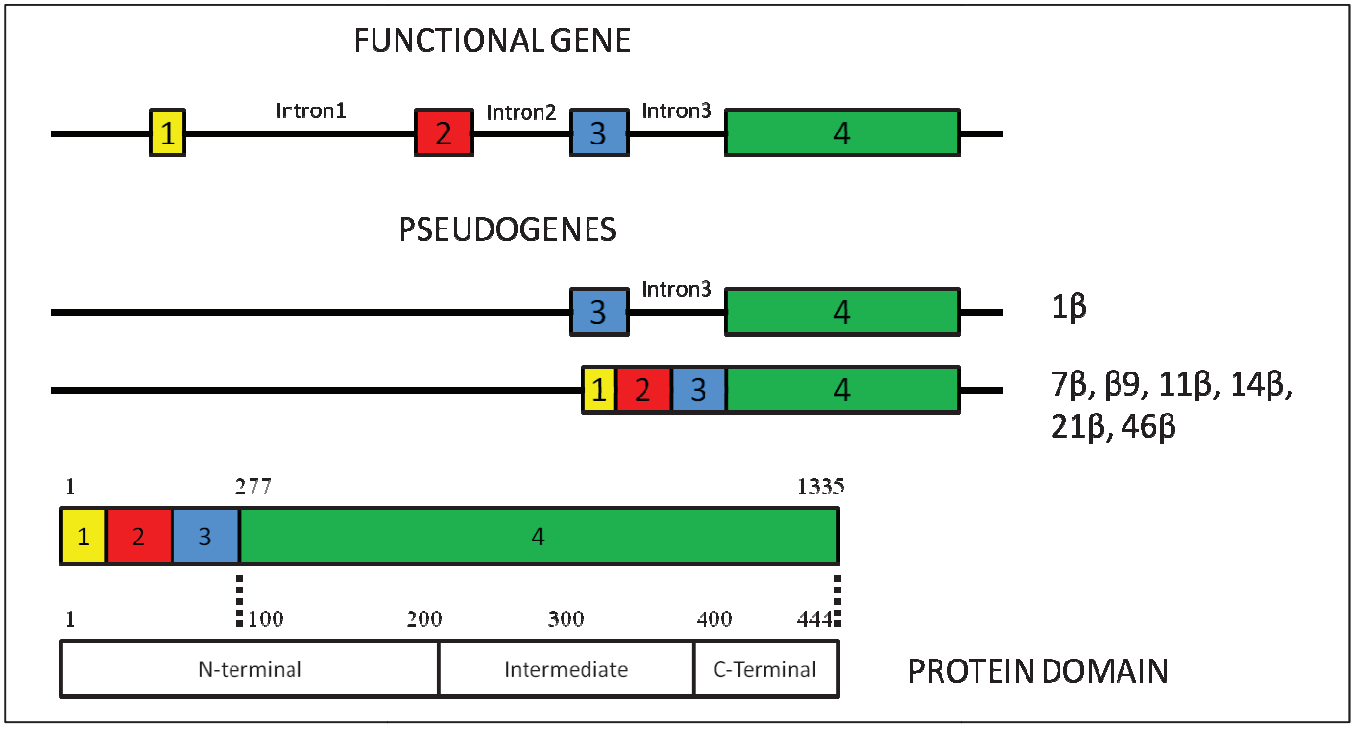


Figure 2. Structure of the class I β tubulin gene, pseudogenes, and their association with the protein domains.

1.1.2.2 Post-translational modifications of α and β tubulins isotypes

In addition to its genetic isotypes, tubulin is subject to many reversible post-translational modifications such as acetylation/ deacetylation, tyrosination/ detyrosination, polyglutamylation, polyglycylation, phosphorylation and palmitoylation. Recently, it was shown that α tubulin was significantly nitrated at tyrosine-224 in grade IV tumor samples of human glioma (Fiore et al. 2006). The carboxyl-terminal of tubulins is the site of most tubulin's post-translational modifications except acetylation and the site of binding of MAPs (Luduena 1998).

Acetylation/Deacetylation

Acetylation occurs at the lysine-40 in the N-terminal of α tubulin by a tubulin acetyltransferase. The modified residue points towards the lumen of the microtubule (Nogales et al. 1999). Acetylation occurs in the flagellar apparatus whereas cytoplasmic tubulins are found in a deacetylated form (Maruta et al. 1986). It occurs also in cultured neurons after polymerization. However deacetylation of acetylated α tubulin is closely coupled to depolymerization (Black and Keyser 1987; Black et al. 1989). It has been shown that acetylation of α tubulins in the flagellum renders it more stable than the majority of cytoplasmic microtubules to both cold and colchicine (Maruta et al. 1986). In addition, cytoplasmic microtubules resistant to nocodazole and colchicine but not cold resistant contain more acetylated α tubulins and are unable to exchange these tubulins with free tubulins (Piperno et al. 1987; MacRae 1997; Rosenbaum 2000). The acetylation maintains the axonal microtubules stable (Cambray-Deakin and Burgoyne 1987). Acetyltransferase, the enzyme responsible for this modification was identified in *Chlamydomonas* in addition to the deacetylase reverse enzymes such as the two histone deacetylase enzymes HDAC6 and SIRT2 (Hubbert et al. 2002; Matsuyama et al. 2002; Zhang et al. 2003). HDAC6-mediated

deacetylation destabilizes microtubules dynamics and promotes tubulin disassembly (Matsuyama et al. 2002).

Tyrosination/Detyrosination

Tubulin detyrosination is maximal in interphase where G1 cells can lack detectable tyrosinated tubulins. Tyrosinated tubulins are present in G2/S and mitosis where they form the soluble pools of tubulins as well as the dynamic astral microtubules (Peris et al. 2006).

After translation, in most of the α tubulins, the tyrosine residue at their C-terminal is removed by the tubulin tyrosine carboxypeptidase (TTCP) and a glutamic acid becomes expressed at the C-terminal. This type of α tubulin is called Glu α tubulin (Gundersen et al. 1987). After that, tyrosination occurs at the C-terminal by addition of tyrosine residue to detyrosinated α tubulin by tubulin tyrosine ligase (TTL) (Westermann and Weber 2003). The tyrosinated microtubules are more sensitive to methyl mercury than detyrosinated microtubules (Lawton et al. 2007). Breast cancer cells resistant to paclitaxel were found to contain increased levels of tyrosinated tubulin (Banerjee 2002). The presence of detyrosinated tubulins is associated with a higher level of microtubule stability and is of high occurrence in breast cancers of poor prognosis and linked to tumor aggressiveness (Cambray-Deakin and Burgoyne 1987; Mialhe et al. 2001). However, the enhanced stability in microtubules is not a direct function of detyrosination level since the presence of detyrosinated tubulins is not sufficient to confer differential stability (Khawaja et al. 1988). Detyrosination plays a signaling role in the cytoskeleton as it induces the vimentin intermediate filaments to co-align with microtubules via a kinesin-dependent mechanism (Gurland and Gundersen 1995; Kreitzer et al. 1999). It was shown that the motor domain of Kif5c, an isoform of kinesin-1, can move detyrosinated microtubules at significantly lower velocities than tyrosinated microtubules (Dunn et al. 2008). CIP-115 and p150 Glued, two proteins that bind to microtubules were found to localize to the ends of tyrosinated microtubules and not to the

ends of detyrosinated ones which might explain the fact that tubulin detyrosination causes spindle abnormalities (Peris et al. 2006). In a recent study, it was shown that tubulin detyrosination inhibits the microtubule disassembly in fibroblasts by inhibiting the depolymerizing activity of kinesin-13 family members of motor proteins such as MCAK. Detyrosination of microtubules suppresses the activity of MCAK *in vitro* mainly because the ADP-Pi and ADP-bound forms of MCAK associate less efficiently with detyrosinated than with tyrosinated microtubules (Peris et al. 2009).

Polyglycylation

Polyglycylation is the covalent attachment of a polyglycine side chain of variable lengths to the carboxyl group of conserved glutamate residues in the C-terminal tail of both the α and β tubulins. It occurs in axonemal microtubules whereas cytoplasmic pool of tubulins is moderately polyglycylated. Glycylation is important and a certain threshold must be reached in order for cells to live. When glycylation is absent, an abnormal and slow growth and motility is observed (Westermann and Weber 2003).

Polyglutamylation

Polyglutamylation occurs when a polyglutamate side chain of variable length is attached to the carboxylate group of glutamate residues in the C-terminal tail of both the α and β tubulins. Polyglutamylation can differentially affect the interaction between microtubules and their associated proteins. It occurs during the genesis of centrioles and is thought to be involved in recruiting proteins to outer centriole walls to anchor centriole microtubules and form the centriolar matrix (Westermann and Weber 2003).

Phosphorylation

In rare cases, tubulin can contain covalently bound phosphates at a serine residue in the C-terminal tail. During the cell cycle, the phosphorylation state of microtubules varies and is higher in the S and M phases of the cell cycle. This might be important for the microtubules

to perform their functions during the cell cycle (Piras and Piras 1975). In addition, phosphorylation of β tubulin by CdK1 (cyclin-dependent kinase) might regulate microtubule dynamics during mitosis (Fourest-Lieuvin et al. 2006). In other cases, phosphorylation can be involved in the differentiation program such as phosphorylation of Class III β tubulin that accompanies neurite growth (Gard and Kirschner 1985).

Palmitoylation

S-palmitoylation is the reversible addition of palmitate to tubulins on cysteine residues via a thioester linkage. In cells mutated for cys-377, the astral microtubules were defective which highlights the importance of the cys-377 of α-tubulin and the lipid–protein interactions via palmitoylated α-tubulin in the function of astral microtubule during mitosis (Caron 1997; Caron et al. 2001).

1.1.2.3 Other tubulins

In addition to the ubiquitous α and β tubulin, the presence of γ tubulin as templating protein for the correct assembly of microtubules is very important (Luduena 1998; Erickson 2000). Other tubulins, δ, ε, ζ, η, θ, ι, and κ have been characterized although their roles in microtubule assembly remain unclear. The similarities of tubulins between humans, *Drosophila*, *Chlamydomonas*, *Trypanosoma* and *Paramecium* are distributed as 89-90 % α tubulin, 88-94 % β tubulin, 72-94 % γ tubulin, 47-57 % δ tubulin and 58 % ε tubulin (Fygenson et al. 2004). δ, ε, ζ and η tubulins are absent in fungi and plants maybe because they lack centrioles and basal bodies (Tuszynski et al. 2006).

γ tubulin (gamma)

γ-tubulin with about 30 % identity to α and β tubulins was discovered in spindle pole bodies in the fungus *Aspergillus nidulans* in 1989 (Oakley and Oakley 1989). This tubulin is localized to centrosomes in higher eukaryotes and involved in microtubule nucleation, stabilization and establishment of microtubule polarity. γ-tubulin located and anchored to

MTOC through pericentrin exists as either a monomer or heterotetramer (two γ-tubulins and two associated proteins) or as part of the large ring complex (Oakley 1992). The tetramer is Y-shaped flexible particle known as the γ-tubulin small complex (γ-TuSC) and in most eukaryotes it associates with accessory proteins to form the γ-tubulin ring complex (γ-TURC) (Kollman et al. 2008). This complex is a "lock washer" ring structure made of 10-13 γ-tubulin molecules that has the same diameter of a microtubule (Desai and Mitchison 1997). Both the γ-tubulin and γ-TuRC nucleate microtubules however the γ-TuSC has weak nucleation ability (Moritz and Agard 2001). In addition, γ-tubulin plays role coordinating late mitotic events in a microtubule-independent pathway by establishing or maintaining a mitotic checkpoint block (Prigozhina et al. 2004).

δ tubulin (delta)

δ tubulin was identified as a flagellar assembly mutant in the green algae *Chlamydomonas reinhardti* and has also been found in *Chlamydomonas Euplotes*, human, rat, *Xenopus*, mouse, dog and Trypanosomids (Dutcher 2001). It is localized between the centrosomes independently of microtubules and differently from γ-tubulin as well as between centrioles within centrosomes (Chang and Stearns 2000).

ε tubulin (epsilon)

ε tubulin was discovered using bioinformatic techniques but its cellular function remains to be elucidated. It is found in *Chlamydomonas*, human, mouse, rat, *Xenopus* and Trypanosomids immunolocalized to the centrioles and basal bodies. ε tubulin localizes to the pericentriolar material and exhibits a cell-cycle-specific pattern of localization. In the beginning of mitosis, ε tubulin associates with only the older of the centrosomes. The newer centrosome acquires ε tubulin only after some maturation process at the end of mitosis (Chang and Stearns 2000).

ζ tubulin (zeta)

ζ tubulin was discovered using bioinformatic techniques but its cellular function remains to be elucidated. It was found localized to the basal body region in *Trypanosoma brucei* and *Leishmania major* as well as in the centriolar region of animal cells (Vaughan et al. 2000).

η tubulin (eta)

η tubulin was discovered due to a mutation in Paramecium that caused a basal body duplication defect and γ tubulin mislocalisation (Ruiz et al. 2000).

1.2. Folding pathway of tubulins

1.2.1. Formation of α/β tubulin heterodimers

Microtubules are made of α/β tubulin heterodimers whose proper folding involves many chaperonins as well as protein cofactors (Tian et al. 1995b; Lewis et al. 1997). After being synthesized, the tubulins are sequestered by cytosolic chaperonins (known as c-cpn, TRiC or CCT for chaperonin containing TCP-1) for their correct folding in an ATP-dependent manner and preparation for further interactions with the tubulin binding cofactors TBCs (Tian et al. 1995a; Lopez-Fanarraga et al. 2001). Eukaryotic CCT, the most complex of all chaperonins is an oligomeric structure built from two identical rings, each composed of single copies of eight different subunits (Lewis et al. 1997; Kubota 2002). During the early S-phase of the cell cycle, after being synthesized, tubulin polypeptides are transferred by the protein prefoldin to the cytosolic chaperonins (Yokota et al. 1999). However, prefoldin does not form a ternary complex with either tubulin or chaperonin. Once the tubulin is in contact with the chaperonin, the prefoldin loses its affinity for the unfolded target protein and automatically detaches from it (Vainberg et al. 1998). Then the tubulin becomes bound to the ADP form of the CCT through the exposed hydrophobic surfaces. ADP-ATP exchange then occurs and

alters the conformation of the chaperonin (Gao et al. 1993; Melki et al. 1993). Interaction of the chaperonin with the tubulin generates a folding intermediate either through transmission of the allosteric change in the chaperonin to the tubulin or through the direct binding of these two. ATP hydrolysis then releases the tubulin into the cytoplasm for additional folding (Lewis et al. 1997).

The post-chaperonin folding pathways of α-tubulin and β-tubulin into the α/β tubulin heterodimers are interdependent and five TBCs (TBCA to TBCE) are involved (Campo et al. 1994). TBCB and TBCA bind to α and β tubulins respectively and the formed tubulin-intermediate-cofactor complexes serve as reservoirs of tubulins in the cytoplasm (Tian et al. 1996; Tian et al. 1997). The α and β tubulins are then delivered to TBCE and TBCD respectively where they form with TBCC a supercomplex (TBCE/α tubulin/TBCC/TBCD/β tubulin). After hydrolysis of GTP by β tubulin, this complex releases native tubulin consisting of α/β tubulin heterodimers polymerizable into microtubules (Tian et al. 1997; Tian et al. 1999). The TBCD/ β tubulin and TBCE/ α tubulin complex also emerge from the backreaction between native tubulin heterodimers and cofactor D and cofactor E, respectively. Therefore, tubulin subunits are cycled continuously (Tian et al. 1997). The GTPase activator activity of TBCC, TBCD and TBCE is of high importance since it might be involved in other roles than the release of native tubulin, such as binding to the GTP cap on polymerizing microtubules **(Figure 3)**.

1.2.2. Tubulin Binding Cofactor C

Tubulin binding cofactor C is encoded by an intronless gene (1038 bps) localized on chromosome 6, on the small arm, band 1, section 2 and sub-band 1. The protein contains 346 amino acids and has a molecular weight of 39 kDa. TBCC has two domains: the N-terminal part and a C-terminal domain. The N-terminal part has a strong coiled-coil sequence signature similar to a spectrin repeat. Spectrin is a cytoskeletal protein that lines the intracellular side of

the plasma membrane and plays an important role in maintenance of plasma membrane integrity and cytoskeletal structure. The C-terminal domain shares a significant similarity with other cytoskeleton-related proteins and moderate similarity with adenyl cyclase-associated protein (CAP) and RP2 protein responsible for the X-linked retinitis pigmentosa (Grynberg et al. 2003). It was shown that this domain with an arginine residue (Arg-118 in RP2 and Arg-262 in TBCC) is responsible for the GTPase activity of both TBCC and RP2 however RP2 cannot participate in the tubulin heterodimers assembly pathway. Mutations in this domain lead to total loss of GTPase activity (Bartolini et al. 2002). Although Arl2 (ADP Ribosylation factor Like protein 2) was found to downregulate the GTPase activity of TBCC, TBCD and TBCE, it does not bind RP2. On the other hand, another protein Arl3 was found to bind RP2 but not TBCC and is thought to be the cause of retinitis pigmentosa (Kuhnel et al. 2006). Unlike predominantly cytoplasmic TBCC, RP2 localizes to the plasma membrane and Arl3 associates with microtubules. Therefore RP2 and Arl3 are bound to the cell membrane with the cytoskeleton in photoreceptors (Grayson et al. 2002).

POR protein, in Arabidopsis thaliana, shares weak amino acid similarity with TBCC but was found to be involved in releasing assembly-competent α/β tubulin heterodimers (Kirik et al. 2002). In Arabidopsis, the same folding pathway as that of mammalian cells is observed; TBCs C to E and Arl2 are crucial for microtubule formation whereas TBCA and TBCB are accessory cofactors (Steinborn et al. 2002). Recently, in a study done on the flagellated Trypanosoma brucei, a family of related TBCC domain-containing (TBCCD1) proteins was described. TBCCD1 lacks a conserved catalytic arginine residue responsible for the GTPase activity (Scheffzek et al. 1998). It was found located at the mature basal body where it performs a flagellum/cilium-specific function (Stephan et al. 2007). Another study showed that TBCDD1 was responsible for the mother-daughter centriole linkage and mitotic spindle orientation (Feldman and Marshall 2009).

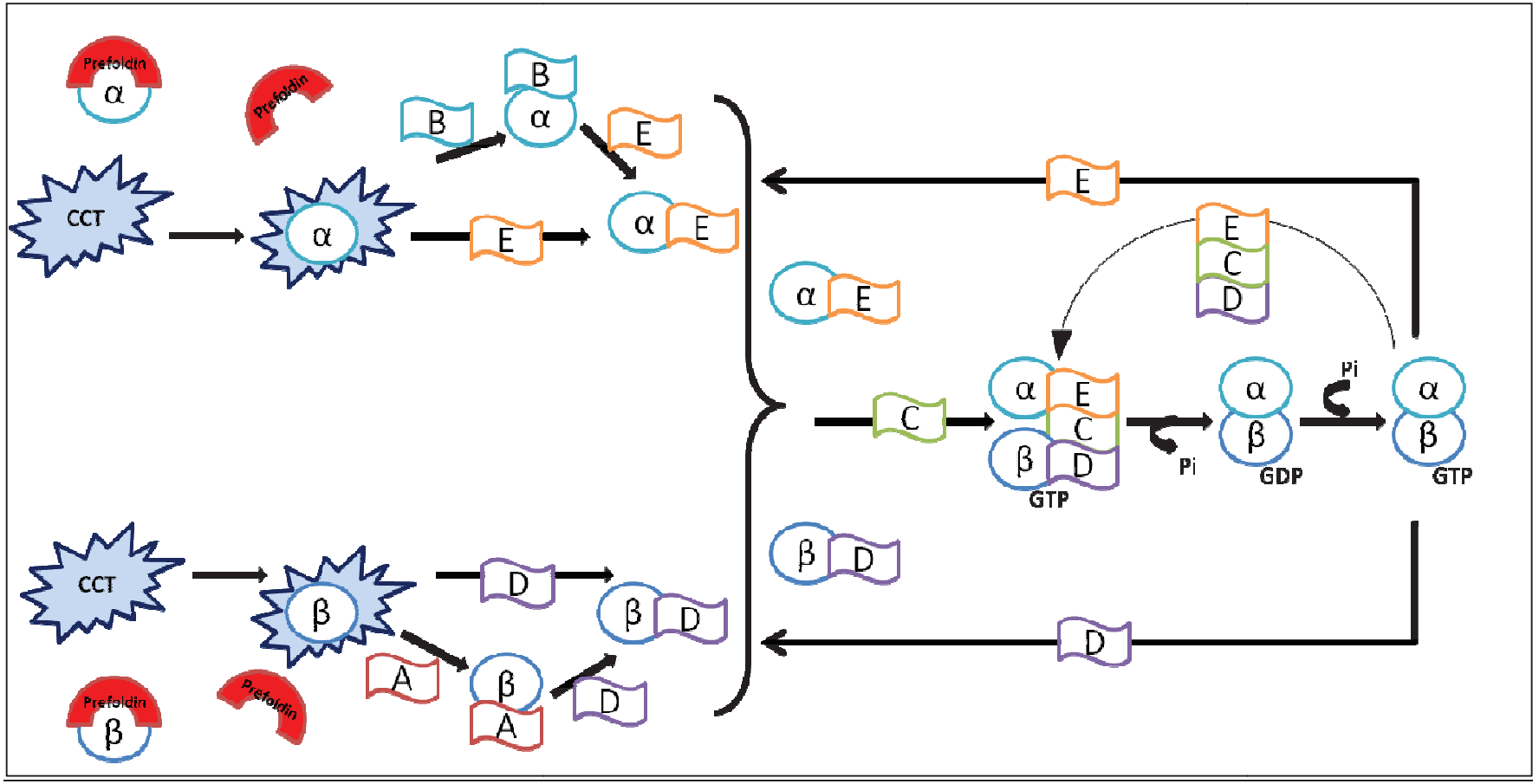


Figure 3. Folding pathway of native α and β tubulins involving chaperonins (prefoldin and CCT) and Tubulin Binding Cofactors (A to E).

1.2.3. TBCs and some related diseases

TBCA

The 3D structure of TBCA is a monomeric structure with a rod-like shape and a three α-helix left-handed coiled-coil that offers a convex surface with many exposed residues on one face of the rod. TBCA interacts with β tubulin via the three α-helical regions but not with the rod-end loops (Guasch et al. 2002). TBCA is a highly conserved protein that forms stable complexes with β tubulin but is not indispensable for the folding pathway. This means that it plays a role as a reservoir for β tubulin in the cytoplasm. In addition, it was shown that similarly to its *S. cerevisiae* yeast homolog Rbl2p, TBCA is involved in meiosis (Archer et al. 1995; Archer et al. 1998). It was found highly expressed in testis from the onset of meiosis through spermatogenesis to differentiating spermatids. This was explained by the fact that TBCA is needed in testis to store or lead to degradation the excess β tubulins issued from excessive microtubule remodeling observed in testis (Fanarraga et al. 1999). TBCA silencing in HeLa cells and MCF7 cells caused a disruption in the microtubule cytoskeleton with decreased levels of both α and β tubulins only in HeLa cells. Although activation of caspase 7 and cleavage of PARP proteins were observed in TBCA knockdown cells, the arrest in G1 phase of the cell cycle was followed by cell death by secondary necrosis (Nolasco et al. 2005).

TBCB

The homolog of TBCB in *S. pombe* fission yeast is the Alp11. Alp11 overexpression results in microtubule disruption and cell death (Radcliffe et al. 1999; Radcliffe and Toda 2000). Alp11 binds free α tubulin at the central coiled-coil region and this binding affinity is increased by its CLIP-170 domain on its C-terminal portion (Radcliffe and Toda 2000). Recently, it was shown that TBCB can be phosphorylated by p21-activated kinase 1 (Pak1) at the 65 and 128 serine residues and this phosphorylation regulates microtubule dynamics. In

addition, this phosphorylation of TBCB is essential to enhance polymerization of new microtubules. Similarly to Pak1, TBCB expression was deregulated in breast cancer specimens. An overexpression was observed for both proteins in five out of eight tumors (Vadlamudi et al. 2005). Another study confirmed the role of phosphorylation by identifying that the nitration of TBCB antagonizes this phosphorylation. Nitration of TBCB at 65 and 98 tyrosine residues decreases the synthesis of new microtubules and regulates microtubule dynamics (Rayala et al. 2007).

Recently many studies were done on TBCB. The first showed that TBCB localizes at the transition zone of the growth cones of growing neurites during neurogenesis. It is involved in microtubule dynamics and control of excessive axonal growth (Lopez-Fanarraga et al. 2007). Another study pointed the involvement of TBCB during microglia transition to a more active, proliferative and macrophage-like state. TBCB is implicated in the microglial cytoskeletal changes as a microtubule density modulator that slightly decreases the density of microtubules to allow the actin-based cell migration and phagocytosis (Fanarraga et al. 2009). Nitrogen-containing bisphosphonates (N-BP) are the best drugs used to treat diseases in which excessive bone resorption occurs such as osteoporosis and cancer-induced bone diseases. TBCB, identified as a novel target of N-BP is involved in the biological processes such as microtubule assembly that are behind the cytotoxicity of N-BP (Bivi et al. 2009).

TBCD

TBCD was found to interact with Arl2 (ADP Ribosylation factor Like protein 2). Arl2 downregulates the GTPase activity of TBCC, TBCD and TBCE and inhibits the binding of TBCD to native tubulin. The overexpression of TBCD in cultured cells results in the destruction of the tubulin heterodimer and microtubules. In this case, GDP-bound Arl2 can prevent this destructive activity of TBCD by binding to it (Bhamidipati et al. 2000).

In addition to its role in the folding pathway of native tubulin, TBCD functions as a centrosomal protein and is essential for the anchorage of γ-TuRC and pericentrin to centrosomes and the nucleation of microtubules at centrosomes. Excess TBCD causes a defect in aster formation but has no effect on the microtubule network. This is because excess TBCD prevents γ-TuRC from binding centrosomes but doesn't prevent them from promoting microtubule growth. In cells depleted for TBCD, multipolar spindles were observed due to centrosomal fragmentation (Cunningham and Kahn 2008). In a more recent study, TBCD was found to be concentrated at the centrosome and midbody and involved in centriologenesis, spindle microtubule dynamics and midbody abscission in human cells. Overexpression of TBCD detaches microtubules from centrosomes and causes G1 arrest. Its inhibition produces defects in microtubule retraction at midbody during cytokinesis (Fanarraga et al. 2010).

Furthermore, TBCD and Arl2 interact to regulate the apical junctional complex AJC (composed of tight and adherens junctions) assembly-disassembly process. TBCD binds to the plasma membrane through its 15 C-terminal amino acids and promote disassembly of the apical junction complex and cell dissociation from the monolayer. The binding of Arl2 to TBCD inhibits this latter activity of TBCD and triggers assembly of AJC (Shultz et al. 2008).

In *S. pombe* fission yeast, the homolog of TBCD, Alp1, was crucial for the formation of G1 and S-phase microtubules and the chromosome segregation in mitosis. Cells with nonfunctional TBCD continue to have normal cytoplasmic microtubules for 2 to 3 hours but then microtubules disintegrate and only short ones remain (Fedyanina et al. 2009).

TBCE

The homolog of TBCE in *S. pombe* fission yeast, Alp21, interacts with TBCB homolog, Alp1 (Radcliffe et al. 1999). Overexpression of TBCE in cultured cells results in

the destruction of the tubulin heterodimer and microtubules (Bhamidipati et al. 2000). Recent work showed that TBCB binds to TBCE and results in a highly efficient dissociation of tubulin heterodimers and leads to complete microtubule loss (Kortazar et al. 2006). TBCE, TBCB and α tubulin form a complex after heterodimer dissociation, whereas the free beta-tubulin subunit is recovered by TBCA (Kortazar et al. 2007).

TBCE has been shown to be involved in certain diseases. TBCE is highly expressed in neurons and mutations in this protein causes progressive motor neuronopathy in mice as well as the syndrome of congenital hypoparathyroidism-mental retardation-facial dysmorphism (HRD or Sanjad-Sakati syndrome) (Martin et al. 2002; Parvari et al. 2002; Tian et al. 2006; Padidela et al. 2009).

E-like

E-like protein was identified based on sequence likeness (23 %) with TBCE and is highly expressed in testis. In cells overexpressing E-like proteins, massive depolymerization of the microtubule cytoskeleton and disruption of the Golgi complex were observed. Microtubule depolymerization is due to the misfolding of functional heterodimers and later their destruction by proteosome. Suppression of E-like increases the cellular content of stable, posttranslationally modified microtubules. E-like cannot bind to microtubules nor participate in the folding pathway of tubulins (Bartolini et al. 2005). It was hypothesized that E-like protein might aid through its action in the regulation of organelle distribution and organization (Keller and Lauring 2005). Recently, it was shown that physiologically relevant levels of Op18/stathmin counteract the tubulin disruptive action of TBCE and E-like proteins in human leukemia cell line (Sellin et al. 2008).

Related diseases

Mutations in microtubule-associated proteins are the causes of several inherited human diseases, such as frontal-lobe dementia with parkinsonia and lissencephaly (tau mutations),

Charcot–Marie–Tooth and Opitz syndrome. All of these diseases cause major brain impairment, consistent with the important role of microtubules in neuronal architecture and axonal transport. In addition to Sanjad-Sakati syndrome, many diseases are caused by mutations in chaperones, such as McKusick–Kaufman syndrome, Spastic ataxia Charlevoix–Saguenay and desmin-related myopathy. Therefore TBCs and chaperones are considered potential therapeutic targets due to their ability to disaggregate and dispose of misfolded proteins (Lewis and Cowan 2002).

1.3. Microtubules

1.3.1. Structure and formation

Tubulin assembly into microtubules is a flexible and polymorphic mechanism that involves interactions between the structures of microtubule intermediate and their state of growing and shortening. This property allows microtubules to play functional roles at the right time and place in the cell (Nogales and Wang 2006).

Structure

Using electron microscopy, microtubules were shown to be cylinders of 25 nm in outside diameter, 5 nm in wall thickness and many micrometers long. They are composed of 13 protofilaments, each composed of a string of globular subunits, mainly the α and β tubulins (Erickson 1975). Sometimes, the protofilaments are composed of post-translationally modified tubulins which can still associate with the classical form of tubulin (Linck 1982). Each tubulin heterodimer of 8 nm in length interacts laterally and longitudinally to form the protofilaments and then the microtubules (Downing and Nogales 1998).

There are two potential lattices (A and B) in which the tubulin subunits can be arranged. It has been reported that the α and β tubulin molecules could either alternate (α-β-α-

β…, A-lattice) or be aligned (α-α-α… or β-β-β…, B-lattice) (Mandelkow et al. 1986). B lattice is predominant, but A lattice bonds between tubulin subunits are found at the seams (Kikkawa et al. 1994). With 13 protofilaments, the A lattice could be helically symmetric, whereas the B-lattice could not. Cytoplasmic microtubules have a B-lattice which means that they lack helical symmetry (Mandelkow et al. 1977). Three parameters define the specificity of lattices: the distance between protofilaments, the distance between subunits along the protofilaments and the angle formed by the oblique line of subunits in adjacent filaments (Erickson 1975).

Structural intermediates

Microtubule assembly and disassembly involve transient polymer intermediates manifested mainly as changes in the curvature of the protofilaments (Nogales and Wang 2006). Within the microtubule, α and β tubulins are in different nucleotide-bound states; a nonexchangeable GTP is bound to α tubulin at the N-site (buried at the monomer-monomer interface within the dimer) and an exchangeable GDP is bound to β tubulin at the E-site (on the surface of microtubule). Tubulin dimers can exist in two conformations: a straight GTP-bound conformation and curved GDP-bound conformation (Nogales and Wang 2006). Within the microtubule, the tubulin-GDP is kept in a straight conformation by the subunits interacting in the lattice (Drechsel and Kirschner 1994; Nogales et al. 2003). Thus the energy of GTP hydrolysis is stored within the lattice as structural strain and the microtubules remain stable due to the cap of tubulin-GTP at the ends of protofilaments (Caplow et al. 1994). Only few GTP containing subunits at the tip of the microtubule are sufficient to stabilize a growing microtubule (Drechsel and Kirschner 1994). Once the cap is lost, the protofilaments curl and peel off revealing the curved conformation of tubulin-GDP and forming closed rings (Downing and Nogales 1998) **(Figure 4)**.

During growth, the microtubule ends undergo conformational changes from blunt to long gently curved sheets. Blunt ends are unstable because the loss of the GTP-tubulin subunits triggers them in a shrinking state, whereas sheets are always stable (Arnal et al. 2000). Microtubules are either stabilized by some components or undergoing dynamic instability by exchanging subunits with free tubulin dimers. This dynamic instability will be discussed later in the text.

Roles of structural intermediates

Microtubules are a molecular machine of their own and not only an architectural scaffold (Howard and Hyman 2003). During depolymerization, tubulin detachment from the ends is carried by protofilaments peeling outwards. This act releases the conformational strain of tubulin-GDP strain stored in the lattice. In the case of a kinetochore complex attached to microtubules, this power released will push it and let it diffuse on the lattice in the direction of depolymerization without energy consumption by the complex (Westermann et al. 2006). In addition, it is suspected that the transient sheets at the end of growing microtubules may be the binding site for microtubule plus-end tracking proteins since they expose the inside surface of the microtubule and provide a new inter-protofilament interface (Al-Bassam et al. 2002; Mahadevan and Mitchison 2005). During microtubule growth, the proteins will detach from closing sheets and reattach to newly formed ones which result in effective transport of plus-end tacking proteins (Akhmanova and Hoogenraad 2005).

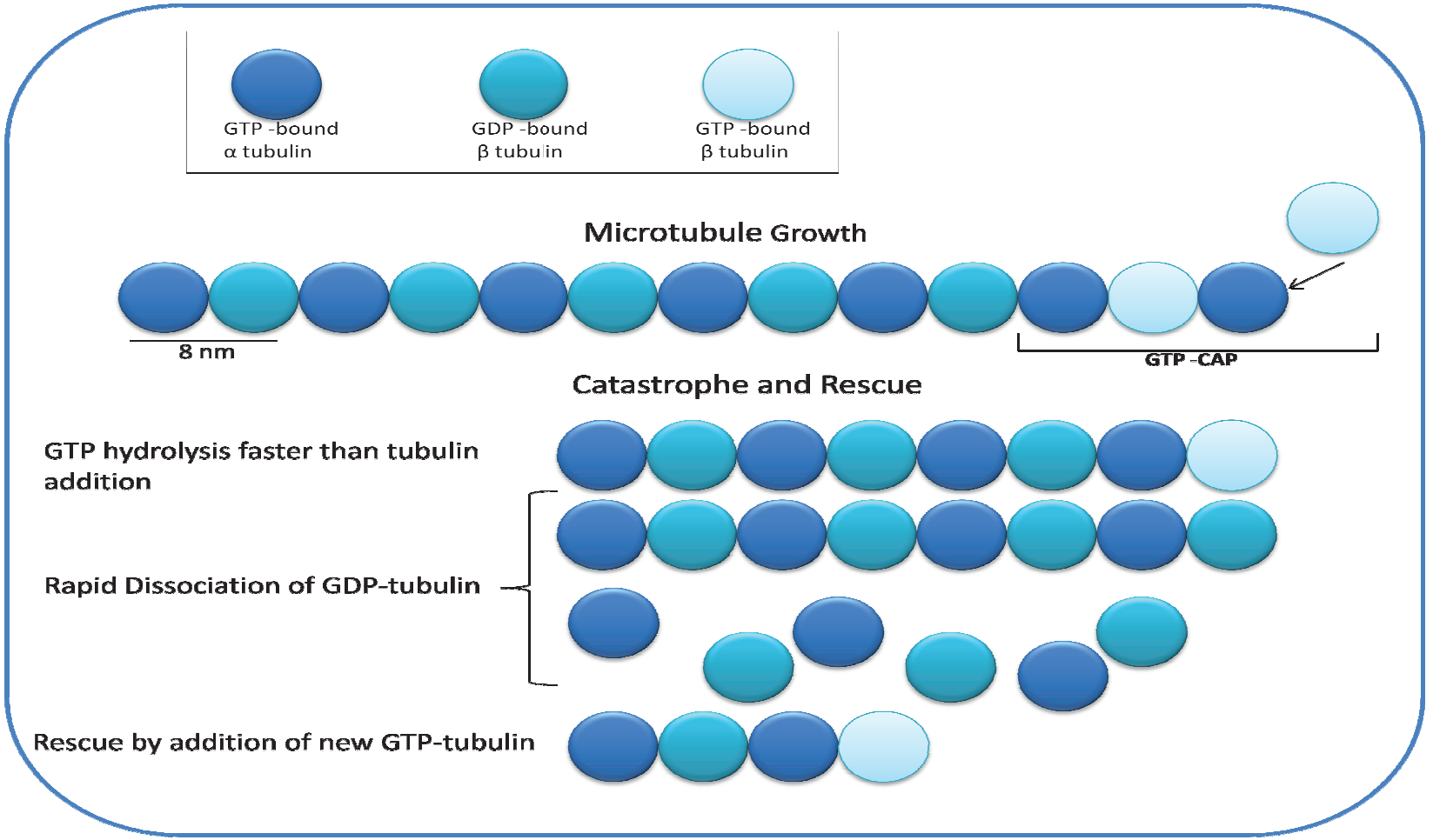


Figure 4: Microtubule protofilaments are made of GTP-α tubulin and GDP-β tubulin. Once the GTP-β tubulin cap is lost, the heterodimers rapidly dissociate.

Formation

The microtubule assembly of tubulin heterodimers starts by polymers nucleation and continues through elongation to reach a plateau (Johnson and Borisy 1977). Microtubule nucleation is initiated in the Microtubule Organizing Center (MTOC) or centrosomes. The common microtubule nucleation is seeded by the γ tubulin ring complex (γ-TuRC) in the centrosomes but it can also occur in its absence (Oakley 2000). The nucleation capacity of purified tubulins is enhanced *in vitro* if γ tubulin is added (Leguy et al. 2000). There are two models for the nucleation by the γ-TuRC. In the first model, γ-TuRC functions as a template for microtubule polymerization and remains attached to minus end. In the other model, the γ-TuRC is unrolled and serve as a protofilament of the nascent microtubule (Erickson and Stoffler 1996). Microtubule nuclei are generated by combination of stable tubulin oligomers and nucleation oligomers are formed of 10 tubulin dimers that associate laterally (Job et al. 2003). The nucleation rate remains constant during elongation phase regardless of the drop in free GTP-tubulin concentration however elongation occurs with different rates even in microtubules nucleated by the same centrosome (Chretien et al. 1995).

In the first stage of microtubule assembly, the microtubules are short and flattened sheets of short and incomplete segments of the microtubule wall are observed. Assembly continues by elongation of these protofilaments and by a slower addition of new filaments to the side of the sheet. The sheet curves as it grows and once the full complement of 13 protofilaments are added; the edges meet and seal to form the intact cylinder. Growth then continues by elongation of the helical lattice of the microtubule (Erickson 1975). At high growth rate and after tubulin conformational changes, the cylinder closure accompanied by GTP-hydrolysis occurs very fast and leaves some GTP-tubulins not hydrolyzed on the terminal subunits. This GTP-cap acquires stability for the cylinder (Drechsel and Kirschner 1994) **(Figure 5A)**. At low growth rate, the conformational change occurs at a rate similar to

that of cylinder formation, cylinder closure accompanied by GTP- hydrolysis reaches the extremity of the sheet leading to week lateral interactions and microtubule shrinking (Arnal et al. 2000) **(Figure 5B)**.

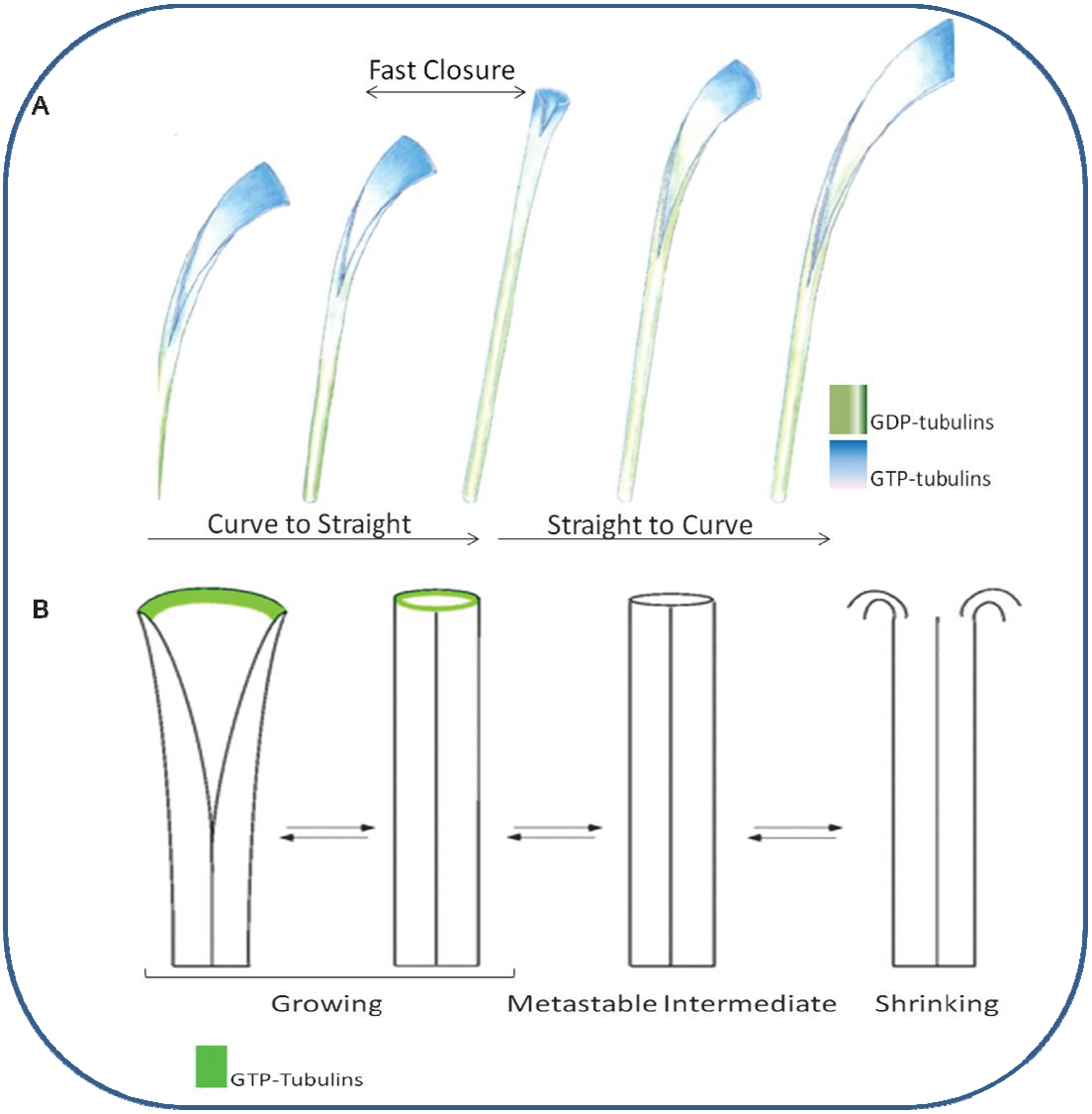


Figure 5. The formation of microtubule cylinders: A) At high growth rate and after tubulin conformational changes, the cylinder closure accompanied by GTP-hydrolysis occurs very fast and leaves some GTP-tubulins not hydrolyzed on the terminal subunits. Adapted from (Chretien et al. 1995). B) Once the GTP-tubulins (green) are lost, lateral interactions become weak and microtubule shrinks. Adapted from (Arnal et al. 2000).

1.3.2. Microtubule dynamics

Microtubules are polar structures formed by the head-to-tail association of αβ heterodimers (Amos and Klug 1974). This polarity causes different polymerization rates at the two ends of microtubule. The faster growing end is referred to as the plus end with β tubulin exposed and the slower growing end is referred to as minus end with α tubulin exposed (Allen and Borisy 1974). In animal cells, minus ends are anchored at centrosomes or microtubule organizing center (MTOC) and plus ends are directed toward the cytoplasm (Howard and Hyman 2003). Two interesting mechanical processes have been described that account for the dynamics that microtubules display in the cell: Treadmilling and dynamic instability. The microtubule dynamics allows them to have flexible spatial arrangements that can change rapidly to accomplish cellular needs and perform mechanical functions.

Initially, the dynamics of microtubules were thought of as simply polymerization steady state where the subunits associate and dissociate at the microtubule ends. In late 1970s and early 1980s, observation of continuous incorporation of tubulin at one end along with a balanced loss of subunits at the opposite end, led to the concept of treadmilling (Margolis et al. 1978). In 1984, a novel mechanism was introduced, designated as dynamic instability. According to this model, the microtubules never reach a steady state but remain in prolonged states of polymerization and depolymerization that interconvert infrequently (Mitchison and Kirschner 1984a; Mitchison and Kirschner 1984b). Since then, additional studies have shown that this mechanism is the predominant one governing the microtubule dynamics **(Figure 6)**.

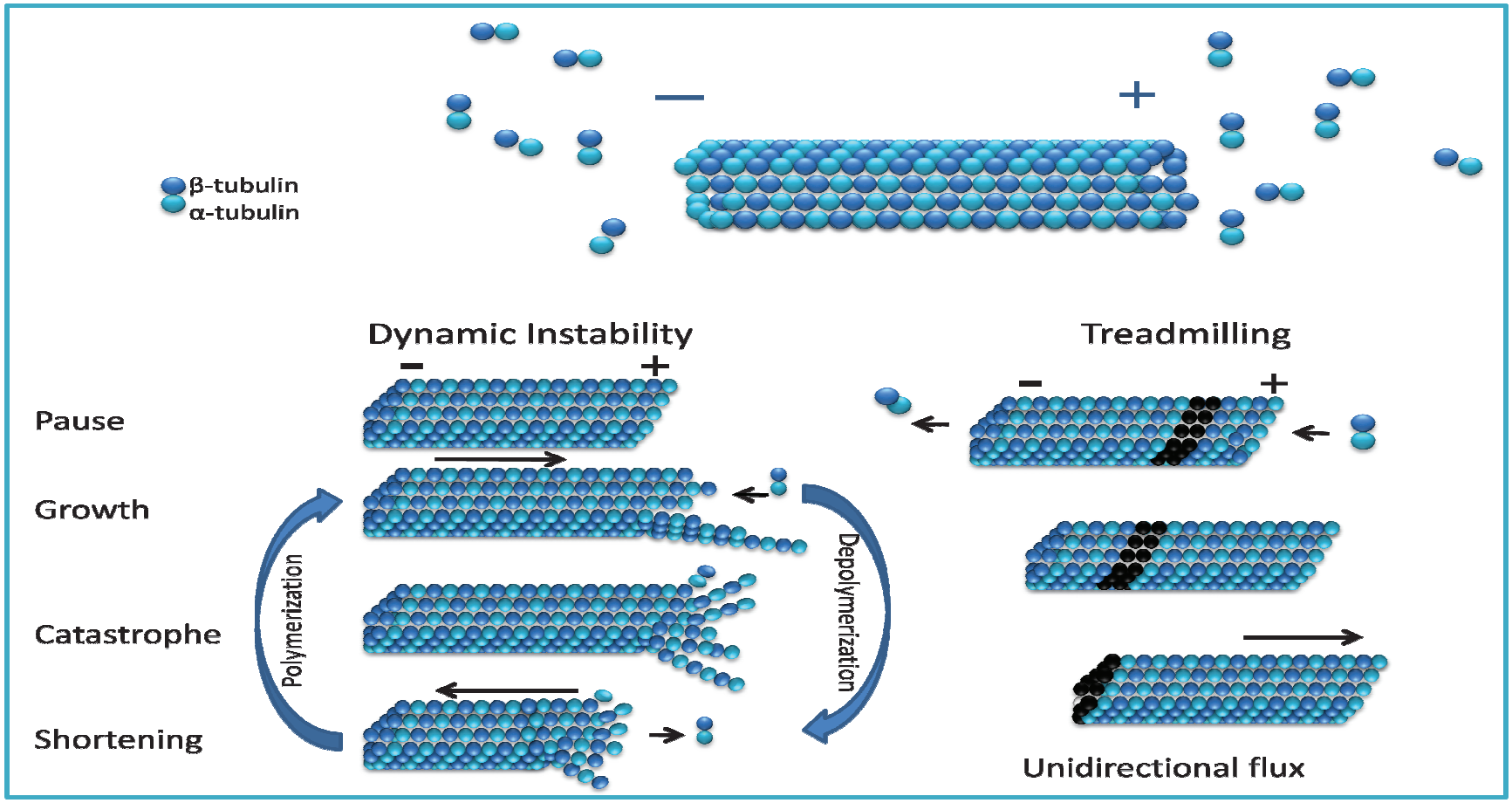


Figure 6. Two interesting mechanical processes have been described for the microtubule dynamics: Treadmilling and dynamic instability. Treadmilling is characterized by the continuous incorporation of tubulin at one end along with a balanced loss of subunits at the opposite end. During dynamic instability, the free end of microtubules undergoes a series of polymerization and depolymerization.

1.3.2.1. Dynamic instability and treadmilling

Dynamic instability

Dynamic instability allows microtubules to search three-dimensional space efficiently, rapidly fill the cytoplasm and bind specific targets (SEARCH-CAPTURE MODEL) (Tanaka and Kirschner 1991). During the polymerization of pure tubulin, plus ends switch between phases of slow growth and rapid shrinking (Mitchison and Kirschner 1984a). The minus ends dynamic instability exists and is similar to that of plus ends but it is not physiologically relevant due to the fact that minus ends are found either capped in centrosomes or depolymerizing when free (Walker et al. 1988; Erickson and O'Brien 1992).

Microtubules generate both pushing force by polymerization and pulling force by depolymerization (Inoue and Salmon 1995). Microtubule polymerization is a bimolecular reaction dependent on free tubulins concentration whereas depolymerization is a unimolecular reaction independent of free tubulins (Walker et al. 1988). Dynamic instability is characterized by four main parameters: the rates of microtubule growth and shortening, the frequency of transition from growth or pause to shortening (called catastrophe) and the frequency of transition from shortening to growth or pause (called rescue). Dynamic instability is powered by GTP hydrolysis that destabilizes the microtubule lattice through the production of weakly interacting GTP-bound tubulins (Caplow et al. 1994). As long as the microtubules are stabilized by the GTP cap, polymerization continues. Once the GTP cap becomes hydrolyzed, the microtubule is said to be in catastrophe and depolymerization occurs until the microtubule rescues and regains its cap (Mitchison and Kirschner 1984a). The pause state of microtubules, frequent *in vivo* but less *in vitro* represents the time microtubules stay neither polymerizing nor depolymerizing (Walker et al. 1988; Shelden and Wadsworth 1993). The parameter “dynamicity” is used to describe the overall rate of tubulin subunits exchange at microtubule ends (Mitchison and Kirschner 1984a).

When the concentration of tubulin dimers is increased, the polymerization rate increases and results in a decrease in the catastrophe frequency (Erickson and O'Brien 1992). The relationships between the polymerization rate and catastrophe frequency on one hand and the rescue frequency and tubulin concentrations on the other remain poorly understood (O'Brien et al. 1990; Walker et al. 1991; Erickson and O'Brien 1992).

Dynamic instability changes in response to cellular factors and regulatory signals. The transition frequencies, the length of microtubules and overall dynamics are the primary targets of these factors (Verde et al. 1992; Gliksman et al. 1993; McNally 1996). Examples of regulatory parameters are the transition from interphase to mitosis, the cellular differentiation and the signal transduction pathways (Bulinski and Gundersen 1991; Verde et al. 1992; Gliksman et al. 1993; Robinson and Vandre 1995; McNally 1996).

Treadmilling

Until recently, treadmilling was considered to be of minor implication in cellular functions. It is characterized by the intrinsic flow of subunits from one polymer end to the other caused by the differences in the critical subunit concentration at the opposite microtubule ends (Margolis and Wilson 1998). In order for treadmilling to work, both ends must be free for subunit gain or loss. Therefore, the minus end is either released from centrosomes or held in place by lateral associations that leave the end free for subunit exchange (Vale 1991; Rodionov and Borisy 1997). GTP hydrolysis is also important in treadmilling; it generates the inequality in the critical subunit concentrations at the two polymer ends which is necessary to create net flow of tubulin form one end to the other (Margolis 1981) . Treadmilling *in vivo* proceed rapidly and at higher rates than *in vitro*. This indicates that treadmilling is controlled by cellular factors that remain unknown. However *in vitro*, stabilized MAP-free steady-state microtubules unable to go into dynamic instability have rapid treadmilling (Panda et al. 1994; Rodionov and Borisy 1997).

1.3.2.2. Microtubule targeting agents

Microtubules represent the single best cancer target identified to date and the treatments targeting microtubules continue to be important and successful chemotherapeutic class of drugs **(Table 3)**. Microtubule dynamics of the spindle is crucial for mitosis and cell proliferation. Thus suppression of microtubule dynamics is sufficient to induce cell death (Wilson and Jordan 1995). At low concentrations, microtubule targeting agents stabilize kinetically microtubule dynamics, inhibit cell proliferation and kill tumor cells without causing depolymerization or polymerization of microtubules. At high concentrations, they either stabilize or destabilize microtubules by either stimulating polymerization or depolymerization, respectively.

Although most antimicrotubule treatments target the dynamics of microtubules, some agents bind tubulins and inhibit microtubule polymerization and induce apoptosis. For example, 3-(Iodoacetamido)-benzoylurea is a tubulin ligand that inhibits microtubule polymerization and induces apoptosis in tumor cells (Jiang et al. 1998). In addition, it has been described that a novel microtubule targeting agent, 1-Aryl-3-(2-Chloroethyl) Ureas (CEU) binds covalently β tubulin, alkylates it and causes depolymerization of microtubules by preventing microtubule assembly. CEU do not significantly alkylate nucleophiles such as DNA, glutathione and glutathione reductase (Legault et al. 2000). Therefore, this finding is promising in order to design drugs that can selectively target proteins and alkylate them, especially tubulins and microtubule related proteins.

Here we will focus on treatments that target the microtubule dynamics through either stabilization or destabilization microtubules.

Table3. Microtubule targeting agents, their diverse binding domain on tubulin and their stages of clinical development

Binding Domain	Drug (commercial name)	Therapeutic Uses	Clinical trials
Taxane Site	Paclitaxel (Taxol)	Ovarian, breast and lung tumors	In clinical Use and Phase I-III trials
	Docetaxel (Taxotere)	Prostate, brain and lung tumors	Phase I-III trials
	Epothilones (A, B and D)	Paclitaxel-resistant tumors	Phase I-III
Colchicine domain	Colchicine	Non-neoplastic diseases	Failed trials (toxicity)
	Combretastatins	Vascular-targeting agent	Phase I-II
Vinca domain	Vinblastine (Velban)	Hodgkin's disease, testicular germ-cell cancer	In clinical Use (in combination)
	Vincristine (Oncovin)	Leukaemia, lymphomas	In clinical Use (in combination)
	Vinorelbine (Navelbine)	Solid tumors, lymphomas, lung cancer	In clinical Use (in combination or single) and Phase I-III trials
	Vinflunine	Bladder, NSCLC, breast cancer	Phase III
	Halichondrins	-	Phase I
	Dolastatins	Vascular-targeting agent	Phase I; Phase II completed

Stabilizing agents

Taxane binding site

Taxanes

Paclitaxel

Paclitaxel is a poison produced by endophytic fungi found in a variety of plants of the Taxaceae including the original source of the drug, the pacific yew *Taxus brevifolia* (Li et al. 1996). In microtubules, GTP is bound to α tubulin however GDP as well as paclitaxel bind to β tubulin. Paclitaxel binds reversibly to the second globular domain of β tubulin on the inside of the microtubule, on the other side from the GTPase domain. Once paclitaxel binds to β tubulin, the polymers formed present high stability even if they contain GDP (Amos and Lowe 1999). The proper binding of paclitaxel to β tubulin depends on the correct residues in the binding pocket and mutations in this area reduce toxicity of paclitaxel. The overall shape of the binding pocket along with neighboring residues influences the binding affinity of paclitaxel (Derry et al. 1997; Nicholov et al. 1997; Nogales et al. 1998a). A recent study described the implication of parkin, E3 ubiquitin ligase responsible for the pathogenesis of Parkinson's disease and cancer in regulating sensitivity to paclitaxel. Earlier, it was described that parkin binds to microtubules and αβ heterodimers and increase the degradation of α and β tubulins (Ren et al. 2003). Recently, it was shown that parkin increases the ability of paclitaxel to trigger multinucleation and apoptosis and render breast cancer cell lines more sensitive to paclitaxel (Wang et al. 2009). In a recent study, it was shown that green tea polyphenol (-)-epigallocatechin gallate (EGCG) may be used as a sensitizer to enhance the cytotoxicity of paclitaxel. EGCG in combination with paclitaxel significantly induced apoptosis and tumor growth in mice was significantly inhibited (Luo et al. 2010).

The mechanism of inhibition of mitosis by paclitaxel is due to inhibition of microtubule dynamics. The rates and lengths of microtubule shortening and growing are

inhibited, the duration of pause is increased and the overall dynamicity is inhibited (Yvon et al. 1999). At low concentration, paclitaxel kinetically suppresses microtubule dynamics without significantly changing the polymer mass (Derry et al. 1995). These concentrations fall in the range of effective plasma paclitaxel concentrations in humans during chemotherapy (Sparreboom et al. 1998). Taxanes have become widely recognized as active chemotherapeutic agents in the treatment of ovarian, lung, metastatic breast cancer and early stage breast cancer with higher overall survival (OS) and disease free survival (DFS) (McGrogan et al. 2008). However some patients treated with taxanes relapse. Paclitaxel is administered in solution with alcohol and purified Cremophor® EL but this solvent causes severe hypersensitivity reactions. Therefore, premedication with dexamethasone is recommended. The elimination half life of paclitaxel is 15 to 50 hours and this drug presents nonlinear pharmacokinetics. Many side effects emerge including alopecia, myelosuppression, gastrointestinal symptoms and febrile neutropenia (Gligorov and Lotz 2004; Roy and Perez 2006).

One of the challenges in treatment of patients with solid malignancies is the difficulty in controlling invasive tumor cells and preventing the spread of metastases. Tumor invasion occurs through migration of cells though lymphatic vessels to bloodstream and then to distant organs. Paclitaxel at concentrations that suppress microtubule dynamics reduced the rate of movement of NRK fibroblasts. This was explained by the fact that these cells with low microtubule dynamics will have limited number of microtubule plus ends that reach the lamillipodium base to regulate its formation (Liao et al. 1995; Mikhailov and Gundersen 1998). In endothelial cells, it was also shown that paclitaxel has antiangiogenic activity not linked to its cytotoxicity, since it occurs at drug concentrations which does not affect proliferation of these cells (Belotti et al. 1996). More studies need to be done to elucidate the role of microtubule dynamics in tumor cell migration, invasion and metastasis.

Microtubules targeting agents activate the apoptotic pathway through intrinsic signaling pathway (as discussed later) as well as through direct action on mitochondria. Paclitaxel binds to mitochondria either through Bcl-2 or through tubulins found on mitochondria (Rodi et al. 1999; Wu et al. 2000; Carre et al. 2002a). Paclitaxel induces changes in mitochondrial transmembrane potential ($\Delta\Psi m$) which mediates the opening of PTPs (permeability transition pores) and the release of cytochrome-*c* (Andre et al. 2000; Varbiro et al. 2001; Carre et al. 2002b; Kidd et al. 2002). In addition, it induces changes in respiration rate of mitochondria and increases cytochrome oxidase-mediated ROS (reactive oxygen species) production (Varbiro et al. 2001). Thus, modulation of the respiration rate and ROS levels as well as cytochrome-*c* release may trigger apoptotic signaling pathways as a result of direct effect of microtubule targeting agents.

Docetaxel

Docetaxel is a second generation taxane that differs from paclitaxel by its linear pharmacokinetics and elimination half life of 1 hour but has the same side effects (Ringel and Horwitz 1991; Gligorov and Lotz 2004). Docetaxel shares the same binding site as paclitaxel though with greater affinity (Diaz et al. 1998). It is used mainly to treat locally advanced or metastatic breast and lung cancer after chemotherapy has failed (Berrieman et al. 2004).

Novel taxanes formulations

ABI-007

ABI-007 is a novel Cremophor®EL-free nanoparticle albumin-bound paclitaxel developed to overcome the hypersensitivity induced by Cremophor®EL. It is administered in every-3-week schedule with a dose of 260 mg/m^2 in consideration of efficacy, toxicities and similarity of pharmacokinetic profile in western studies (Yamada et al. 2010).

CT-2103

CT-2103 (Cell Therapeutics) is a water-soluble macromolecular conjugate of paclitaxel to a polyglutamate backbone, designed to enhance tumor permeability and to improve intratumoral delivery of paclitaxel. However, high rate of cumulative neurotoxicity was observed in a clinical study (Veronese et al. 2005).

Epothilones

Epothilones, novel antimicrotubule agents and macrolide antibiotics, are produced by myxobacterium *Sorangium (Polyangium) cellulosum*. They bind to microtubules near the taxane-binding site, stabilize microtubules and cause cellular arrest similarly to taxanes (Gerth et al. 1996; Kamath and Jordan 2003).

Epothilone A and B

Epothilones A and B are cytotoxic *in vitro* (Gerth et al. 1996). Naturally occurring epothilone B, patupilone (EPO906) is 20 times more cytotoxic than paclitaxel and active on taxane-resistant cells. Clinical studies showed pharmacokinetic difficulties and metabolic instability of natural epothilones therefore synthetic and semisynthetic derivatives have been developed (Morris and Fornier 2008). Ixabepilone (BMS-247550) is a semisynthetic derivative of natural epothilone B and is widely used in clinical cases. *In vitro* studies showed that ixabepilone is 2.5 times more cytotoxic than paclitaxel and its cytotoxicity is maintained in taxane-resistant cell lines such as βIII tubulin expressing malignancies (Lee et al. 2001; Dumontet et al. 2009). In October 2007, ixabepilone was approved by the Food and Drug Administration for metastatic breast cancer (Lee et al. 2001; Morris and Fornier 2008).

Other analogues of Epothilone B are being developed. BMS-310705 is a semisynthetic analogue of epothilone B, more soluble than ixabepilone. It is used in phase I studies but causes diarrhea and neurotoxicity (Sessa et al. 2007). ABJ-879, semisynthetic analogue of epothilone B has higher activity than paclitaxel even in multidrug resistant cell lines but no

clinical studies have been done yet on it (Wartmann et al. 2004). ZK-EPO is a fully synthetic epothilone that has remarkable activity and can overcome multidrug resistance. Its most notable toxicity is neurotoxicity (Klar et al. 2006).

Epothilone D

Epothilone D, also known as desoxyepothilone B, is less toxic than the other epothilone analogs. It is more potent than paclitaxel, but has less antiproliferative activity than the other epothilone analogs (Wang et al. 2005). KOS-862, a derivative of epothilone D, has been successfully combined with drugs like gemcitabine, trastuzumab and carboplatin in phase I studies (Marshall et al. 2005; Monk et al. 2005 ; Cortes et al. 2006). The clinical use of KOS-1584, another derivative of epothilone D, has been encouraging in NSCLC, ovarian, head and neck cancer but diarrhea has emerged as a dose-limiting toxicity (Stopeck et al. 2007).

Discodermolide and dictyostatin

Discodermolide and dictyostatin have antiproliferative activity even in cells with mutated β tubulins (Madiraju et al. 2005; Rowinsky and Calvo 2006). Discodermolide is produced by a deep-sea sponge *Discoderma dissoluta* as a defense mechanism. *In vitro* studies showed promising activity and synergy with paclitaxel which means that they don't have same binding sites on microtubules (Mita et al. 2004). Dictyostatin is structurally related to discodermolide and extracted from a marine sponge of the genus *Spongia* (Madiraju et al. 2005). It showed encouraging results in clinical studies however pulmonary toxicity was observed in some cases (Rowinsky and Calvo 2006).

Laulimalide- binding site

Laulimalide

Laulimalide is a structurally complex substance derived from marine sponges and has antiproliferative activity on paclitaxel-resistant cells (Liu et al. 2007). It binds to microtubules

at a site that is distinct from the taxoid site and can also bind to α tubulin binding sites (Pryor et al. 2002; Wilmes et al. 2007). It is not widely used due to the low efficiency and high toxicity observed on mice xenografts (Liu et al. 2007).

Peloruside A

Peloruside A is a metabolite of the New Zealand marine sponge *Mycale hentscheli* that share similar structure with epothilones (Wilmes et al. 2007). It binds to α tubulin laulimalide-binding site therefore can be used in combination with taxanes which gives a synergic antiproliferative activity (Hamel et al. 2006; Wilmes et al. 2007). In a recent study, it was shown that peloruside A involves c-Myc (Myelocytomatosis) and influences the proteome of HL-60 cells involved in the pathways of microtubules and apoptosis (Wilmes et al. 2010).

Other agents

Other agents of different mechanisms of action are being discovered. Cyclostreptin was found in the broth of a bacterium from the *streptomyces* species (Buey et al. 2007). Even if less cytotoxic than paclitaxel *in vitro*, it is active on paclitaxel-resistant cell lines through its covalent cross-linking with β-tubulin (Buey et al. 2007). Eleutherobin and sarcodictyins A and B are other taxane-binding site drugs naturally derived from marine soft coral (Hamel et al. 1999).

Destabilizing agents

Colchicine-binding site

At low concentrations, Colchicine can stabilize microtubules without causing important depolymerization but by increasing the percentage of time spent in the pause state. It binds to soluble tubulin and then the tubulin-colchicine complexes become incorporated at the microtubule ends (Panda et al. 1995). Colchicine has no clinical use in malignancy but many orally available compounds are under investigation for potential cytotoxicities. These

compounds act at the colchicine-binding site of tubulin and include 2-methoxyestradiol, sulfonamide derivatives and synthetic derivatives of *Aspergillus* species (Yee et al. 2005; Kamath et al. 2006; Nicholson et al. 2006). At the lowest effective concentrations, 2-methoxyestradiol, a metabolite of estradiol-17β, suppresses microtubule dynamics and arrests mitosis without depolymerizing microtubules. However its activity is decreased by the presence of microtubule-associated proteins (Kamath et al. 2006).

The principle activity of microtubule targeting agents is as antiproliferative agents. Novel agents (combretastatins and N-acetylcolchicinol-O-phosphate) that resemble colchicine and bind to colchicine domain on tubulin are undergoing clinical testing as vascular-targeting agents. The efficacy of a microtubule-targeting agent as either antiproliferative or as a vascular-targeting agent depend on the pharmaco-dynamic and pharmacokinetic characteristics, on the reversibility of binding to tubulin and long-term retention in cells (Jordan and Wilson 2004). The ones that enter cells rapidly, reversibly bind to tubulin or microtubules, rapidly depolymerize microtubules and rapidly get metabolized or excreted might act best as antivascular agents. Those that are retained in cells and induce long-term mitotic block might work best as antiproliferative agents that induce apoptotic cell death (Jordan and Wilson 2004).

Nocodazole

Nocodazole (methyl [5-(2-thienylcarbonyl)-1Hbenzimidazol-2-yl]) is a synthetic drug that has antimitotic and antitumor activities. It targets the arg-390 residue of the β tubulin (Decordier et al. 2002). Its activity is reversible, rapid and dose dependent (Jordan et al. 1992). At high concentrations, it depolymerizes microtubules and inhibits polymerization (Friedman and Platzer 1978; Ireland et al. 1979). However at low concentrations, it inhibits microtubule dynamics both in vivo and in vitro (Vasquez et al. 1997). Nocodazole is like colchicine in its stimulation of tubulin-dependent GTP hydrolysis (Lin and Hamel 1981).

Vinca-binding site

Vinca alkaloids derived from the periwinkle leaves (catharanthus roseus (L.), known as *Vinca rosea*). *Vinca* alkaloids include first generation and second generation *Vinca* alkaloids. Vinblastine and vincristine are first generation whereas vinorelbine and Vinflunine are second generation vincas. *Vinca* alkaloids have a well-established role in many malignancies especially haematological cancers and the first generation ones are most commonly used in combination therapy. Peripheral neuropathy and reversible myelosuppression are their principle side effect. Neurotoxicity may emerge from the alteration in microtubule dynamics that disrupted the axonal flow and caused defects in axonal processes (Sahenk et al. 1994; Gidding et al. 1999; Jordan and Wilson 2004). At relatively high concentrations, they inhibit microtubule polymerization (Binet et al. 1990; Jordan et al. 1991; Kruczynski et al. 1998). However at low concentration, they suppress microtubule dynamics without any effect on microtubule mass or spindle microtubule organization (Jordan et al. 1985; Toso et al. 1993; Dhamodharan et al. 1995).

Vinblastine

Vinblastine (0.5-1 μM) binds to microtubule ends with relatively high affinity and greatly decreases the rate of both growing and shortening at the microtubule ends and increase the duration of pause (Toso et al. 1993). It inhibits chromosome congression (prometaphase movement) and metaphase-anaphase transition by suppressing microtubule dynamics (Jordan et al. 1991; Jordan and Wilson 1998). It is used in the treatment of Hodgkin's disease, some lymphomas, advanced testicular cancer, and Kaposi's sarcoma (Berrieman et al. 2004).

Vincristine

Vincristine is used in the treatment of acute lymphoblastic leukaemia in children, some lymphomas, neuroblastoma, Wilm's tumor and some sarcomas. It is also investigated

for the use in treatment of colorectal cancer and cancers of breast, ovary, cervix and lung (Berrieman et al. 2004).

Vinorelbine

Vinorelbine, a semisynthetic derivative of natural product vinblastine, showed improved efficacy and less toxicity in clinical studies. Unlike other vincas, vinorelbine can be used alone or in combination with cisplatin and is important in treatment of advanced NSCLC, breast cancer, cisplatin-resistant ovarian cancer and Hodgkin's lymphoma (Berrieman et al. 2004).

Vinflunine is a novel difluorinated vinorelbine derivative (20′,20′-difluoro-3′,4′-dihydrovinorelbine) with high clinical efficiency in NSCLC and greater *in vitro* activity than the other *Vinca* alkaloids (Bennouna et al. 2006). Vinflunine showed reduced neurotoxicity relative to other vinca alkaloids (Lobert et al. 1998).

Synthetic Vinca alkaloids

Halichondrin B is a large polyether macrolide derived from the marine sponge *Halichondria okadaic* (Jordan et al. 2005). Crytophycins are naturally occurring compounds isolated from blue-green algae (D'Agostino et al. 2006). Synthetic derivatives of these that act at the *Vinca* site are being tested. For example, eribulin mesylate (E7389), a synthetic derivative of halichondrin is used in phase II trials in breast, prostate and NSCLC. It strongly disrupts the mitotic spindle by binding directly to microtubules or to free tubulins and slows down the growth rate of microtubules. This slowed growth prevents microtubules from reaching kinetochores and the cell from passing through the metaphase-anaphase checkpoint. Therefore the cell will remain blocked in mitosis (Jordan et al. 2005). LY355703 is a synthetic cryptophycin derivative which has shown some activity in platinum-refractory ovarian cancer (D'Agostino et al. 2006).

Dolastatins

Dolastatins were originally isolated from the Indian Ocean mollusk, *Dolabella auricularia* (the sea hare). They have anticancer activity and act by binding near the vinca binding site (Riely et al. 2007). Many compounds have been tested and showed no clinical efficacy. TZT-1027, a derivative of dolastain-10, showed no activity in NSCLC in a phase II trial despite promising results in preclinical studies (Riely et al. 2007). Tasidotin (ILX651), a synthetic derivative of dolastatin-15, is potentially more promising since it inhibits microtubule nucleation at low concentrations. Phase II studies in melanoma and NSCLC are ongoing (McDermott et al. 2005).

1.3.2.3. Microtubule dynamics and cell signaling

Microtubule functions are regulated by many intracellular signaling pathways such as Rho GTPases, a GTP binding enzyme important in migration and stress fiber formation and MAPKs (mitogen-activated protein kinases). Conversely, microtubule dynamics influences cell signaling since modulation of dynamics affects the binding, sequestration and activation of many proteins. For example, antimicrotubule agents activate the nuclear factor κB (NF-κB) signaling pathway through degradation of NF-κB inhibitor (IκB) located on intact microtubules (Bourgarel-Rey et al. 2001; Huang et al. 2004; Mistry et al. 2004). The same applies to the members of the apoptotic machinery, such as Bim that once dissociated from microtubules, can initiate apoptosis (Puthalakath et al. 1999).

Microtubules can mediate transport of vesicles and organelles in a well-defined manner to particular targets. This transport is very important for information transduction and involves microtubule motor proteins, kinesin and dynein (Guzik and Goldstein 2004). The sliding of these motor proteins is highly regulated by the microtubule dynamics and by the

presence of destabilizing and stabilizing microtubule related proteins (Lopez and Sheetz 1993; Seitz et al. 2002).

1.3.3. Microtubule related proteins

1.3.3.1. Microtubules associated proteins (MAPs)

Microtubule stability is promoted mainly by MAPs. One major family of MAPs is that of structural MAPs which includes large proteins MAP1A, MAP1B, MAP1C, MAP2, MAP4 and smaller ones MAP2C, Tau. This family is known to cross-link, stabilize and control assembly of microtubules in the cytosol (Maccioni and Cambiazo 1995; Honore et al. 2005). MAP1A and MAP1B are large, filamentous molecules found in axons and dendrites of neurons (Pedrotti et al. 1996; Chien et al. 2005). MAP2 is found in dendrites where it forms fibrous cross-bridges between microtubules and between microtubules and intermediate filaments and in non-neuronal tissues such as hair follicles (Hallman et al. 2002). It is distributed in an inhomogeneous manner along microtubule length and forms high- density regions where it stops depolymerization (Itoh and Hotani 2004). Neuronal MAPs weakly increase the polymerization rate, strongly suppress catastrophes and promote rescues (Drechsel et al. 1992; Pryer et al. 1992; Trinczek et al. 1995). MAP4 is found in neuronal and non-neuronal cells. Unlike neuronal MAPs, it promotes microtubule assembly *in vitro*, by strongly enhancing the rescue frequency without decreasing the catastrophe frequency (Ookata et al. 1995).

Tau is smaller than the other MAPs and present in axons and dendrites as well as in endothelial cells (Tar et al. 2004). The impact of MAPs on the different parameters of the dynamic instability varies from one MAP to another and from one isoform to another within a given MAP. This is the case of Tau that exists in six isoforms due to alternative splicing of mRNA. These isoforms differ in the number of repeats in the microtubule binding domains, either a three or four- repeat site. That of four-repeat domain reduced the rates of both

growing and shortening events whereas the three-repeat suppressed only shortening events in MCF7 cells (Bunker et al. 2004). A study *in vitro* showed that the ratio of Tau- tubulin influences the growth rate in four and three-repeat Tau isoforms. When this ratio is low, the three-repeat reduced growth rate and the four-repeat increased it, whereas at high ratio, both repeats increase the growth rate. In addition, tau is able to inhibit the destabilizing activity of XKCM1, a kinesin (Noetzel et al. 2005).

In general, phosphorylation regulates most MAPs as it detaches them from the microtubule lattice and/or tubulin and decreases microtubule stability. The most important kinases that regulate microtubule dynamics include the Cdc2 kinase family, Cdk5 in neurons, PAKs and mitogen-activated protein kinases (ERK, JNK) (Honore et al. 2005). In addition to phosphorylation, another mechanism regulates the interaction between microtubules and MAPs. Modulin is a protein capable of modulating the binding of multiple MAPs to microtubules *in vitro* (Ulitzur et al. 1997). Type 2A phosphatase (PP2A) binds to microtubule and plays a role in sequestering intracellular pools of enzymes and promoting kinase-mediated phosphorylation of MAPs. Once microtubules depolymerize, PP2A detaches, becomes activated and dephosphorylates MAPs. Overall microtubule dynamics is therefore a result of a balance between the effects of these regulatory kinases and phosphatases (Merrick et al. 1996; Sontag et al. 1999).

1.3.3.2. Destabilizing and stabilizing plus end binding proteins (+TIPs)

A large number of proteins interacts specifically with microtubule plus ends. These proteins are divided into microtubule stabilizing +TIPs called also microtubule plus end tracking proteins and microtubule destabilizing +TIPs.

Destabilizing +TIPs

The MCAKs (mitotic centromere-associated kinesins) belongs to the Kin I subfamily of kinesin-related proteins, a unique group of motor proteins that are motile but instead destabilize microtubules (Desai et al. 1999). These proteins are capable of traveling to both the minus and plus ends of microtubules whereas most motors are unidirectional. Thus they can catalytically depolymerize a microtubule from both ends making it a very efficient process (Hunter et al. 2003). MCAKs are recruited to the centromere at prophase and remain centromere associated until after telophase. The localization of MCAKs at kinetochores suggests that they could trigger depolymerization during mitosis (Maney et al. 1998). MCAKs walk to growing end, bind to it and convert it to a shrinking one by causing bending and curl formation at this end which weakens the association of the terminal GTP-tubulin dimer and catalyses its dissociation into solution (Howard and Hyman 2003).

Stabilizing +TIPs

Microtubule plus end tracking proteins is a diverse group of proteins that bind independently to microtubule but can also interact one with the other which shed light on the potential existence of a plus end complex. These proteins influence the microtubule structure and accessibility by binding to its plus ends. Here we introduce some of these proteins.

The first protein described was CLIP-170 (Cytoplasmic Linker Protein of 170 kDa), a linker between membranes and microtubules (Perez et al. 1999). This protein is responsible for the microtubule rescue events that occur at the cell periphery to allow the microtubules to adapt rapidly to changes in cell shape (Komarova et al. 2002). CLIP-170 most likely targets the sheet structure of microtubules and dissociates from it as the sheet closes into a tube (Perez et al. 1999). CLIP-170 can interact with dynein and dynactin to promote microtubule capture at the cortical sites (Goodson et al. 2003; Sheeman et al. 2003). CLASP proteins target microtubule ends by binding to CLIP-170.

EB1 (End Binding Protein 1) binds to the tips of growing microtubules and regulate dynamics. During mitosis, it stabilizes the polymer by binding to elongating kinetochores microtubules and preventing catastrophes and during interphase, it decreases pause duration (Rogers et al. 2002; Tirnauer et al. 2002). EB1 can recruit APC (adenomatous polyposis coli) to the microtubule end. APC is a large multidomain protein thought to function as a tumor suppressor through its involvement in the Wnt/ β-catenin signaling pathway (Mimori-Kiyosue and Tsukita 2001). EB1 interacts with dynactin, a protein that has microtubule nucleation effect and enhances microtubule stability (Ligon et al. 2003).

Another family of proteins was described, the Dis1/TOG (defect in sister chromatids disjoining/ tumour overexpressing gene) family. These proteins can bind directly to microtubules and promote microtubule polymerization at both ends (Charrasse et al. 1998). CLASP1 and CLASP2 (CLIP associated proteins) stabilize microtubules by promoting pause and restricting growth and shortening events. They form complex with EB1 protein and mediate interaction between the microtubules and the cell cortex (Mimori-Kiyosue et al. 2005).

1.3.3.3. Stathmin

The oncoprotein 18 (op18)/stathmin regulates microtubule dynamics both during interphase and mitosis and represents a major microtubule destabilizing phosphoprotein that promotes microtubule depolymerization by promoting catastrophe in the mitotic spindle and sequestration of tubulins in interphase (Rubin and Atweh 2004). Stathmin is highly expressed in breast cancer and acute leukaemia (Melhem et al. 1997; Bieche et al. 1998). Its activity is regulated at the transcriptional level by p53 and E2F and at the protein level by phosphorylation (Ahn et al. 1999; Polager and Ginsberg 2003). Once phosphorylated by Cdc2 kinases or p21-associated kinases (PAKs), stathmin can no longer bind tubulin and destabilize microtubules (Daub et al. 2001; Bokoch 2003). Stathmin is highly phosphorylated and

inactive in mitotic cells and more functional in interphase cells (Marklund et al. 1996). During mitosis, the short steady-state length of microtubules is maintained by PP2A that regulates microtubule dynamics though regulation of microtubule-destabilizing activity and phosphorylation of stathmin. Upon completion of mitosis, PP2A dephosphorylates stathmin that becomes active again (Tournebize et al. 1997). The inhibitor of PP2A, okadaic acid increases microtubule dynamics through an increase in elongation velocity (Howell et al. 1997). Overexpression of stathmin was found to be involved in decreasing the response of cells to paclitaxel and vinblastine by promoting microtubule destabilization and delaying entry into mitosis (Alli et al. 2002). Inhibition of stathmin induced microtubule polymerization, promoted G2/M transition and sensitized breast cancer cells to paclitaxel and vinblastine (Alli et al. 2007).

1.3.3.4. p53

p53 is physically associated with microtubules via dynein and localizes to cellular microtubules (Giannakakou et al. 2000). It is a transcription factor that once activated can induce the expression of several genes including p21, Bax, Mdm2 and Gadd45 (Levine 1997). p53 mRNA is long-lived however the protein is short-lived and present at low levels in the nucleus of normal cells; however in cases of DNA damage, oxidative stress or inhibition of microtubule dynamics, the protein becomes phosphorylated and stabilized. In addition, p53 is transported to the nucleus via the interphase microtubule minus end-directed motor dynein after DNA damage. Thus, nuclear p53 activity depends on microtubule integrity (Giannakakou et al. 2000; Giannakakou et al. 2001; Giannakakou et al. 2002). p53, also called tumor suppressor protein, is translocated to the nucleus and also to the mitochondria following suppression of microtubule dynamics by microtubule targeting agents (Giannakakou et al. 2002). p53 has been described to induce apoptosis through a

transcription-independent mechanism by directly interacting with Bax, Bad or Bak and by directly mediating mitochondrial membrane permeability (Perfettini et al. 2004; Jiang et al. 2006).

It was previously reported that chemoresistance present in p53-deficient cells is attributed to defective apoptotic pathways (Galmarini et al. 2001). It was later demonstrated that extensive alterations in microtubule composition and dynamics in mut-p53 cells contribute also to the resistant phenotype with respect to antimicrotubule agents (Galmarini et al. 2003). In addition p53 gene mutations are among the most common mutations identified in human breast tumors. 30 % of primary breast carcinomas have missense mutations in p53 and half of the 70 % of the carcinomas highly express the wild-type p53 protein (Ozbun and Butel 1995).

1.3.3.5. ADP Ribosylation factor Like protein 2 (Arl2)

ADP Ribosylation factor Like protein 2 (Arl2) is a 21 KDa GTPase belonging to the ADP ribosylation factor (ARF) family (Kahn et al. 1991; Kahn et al. 2005). Several genetic studies suggest that Arl2 plays a role in microtubule dynamics (Hoyt et al. 1997; Radcliffe et al. 2000; Antoshechkin and Han 2002). In mammalian cells, Arl2 binds to tubulin binding cofactor D (TBCD) and inhibits the heterodimer- dissociating activity of TBCD (Bhamidipati et al. 2000). More recently, it has been reported that Arl2 inhibited TBCD-dependent cell dissociation from the monolayer and disassembly of the apical complex (Shultz et al. 2008).

The entire cytosolic pool of Arl2 is complexed with TBCD and with the heterotrimeric protein phosphatase 2A (PP2A) (Shern et al. 2003). PP2A is considered to behave as a tumor suppressor protein (Janssens et al. 2005). It is one of the major serine/threonine phosphatases of mammalian cells involved in the regulation of several fundamental cell processes such as cell cycle progression, DNA replication, apoptosis and protein synthesis (Beghin et al. 2009).

In MCF7 and MDA-MB 231 cells expressing different levels of Arl2, it was shown that alterations of cellular Arl2 protein content were associated with modifications of polymerization-competent α/β tubulin heterodimer levels resulting in altered MT dynamic properties and with modifications of mitotic progression as well as with modifications of the content, localization and activity of the catalytic subunit of PP2A (PP2Ac) with no significant changes in PP2Ac mRNA levels (Beghin et al. 2007b). In addition, Arl2 content was associated with the degree and distribution of phosphorylated p53 which binds microtubules (Beghin et al. 2008). Finally, Arl2 content is a major determinant of aggressive phenotype and tumorigenicity in breast cancer cell models, and low Arl2 expression levels appear to be correlated with enhanced aggressivity in the clinic (Beghin et al. 2009)

1.3.4. Functions of microtubules

Microtubules are the most rigid of the intracellular cytoskeletal filaments. This rigidity is length dependent and crucial for microtubules to maintain the cell shape, to create tensile forces and control long-range transport (Hawkins et al. 2009). In nondividing cells, microtubules organize the cytoplasm, position the nucleus and other organelles, and serve for intracellular transport and for structuring of flagella and cilia. During cell division, the dynamic microtubule spindle physically aggregates the chromosomes and orients the plane of cleavage (Kerapov 1976; Desai and Mitchison 1997).

Major component of cytoskeleton

Microtubules constitute a major component of the cytoskeleton of mammalian cells and play a crucial role in the control of cell morphology. They provide physical strength and determine the shape and internal arrangement of the tracks where the organelles move and delineate the spindle which separates chromosomes during mitosis (Thyberg and Moskalewski 1999). It was shown in epithelial cells that disruption of microtubules alters polarity of basement membrane proteoglycan secretion (De Almeida and Stow 1991).

In addition to these functions, microtubules are required to preserve normal structure and function of the Golgi complex. A close relation exists between the Golgi elements and the stable subpopulation of microtubules since microtubules functions as tracks for transport of Golgi elements and as framework to which Golgi elements are anchored. Golgi stacks are usually arranged in the region around the centrosomes close to the origin of microtubules. During prophase, the cytoplasmic microtubules depolymerize and consequently the Golgi stacks become smaller and perinuclear. When antimicrotubule drugs are added and microtubules are lost, Golgi stacks break up into smaller fragments, move around in the cytoplasm and associate with the endoplasmic reticulum (Thyberg and Moskalewski 1999).

An unexpected relationship exists between the role of microtubules in cell spreading and the tension state of cell–matrix interactions. At a low tension state (absence of stress fibers and focal adhesions), fibroblasts spread with dendritic extensions whose formation requires microtubules; at a high tension state (stress fibers and focal adhesions), cells spread with lamellipodial extensions and microtubules are required for cell polarization but not for spreading (Rhee et al. 2007).

Intercellular contacts

Epithelial tight junction (TJ) and adherens junction (AJ) form the apical junctional complex (AJC) which regulates cell-cell adhesion, paracellular permeability and cell polarity. Major proteins of epithelial AJs include E-cadherin and members of nectin protein family (Takai and Nakanishi 2003; Gooding et al. 2004). The actin and microtubule cytoskeleton determine cell shape and polarity, and promote stable cell-cell and cell-matrix adhesions through their interactions with cadherins and integrins, respectively (Hall 2009). The potential link between cadherins and microtubules was described but has not been extensively investigated. It was shown that by blocking microtubule dynamics, the ability of cells to concentrate and accumulate E-cadherin at cell-cell contacts was perturbed. The transport of E-

cadherin to the plasma membrane or the amount of E-cadherin expressed at the cell surface was not affected. Microtubule dynamics allows cells to concentrate E-cadherin at cell-cell contacts by regulating the regional distribution of E-cadherin once it reaches the cell surface (Stehbens et al. 2006). In addition, it was shown that the microtubules regulate the formation of contractile-F-ring, the disruption of AJC and the internalization of AJ/TJ proteins in calcium depleted epithelial cells. Stabilization of microtubules attenuated disassembly of AJC and translocation of E-cadherin from cell-cell contacts into the cytosolic ring-like structure as well as prevented the formation of apical contractile F-actin rings (Ivanov et al. 2006).

Flagella and cilia

Eukaryotic flagella are made of a bundle of nine fused pairs of microtubule doublets that surround two central single microtubules. The "9+2" structure is called an axoneme. At the base of a eukaryotic flagellum is a basal body which is the microtubule organizing center (MTOC) and is structurally identical to centrioles. A plasma membrane encloses the flagellum and makes it accessible to the cell's cytoplasm. Eukaryotic flagella and cilia are ultrastructurally identical but differ in their beating pattern. In the case of flagella (e.g. the tail of a sperm) the motion is propeller-like. In contrast, beating of motile cilia consists of coordinated back-and-forth cycling of many cilia on the cell surface (Haimo and Rosenbaum 1981).

Molecular machines

Polymerizing microtubules can deform membranes or induce microtubule bending through the pushing force they produce (Hotani and Miyamoto 1990; Elbaum et al. 1996). Depolymerizing microtubules can move beads that remain attached to their ends through pure kinesins (Coue et al. 1991). Once assembled, as they shrink and grow, microtubules provide tracks for the transport of organelles and chromosomes along with the help of motor proteins that move along their lateral surfaces or through interactions between microtubules and the

plasma membrane (Hirokawa et al. 1998). They also act as network for vesicles transport which is of high relevance to the field of drug delivery as well as other intracellular transport. Vesicle transport represents an endocytic pathway to enhance drug delivery to a target organ (Hamm-Alvarez 1998).

Microtubule motor proteins, kinesin and dynein, travel in a specific direction along a microtubule because of the polarity of microtubules and the orientation by which the head of the motor protein bind to the microtubule. Motor proteins are powered by the hydrolysis of ATP and convert chemical energy into mechanical work (Rice et al. 1999). Most kinesins walk towards the plus end of a microtubule and transport cargo form the center of the cell to the periphery in an anterograde transport. Few exceptions of kinesins, Kinesin-14 family proteins walk in the opposite direction, toward microtubule minus ends (Ambrose et al. 2005). Another motor protein known as dynein, moves towards the minus end of the microtubule. Axonemal dynein is responsible for the movement of eukaryotic cilia and flagella and cytosolic dynein thus transports cargo along microtubules (vesicles, organelles and chromosomes) from the periphery of the cell towards the centre in a retrograde transport (Wade 2009).

For proper functioning and survival, cells require many enzymes, organelles and cytoskeleton element. The proper transport and distribution of these elements require an appropriate transport system that can afford long-distance transmission in some cases. For example, axons in neurons can extend distances and therefore needs long-range microtubule-dependant transport. The organization of microtubules is nearly uniform with plus ends facing towards the axonal terminus and the minus ends towards the cell body (Guzik and Goldstein 2004). Studies showed that signaling endosomes that transduce survival signal from axonal terminal to the cell body are transported via retrograde microtubule-dependent transport (Delcroix et al. 2003; Ye et al. 2003; Howe and Mobley 2004).

Microtubules are major actors in the movement of chromosomes during metaphase and anaphase of mitotic cell division (Rieder and Salmon 1998). The microtubule plus end attached to chromosome via the kinetochore undergo growth and shrinkage that allows the chromosome to move away from or toward the pole where the minus end of microtubule is attached (Inoue and Salmon 1995).

Cellular functions depending on microtubule dynamics

Among the various cell functions regulated by microtubule dynamics, the most important ones involved in tumor progression are the control of cell proliferation and migration (Honore et al. 2005).

Mitosis and cell proliferation

Three distinct subsets of microtubules exist: the kinetochores microtubules (kMTs) with their plus ends attached to sister chromatids at the kinetochores, interpolar or pole-pole microtubules which overlap microtubules from opposite pole at the spindle midzone and astral microtubules that extend away from the spindle. Studying the dynamics of microtubules during mitosis is hard to achieve due to the brief time window and the problems of image resolution (Kline-Smith and Walczak 2004). Microtubule turnover is much more important during mitosis (half-time of 10s) than in interphase cells in order to establish the mitotic spindle and maintain it (Saxton et al. 1984). Prior to nuclear envelope breakdown (NEB), interpolar and astral microtubules are formed (Piel et al. 2001). Assembly of mitotic spindle is not as critically dependent upon dynamics of its microtubules as is spindle function at metaphase (Hoyt et al. 1991; Li and Murray 1991). Upon NEB, microtubule dynamics increase dramatically (20 to 100-fold) and during prometaphase microtubule plus ends search the cytoplasm to link with a chromosomal kinetochore (McIntosh et al. 2002). This linkage is stabilized by CENP-E (centromere protein E) and the chromosome position within the spindle

appears to be a critical determinant of CENP-E function at kinetochores (McEwen et al. 2001). Chromosome-associated motors called chromokinesins produce forces at chromosome arms to trigger their proper alignment and orientation. During anaphase, depolymerization at plus ends of kMTs drive chromosome segregation (Mazumdar and Misteli 2005).

The kMTs are continuous from the centrosomes to the kinetochores unlike the astral and interpolar microtubules that have many free ends regulated by the microtubule related proteins (McDonald et al. 1992; Rusan et al. 2001). The dynamics of astral microtubule plus ends in prometaphase is highly increased with a decrease in rescue frequency, an increase in catastrophe frequency, a decrease in pause time and no changes in growth and shortening rates (Rusan et al. 2001). During anaphase, the overlapping microtubules polymerize at plus ends, elongate the spindle and prepare for cytokinesis (Higuchi and Uhlmann 2005).

Cell migration

Cells migrate during development as well as during life in response to extracellular stimuli or in case of wound repair or immune response. In pathological cells like cancerous cells, migration is involved in neoangiogenesis and metastasis. Migration is characterized by a sequence of events including formation of pseudopodial protrusions, attachment and translocation of cell body (Lauffenburger and Horwitz 1996). A majority of cell types does require microtubules for directional locomotion (Vasiliev et al. 1970). Fibroblasts, require microtubules, specifically dynamic ones for spontaneous and wound-healing migration (Vasiliev et al. 1970; Gail and Boone 1971).

In migrating cells, a very stable microtubule array becomes polarized at the leading edge. The plus ends are maintained stable by the binding of EB1 (the end binding 1) /APC (adenomatous polyposis coli) and by integrin- and focal adhesion kinase (FAK)-facilitated Rho-mDia signaling pathways (Palazzo et al. 2004). These stable microtubules can function as specialized tracks for vesicle and cytoskeletal trafficking (Yvon et al. 2002). Microtubule

dynamics is higher at the trailing edge than at the leading edge in cells. Microtubules at the leading edge have lower shortening rate, lower catastrophe frequency and higher duration of pause as compared to trailing edge. Thus in migrating cells, a gradient of microtubule dynamic instability occurs form the leading edge to the trailing edge, and this is crucial for cell polarity and motility (Salaycik et al. 2005).

1.3.5. Cell cycle and microtubules

1.3.5.1. Cell cycle checkpoints and breast cancer

During cell cycle, cells must complete a series of events that control the faithful replication of the genome prior to cell division. Cell cycle progression is controlled by the activity of many protein kinase complexes, each consisting of a cyclin and a cyclin-dependent kinase (CDK). The regulatory pathways mediated by these proteins complexes are referred to as cell cycle checkpoints (Tannoch et al. 2000). The checkpoints are responsible in arresting the cell cycle temporarily in order for any cellular damage to be repaired, for any cellular stress to be dissipated or for growth factors, nutrients and hormones to be available. Checkpoints can also activate pathways that lead to programmed cell death if damages are not repaired (Hartwell and Weinert 1989). For CDKs to become activated they must bind a cyclin, undergo site-specific phosphorylation by CDK-activating kinases (CAK) and dephosphorylation by a member of the CDC25 phosphatase family. CAK adds an activating phosphate to the complex, while wee (Western Equine Encephalitis) adds an inhibitory phosphate; the presence of both activating and inhibitory phosphates renders the complex inactive. Cdc25 is a phosphatase that removes the inhibitor phosphate added by Wee, rendering the complex active (Solomon et al. 1993; Arellano and Moreno 1997).

The transition through G2- and M-phases is controlled by the activity and phosphorylation status of the cyclin A/ p34 CDC2 (CDK1) and cyclin B1/p34 CDC2 (CDK1) complexes (Nurse 1990). Cyclin A binds preferentially to CDK2 throughout G1 and S phase

in human cells and it assembles with CDK1 only after complex formation with CDK2 reaches a plateau during late S and G2 phases (Merrick et al. 2008). Phosphorylated inactive cyclin B1/CDK1 complexes accumulate during G2 phase and the transition into mitosis occurs once this complex is dephosphorylated by CDC25C phosphatase (Hwang and Muschel 1998). The exit from mitosis occurs when the CDC2 kinase loses its activity after ubiquitination and degradation of cyclin B1 and cyclin A by anaphase-promoting complex (APC) (Cohen-Fix and Koshland 1997). Progression through the G1- and S-phases is mediated by the activity of the cyclin D1/CDK4, 6, cyclin E/CDK2, and cyclin A/CDK2 complexes (Sherr 1994). CDKs are negatively regulated by a group of functionally-related proteins called CDK inhibitors, the INK4 and Cip/Kip inhibitors. InK4 family inhibits CDK4 and CDK6 during G1 phase whereas Cip/Kip family (p21, p27 and p57) can inhibit CDK activity during all phase of the cell cycle (Sherr and Roberts 1995; Xiong 1996). During G1 progression, pRb (retinoblastoma protein) gets phosphorylated by cyclin D1/CDK4, 6 and cyclin E/CDK2 complexes. When hypophosphorylated, pRb functions as a transcriptional repressor by binding to the E2F- family of transcriptional factors and inhibiting the E2F-dependent transcription of genes such as S-phase genes cyclin E and cyclin A. When hyperphosphorylated by cyclin/CDK, pRB dissociates from E2F and the cell enters the S-phase (Sellers and Kaelin 1996; Sladek 1997; Knudsen et al. 1998). In case any DNA damage occurs, the cell cycle is arrested in G2 phase to prevent the passage of mutated DNA to daughter cells in mitosis. The checkpoints responsible for this are the phosphoinositide-3 kinase (PI-3K) family. Once activated, these kinases can activate and phosphorylate p53 and other proteins (Kapoor and Lozano 1998). It was shown that p53 and p21 were important to maintain cells with DNA damage arrested in G2 phase and therefore their absence in tumor cells causes an accelerated passage into mitosis (Bunz et al. 1998).

The modifications in expression levels of the checkpoints and related proteins are frequently observed in breast cancerous cells. Some oncogenes such as MCT-1 (multiple copies in T-cell malignancy) can regulate cell cycle checkpoints and transform human mammary epithelial cells (Hsu et al. 2005). p53 gene mutation is one of the most common mutations identified in human breast tumors (Osborne et al. 1991). Some breast carcinomas highly express wild-type p53 however the localization of p53 is modified and during S-phase they remain in the cytoplasm instead of going into the nucleus (Takahashi and Suzuki 1993). In MCF7 breast carcinoma cells, the overexpression of Mdm2 (murine double minute) blocks the activation, the cell cycle arrest and the apoptotic functions of p53. Mdm2 is a cellular protein that binds p53 and targets it for degradation. Therefore in cancerous cells overexpressing Mdm2, the interaction between p53 and Mdm2 must be interrupted to restore the function of p53 (Oliner et al. 1993; Suzuki et al. 1998). 20 % of human breast cancers present loss in normal pRb function and the 80 % remaining present alterations in the components that regulate pRb. For instance, cyclin D1 and cyclin E overexpression, CDK4 gene amplification or the deletion of p16 are all observed in primary breast carcinomas (Varley et al. 1989; Ravaioli et al. 1998). Cyclin D1 is highly implicated as a mediator of hormonal regulation of cell proliferation in breast epithelial cells (Prall et al. 1998). The overexpression of p27 in breast tumor cells has been associated with decreased cyclin D1 protein expression and increased apoptosis (Katayose et al. 1997; Sgambato et al. 1997). Cyclin E was described as a dominant oncoprotein and as a “master cyclin”. A high level of cyclin E has been associated with the initiation or progression of breast cancer (Moroy and Geisen 2004). CDC25B, a protein that functions in a similar manner as CDC25C phosphatase and regulate cell cycle progression by the removal of inhibitory phosphorylation from cyclin/CDK complexes, is overexpressed in 30 % of primary breast cancers (Galaktionov et al. 1995). BRCA1 and BRCA2 are involved in the DNA damage response checkpoint

pathways in breast tumor cells (Zhang et al. 1998b). They are highly expressed in the G1/S transition as well as the G2 of the cell cycle and they function as p53 coactivator in transcriptional pathways resulting in apoptosis (Zhang et al. 1998a). In conclusion, the high frequency of alterations in p53, pRb and the signaling pathways in breast cancer cells that regulate them is enough to cause a defective G1/S checkpoint function in the majority of human breast carcinomas. Cancerous cells with defective checkpoint function can respond better to anticancer treatments especially the ones that target the cell cycle. In addition, treatments of cancerous cells that inhibit the checkpoints can be of high importance in order to increase the progression time from one phase to another in the cell cycle (Soni and Jacobberger 2004).

1.3.5.2. Spindle assembly checkpoint and timing of cell cycle

Spindle checkpoints influence the timing of mitosis and the overall duration of the cell cycle. They are involved in the mechanisms that control the time when the cell will initiate anaphase, finish mitosis and start the next cell cycle (Sluder 1979). For the M-phase of the cell cycle, the DNA structure checkpoints and the spindle assembly checkpoint (SAC) are the most important checkpoints. Inhibition of members of the SAC such as Mad2 significantly accelerates mitosis. Therefore, it can be suggested that SAC sets the duration of mitosis even in cells in which the chromosome segregation is proceeding normally (Meraldi et al. 2004). After duplication in the S-phase, centrosomes remain closely paired and continue to function as microtubule organizing center during G2. After G2, they recruit additional tubulin ring complexes in order to increase the microtubule nucleation activity. During mitosis, the spindle checkpoint monitors the correct formation of the microtubule spindle, the correct alignment of chromosomes on the spindle and their correct attachment to kinetochores (Nigg 2001). Spindle formation and microtubule movement depend on three parameters: the dynamic instability of microtubules, the ratio of stabilizing and destabilizing microtubule

binding proteins and the action of microtubule motor proteins (Andersen 1999). The transition into mitosis is triggered by several kinases including cyclin A/CDK1 and mitogen-activated kinase (MAP kinase) that control microtubule-associated proteins and microtubule-destabilizing proteins by phosphorylation. Stathmin is one target of cyclin B/CDK1 that once phosphorylated, it becomes inactivated and can no more destabilize the microtubule (Larsson et al. 1997).

Mediators of the SAC pathway include the mitotic arrest deficient Mad (1-3) and budding uninhibited by benzimidazoles Bub (1-3) proteins (Sorger et al. 1997). Mad2 localizes to the kinetochores during prometaphase and remains there till the alignment of chromosomes in metaphase. It interacts with APC machinery to allow the entry into anaphase. Bub1 and Bub3 localize to the kinetochores and regulate chromosome/kinetochore interactions. They are also responsible in arresting the cell cycle after disruption of microtubule dynamics during mitosis. In some cancerous cells, such as human colon carcinoma cells, mutations in Bub1 inactive it and cause a disruption in the spindle checkpoint (Sorger et al. 1997). Other proteins of the SAC include the microtubule motor proteins dynein, the cytoplasmic linker protein (CLIP) 170 and CENP (McGrogan et al. 2008). At the onset of anaphase, the spindle assembly checkpoint determines the timing of anaphase onset and monitors the attachment of microtubules kinetochores and /or the generation of tension that results from bipolar attachment of sister chromatids. In anaphase, the separation of sister chromatids results from a loss of sister-chromatid cohesion rather than from an increase of forces moving towards the poles. CDK1 phosphorylates the majority of cohesins, the proteins responsible for the sister-chromatid cohesion. The remaining cohesins are removed by APC at the metaphase-anaphase transition (Waizenegger et al. 2000). At metaphase-anaphase transition, Cdc20 dissociated from Mad2 activates APC that degrades

many anaphase onset inhibitors, mitotic cyclins and mitotic kinases and remains active until the G1 phase (Morgan 1999).

In addition, a third checkpoint exists, the spindle positioning checkpoint. It links CDK1 inactivation and mitotic exit to proper orientation of the mitotic spindle (Nigg 2001). It involves Bub2p, a spindle-pole-associated subunit of a two-component GTPase-activating protein (GAP). This protein leads to inactivation of CDK1 through the stabilization of CDK1-inhibitor (Hoyt 2000). After an aberrant mitosis, the checkpoint proteins involved in the entry into G1/S such as p53, p21 and pRb are also important to prevent passage into S-phase (Stewart and Pietenpol 1999). Therefore cancerous cells that lack these proteins, can exit aberrant mitosis by re-entering G1 with 4N DNA content and entering S-phase. The results of this process called endoreduplication are polyploid cells (Sorger et al. 1997).

1.3.5.3. Mitotic arrest and cell fate

After mitotic arrest induced by microtubule targeting agents, cell fate can be diverse. If the cell cannot exit mitosis, it undergoes apoptosis. If the cell exits mitosis into G1, it is either arrested in G1 or exits into apoptosis or necrosis or even continues into S-phase. So the cell fate is really complicated and depends on many factors. During mitotic arrest, transcription is inhibited and therefore many antiapoptotic proteins are depleted. If the mitotic arrest is prolonged, cell undergoes apoptosis I. If at the same time cyclin B is inhibited; the cell can exit mitosis without division (mitotic slippage) and become tetraploid. But in this case, transcription resumes and overexpression of p53 induces p21 and Bax (proapoptotic proteins). Depending on the relative effect of these two proteins, cells are then either arrested in G1 due to p21 or go into apoptosis due to Bax (Blagosklonny 2007).

BREAST CANCER

"Within the European Union, every 2.5 minutes a woman is diagnosed with breast cancer. Every 7.5 minutes a woman dies from the disease" (Stella Kyriakides).

Breast cancer is the most common cancer in women, the second most common cause of cancer death in women in United States and the most common cause of death in women between the ages of 45 and 55. Although is a common form of cancer in women, male breast cancer does occur and accounts for 1% of all cancer deaths in men. In 2006, in Europe, new cases of breast cancer (13.5% of total cancers) along with more mortality were reported.

2.1. Breast anatomy

Breast is a gland that produces milk. It is on the chest muscles covering the ribs. Each breast is made of 15 to 20 lobes themselves made of many smaller lobules. Lobules contain many tiny glands responsible for the production of milk that will be drained through the ducts to the nipple. The nipple is in the middle of a dark area of skin called the areola. Between the lobules and the ducts, a fatty and connective tissue fills the space. Breast also contains lymph vessels that lead to small and round lymph nodes. They are grouped near the breast in the axilla (underarm), above the collarbone, in the chest behind breastbone and in many other places. They represent a trap for bacteria, cancer cells and harmful substances. The anatomy of a normal breast is illustrated **in the figure 7**.

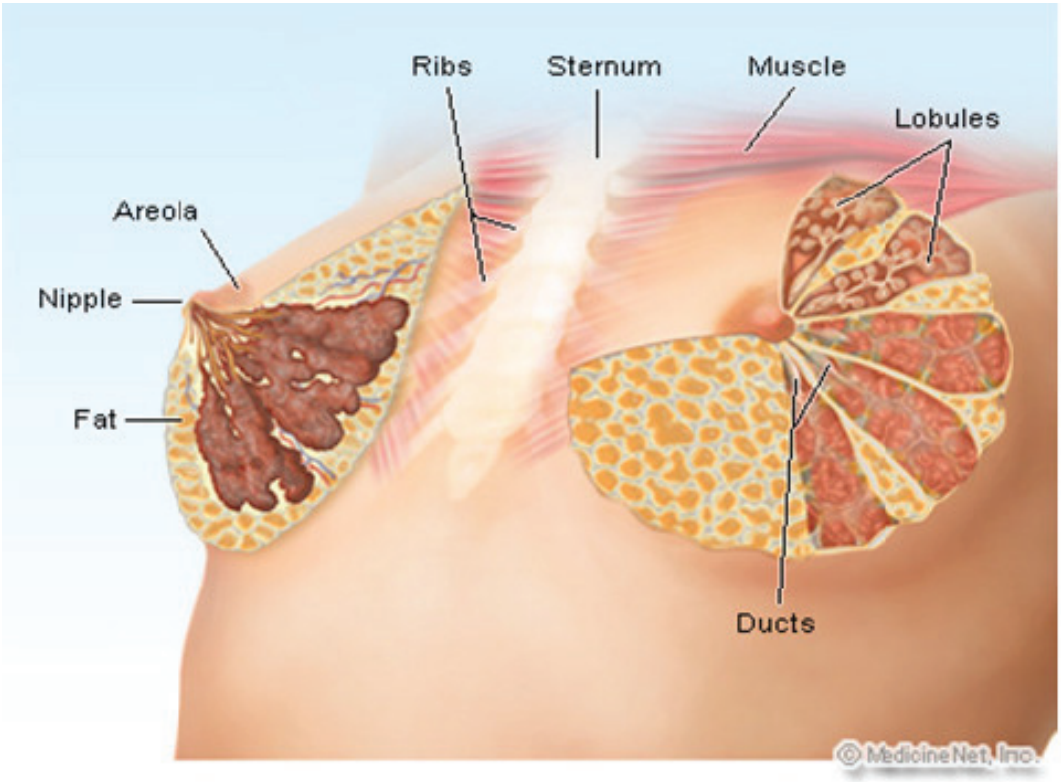


Figure 7: Picture of the anatomy of the normal breast. Adapted from MedicineNet

2.2. Epidemiology and types of breast cancer

Cancer

Cancer begins when cells grow and divide in a random manner without any need or control by the body. These cells form tissues along with older cells that fail to die. This mass of tissue is called tumor. Tumors can be either benign or malignant. The benign tumors rarely threaten lives, are not invasive and when removed, they usually don't grow again. The malignant tumors called cancer are serious, life-threatening, invasive and sometimes metastatic.

Breast Cancer

When breast cancer cells spread, they can be found in lymph nodes near the breast as well as to bones, liver, lungs and brain. The formed tumor contains the same type of abnormal cells as the original tumor and is treated as a breast cancer and not as a bone, liver, lung or

brain cancer. Breast cancer is not contagious and the exact causes of this disease remain unclear. However, a woman can be more exposed to have breast cancer than others due to certain risk factors that will be discussed later. The incidence of breast cancer significantly increased in the last two decades but the mortality rate remained stable pointing out the efficacy of therapies being used.

Ninety five percent of breast cancers are adenocarcinoma and develop from either the lobules (lobular carcinoma in situ LCIS) or the ducts (ductal carcinoma in situ DCIS). The ductal cancers also called intraductal carcinoma are more frequent than lobular cancers. These cancers can become invasive and spread to lymph nodes. Other pathologies exist including Paget disease, medullary carcinomas, sarcomas and non-cancerous lesions.

Classifications of breast cancer

Breast cancer is classified into stages based on the size of the tumor and whether the cancer is spread or not. The stage is often not known until after surgery to remove the tumor in the breast and the lymph nodes under the arm. Recurrent cancer is when cancer comes back after a period of time.

Stage 0

Two types of breast cancer are characterized, the lobular carcinoma in situ (LCIS) where the abnormal cells line the lobule and the ductal carcinoma in situ (DCIS) where the abnormal cells line the duct. The LCIS type is rarely invasive whereas the DCIS can move from being intraductal carcinoma to invasive cancer if not treated.

Stage I (T1 N0 M0)

This is the early stage of invasive breast cancer where the tumor is no more than 2 centimeters.

Stage II (T0 N1 M0, T1 N1 M0, T2 N1 M0)

In this stage, there are three cases: either the tumor is no more than 2 cm but has spread to the lymph nodes under the arm or the tumor is of 2-5 cm and may have spread to the lymph nodes under the arm or the tumor is more than 5 cm but has not spread.

Stage III (A/T3 N0 M0, T3 N1 M0, T0-3 N2 M0 ; B/ T4 Any N M0, Any T N3 M0)

This stage is locally advanced cancer divided into stage IIIA, IIIB and IIIC. Stage IIIA is characterized by either a tumor of less than 5 cm that has spread to underarm nodes connected to other nodes/structures or to lymph nodes behind the breastbone, or by a tumor of more than 5 cm that has spread to lymph nodes behind the breastbone or to underarm nodes connected or not to other nodes/structures. Stage IIIB is characterized by a tumor of any size spread either to the lymph nodes under the arm or to the underarm nodes connected to other nodes/structures or to lymph nodes behind the breastbone. Inflammatory breast cancer (swollen and red breast) is a rare type of cancer that once diagnosed it means at least stage IIIB is reached. Stage IIIC is characterized by a tumor of any size spread either to the lymph nodes behind the breastbone and under the arm or to the lymph nodes under or above the collarbone.

Stage IV (Any T Any N M1)

This is called distant metastatic cancer where cancer has spread to other parts of the body.

Another TNM classification system by the UICC (Union internationale contre le cancer) exists based on the size T, number of lymph nodes invaded N and level of metastasis M. The corresponding TNM code was mentioned between parentheses for all stages and further detailed in the following **table 4**.

Table 4: TNM Classification System

Dimensions of the tumor	T0	No evidence of primary tumor
	Tis	In situ cancer
	T1	Tumor 2 cm or less
	T2	Tumor more than 2 cm but not more than 5 cm in its greatest dimension
	T3	Tumor more than 5 cm in its greatest dimension
	T4	Tumor of any size with direct extension to chest wall or skin
Regional Lymph Nodes	N0	Homolateral axillary nodes not considered to contain growth
	N1	Movable homolateral axillary nodes considered to contain growth
	N2	Homolateral axillary nodes containing growth and fixed to one another or to other structures
	N3	Homolateral supraclavicular or infraclavicular nodes containing growth, or edema of the arm
Distant metastasis	M0	No evidence of distant metastasis
	M1	Distant metastasis present, including skin involvement beyond the breast area

2.3. Risk factors

Risk factors have been determined thanks to many studies. They include age, personal history of breast cancer, family history, breast changes, gene changes, reproductive and menstrual history, race, radiation therapy to the chest, breast density, diethylstilbestrol, excessive weight or obesity after menopause, lack of physical activity and alcohol consumption.

Factors that increase the risk of breast cancer are detailed as follows:

- Age: more frequent after menopause and in women over 60.
- Personal history of breast cancer: having breast cancer in one breast increases the risk of getting it in the second one.
- Family history: if one member of the family had breast cancer, especially before the age of 40.
- Breast changes: if the breast contains abnormal types of cells (atypical hyperplasia).
- Gene changes: such as BRCA1, BRCA2 and others.
- Reproductive and menstrual history: women with no children, women who had their first child at a relatively old age, women who had their first menstrual cycle before the age of 12 or went through menopause after 55, and women treated with estrogen and progestin after menopause.
- Race: more in white women then Asian, Latina, or African American women.
- Radiation therapy to the chest: if radiation therapy was administered before age 30 for treatment of other diseases.
- Breast density: if breast tissue is dense and fatty.
- Diethylstilbestrol (DES): women who have taken DES during pregnancy.
- Excessive weight or obesity after menopause
- Lack of physical activity

- Alcohol consumption

Although many risk factors can be avoided, some of these cannot such as family history. One must discuss with doctors this concern and plan a schedule for checkups. Screening is very important to prevent breast cancer or to diagnose it in its primary stages. These include screening mammogram, clinical breast exam and breast self-exam. Mammogram x-rays uses small doses of radiation not harmful to detect breast lump, cluster of tiny specks of calcium (microcalcifications) or cancer but it has many technical limitations. It sometimes shows things that turn out not to be cancer (false positive) or at contrary misses some cancers (false negative) or doesn't detect cancers that have already spread. It should be done every 1-2 years for women in 40s and above and for younger if at risk. In case of abnormal cells, further tests must be done (hormone receptor test and Human epidermal growth factor recept-2 HER2 test) and biopsy (fine-needle, core biopsy or surgical biopsy) is needed. Another way to screen is by clinical breast exam where the doctor checks for differences in the size and shape of the breast as well as in the skin texture and the presence of a lump or thickness in or near the breast or in the underarm areas. In addition, he checks if the nipple is tender, turned inward into the breast or discharges fluids if squeezed. Finally, woman can do self-exam to check any changes in her breasts.

2.4. Treatments

Cancer treatments can be either local or systemic therapy depending on the size of tumor, stage of cancer and state of the patient. In the first case, surgery and radiation remove the cancer from the breast or control its growth in other areas where it has spread. In the case of systemic therapy, chemotherapy, hormone therapy and biological therapy are used to enter the blood stream, destroy and control the cancer growth in the body. It is either used before or after local therapy and in cases of spread cancers.

Surgery

This is the most common treatment. It consists of either breast-sparing surgery (also called breast-conserving surgery, lumpectomy, segmental mastectomy and partial mastectomy) or mastectomy. Breast-sparing surgery removes limited amount of healthy tissue and can consist in excisional biopsy that removes the whole lump. In addition, most of the time, underarm lymph nodes are removed (axially lymph nodes dissection) to test whether malignant cells have reached these nodes. Women are then given radiation to destroy any remaining cells.

In the case of mastectomy, the whole breast or a large part of it is removed as well as the lymph nodes under the arm. Radiation is also given sometimes after the surgery.

Radiation therapy

It uses high-energy rays to kill cancer cells and is given for most women who had breast-conserving surgery and some women who had mastectomy. Radiotherapy can be given either as external therapy for 5 days a week for several weeks or internal (implant) radiations for several days.

Chemotherapy

It uses anticancer drugs as one drug or as combinations in pills or intravenous injections to kill cancer cells. The drug reaches cancer through the blood stream. This type of treatment has many side effects such as hair loss, weak immunity, poor appetite, vomiting, nausea, diarrhea, mouth sore and other side effects influencing fertility.

Anticancer drugs target DNA replication (intercalating agents such as anthracyclins (mainly doxorubicin) and antimetabolites (mainly 5-fluorouracil) and DNA transcription (alkylators: cyclophosphamide), tubulins/microtubules (taxanes and vinca alkaloids) and protein synthesis.

Hormone therapy

Seventy percent of breast cancers are hormone-dependent in the sense that they use natural estrogen and progesterone to grow. Hormone therapy blocks the effect of hormones. Three categories of molecules are available: the selective estrogen receptor modulator SERM (tamoxifen) that compete with estrogen to bind to estrogen receptor ER, the estrogen pure antagonists (fulvestrant) that bind the ER and degrade it and anti-aromatases (letrozole) that prevent conversion of androstenedione and testosterone to estrone and estradiol.

Biological therapy

In this therapy, the immune system is targeted to help it fight the cancer. Examples are the monoclonal antibodies such as trastuzumab that targets the ERB2 receptors (HER2 extracellular membrane receptor) of cancerous cells and is given to patients whose tumors express high levels of HER2.

In conclusion, once the tumor is surgically removed, patients with stage 0 LCIS are either not treated or given tamoxifen as preventive therapy however DCIS is treated with breast-sparing surgery and radiation or mastectomy and patients are given tamoxifen in case of invasive risk. Stages I and II patients are treated by breast-sparing surgery and radiation. Mastectomy and nodes removal are frequently performed for stage II and IIIA disease. Stage IIIB and IIIC are treated with chemotherapy followed by breast-sparing surgery or mastectomy then lymph nodes removal and radiation. Stage IV is given chemotherapy and hormone therapy along with radiation.

2.5. Factors associated with a resistance phenotype

One of the most widely studied resistance mechanisms is the multidrug resistance (MDR) phenotype that is associated with reduced drug accumulation due to overexpression of ABC-transporters (ATP-binding cassettes) that causes drug efflux. However, in human cancers treated with antimicrotubule agents, other more active mechanisms of resistance are

being studied (Pasquier and Kavallaris 2008). One of these mechanisms is the alterations of tubulin and microtubule system including the changes in expression of tubulin isotypes, the mutations of tubulins and posttranslational modifications (Drukman and Kavallaris 2002; Verrills and Kavallaris 2005).

In breast cancer cells resistant to docetaxel, the expression level of different β tubulins isotypes was modulated (Shalli et al. 2005). High expression level of βIII tubulin is observed in many drug-resistant cell lines such as paclitaxel-resistant ovarian cancers (Kavallaris et al. 1997). It was shown that the resistance to paclitaxel in Chinese hamster ovary cells is due to the fact that the overexpression of βIII tubulin reduces the ability of paclitaxel to suppress microtubule dynamics (Kamath et al. 2005). Clinical evidence highlights the involvement of βIII tubulin in the resistance phenotype of lung, breast and ovarian cancers to taxanes and vinca alkaloids (Ferrandina et al. 2006). It has been described that the dynamics of microtubules assembled from βIII or βIV tubulins were less sensitive to suppression by paclitaxel than those of microtubules made from βII tubulins (Derry et al. 1997).

Mutations affecting either β tubulins or α tubulins have been also reported to be associated with resistance to taxanes, vinca alkaloids, epothilones and 2-ME2 *in vitro* (Verrills and Kavallaris 2005; Yin et al. 2010). **Table 5** summarizes some mutations and polymorphisms in the class I β tubulin gene that were found in human cell lines and that were in certain case related to drugs resistance (Berrieman et al. 2004).

Table 5. Polymorphism and mutations found in the class I β tubulin gene in human cell lines (Berrieman et al. 2004)

Exon/Codon	Base change	Amino acid change	Mutation or polymorphism	Cell line
4/173	CCC→**G**CC	P→A	Mutation	Epothilone A- resistant HeLa cells
4/180	GTC→GT**T**	V→V	Polymorphism	2 NSCLC cell lines
4/195	AAT→AA**C**	N→N	Polymorphism	2 NSCLC cell lines
4/217	CTG→CT**A**	L→L	Polymorphism	Lymphoblastoid, NSCLC, 4 SLC cell line
4/240	CTC→**A**TC	L→ I	Mutation	Vincristine-resistant leukaemia
4/270	TTT→**G**TT	F→V	Mutation	Paclitaxel-resistant ovarian cancer
4/274	ACC→A**T**C	T→ I	Mutation	Epothilone A-resistant ovarian cancer
4/282	CGA→C**A**A	R→Q	Mutation	Epothilone B-resistant ovarian cancer
4/292	CAG→**G**AG	Q→E	Mutation	Epothilone B-resistant NSCLC
4/350	AAG→AA**T**	K→N	Mutation	Indanocine-resistant leukaemia
4/364	GCA→**A**CA	A→T	Mutation	Paclitaxel-resistant ovarian cancer
4/400	GGC→GG**T**	G→G	Polymorphism	Lymphoblastoid
4/422	TAT→T**G**T	Y→C	Mutation	Epothilone B-resistant cervical cancer

The presence of β tubulin mutations in clinical samples remains controversial **(Table 6)**. It was first linked to patient survival after paclitaxel chemotherapy and in a clinical study with 49 patients, it was shown that 16 patients had β tubulin mutations and didn't respond to paclitaxel whereas out of the 33 patients with no mutations, 13 patients responded either partially or completely to paclitaxel (Monzo et al. 1999). Many studies criticized this latter one and noted that the exonic primers used might have also amplified pseudogenes and led to erroneous conclusions (Kelley et al. 2001; Sale et al. 2002). The results of Monzo and colleagues (Monzo et al. 1999) were verified using intronic primers and surprisingly the β tubulin mutations were not found to be as frequent as claimed. Many studies showed that clinical samples of either naïve or drug-resistant cancers have no sequence variations other than known polymorphisms or silent mutations. With respect to drug resistance, polymorphisms in the β tubulin gene are rare and when they occur they do not change the encoded aminooacid responsible for the protein product where the drug binds (Sale et al. 2002). The lack of data with respect to mutations in clinical samples suggest that other mechanisms such as drug efflux or detoxification or changes in expression levels of different β tubulin isotypes should be given priority in the study of antitubulin drug resistance.

Finally, posttranslational modifications of tubulin peptides have also been identified in cells resistant to antimicrotubule agents. Acetylation and detyrosination of α tubulins are frequent in stable microtubules although no clear evidence on the whether they are the cause or consequence of this stability (Bulinski and Gundersen 1991). Post-translational modifications at the C-terminal domain of the class I β tubulin including glutamylation were reported in vincristine-resistant leukaemia cells that acquired an increased microtubule polymer content and a stabilization of microtubules (Kavallaris et al. 2001).

Table 6. Polymorphism and mutations found in the class I β tubulin gene in human clinical samples (Berrieman et al. 2004)

Exon/Codon	Base change	Amino acid change	Mutation or polymorphism	Sample
1/4	ATC→A**C**C	I→T	Mutation	1 NSCLC
1/11	CAA→CA**G**	E→E	Polymorphism	1 NSCLC
4/127	TGT→T**T**T	C→F	Mutation	1 NSCLC
4/131	CAG→**A**AG	Q→K	Mutation	2 NSCLC
4/147	ATG→AT	Frameshift	Mutation	3 NSCLC
4/180	GTC→**A**TC	V→I	Mutation	2 NSCLC
4/180	GTC→GT**T**	V→V	Polymorphism	3 NSCLC
4/183	TAC→TA**A**	Y→Stop	Mutation	2 NSCLC
4/195	AAT→AA**C**	N→N	Polymorphism	3 NSCLC
4/217	CTG→CT**A**	L→L	Polymorphism	1 Ovarian, 1 NSCLC, 22 Breast
4/235	TGT→**G**GT	C→G	Mutation	1 NSCLC
4/243	CCT→**T**CT	P→S	Mutation	1 NSCLC
4/245	CAG→**A**AG	Q→K	Mutation	1 NSCLC
4/245	CAG→**G**AG	Q→E	Mutation	2 NSCLC
4/246	CTC→CTC**C**	Frameshift	Mutation	1 NSCLC
4/250	CTC→C**G**C	L→R	Mutation	1 NSCLC
4/260	TTC→**G**TC	F→V	Mutation	1 NSCLC
4/306	CGC→**T**GC	R→C	Mutation	Breast
4/437	GGT→**A**GT	G→S	Polymorphism	Breast

In order for antimicrotubule agents to efficiently function and target microtubules, spindle microtubules must be well assembled and attached to the kinetochore of each sister chromatid (Zhou et al. 2002). The spindle assembly checkpoint (SAC) monitors the correct progress of this event and therefore is considered crucial to ensure an appropriate response to treatments (Musacchio and Hardwick 2002). In addition, the expression levels of microtubule associated proteins (MAPs, Stathmin, Tau, etc…) play important roles in the resistance phenotype of cancer cells. For instance, an increase in stabilizing proteins or a decrease in destabilizing proteins lead to enhanced stability that counteracts the action of destabilizing drugs (Kavallaris et al. 2001; Don et al. 2004). In other cases, an increase in expression levels of these proteins reduces the ability of drugs to reach the binding sites on the wall of the microtubule and therefore decrease the response to treatment in cancer cells (Rouzier et al. 2005). Resistance to antimicrotubule agents is multifactorial and the clinical relevance of various mechanisms remains to be unraveled.

2.6. Microtubules, apoptosis and cancers

Regulation of microtubule dynamics is crucial for mitosis, cell migration, cell signaling and transport. Microtubule-targeted drugs (MTDs) constitute a major anticancer drug family with antimitotic and antiangiogenic properties and can inhibit tumor progression through altering microtubule dynamics in both rapidly dividing tumor cells such as leukemias and lymphomas as well as in slow growing tumors such as breast, lung and ovary cancers (Honore et al. 2005). Anti-mitotic and anti-cancer activity of MTDs that are used at low but clinically relevant concentrations is largely due to the suppression of microtubule spindle dynamics rather than to their effects on the microtubule polymer mass (Jordan 2002; Honore et al. 2003; Kamath and Jordan 2003). Interestingly, tumor cells resistant to anti-mitotic agents because of their increased microtubule dynamics are still blocked at the metaphase/anaphase transition and display abnormal mitotic spindles in the absence of these agents. Resistant lung cancer cells, A549-T12 when grown with low paclitaxel concentrations,

recover a normal mitotic spindle with normal dynamics compatible with cellular functions and are able to proliferate normally (Goncalves et al. 2001). All this suggest that both excessively rapid dynamics and suppressed dynamics cause impaired mitotic spindle function and inhibit cell proliferation.

MTDs are potent inducers of apoptosis, programmed and tightly regulated cell death which is insufficiently activated in cancer cells. After MTDs inhibit microtubule dynamics, the spindle checkpoint keeps APC/C inactive and prevents cyclin B destruction. Therefore the cell cannot get out of mitosis and the passage through the cell cycle is altered (Rieder and Maiato 2004). MTDs can either cause mitotic arrest followed directly by apoptosis or preceded by exit from mitosis into a multinucleate interphase or a blockage in G0 phase (Wang et al. 2000). The slowing or complete block in mitosis, the occurrence of apoptosis and the pathways involved in triggering apoptosis differ from the type and concentrations of the MTD and depend on the cancer cell line. Some breast cancer cell lines are blocked in mitosis when exposed to high concentrations of nocodazole and vincristine whereas others are arrested in G1 and G2 (Blajeski et al. 2002). In colon adenocarcinoma, primary HT29-D4 cells treated with paclitaxel are driven to apoptosis after mitotic arrest unlike the differentiated HT29-D4 cells that need very high concentrations of paclitaxel and are driven to apoptosis without any effect on the mitotic spindle or any mitotic block (Carles et al. 1999).

Although MTDs differently disturb microtubule network integrity, most of these agents trigger similar molecular mechanisms to promote apoptosis of tumor cells. Apoptosis is induced through two main signaling pathways: the extrinsic pathway also called the death receptor pathway and the intrinsic pathway independent of receptors and triggered for instance by ultraviolet radiation, chemotherapeutic drugs, viruses, bacteria or growth factors (Strasser 1995). The extrinsic pathway involves the formation of death-inducing signaling complex (DISC) which activates caspases-8 and apoptotic signaling pathways and is observed in apoptosis induced by doxorubicin in neuroblastoma, for example (Fulda et al.

1998). MTDs do not activate this latter pathway even though they can modify the level of expression of death receptors and their ligands (Kim et al. 2003).

MTD cytotoxicity involves the intrinsic pathway in which cellular apoptotic signals converge on the mitochondria with a release of apoptogenic proteins through an increase in mitochondrial membrane permeability. In addition, the intrinsic pathway involves members of the Bcl-2 family proteins and is controlled by the ratio of proapoptotic/antiapoptotic proteins. Apoptosis is then triggered either through caspase activations or in a caspase-independent manner. The expression levels of Bcl-2-like proteins in cancer cells are important factors to predict response to treatments since overexpression of these proteins suppress apoptosis induced by MTDs (Ibrado et al. 1997). It is widely believed that after microtubule disruption by MTDs, antiapoptotic proteins become hyperphosphorylated and lose their ability to interact with proapoptic proteins therefore increase their levels in the cells and lead to apoptosis (Salah-Eldin et al. 2003). Hyperphosphorylation of Bcl-2 occurs in normally cycling cells at the G2/M phase but interestingly when the MTDs concentration is high enough to cause a long duration and irreversible block, the mitosis-associated signals can distinguish the preapoptotic stage of the cell from its normal cycling one. The persisting hyperphosphorylation of Bcl-2 is thus considered to constitute an apoptotic signal (Fan et al. 2000).

Upstream of mitochondria, microtubule disruption could release microtubule-sequestered factors such as Bim and survivin. Bim, a proapoptotic protein, is freed and translocated to mitochondria where it neutralizes Bcl-2 (antiapoptotic) or activates Bax (proapoptotic) after treatment with paclitaxel (Puthalakath et al. 1999; Li et al. 2005). An apoptosis inhibitor (IAP), survivin, associates with microtubules and participates in mitotic spindle function. Disruption of survivin–microtubule interactions results in loss of survivin's anti-apoptosis function and increased caspase-3 activity, a mechanism involved in cell death, during mitosis (Li et al. 1998). Survivin localizes with centrosomes during interphase then moves to centromeres during mitosis. In paclitaxel treated cells, it relocalizes to α-tubulin in

microtubules both in interphase and mitosis (Jiang et al. 2001) and is induced as an early event independent of G2/M arrest (Ling et al. 2004). Expression of survivin gene correlates with paclitaxel resistance in human ovarian cancer (Zaffaroni et al. 2002) and forced expression of survivin in epitheloid carcinoma cells influences microtubule dynamics and stabilizes microtubules against nocodazole-induced depolymerization (Giodini et al. 2002). Survivin induction is thought to be the mechanism by which cancer cells evade apoptosis. In conclusion, microtubule-regulating proteins and proteins sequestered or transported by microtubules are likely to be important factors linking the suppression of microtubule dynamics and induction of apoptosis.

PURPOSE OF THE THESIS

Cell behavior and function depend to a large extent on the cytoskeleton that consists of three interconnected filament systems: intermediate filaments (IFs), actin-containing microfilaments (MFs) and microtubules (MTs). Microtubules are crucial structures for living cells as they are involved in many biological functions including cell motility, cell division, intracellular transport, cellular architecture as well as other cell types specific functions. Their dynamic property involved in cell division makes out of microtubules major therapeutic targets for anticancer drugs in treatment of breast cancer. Microtubules are composed of αβ tubulin heterodimers arranged to form polar hollow cylinders. The folding pathway of α and β tubulins involves many tubulin binding cofactors (A to E). Tubulin binding cofactor C (TBCC) is crucial to obtain the αβ tubulin heterodimers that polymerize into microtubules. Since little is known about this protein with respect to breast cancer, we were interested in studying the impact of alterations in TBCC expression on the phenotype, microtubule content and dynamics as well as on the response to chemotherapy in breast cancer cells.

First, we established stable transfectants with different expression levels of the protein TBCC by transfection of MCF7 cells with vector either expressing the sens of *TBCC* or the antisens of *TBCC*. These cells were named MC- for the cells with low TBCC level, MC+ for the cells with high TBCC levels and MP6 for the control cells. Second, these cells were characterized both *in vitro* and *in vivo*. We found that the cells with low TBCC level acquired a higher proliferative capacity both *in vivo* and *in vitro* whereas the ones overexpressing TBCC were less proliferative than the control cells and had a lower tumor growth capacity. The alterations in TBCC expression levels influenced the distribution in the cell cycle and TBCC overexpression was accompanied by an increase in percentage of cells in the G2/M phase of the cell cycle and a lengthening of mitosis. In parallel, the cells with reduced TBCC expression level presented a higher percentage of cells in the S-phase of the cell cycle and short durations of both mitosis and S-phase. Alterations in TBCC had major impact on the

content and dynamics of microtubules. The cells overexpressing TBCC presented high content in the nonpolymerizable fraction of tubulins and lower fractions of both the polymerizable and the microtubule fractions of tubulins as well as decreased microtubule dynamicity. The cells with low TBCC level presented low content in nonpolymerizable fraction of tubulins along with high contents in polymerizable and microtubule fractions of tubulins as well as high microtubule dynamicity. The influence that TBCC had on microtubules is through its role in the folding pathway where it has influenced the formation of native heterodimers of tubulins and consequently influences the dynamic instability of microtubules. The modified dynamicity of microtubules affected the sequence of both mitotic and S-phase. Finally, the new distribution into the cell cycle provided new prolife of response to treatments that target different stages of the cell cycle. The cells overexpressing TBCC presented enhanced chemosensivity to antimicrotubule agents, paclitaxel and vinorelbine, that target the mitotic phase of the cell cycle. The cells with low TBCC levels were highly sensitive to the S-phase targeting agent, gemcitabine without acquiring resistance to antimicrotubule agents.

These results underline the importance of the microtubular network in the tumor cell aggressivity phenotype and response to treatments. While currently available compounds mostly target microtubule dynamics, another possibility could be to alter tubulin pools in tumor cells. Another perspective for these results would be to look for potential partners of TBCC. It would be interesting to investigate if TBCC interacts or binds with microtubule binding proteins and to try to synthesize a ligand that can stabilize this protein inside the cytoplasm so that it would be continuously active.

MATERIALS AND METHODS

1. CELLULAR TOOLS AND REAGENTS

1.1. Cell Culture

1.1.1. Cellular Model: Human Breast Adenocarcinoma (MCF7)

MCF7 (human breast adenocarcinoma) is a breast cancer cell line isolated in 1973 from a 69 year old Caucasian woman. MCF-7 stands for Michigan Cancer Foundation-7, the institute in Detroit where the cell line was established in 1973 by Herbert Soule and colleagues. MCF7 and two other breast cancer cell lines, T47D and MDAMB-231, account for more than 2/3 of all abstracts reporting studies on breast cancer cell lines. MCF7 retained several characteristics of differentiated mammary epithelium including the ability to process estradiol via cytoplasmic estrogen receptors and the ability of forming domes in 3D growth.

Characteristics of MCF7 cells

MCF7 cells were established from a pleural effusion. They belong to the invasive ductal carcinoma IDC type of breast cancer. These cells are estrogen receptor-positive (ER+) and progesterone receptor-positive (PgR+). MCF7 cells contain wild type p53 but are caspase-3 deficient (Janicke et al. 1998). Tumor necrosis factor alpha (TNF alpha) inhibits the growth of MCF7 cells. Treatment with anti-estrogens can modulate the secretion of insulin-like growth factor binding proteins. Cytogenetic analysis revealed a modal chromosomal number of 82 that ranges from 66 to 87 (from hypertriploidy to hypotetraploidy). MCF7 cells lack chromosome 20. MCF7 cells are pseudo-circular adherent cells of epithelial morphology. They grow as clusters of small individual cells.

1.1.2. Culture conditions

MCF7 cells were grown in DMEM supplemented with penicillin (200 UI/ml), streptomycin (200 µg/ml) and fetal bovine serum (10%) at 37°C in a humidified atmosphere containing 5% CO_2. The stable transfectants obtained through blasticidin selection (20 µg/ml) (KN-1004, Euromedex, France) were grown in the above mentioned medium to which blasticidin was added once per week.

1.2. Reagents

Reagent	Supplier	Reagent	Supplier
Paclitaxel	Bristol-Myers Squibb New York, USA	Blasticidin	KN-1004, Euromedex France
Vinorelbine	Pierre Fabre médicaments Boulogne, France	Oligofectamine	Invitrogen, Cergy Pontoise, France
Gemcitabine	Lilly, IN USA	Paraformaldehyde	Merck Fontenay-sous-Bois, France
BrdU Assay 5-bromo-2-deoxyuridine	Roche Basel, Switzerland	DAPI diaminido-phenyl-indol	Roche Manheim, Germany
MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]	Sigma-Aldrich St Quentin Fallavier, France	Matrigel	BD Biosciences Belgium
PI Propidium Iodide	Interchim Montluçon, France	2' Deoxycytidine	Sigma-Aldrich St Quentin Fallavier, France
Trizol	Invitrogen Cergy Pontoise, France	Thymidine	Sigma-Aldrich St Quentin Fallavier, France
BrdU powder 5-bromo-2-deoxyuridine	B5002, Sigma Aldrich St Quentin Fallavier, France	Chloroform	Biosolve Netherlands

2. MOLECULAR BIOLOGY

Construction of sense and antisense TBCC vectors

2.1. mRNA extraction and reverse transcription

The mRNA was extracted from hTerT-HME-1 human mammary epithelium cells (from the American Type Culture Collection, ATCC). Cells were suspended in 1 ml of Trizol, vortexed and incubated for 5 min at room temperature. Then 200µl of chloroform were added and the tubes were vortexed and incubated 3 min at room temperature. After incubation, the tubes were centrifuged at 15,000 x g for 15 minutes at 4°C to separate the content into different layers. The superior aqueous layer was removed without touching the protein-containing interface and 500µl of isopropanol were added to it. The tube was mixed by inversion and incubated for at least one hour at -20°C. Later on, tubes were centrifuged at 15,000 x g for 15min at 4°C and the supernatant was discarded. The pellet was cleaned by resuspension in 1ml of 70% cold ethanol and centrifugation at 12,000 x g, for 5min at 4°C. The pellet was air dried for 15 to 30 minutes and resuspended in 20µl DEPC water (Euromedex, France).

2 µg of the extracted mRNA were reverse transcribed into cDNA in a reaction using Moloney leukaemia virus reverse transcriptase M-MLV RT (Invitrogen, Cergy Pontoise, France), 5X buffer, random primers (Invitrogen), 10mM dNTP (Invitrogen), and DTT (Dithiothreitol, Invitrogen) for 1 hour at 37°C.

2.2. PCR, Polymerase Chain Reaction

The cDNA obtained was then amplified by the full length *TBCC* forward GCCAATATGGAGTCCGTCAG and reverse CAACTGCTTAGTCCCACTGGA primers using a high fidelity polymerase according to the manufacturer's instructions (Jena

Bioscience, Germany). The PCR conditions were 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min.

2.3. Cloning of human *TBCC* cDNA

The amplicon was run on 1% agarose gel and the 1 kbps DNA band corresponding to *TBCC* was cut with the gel. The DNA was purified from the agarose gel using the GENECLEAN® II kit (Qbiogene, Inc., Carlsbad, California). *TBCC* was then subcloned into pGEM®-TEasy (Promega, France) and digested with EcoRI (Fermentas, France) to acquire the EcoRI restriction sites. The digested fragment was the cloned in pcDNA6/ V5-His A plasmid (Invitrogen) that was also digested with EcoRI. At this level, the *TBCC* insert could have been ligated either in the sense or antisense orientation into the pcDNA6/ V5-His A.

In order to obtain the sense and the antisense *TBCC* plasmids, we transformed DH5α competent bacteria (Invitrogen, Gibco) with the ligation products and spread the bacteria on LB agar (+ 100µg/ml ampicillin). The colonies obtained were then amplified and plasmids were extracted by minipreps using the NucleoSpin kit (Macherey-Nagel). The plasmids were then digested using PvuI (Fermentas, France) and BamHI (Fermentas, France) restriction enzymes. The orientation of the insert was determined based on the fragment sizes. The plasmid containing the insert in the sense orientation was cut into 895, 1746, 2095 and 1485 bps fragments whereas the antisens plasmid was cut into 225, 2416, 2095 and 1485 bps fragments. The sense and antisense plasmids were sent to Genome Express (France) for sequencing. The sequencing results confirmed the suspected orientations and that *TBCC* was devoid of any mutation as compared to the sequence NM_003192. These two plasmids were named pcDNA6/C+ for the sense and pcDNA6/C- for the antisense.

Expression level of *TBCC* in breast cancer cell lines

2.4. Quantitative Real Time- PCR

In order to study the expression level of *TBCC* in different breast cancer cell lines, cDNAs from MCF7, HME, UACC812, MDAMB361, MDAMB453, MDAMB436, BT20, HS578T, MDAMB157, CAL51, HBL100, T47D, BT474 and MDAMB231 were generously provided by Centre Léon Bérard. The cDNA levels were normalized to the expression of 18S ribosomal gene using the pre-developed TaqMan assay reagents control kit (Applied biosystem, Foster City, Canada) with denaturation/annealing at 95 °C for 15 seconds and extension at 60 °C for 1 minute. The cDNA mentioned above were mixed with *TBCC* forward (CAAGAAGGCGGAAGGAGAC) and reverse (GGGACTCCAGGTTGGAGAA) primers (300 nM each), $MgCl_2$ (2 mM), deoxynucleotide triphosphates (500 µM) and LC-FastStart DNA Master SyBRGreen (0.67 µl) (Roche, Meylan, France). PCR conditions were 50 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds and extension at 72 °C for 10 seconds. Results were analyzed using Relquant software (Roche, Meylan, France) as indicated in the user's manual and the results were expressed in arbitrary units using the HME cells as reference.

3. CELL BIOLOGY

3.1. Transient transfections of small interfering RNAs (siRNAs)

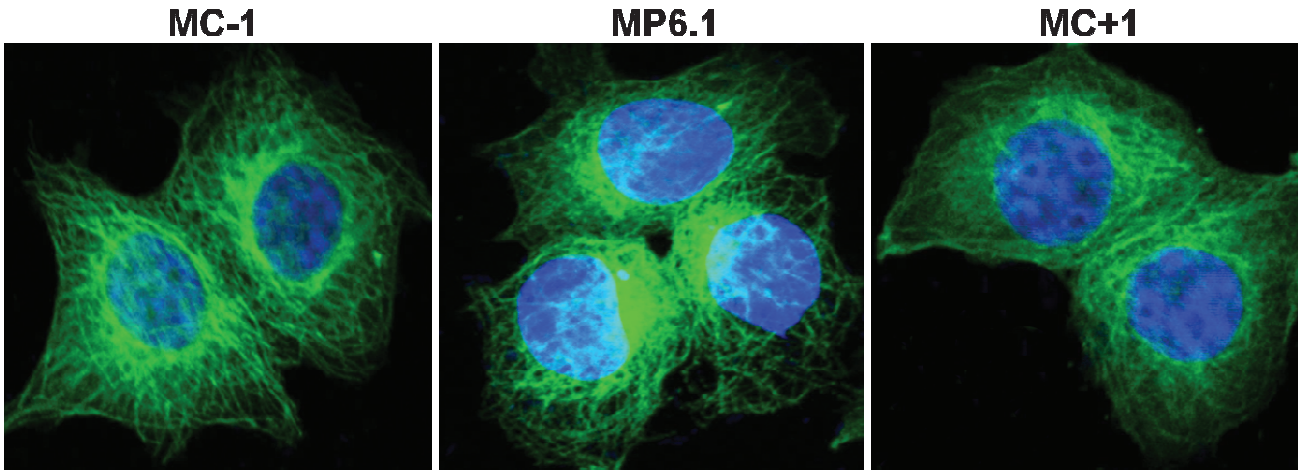
A desalted duplex siRNA targeting TBCC 5'-CUGAGCAACUGCACGGUCA-3' and its corresponding scrambled sequence were designed by Sigma-Aldrich (St Quentin Fallavier, France). The siRNAs (200 nM) were transfected into 25 x 10^4 of MCF7, MP6.1 or MC+1 cells using oligofectamine (Invitrogen, Cergy Pontoise, France) according to the manufacturer's protocol on two consecutive days. Protein analyses by western blot or flow cytometry experiment were done on the third day after transfection.

3.2. Stable transfections

MCF7 (human mammary adenocarcinoma, ATCC) cells were transfected with empty pcDNA6, pcDNA6/C+ or pcDNA6/C- using lipofectin (Invitrogen, Cergy Pontoise, France) following the manufacturer's instructions. The stable transfectants obtained through blasticidin selection (20 μg/ml) (KN-1004, Euromedex, France) were named MP6 (pcDNA6), MC+ (pcDNA6/C+) and MC- (pcDNA6/C-). Cloning of the each of the batch populations was performed as mentioned in the following paragraph.

3.3. Clones of *TBCC* variants

The populations of MP6 (MCF7 + empty plasmid pcDNA6/ V5-His A), MC+ (MCF7 + sens TBCC plasmid) and MC- (MCF7 + antisens TBCC plasmid) were seeded in a 96-well plate in a dilution of 0.1 cell/well. After one week of culture, the wells containing only one cell were labeled and monitored until they formed a clone. 3 clones of MP6, 7 clones of MC+ and 7 clones of MC- were retained; wells were trypsinated and seeded again in a 24-well plate each. Clones were gradually transferred from 24-well plate to 12-well, 6-well until T25 flasks, with continuous selection with blasticidin. Finally, the expression levels of TBCC in the MC+ and MC- cells were compared to the 3 clones of MP6 by western blot. The 3 control clones of MP6 were randomly named MP6.1, MP6.2 and MP6.3. 3 clones of MC+ significantly overexpressed TBCC with respect to the 3 control clones and were named MC+1, MC+2 and MC+3 , from the highest to the lowest difference in expression. 3 clones of MC- were characterized with significant lower TBCC levels with respect to the control clones and were named MC-1, MC-2 and MC-3.



Double staining (Green, β tubulin-FITC; Blue, DAPI)

3.4. Protein extraction and western blot analysis

Proteins were extracted using a lysis buffer (20 mM Tris-Hcl pH 6.8, 1 mM MgCL2, 2 mM EGTA, 0.5% Nonidet P40) to which 0.01 % of protease inhibitors (STI, leupeptin, aprotinin, benzamidin, PMSF and TPCK) were added. The lysate was put on ice for 1 hour then centrifuged at 12,000 x g for 15 min at 4°C. The supernatant was then removed and proteins were quantified by the Bradford assay (Coomassie reagent) using the bovine serum albumin as a protein concentration standard. 100 µg of proteins were then run on 12 % acrylamide gel and then transferred on a PVDF membrane using the program 3 of the iBlot dry blotting system (Life technology Invitrogen, Cergy Pontoise, France). After incubation with the primary and secondary antibodies, signal was detected by chemiluminescence assay (ECL Amersham Life Science UK). Densitometric quantification was performed with Image J (NIH, USA) software and the values were normalized against the β-actin expression.

The primary antibodies used were anti β III-tubulin (clone Tuj1, 1/2500; Covalab, Lyon, France), anti p53 (clone DO7, 1/1000; Dako, Denmark), anti cyclin E (CC05, 1/500, Calbiochem Oncogene), anti cyclin B1 (sc-245, 1/500, Santa Cruz Biotechnology), anti p21 (sc-397, 1/500, Santa Cruz Biotechnology), anti Cdk2 (sc-748, 1/500, Santa Cruz Biotechnology), anti Cdc2 p34 (sc-56261, 1/500, Santa Cruz Biotechnology), anti β-actin (clone AC-15, 1/5000), anti α-tubulin (clone DM1A, 1/1000), anti β-tubulin (clone 2.1,

1/1000), anti tyrosinated α-tubulin (clone TUB-1A2, 1/1000) and acetylated α-tubulin (clone 6-11B-1, 1/1000) from Sigma Aldrich (St Quentin Fallavier, France). The polyclonal antibodies against TBCC (1/800) and TBCD (1/3000) were generously provided by N. Cowan (New York University Medial Center, USA) and those against Arl2 (1/1000) and Glu-tubulin (1/1000) were generously provided by R. Kahn (Emory University School of Medicine, Atlanta, USA) and L. Lafanechère (Centre de Criblage pour des Molécules Bio-Actives, Grenoble), respectively.

All primary antibodies were incubated overnight at 4°C except for the β-actin that was incubated only one hour. Secondary antibodies were anti-mouse (1/5000, Sigma Aldrich, St Quentin Fallavier, France) and anti-rabbit (Caltag Laboratories, Burlingame, CA) and were incubated 1 hour at room temperature. The anti-rabbit secondary antibody used is a biotinylated one so the membranes were incubated with streptavidin for 1 hour.

3.5. Proliferation tests: BrdU and MTT

Cell proliferation was estimated using MTT and BrdU assay. For the MTT tests, cells (13×10^4) were seeded in a 6-well plate and incubated at 37 °C. Every 24 h and for one week, MTT (3 mg) was added to each well of each plate. After 2 hrs of incubation at 37 °C, supernatants were removed, formazan crystals solubilized with 3 ml of isopropanol-HCl-H_2O (90:1:9, v/v/v) and plates scanned. The absorbance was measured spectrophotometrically with a microplate reader (Labsystem Multiskanner RC) and the MTT values were obtained as subtraction of absorbances read at 540 and 690 nm.

The non-radioactive BrdU–based cell proliferation assay (Roche, Basel, Switzerland) was performed according to the manufacturer's protocol. Exposed and unexposed cells (5×10^3 cells per well) were seeded in a 96-well plastic plate and the assay was performed after 48, 72, 96 and 168 hours. Cells were exposed to either 0.5 nM, 1 nM or 3 nM of gemcitabine (Lilly, IN, USA) for one week. BrdU incorporation into the DNA was determined by measuring the absorbance at 450 on an ELISA plate reader.

3.6. Flow cytometry analysis of the cell cycle

Cells were incubated 24 h with either 10 nM paclitaxel (Bristol-Myers Squibb, New York, USA) or 1 nM vinorelbine (Pierre Fabre medicaments, Boulogne, France). Exposed and unexposed cells were then collected and incubated 1 hour at 4 °C with propidium iodide (0.05 mg/ml) solution containing Nonidet- P40 (0.05%). Cells were analyzed using a FACS Calibur flow cytomoter (BD Biosciences Europe, Erembodegem, Belgium) and cell cycle distribution was determined using Modfit LT 2.0 ^{TM} software (Veritysoftware Inc, Topsham, USA). For the siRNA-transfected clones MP6.1 and MC+1, the cell cycle was studied 48 h after the first transfection.

S-phase synchronized cells were obtained by a double thymidine block. Cells were blocked for 17 hours with 2mM of thymidine then washed twice with PBS to remove all traces of thymidine. Cells were released with 24 μM of deoxycytidine for 9 hours then washed twice with PBS and blocked again in thymidine for 17 hours. After that, cells were released again with deoxycytidine and collected every 2 hours to be analyzed by flow cytometry.

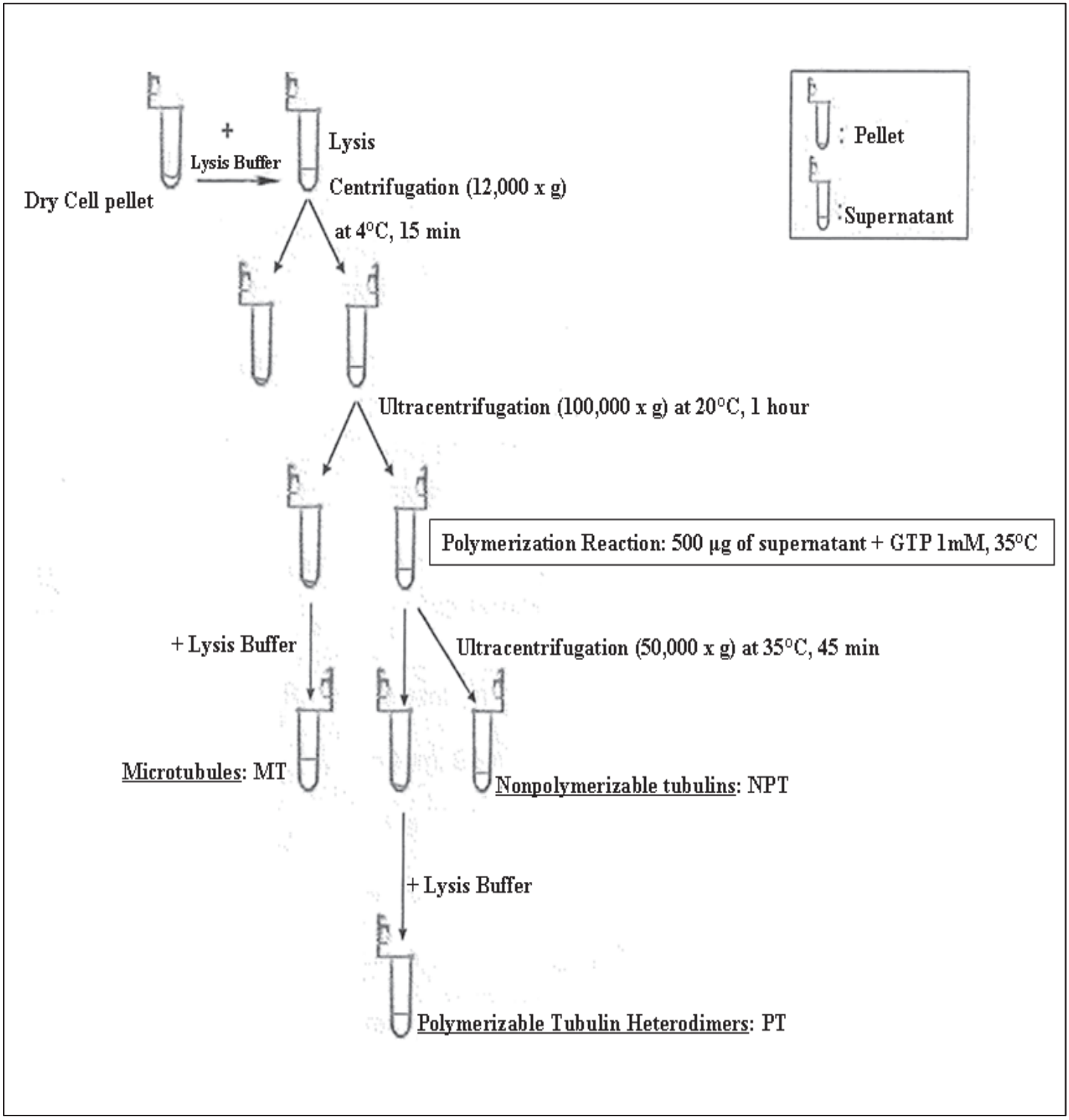
3.7. Detection of apoptosis by annexin-V staining

Apoptotic cells were detected using the Annexin-V-FLUOS Staining Kit (Roche Manheim, Germany). Briefly, cells were harvested, washed twice, labeled with fluorescein isothiocyanate (FITC)-Annexin-V and analyzed by flow cytometry as described by the manufacturer (Roche Manheim, Germany).

3.8. Separation and quantification of the different tubulin pools

Cells (20 x 10^6) were harvested and lysed in 200 μl of buffer (100 mM Pipes, pH 6.7, 1 mM EGTA, and 1 mM $MgSO_4$) by two freeze-thaw cycle. Lysed cells were centrifuged at 12,000 x g for 15 minutes at 4°C. The supernatant was then ultracentrifuged (100,000 x g for 1 h at 20°C) and separated into a supernatant containing “soluble tubulins” and a pellet

containing “microtubules”. The microtubule fraction was resuspended in 100 µl of lysis buffer and 100 µl of the supernatant (500 µg) were incubated with 1 mM of GTP at 35°C for 30 minutes to allow tubulin polymerization then ultracentrifuged at 50,000 x g for 45 minutes at 35°C. The resulting pellet contained the “polymerizable tubulin” (PT) heterodimers and the supernatant contained “nonpolymerizable tubulin” (NPT) heterodimers, included tubulin peptides complexed with tubulin binding cofactors. The different fractions of tubulins were run on silver stained gels following manufacturer’s recommendations (Amersham Biosciences AB, Sweden). After coloration, the single band observed at 55 kDa for the polymerizable tubulin heterodimers confirmed the success of purification (data not shown). The experiment was performed in triplicate using MP6.1, MC- and MC+1. Western blots were then done for α and β tubulins and densitometric quantification of western blots was performed with ImageJ software (NIH, USA). The procedure of purification is illustrated in the following figure.



Procedure for purification of different tubulin fractions: NPT nonpolymerizable, PT polymerizable, MT microtubule

4. MICROSCOPIC ANALYSIS

4.1. Analysis of microtubule dynamics

4.1.1. Transient tansfection of cells with pAcGFP1-Tubulin

Cells (3 x 105) were seeded in 6-well plate with circular glasses of 24 mm in their bottom and transfected with the pAcGFP1-tubulin vector (Clontech) using lipofectin (Invitrogen) following the manufacturer's instructions.

4.1.2. Time-lapse microscopy

After 48 hours, the glasses containing the cells were placed in culture medium maintained at 37 °C in a 5% CO_2 atmosphere in a time lapse inverted microscope (Olympus IX50) at the Centre Commun de Quantimétrie (Lyon, Université Claude Bernard, France). Cells were imaged with a numerical CFW-1308M 1360X1024 camera (Scion, Frederick, USA) driven by imageJ software (NIH, Bethesda, USA) using a 40x oil immersion lens (Zeiss, Göttingen, Germany). 30 pictures of the cell's microtubules were taken at 4 seconds intervals. The positions of the plus-ends of individual MT in peripheral lamellar regions of cells were tracked over time using ImageJ® software and graphed using Microsoft® Excel spreadsheet as position versus time to generate a 'life-history plot' for each MT. Growth and shortening rates and durations were derived by regression analysis. A difference of >0.5 μm between any two consecutive points was considered as a growth or shortening event. Transitions into depolymerisation or shortening are termed catastrophes, and transitions from shortening to growth or pause are called rescue. The catastrophe and rescue frequencies per unit time were calculated by dividing respectively the number of transitions from growth and pause to shortening and the number of transitions form shortening and pause to growth by the sum of the time in growth and pause. Dynamicity represents total tubulin exchange at the MT end and was calculated by dividing the sum of total length grown and shortened by the MT

life span. The experiment was performed twice on 50 microtubules of the clones MP6.1, MC-1 and MC+1.

4.2. Mitosis duration analysis

Cells (3 x 10^5) were seeded in a 35 mm cell culture dish, placed in culture medium maintained at 37°C in a 5% CO_2 atmosphere and observed using an inverted time lapse microscope (Olympus IX50) at the Centre Commun de Quantimétrie (Université Claude Bernard Lyon, France). Images were acquired every 2 minutes for 24 hours using a numerical CFW-1308M 1360X1024 camera (Scion, Frederick, USA) driven by ImageJ software (NIH, Bethesda, USA). 30 complete mitoses were analysed for each of the MP6.1, MC- and MC+1 clones using ImageJ software (NIH, Bethesda, USA).

4.3. Short pulse BrdU incubation

Cells were incubated with 10 μM of BrdU (B5002, Sigma Aldrich) for 5, 15, 30 and 60 minutes without washing cells prior to incubation because this will slow down the growth of the cells during the incorporation phase of the procedure. Slides with these cells were then prepared using cytospin4 by Thermo Scientific. Cells were then labelled with anti-BrdU (347583, Becton Dickinson) and propidium iodide for microscopic analysis following the manufacturer's instructions.

4.4. Immunofluorescence using confocal fluorescent microscopes

Cells were fixed by 4% paraformaldehyde during 15 minutes at room temperature and permeabilized using a PBS-Triton X-100 0.1% solution. Non specific sites were blocked using a solution containing 0.1% bovine serum albumin and 1% fetal calf serum. Cells were incubated with either a 1:100 of an antibody against β-tubulin (clone 2.1, Sigma Aldrich), a 1:30 dilution of a monoclonal antibody against TBCC (Abnova, Taiwan) or a 1:50 dilution of

a monoclonal antibody against γ-tubulin (clone GTU-88, Sigma Aldrich) followed by a secondary FITC-antibody (Dako, Denmark). DNA staining was performed using diaminido-phenyl-indol (DAPI) (Roche, Manheim, Germany).

Images were obtained using a laser scanning confocal TCS Sp2 DMRXA microscope x63 objective (Leica Microsystems; Wetzlar, Germany) at the Centre Commun de Quantimétrie (Université Claude Bernard Lyon, France).

5. IN VIVO STUDIES

Tumor growth and response to treatment

Female CB17/SCID mice purchased from Charles River Laboratories (Arbresle, France) were bred under pathogen-free conditions at the animal facility of our institute. Animals were treated in accordance with the European Union guidelines and French laws for the laboratory animal care and use. The animals were kept in conventional housing. Access to food and water was not restricted. All mice used were 5 to 6 weeks old at the time of cells injections. This study was approved by the local animal ethical committee. Mice were divided into nine groups of six mice each which corresponds to the injections of MP6.1, MP6.2, MP6.3, MC-1, MC-2, MC-3, MC+1, MC+2 and MC+3 cells. 3 x 10^6 cells were injected subcutaneously in mice with 50 % matrigel (BD Biosciences, Belgium). The six mice were divided into two groups of treated and untreated mice. In the treated groups, paclitaxel was injected intraperitoneally in a dose of 10 mg/kg on the same day and a week after. Mice were weighed and the tumor size was measured twice per week with an electronic caliper. The volume was then computed by considering the tumor as a sphere with the formula 4/3 (3.14 x r^3), r as the mean radius. Animals were euthanized either when one of the diameters of the tumor exceeded 17 mm, or if any potential suffering of the animal was observed or if weight loss exceeded 10 %.

Western blot analysis of tumors

Tumors were collected from different clones and divided into three samples MP6, MC- and MC+. Protein extraction was done by suspending tumors in lysis buffer and sonicating them. Western blot was done as described in the section 3.3.

PERSONAL RESULTS

PART 1

Development of variants of breast cancer cells expressing tubulin binding cofactor C

DEVELOPMENT OF VARIANTS OF BREAST CANCER CELLS EXPRESSING TUBULIN BINDING COFACTOR C

1.1. Introduction

In order to study the impact of modifying TBCC expression level on breast cancer cells, we used the MCF7 cells as a model and established transfectants with high levels of TBCC and others with low levels of TBCC as compared to MCF7 cells transfected with the control plasmid.

1.2. Results

1.2.1. Establishment of control MP6, MC+ and MC- populations

The empty pcDNA6, pcDNA6/C+ and pcDNA6/C- were stably transfected in MCF7 cells and the selected populations were tested for TBCC expression level by western blot. The population of cells transfected with the antisens of *TBCC* (MC-) presented a lower TBCC protein expression level than the control population (MP6) with a decrease of 30 %. The population of cells transfected with the sens of *TBCC* (MC+) presented a higher TBCC protein expression level than the control population (MP6) with an increase of 20 % (figure1).

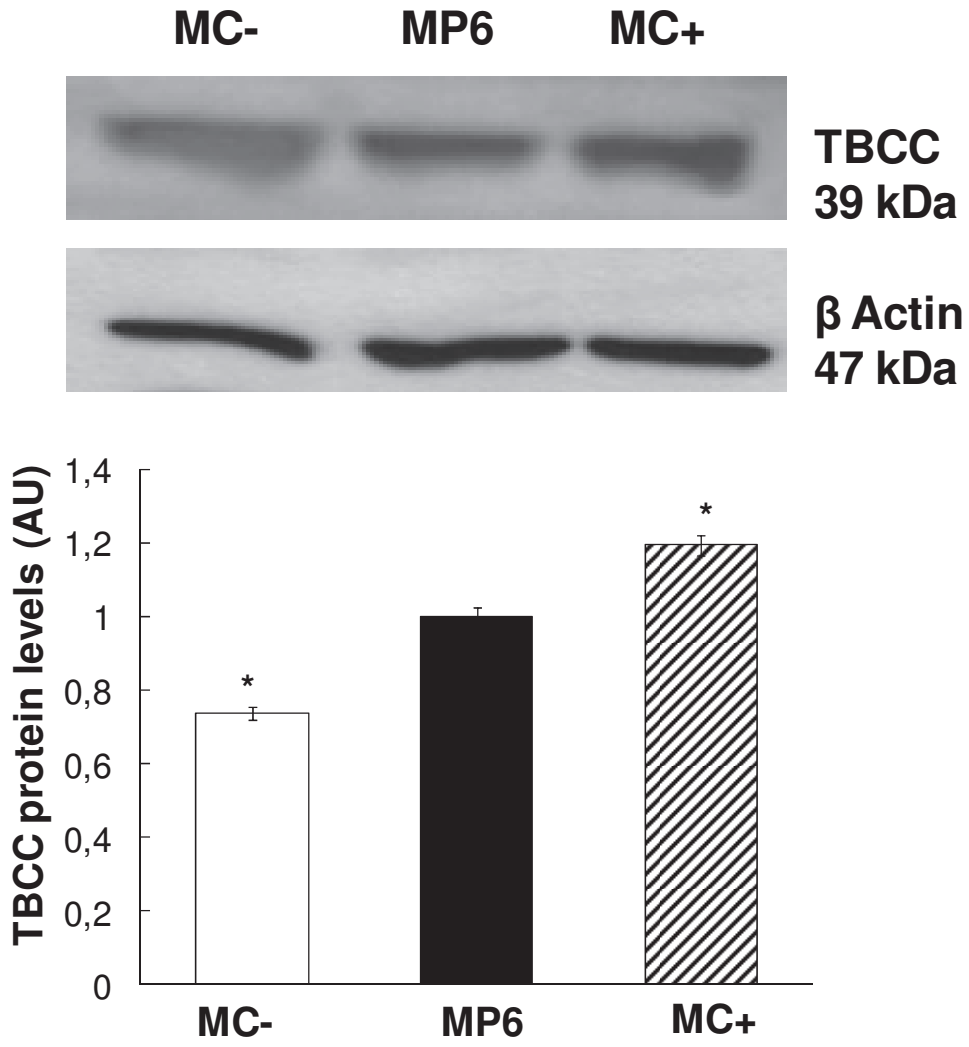
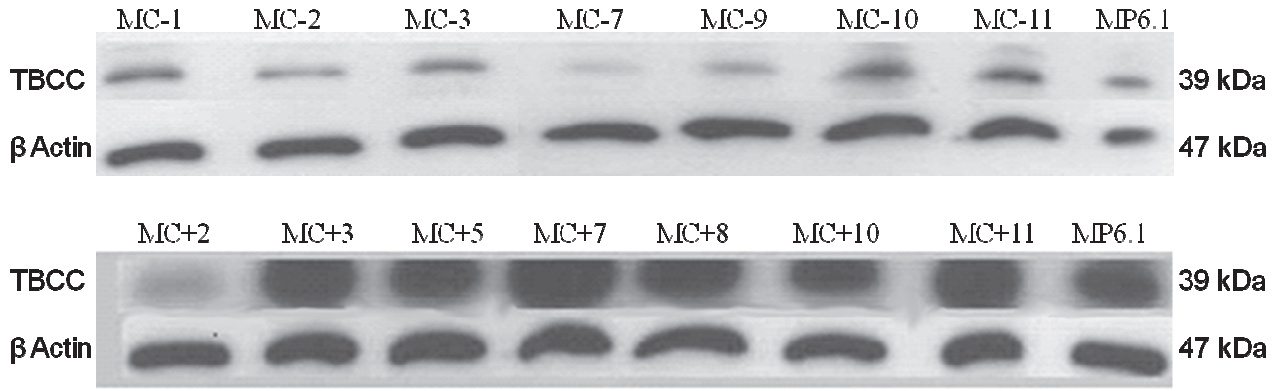


Figure 1. Expression level of TBCC in the stable transfectants of MCF7 cells
Western blot analysis of total cell extracts of MC- (MCF7 transfected with pcDNA6/C-), MC+ (MCF7 transfected with pcDNA6/C+) and MP6 (MCF7 transfected with empty) (Upper panel). TBCC protein levels quantified by ImageJ software (lower panel). Results presented are the average values of three western blots. Bars represent standard deviation.*: Values differ significantly from MP6 at ≥ 95% confidence level by Student's t-test.

1.2.2. Expression level of TBCC in the different clones

After cloning the MP6, MC- and MC+ populations, 7 clones of MC+ and MC- were tested for TBCC expression levels as compared to TBCC expression in one of the MP6 clones. At first the seven clones were numbered randomly. Then, after characterizing the expression levels of TBCC, we selected three MC- and three MC+ clones and named them 1,

2 and 3: MC-1, MC-2 and MC-3 from the lower TBCC level to the highest and MC+1 , MC+2 and MC+3 from the highest TBCC level to the lowest. The modification of nomenclature was for simplification purpose only. Three control clones were selected randomly and named MP6.1, MP6.2 and MP6.3 (figure 2). The 9 clones issued will be described and studied in the following results sections.



Clones	Preliminary nomenclature	Nomenclature based on TBCC expression		Preliminary nomenclature	Nomenclature based on TBCC expression	
	MC-7	MC-1	Least	MC+7	MC+1	Highest
	MC-2	MC-2	to	MC+8	MC+2	to
	MC-9	MC-3	Highest	MC+11	MC+3	Least

Figure 2. Expression level of TBCC in the clones of MC-, MC+ and MP6. TBCC protein levels were determined as compared to one clone MP6.1 of the control cells. The initial nomenclature was a preliminary one given randomly to the clones then it was modified based on the expression levels. The order 1, 2 and 3 was given based on the differential expression levels of TBCC as shown in the table.

1.3. Conclusion

The 9 clones MP6.1, MP6.2, MP6.3, MC-1, MC-2, MC-3, MC+1, MC+2 and MC+3 were used for further characterization and will be developed in the coning sections. We must insist however on the fairly low differences in TBCC content (30% decrease in MC- clones and 20% increase in MC+ clones) observed between transfected cells and controls, suggesting that higher levels in MC+ or lower levels in MC- clones of TBCC expression may be incompatible with cell survival.

PART 2 ARTICLE 1

Tubulin Binding Cofactor C (TBCC) suppresses tumor growth and enhances chemosensitivity in human breast cancer cells.

(Hage-Sleiman et al. *BMC Cancer* 2010, **10**:135)

Tubulin Binding Cofactor C (TBCC) suppresses tumor growth and enhances chemosensitivity in human breast cancer cells.

Objective of this study

Since tubulin binding cofactor (TBCC) is crucial for the proper folding of tubulins and their polymerization into microtubules and since little is known about this protein with respect to breast cancer, we were interested in studying the impact of TBCC overexpression on the phenotype of tumor cells as well as on microtubule content and dynamics and response to antimicrotubule drugs. We have shown that TBCC overexpression decreased the content in polymerizable tubulins and microtubules as well as the microtubule dynamics. These modifications influenced cell cycle distribution of the MCF7 cells. We observed a higher percentage of cells in G2-M phase with a shorter mitosis and an enhanced sensitivity to antimicrotubule agents.

Hage-Sleiman et al. BMC Cancer 2010, 10:135
http://www.biomedcentral.com/1471-2407/10/135

BMC Cancer

RESEARCH ARTICLE Open Access

Tubulin binding cofactor C (TBCC) suppresses tumor growth and enhances chemosensitivity in human breast cancer cells

Rouba Hage-Sleiman*, Stéphanie Herveau, Eva-Laure Matera, Jean-Fabien Laurier, Charles Dumontet

Abstract

Background: Microtubules are considered major therapeutic targets in patients with breast cancer. In spite of their essential role in biological functions including cell motility, cell division and intracellular transport, microtubules have not yet been considered as critical actors influencing tumor cell aggressivity. To evaluate the impact of microtubule mass and dynamics on the phenotype and sensitivity of breast cancer cells, we have targeted tubulin binding cofactor C (TBCC), a crucial protein for the proper folding of α and β tubulins into polymerization-competent tubulin heterodimers.

Methods: We developed variants of human breast cancer cells with increased content of TBCC. Analysis of proliferation, cell cycle distribution and mitotic durations were assayed to investigate the influence of TBCC on the cell phenotype. *In vivo* growth of tumors was monitored in mice xenografted with breast cancer cells. The microtubule dynamics and the different fractions of tubulins were studied by time-lapse microscopy and lysate fractionation, respectively. *In vitro* sensitivity to antimicrotubule agents was studied by flow cytometry. *In vivo* chemosensitivity was assayed by treatment of mice implanted with tumor cells.

Results: TBCC overexpression influenced tubulin fraction distribution, with higher content of nonpolymerizable tubulins and lower content of polymerizable dimers and microtubules. Microtubule dynamicity was reduced in cells overexpressing TBCC. Cell cycle distribution was altered in cells containing larger amounts of TBCC with higher percentage of cells in G2-M phase and lower percentage in S-phase, along with slower passage into mitosis. While increased content of TBCC had little effect on cell proliferation *in vitro*, we observed a significant delay in tumor growth with respect to controls when TBCC overexpressing cells were implanted as xenografts *in vivo*. TBCC overexpressing variants displayed enhanced sensitivity to antimicrotubule agents both *in vitro* and in xenografts.

Conclusion: These results underline the essential role of fine tuned regulation of tubulin content in tumor cells and the major impact of dysregulation of tubulin dimer content on tumor cell phenotype and response to chemotherapy. A better understanding of how the microtubule cytoskeleton is dysregulated in cancer cells would greatly contribute to a better understanding of tumor cell biology and characterisation of resistant phenotypes.

Background

Microtubules are crucial structures for living cells as they are involved in many biological functions including cell motility, cell division, intracellular transport, cellular architecture as well as other cell types specific functions [1]. Their dynamic property involved in cell division makes out of microtubules major targets for anticancer drugs. The drugs commonly used are divided into two main families of taxanes and vinca alkaloids which are known to suppress microtubule dynamics by stabilizing or destabilizing the microtubules respectively and thus inhibiting the metaphase anaphase transition, blocking mitosis and inducing apoptosis [2]. Taxanes and vinca alkaloids are among many drugs used to treat breast cancer [3]. However, resistance to anticancer drugs is appearing, inducing a need to understand and identify

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the mechanisms behind of it. One of the mechanisms responsible of the resistance phenotype is the alteration in the dynamic properties of microtubules. Microtubules have two main dynamic behaviors. First they exhibit a dynamic instability which consists of apparently random transitions between slow elongation and rapid shortening states [4]. Another important property is the treadmilling by which tubulin subunits continuously flux from one end of the polymer to the other, due to net differences in the critical subunit concentrations at the opposite microtubule ends [5].

Microtubules are made of α/β-tubulin heterodimers whose proper folding involves many chaperonins as well as protein cofactors [6,7]. After being synthesized, the tubulins are sequestered by cytosolic chaperonins for their correct folding and preparation for further interactions with the tubulin binding cofactors TBC [8,9]. The proper folding pathways of α-tubulin and β-tubulin into the dimers are interdependent and five TBC (TBCA to TBCE) are involved. TBCB and TBCA bind to α- and β-tubulins respectively and the formed complexes serve as reservoirs of tubulin peptides in the cytoplasm [10,11]. The α- and β-tubulins are then delivered to TBCE and TBCD respectively where they form a supercomplex with TBCC (TBCE/α-tubulin/TBCC/TBCD/β-tubulin). After hydrolysis of GTP by β-tubulin, this complex releases activated α/β-tubulin heterodimers which can readily polymerize into microtubules [10,12,13].

Little is known regarding the role of TBCs in cancer. It has been shown that the inhibition of TBCA in MCF7 and HeLa cells modified the microtubule structures, caused cell cycle arrest in G1 and cell death [14]. TBCB and TBCE have been found to physically interact and induce microtubule depolymerization *in vitro* [15]. In addition to their roles in the proper folding of microtubules, these cofactors might have other roles involving microtubules. It was recently shown that microtubules contribute to the mechanism of cell detachment through TBCD by transporting it to the cell membrane where it interacts with adherent and tight junctions [16]. It has been described that TBCD interacts with Arl2, ADP ribosylation factor like 2 (Arl2) which dissociates it from the α/β tubulin heterodimers [17]. A study in *Arabidopsis thaliana* showed that TBCC plays an important role in releasing competent α/β-tubulin polymerizable heterodimers [18,19]. Another study performed in X-linked retinitis pigmentosa 2 (RP2) showed that RP2 responsible for the progressive degeneration of the photoreceptor cells and TBCC have similar sequences. Both of these proteins were found to be activators of GTPases but only TBCC is capable of catalyzing the heterodimerization of tubulins [20].

Since TBCC is crucial for the proper folding of tubulins and their polymerization into microtubules and since little is known about this protein with respect to breast cancer, we were interested in studying the impact of TBCC overexpression on the phenotype of tumor cells as well as on microtubule content and dynamics and response to antimicrotubule drugs. We have found that overexpressing TBCC influenced cell cycle distribution of breast cancer cells in our model along with an increase in percentage of cells in G2-M phase of cell cycle and a slower mitosis. The dynamics of microtubules were reduced and the content of polymerizable tubulins was decreased. Finally, cells overexpressing TBCC were more sensitive to microtubules targeting agents both *in vivo* and *in vitro*.

Methods

Plasmid construction

The pcDNA6/V5-His A plasmid was used to clone the 1 kb human *TBCC* cDNA (NM_003192) extracted from hTerT-HME-1 human mammary epithelium cells (ATCC). The mRNA was extracted using Trizol reagent (Invitrogen, Cergy Pontoise, France) following the manufacturer's instructions. Reverse transcription into cDNA was then done using Moloney leukaemia virus reverse transcriptase (Invitrogen, Cergy Pontoise, France) for 1 hour at 37°C as described in the manufacturer's manual. The cDNA obtained was then amplified by the full length *TBCC* forward GCCAATATGGAGTCCGTCAG and reverse CAACTGCTTAGTCCCACTGGA primers using a high fidelity polymerase according to the manufacturer's instructions (Jena Bioscience, Germany). The PCR conditions were 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min. Purified amplicon was subcloned into pGEMTeasy and thereafter into pcDNA6 in the sense orientation (designated as pcDNA6/C+) using EcoRI (Fermentas, France).

Cell culture and transfections

MCF7 (human mammary adenocarcinoma, ATCC) cells and transfectants were grown in DMEM supplemented with penicillin (200 UI/ml), streptomycin (200 μg/ml) and fetal bovine serum (10%) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were transfected with pcDNA6/C+ or empty pcDNA6 using lipofectin (Invitrogen, Cergy Pontoise, France) following the manufacturer's instructions. The stable transfectants were obtained through blasticidin selection (20 μg/ml) (KN-1004, Euromedex, France). Cloning of the populations of cells was performed for each of the two batch populations and 3 clones representative of each population were selected for further characterization, on the basis of their differential levels of expression of the protein TBCC. The clones designated MC+1, MC+2 and MC+3 represent the clones overexpressing TBCC. The clones designated MP6.1, MP6.2 and MP6.3 represent the control clones.

Western blot analysis

Protein extraction and western blot analysis were performed as described previously [21]. The antibodies used were anti β III-tubulin (clone Tuj1, 1/2500; Covalab, Lyon, France), anti p53 (clone DO7, 1/1000; Dako, Denmark), anti β-actin (clone AC-15, 1/5000), anti α-tubulin (clone DM1A, 1/1000), anti β-tubulin (clone 2.1, 1/1000), anti tyrosinated α-tubulin (clone TUB-1A2, 1/1000) and acetylated α-tubulin (clone 6-11B-1, 1/1000) from Sigma Aldrich (St Quentin Fallavier, France). The polyclonal antibodies against TBCC (1/800) and TBCD (1/3000) were generously provided by N. Cowan (New York University Medial Center, USA) and those against Arl2 (1/1000) and Glu-tubulin (1/1000) were generously provided by R. Kahn (Emory University School of Medicine, Atlanta, USA) and L. Lafanechère (Centre de Criblage pour des Molécules Bio-Actives, Grenoble), respectively. Expression levels of the proteins were standardized against the β-actin.

RNA interference assays

A desalted duplex siRNA targeting *TBCC* 5'-CUGAGCAACUGCACGGUCA-3' and its corresponding scrambled sequence were designed by Sigma-Aldrich (St Quentin Fallavier, France). The siRNAs (200 nM) were transfected into 25×10^4 of MCF7, MP6.1 or MC+1 cells using oligofectamine (Invitrogen, Cergy Pontoise, France) according to the manufacturer's protocol on two consecutive days. Protein analyses by western blot or flow cytometry experiment were done on the third day after transfection.

Cell proliferation analysis

Cell proliferation was estimated using the methylthiazoletetrazolium (MTT) and BrdU assay. For the MTT test, cells (13×10^4) were seeded in a 6-well plate and incubated at 37°C. Every 24 h and for one week, MTT (3 mg) was added to each well of each plate. After 2 hrs of incubation at 37°C, supernatants were removed, formazan crystals solubilized with 3 ml of isopropanol-HCl-H_2O (90:1:9, v/v/v) and plates scanned. The absorbance was measured spectrophotometrically with a microplate reader (Labsystem Multiskanner RC) and the MTT values were obtained as subtraction of absorbances read at 540 and 690 nm wavelengths. The nonradioactive BrdU-based cell proliferation assay (Roche, Basel, Switzerland) was performed according to the manufacturer's protocol. Treated and untreated cells (5×10^3 cells per well) were seeded in a 96-well plastic plate and the assay was performed after 48, 72, 96 and 168 hours. Treated cells were exposed to either 0.5 nM or 1 nM of gemcitabine (Lilly, IN, USA) for one week. BrdU incorporation into the DNA was determined by measuring the absorbance at 450 on an ELISA plate reader.

Analysis of cell cycle distribution by flow cytometry

Cells were incubated 24 h with either 10 nM Paclitaxel (Bristol-Myers Squibb, New York, USA) or 1 nM vinorelbine (Pierre Fabre medicaments, Boulogne, France). Treated and untreated cells were then collected and incubated 1 hour at 4°C with propidium iodide (0.05 mg/ml) solution containing Nonidet-P40 (0.05%). Cells were analyzed using a FACS Calibur flow cytomoter (BD Biosciences Europe, Erembodegem, Belgium) and cell cycle distribution was determined using Modfit LT 2.0™ software (Veritysoftware Inc, Topsham, USA). For the siRNA's transfected clones, the cell cycle was studied 48 h after the first transfection.

Long time-lapse microscopy and analysis of mitosis

Cells (3×10^5) were seeded in a 35 mm cell culture dish, placed in culture medium maintained at 37°C in a 5% CO_2 atmosphere and observed using an inverted time lapse microscope (Olympus IX50) at the Centre Commun de Quantimétrie (Université Claude Bernard Lyon, France). Images were acquired every 2 minutes for 24 hours using a numerical CFW-1308M 1360X1024 camera (Scion, Frederick, USA) driven by ImageJ software (NIH, Bethesda, USA). 30 complete mitoses were analysed for each of the MP6.1 and MC+1 clones using ImageJ software (NIH, Bethesda, USA).

Immunofluorescence

Cells (MP6.1 and MC+1) exposed or not to 10 nM of paclitaxel for 24 hours were fixed by 4% paraformaldehyde during 15 minutes at room temperature and permeabilized using a PBS-Triton X-100 0.1% solution. Non specific sites were blocked using a solution containing 0.1% bovine serum albumin and 1% fetal calf serum. Cells were incubated with either a 1:100 of an antibody against β-tubulin (clone 2.1, Sigma Aldrich) or a 1:30 dilution of a monoclonal antibody against TBCC (Abnova, Taiwan) followed by a secondary FITC-antibody (Dako, Denmark). DNA staining was performed using diaminido-phenyl-indol (DAPI) (Roche, Manheim, Germany).

Images were obtained using a laser scanning confocal TCS Sp2 DMRXA microscope x63 objective (Leica Microsystems; Wetzlar, Germany) at the Centre Commun de Quantimétrie (Université Claude Bernard Lyon, France).

Separation and quantification of soluble unfolded tubulins, polymerizable αβ-tubulin heterodimers and microtubules

Cells (20×10^6) were harvested and lysed in 200 μl of buffer (100 mM Pipes, pH 6.7, 1 mM EGTA, and 1 mM $MgSO_4$) by two freeze-thaw cycle. Lysed cells were centrifuged at 12,000 × g for 15 minutes at 4°C. The supernatant was then ultracentrifuged (100,000 × g for 1 h at

20°C) and separated into a supernatant containing "soluble tubulins" and a pellet containing "microtubules". The microtubule fraction was resuspended in 100 μl of lysis buffer and 100 μl of the supernatant were incubated with 1 mM of GTP at 35°C for 30 minutes to allow tubulin polymerization then ultracentrifuged at 50,000 × g for 45 minutes at 35°C. The resulting pellet contained the "polymerizable tubulin" (PT) heterodimers and the supernatant contained "nonpolymerizable tubulin" (NPT) heterodimers, included tubulin peptides complexed with tubulin binding cofactors. The different fractions of tubulins were run on silver stained gels following manufacturer's recommendations (Amersham Biosciences AB, Sweden). After coloration, the single band observed at 55 kDa for the polymerizable tubulin heterodimers confirmed the success of purification (data not shown). The experiment was performed in triplicate using the two cell lines MP6.1 and MC+1. Densitometric quantification of western blots was performed with ImageJ software (NIH, USA).

Time lapse fluorescent microscopy and analysis of microtubule dynamics

Cells (3×10^5) were seeded in 6-well plate with circular glasses of 24 mm in their bottom and transfected with the pAcGFP1-tubulin vector (Clontech) using lipofectin (Invitrogen) following the manufacturer's instructions. The glasses containing the cells were placed in culture medium maintained at 37°C in a 5% CO_2 atmosphere in a time lapse inverted microscope (Olympus IX50) at the Centre Commun de Quantimétrie (Lyon, Université Claude Bernard, France). Cells were imaged with a numerical CFW-1308M 1360X1024 camera (Scion, Frederick, USA) driven by imageJ software (NIH, Bethesda, USA) using a 40× oil immersion lens (Zeiss, Göttingen, Germany). 30 pictures of the cell's microtubules were taken at 4 seconds intervals. The positions of the plus-ends of individual MT in peripheral lamellar regions of cells were tracked over time using ImageJ® software and graphed using Microsoft® Excel spreadsheet as position versus time to generate a 'life-history plot' for each MT. Growth and shortening rates and durations were derived by regression analysis. A difference of >0.5 μm between any two consecutive points was considered as a growth or shortening event. Transitions into depolymerisation or shortening are termed catastrophes, and transitions from shortening to growth or pause are called rescue. The catastrophe and rescue frequencies per unit time were calculated by dividing respectively the number of transitions from growth and pause to shortening and the number of transitions form shortening and pause to growth by the sum of the time in growth and pause. Dynamicity represents total tubulin exchange at the MT end and was calculated by dividing the sum of total length grown and shortened by the MT life span. The experiment was performed twice on 50 microtubules of the two clones MP6.1 and MC+1.

In vivo growth analysis

Female CB17/SCID mice purchased from Charles River Laboratories (Arbresle, France) were bred under pathogen-free conditions at the animal facility of our institute. Animals were treated in accordance with the European Union guidelines and French laws for the laboratory animal care and use. The animals were kept in conventional housing. Access to food and water was not restricted. All mice used were 5 to 6 weeks old at the time of cells injections. This study was approved by the local animal ethical committee. Mice were divided into six groups of six mice each which corresponds to the injections of MP6.1, MP6.2, MP6.3, MC+1, MC+2 and MC+3 cells. 3×10^6 cells were injected subcutaneously in mice with 50% matrigel (BD Biosciences, Belgium). The six mice were divided into two groups of treated and untreated mice. In the treated groups, paclitaxel was injected intraperitoneally in a dose of 10 mg/kg on the same day and a week after. Mice were weighed and the tumor size was measured twice per week with an electronic caliper. The volume was then computed by considering the tumor as a sphere with the formula 4/3 ($3.14 \times r^3$), r as the mean radius. Animals were euthanized either when one of the diameters of the tumor exceeded 17 mm, or if any potential suffering of the animal was observed or if weight loss exceeded 10%.

Results

TBCC protein expression in stable clones

Six stable clones of MCF7 cells overexpressing the protein TBCC (designated MC+) were established and characterized compared to clones of control cells transfected with the empty vector (designated as MP6). Among these, three clones with high levels of TBCC, with respect to three clones of control cells, were named MC+1, MC+2 and MC+3 in decreasing order of expression and further explored (Figure 1). Three clones obtained from MCF7 transfected with the empty vector were designated MP6.1, MP6.2 and MP6.3 and used as controls.

Impact of TBCC overexpression on the expression level of related proteins

We have investigated the effect of TBCC overexpression on the protein content of TBCD and ADP-ribosylation factor-like 2 (Arl2) which are two essential partners of TBCC in the folding pathway of α/β-tubulin dimers. TBCD was increased in the MC+1 clone but not in the two other clones MC+2 and MC+3. Conversely Arl2 was increased in the three clones of MC+ with respect

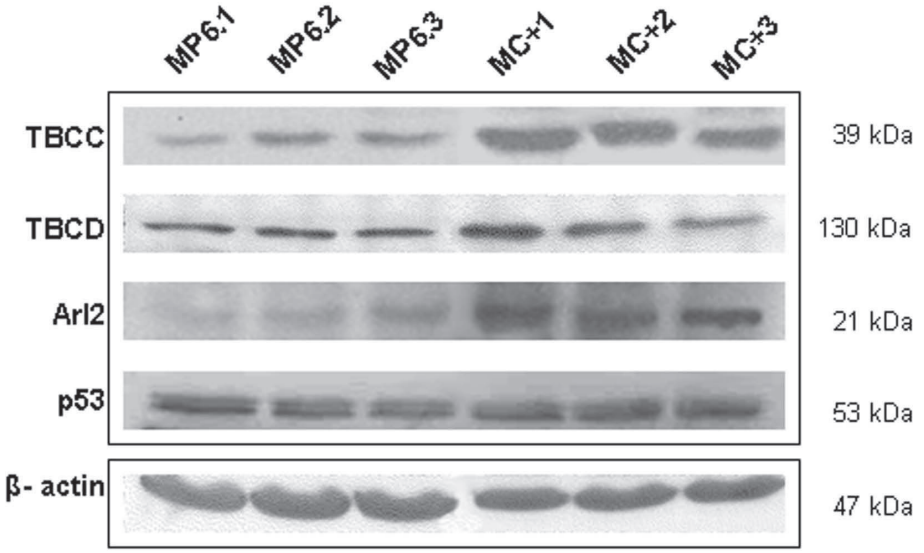

Figure 1 Effect of tubulin binding cofactor C (TBCC) on the expression level of related proteins. Western blot analysis of total cell extracts of the clones showing the effect of overexpression of TBCC on the expression level of TBCC, TBCD, Arl2 and p53. β-actin was used as a reference protein. MC+1, MC+2 and MC+3 represent MCF7 cells stably transfected with pcDNA6/C+. MP6.1, MP6.2 and MP6.3 represent MCF7 cells stably transfected with empty pcDNA6.

to the three control clones of MP6 (Figure 1). Finally, we studied the expression level of the tumor suppressor protein p53 and found that it was slightly increased in the three clones of MC+ cells when compared to MP6 cells (Figure 1).

Influence of TBCC protein content on proliferation rate

When we tested by MTT assay the proliferation rate of the MC+1 and MP6.1 cells *in vitro*, we found that the MC+1 cells had a slightly higher proliferation rate *in vitro* than the control cells at 120 and 144 hours but not at earlier timepoints. This result was visually confirmed by comparing the number of dividing cells stained in blue due to the formation of formazan crystals by their active mitochondria. These observations were confirmed by quantification of the absorbance optical density and the curve plotted (Figure 2A) shows the significant differences between these two cell lines. These results were confirmed on the MC+2, MC+3, MP6.2 and MP6.3 clones. When we tested the proliferation rate of MC+1 and MP6.1 cells using the BrdU incorporation assay, we found no significant difference in the proliferation rate between the two clones. This was confirmed on the other clones MC+2, MC+3, MP6.2 and MP6.3 (Figure 2B).

Influence of TBCC protein content on cell cycle distribution and mitotic duration

To explore if the difference in proliferation rates is correlated to a difference in the cell cycle distribution, we investigated the percentage of cells in S and G2-M phases by propidium iodide and flow cytometry in MC+1 and MP6.1 cells (Figure 2C). We observed that MC+1 cells had a lower percentage of cells in S-phase 11.7 ± 2.6 as compared to MP6.1 cells 16.7 ± 0.78, a significant decrease of 30%, $p < 0.05$ (Figure 2C). This difference was also accompanied by a higher percentage of cells in G2-M phase 45 ± 3.9 as compared to the MP6.1 30 ± 1.27, a significant increase of 50%, $p < 0.05$ (Figure 2C). This experiment was performed on the MP6.2, MP6.3, MC+2 and MC+3 clones and the above results were confirmed.

In order to correlate these phenotypic behaviours to the increased content of TBCC protein, we inhibited the protein expression of TBCC by transient transfection using siRNA targetting TBCC in MP6.1 and MC+1 cells. The siRNA targetting TBCC we designed was validated by western blot on MCF7 cells. This sequence inhibited TBCC (-48%, $p < 0.01$) compared to the siRNA scrambled (data not shown) and was used to inhibit TBCC in MC+1 and MP6.1. In both the MC+1 and MP6.1 cells, the inhibition of TBCC protein caused a significant increase in percentage of cells in S-phase and a significant decrease in percentage of cells in G2-M phase (Figure 2C).

To explain the increased G2-M percentage observed in MC+1 cells, we investigated the durations of prophase-metaphase, anaphase-telophase and complete mitosis in comparison to controls. We found that MC+1 cells took more time to complete mitosis (71 minutes as

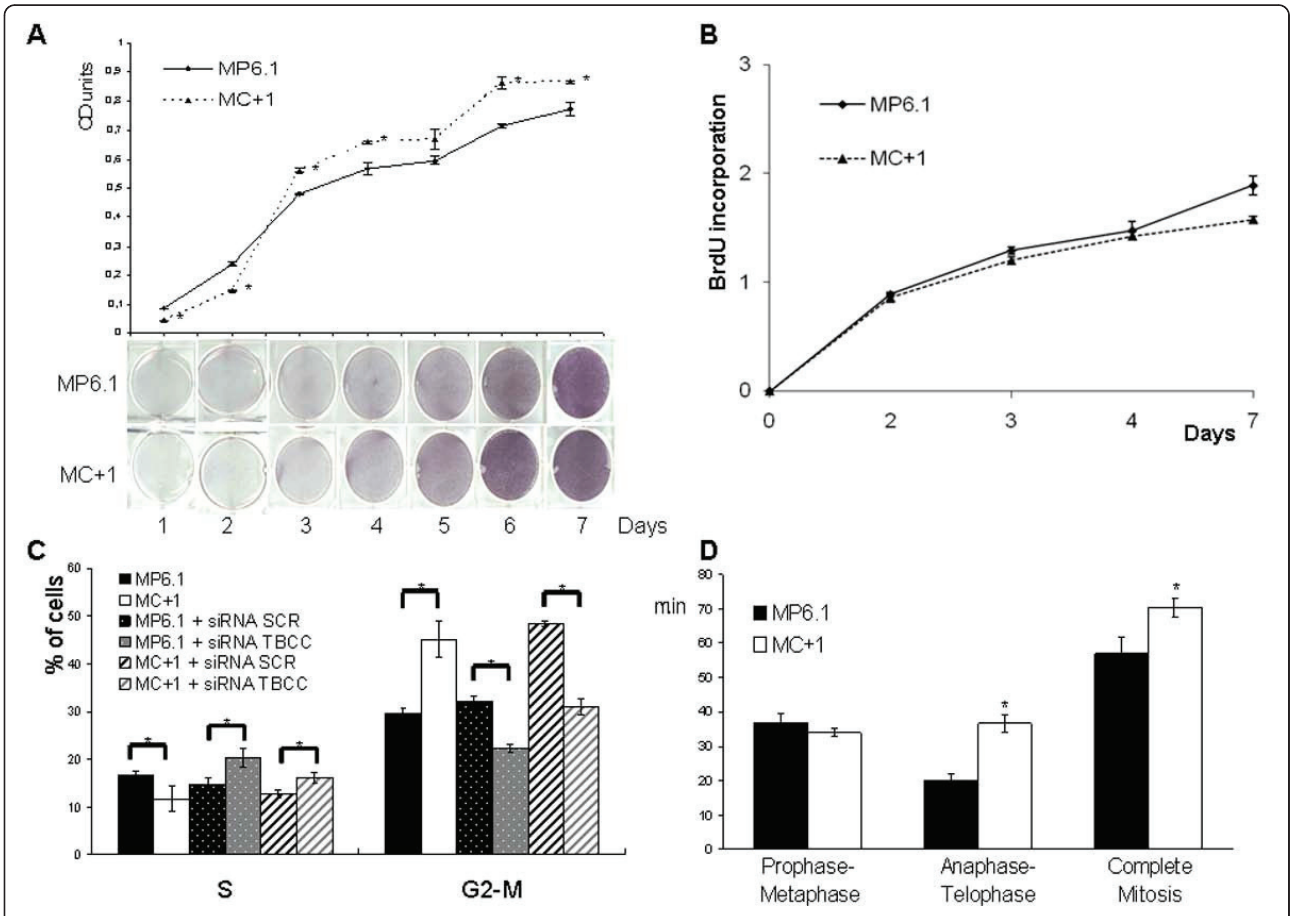

Figure 2 Effect of TBCC on proliferation rate, cell cycle distribution and mitotic duration of MC+1 and MP6.1 cells. (A) Optical density of absorbances measured at 540 and 690 nm of solubilized formazan crystals in MC+1 and MP6.1 formed after addition of MTT. The figure below the graph represents the MC+1 and MP6.1-containing wells colored with formazan crystals after addition of methylthiazoletetrazolium (MTT) every day for a total duration of one week. Results presented are the average values of three experiments. *: Values differ significantly from MP6.1 at ≥ 95% confidence level by Student's *t*-test. Bars represent standard deviation. (B) Proliferative capacity (BrdU labelling) of MC+1 and MP6.1 cells for one week. Results presented are the average values of three experiments. Bars represent standard deviation. (C) Cell cycle distribution of MC+1 and MP6.1 cells after incubation with propidium iodide. Percentage of cells in the S- and G2-M phase of the cell cycle in MC+1, MP6.1, MC+1 + siRNA SCR, MC+1 + siRNA TBCC, MP6.1 + siRNA SCR and MP6.1 + siRNA TBCC. The cells represented as +siRNA SCR and +siRNA TBCC are cells transiently double transfected with siRNA scrambled and siRNA targeting TBCC, respectively. Experiment was done 48 hours after first transfection. Results presented are the average values of three experiments. *: Values differ significantly at ≥ 95% confidence level by Student's *t*-test. Bars represent standard deviation. (D) Durations of prophase-metaphase, anaphase-telophase and complete mitosis in MC+1 and MP6.1 cells in minutes after 24 hours of time-lapse microscopic analysis. *: Values differ significantly from MP6.1 at ≥ 95% confidence level by Student's *t*-test. Bars represent standard deviation.

compared to 57 minutes in MP6.1 cells). This longer time was due mainly to longer anaphase-telophase duration as 37 minutes compared to 20 minutes in MP6.1 and not to a difference in the prophase-metaphase (Figure 2D). The cells overexpressing TBCC proceed slowly through the mitosis which can be an explanation for the high percentage of cells in G2-M phase of cell cycle.

In vivo progression of tumors

In order to study the impact of TBCC on tumorigenesis of breast cancer cells, we injected the six clones MP6.1, MP6.2, MP6.3, MC+1, MC+2 and MC+3 into SCID mice and monitored their *in vivo* progression and tumor formation. The control clones were able to form tumors of 200 to 250 mm^3one month later. However, the MC+ clones presented lower capacities of growth *in vivo* with the tumors of volumes ranging form 1 to 50 mm^3 (Figure 3).

Localisation of TBCC in the cytoplasm

The impact of TBCC overexpression on the cytoplasmic distribution of TBCC was studied by immunofluorescence on MP6.1 and MC+1 cells. In a first step we observed that the distribution of β-tubulins in the

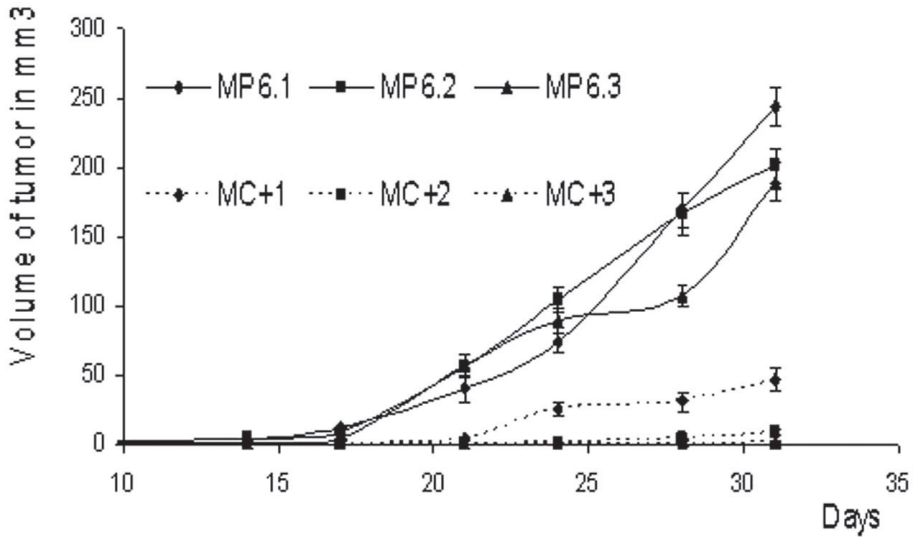

Figure 3 In vivo tumor progression. Progression of tumors growth measured at 2-3 days interval for one month after subcutaneous injections of each of MC+1, MC+2, MC+3, MP6.1, MP6.2 and MP6.3 cells. Results presented are average values of three mice. Bars represent standard deviation.

cytoplasm was not affected by the overexpression of TBCC. This was done by immunofluorescence using an antibody against β-tubulin and the images show similar cytoplasm staining between MP6.1 and MC+1 cells (Figure 4A). TBCC was similarly distributed in both normally and highly-expressing cells. The staining was found to be cytoplasmic however it was not observed in all cells of each cell line (Figure 4B). We found more stained cells in MC+1 cells than in control cells. After overlaying fluorescent images of TBCC with DAPI images we observed that the cells that were strongly stained for TBCC were cells in mitosis. This result suggested that TBCC was more highly expressed in cells undergoing mitosis. This was confirmed (Figure 4C) in MC+1 cells exposed to paclitaxel 10 nM for 24 hours. Paclitaxel is known to stabilize microtubules and causes cell arrest in metaphase. We observed by immunofluorescence microscopy that the cells blocked in mitosis were strongly stained with anti-TBCC antibody.

Effect of TBCC content on different tubulins and on subcellular tubulin fractions

We studied the impact that TBCC overexpression might have on the total α and β-tubulins as well as on different posttranslational modifications of α-tubulin such as acetylated, tyrosinated and detyrosinated (Glu) α-tubulins and finally on the βIII-tubulin isotype. We observed no significant difference in the expression of total α-tubulin and tyrosinated α-tubulin between the clones overexpressing TBCC and the controls (Figure 5A). Regarding Glu α-tubulin and the βIII-tubulin, we observed heterogeneity of expression among both the clones of MP6 and MC+ which made impossible to conclude of a simple profile related to TBCC status (Figure 5A). However, concerning β-tubulin and acetylated α-tubulin we observed an increase in expression in the MC+ clones with respect to the MP6 clones.

In addition, we investigated the different pools of soluble polymerizable αβ-heterodimers (PT) and soluble non-polymerizable tubulins (NPT) as well as the microtubule tubulins (MT) in MC+1 and MP6.1 cells. The different pools of tubulins were obtained after fractionation of MP6.1 and MC+1 lysates by series of ultracentrifugations. We have observed that the modification of the TBCC level in MCF7 cells did not alter the total pool of α-tubulin protein although it increased the total pool of β-tubulin (Figure 5B). The overexpression of TBCC protein strongly increased the NPT fraction and strongly decreased the PT fraction, while it had only a minor effect on the tubulin content of microtubules which was slightly decreased. The profile of expression of the NPT and PT fractions was similar for both the α-tubulins and the β-tubulins but was less marked in the case of α-tubulins (Figure 5B). Quantification of expression levels of α and β-tubulins in the different fractions confirmed the results observed in the immunoblots (Figure 5C).

Overexpression of TBCC decreases microtubule dynamics

The effects of overexpression of TBCC on microtubule dynamic instability were determined in MC+1 cells 48 h after transient transfection with pAcGFP1-α tubulin. The microtubules in MC+1 cells grew and shortened more slowly and for shorter lengths than those in

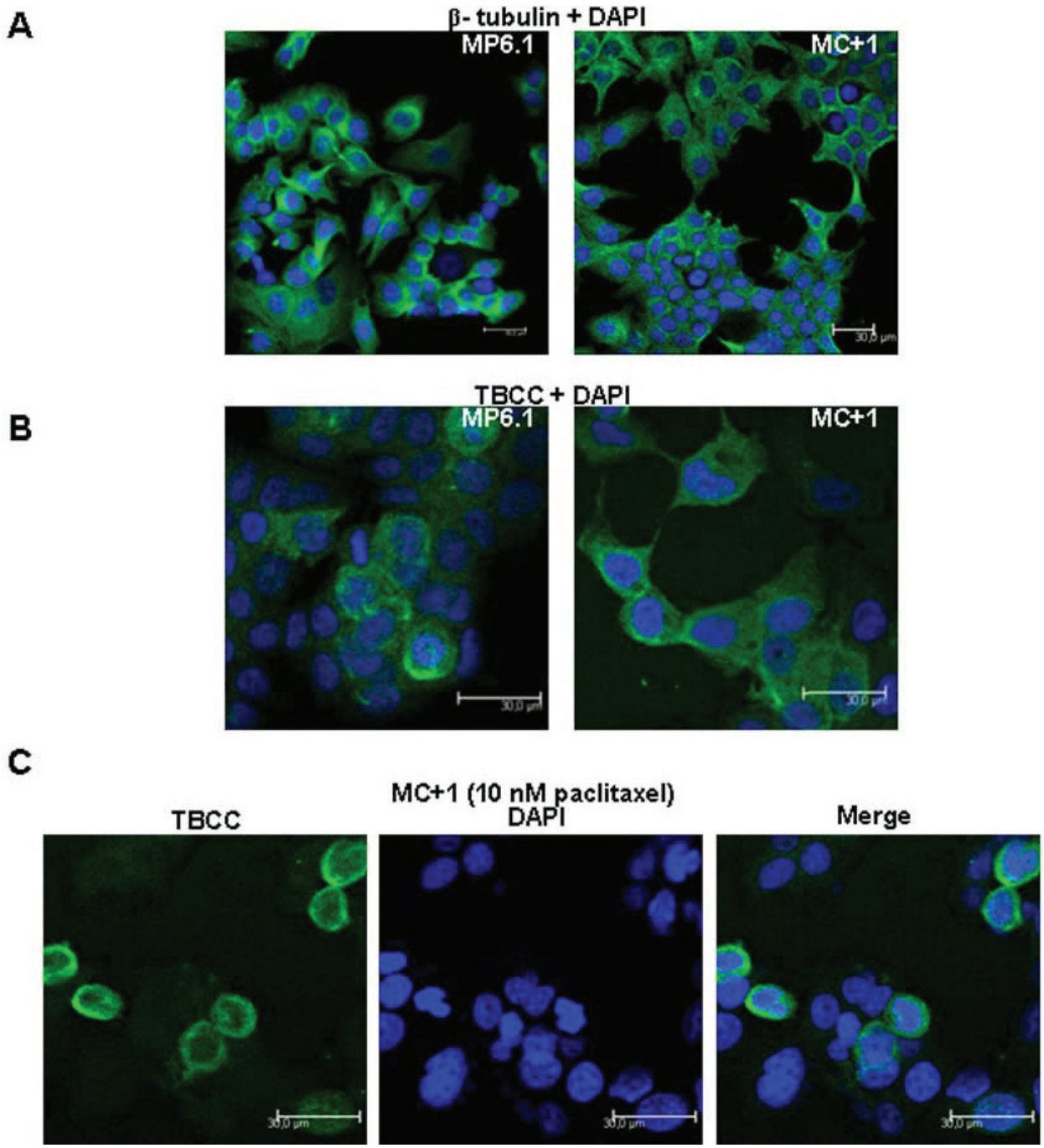

Figure 4 Localization of TBCC in MC+1 and MP6.1 cells. (A) Representative images of MC+1 and MP6.1 cells after DNA (DAPI, blue) and β-tubulin (FITC, green) staining (B) Representative images of MC+1 and MP6.1 cells after DNA (DAPI, blue) and TBCC (FITC, green) staining (C) Representative images of DNA (DAPI, blue) and TBCC (FITC, green) staining in MC+1 cells exposed 24 hours to 10 nM of paclitaxel.

MP6.1 cells and presented significantly less dynamicity. The parameters for dynamic microtubules in MP6.1 and MC+1 cells were determined (Table 1). The mean growth rate was significantly decreased from 16.41 ± 1.99 μm/min in MP6.1 cells to 10.54 ± 0.37 μm/min in MC+1 cells, corresponding to a decrease of 36%, $p < 0.05$. The mean shortening rate and the mean lengths of individual growth and shortening were slightly decreased. The mean shortening duration was slightly increased compared to the mean growth duration and mean pause duration that were significantly increased from 0.15 ± 0.02 min and 0.25 ± 0.01 min in control

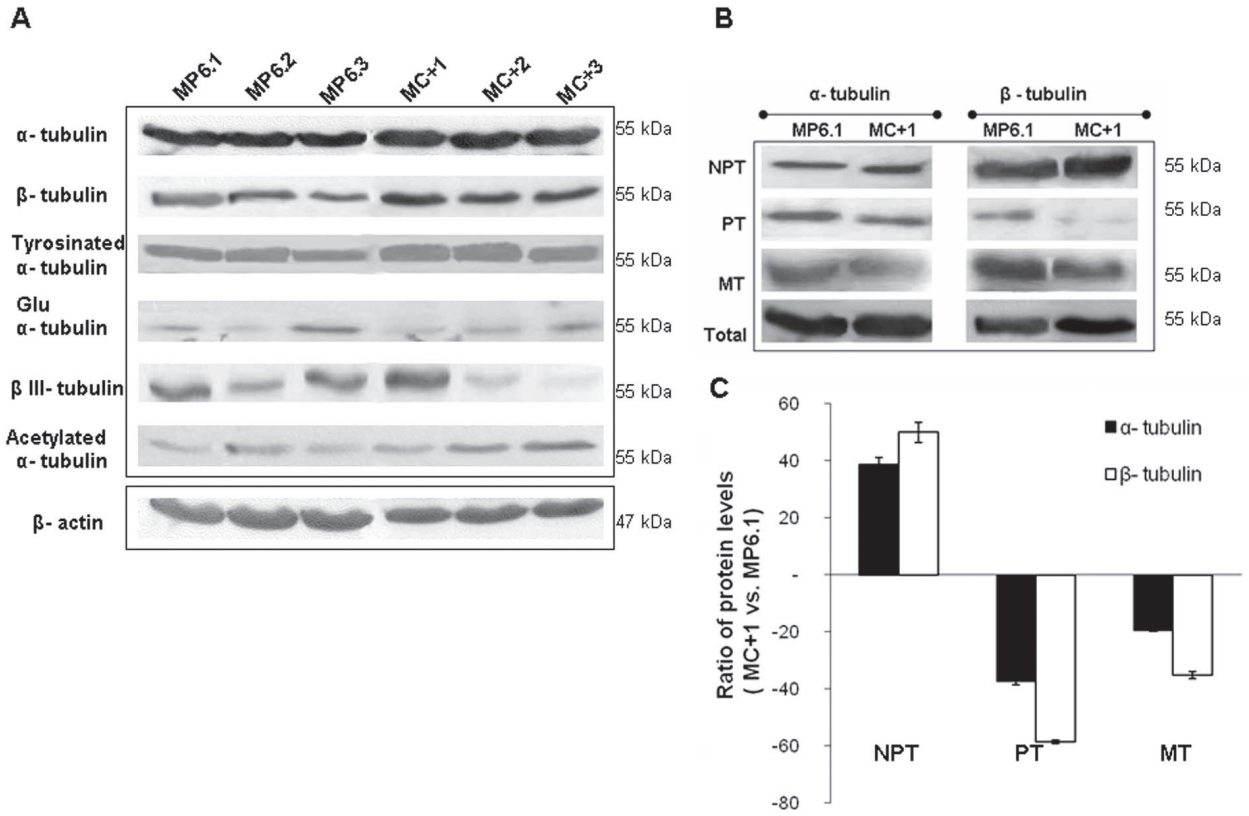

Figure 5 Effect of TBCC on different tubulins content and on subcellular tubulin fractions. (A) Western blot analysis of total cell extracts of the MC+1, MC+2, MC+3, MP6.1, MP6.2 and MP6.3 clones showing the effect of overexpression of TBCC on the expression level of α-tubulin, β-tubulin, tyrosinated α-tubulin, detyrosinated (glu) α-tubulin, β III-tubulin and acetylated α-tubulin. β-actin was used as a reference protein. (B) Representative blots corresponding to the expression levels of α-tubulin and β-tubulin in nonpolymerizable tubulin heterodimers (NPT), polymerizable tubulin (PT) heterodimers, microtubule heterodimers (MT) and total pool of tubulins in MC+1 and MP6.1 cells. These fractions were obtained after series of ultracentrifugations. (C) Protein ratios (MC+1 vs. MP6.1) of both α-tubulins and β-tubulins levels in NPT, PT and MT fractions. Results presented are the average values of three experiments. Bars represent standard deviation.

cells to 0.22 ± 0.01 min and 0.37 ± 0.02 min in MC+1 cells, an increase of 47%, $p < 0.05$ and 48%, $p < 0.05$, respectively. The MC+1 cells presented a slower growth movement than the control cells which is the cause of a slow growth rate and a lower dynamicity. The frequencies of catastrophe and rescue are important parameters in studying the dynamicity of microtubules. The catastrophe frequency is the frequency with which the microtubules switch from either pause or growth to shortening. The rescue frequency is the frequency with which the microtubules switch from shortening to either growth or pause. As seen above, the MC+1 cells presented a shortening rate similar to that of the control cells but with a decreased growth rate. This suggests that the microtubules shortened in MC+1 cells are either taking longer time to grow (high growth duration) or staying in a pause phase (high pause duration). The time-based catastrophe frequency was slightly increased in MC+1 cells unlike the rescue frequency that was increased significantly by 54%, $p < 0.05$ as compared to control cells. This can be explained by the fact that the microtubules of MC+1 cells have tiny movements of growth and shortening for short distances (less than 0.5 μm) not counted in the growing and shortening events and in the overall dynamicity of the microtubule. The dynamicity was significantly decreased from 8.80 ± 1.02 μm/min in MP6.1 to 6.09 ± 1.06 μm/min in MC+1 cells. This decrease of 31%, $p < 0.05$ is representative of less overall distances travelled by the microtubule either in a growing or in a shortening event for 2 minutes.

In vitro response to Gemcitabine and antimicrotubule compounds

Since TBCC is a major protein involved in the proper folding pathway of tubulins into microtubules, we investigated the response of the TBCC overexpressing breast cancer cells to antimicrotubule agents. These agents block the cell cycle in G2-M phase. We incubated MC+1 and MP6.1 cells 24 hours with non-toxic doses of 10 nM paclitaxel or 1 nM vinorelbine, and observed

Table 1 Parameters of microtubule dynamics in MC+1 and MP6.1 cells

Parameters		MP6.1	MC+1	Change
Mean rate (μm/min ± SE)	Growth	16.41 ± 1.99	10.54 ± 0.37 *	-36%
	Shortening	15.25 ± 2.14	14.35 ± 1.53	
Mean length (μm ± SE)	Growth	2.38 ± 0.49	2.14 ± 0.51	
	Shortening	2.40 ± 0.39	2.29 ± 0.10	
Mean duration (min ± SE)	Growth	0.15 ± 0.02	0.22 ± 0.01 *	47%
	Shortening	0.16 ± 0.01	0.17 ± 0.01	
	Pause	0.25 ± 0.01	0.37 ± 0.02 *	48%
Mean frequency (min^{-1}± SE)	Rescue	9.20 ± 0.53	14.20 ± 0.24 *	54%
	Catastrophe	4.64 ± 0.70	5.38 ± 0.56	
Dynamicity (μm/min ± SE)		8.80 ± 1.02	6.09 ± 1.06 *	-31%

*: Values differ significantly from MP6.1 at ≥ 95% confidence level by Student's *t*-test. Values are represented as mean ± SD

that the cells overexpressing TBCC were more sensitive to the G2-M blockage caused by these treatments with respect to the MP6.1 cells. MC+1 cells presented 79 and 83% of cells in G2-M after paclitaxel and vinorelbine exposure, respectively. The control cells were also blocked but to a maximum percentage of 55% (Figure 6A). The percentage of MC+1 and MP6.1 cells in the Sub-G0 phase of the cell cycle after 24 hours of treatment with 10 nM of paclitaxel and 1 nM of vinorelbine increased. However this increase was not statistically significant and was slightly higher in MC+1 cells than in MP6.1 cells (Figure 6B). In addition to antimicrotubule agents, we investigated the response of the cells to gemcitabine, an S-phase specific antimetabolite and we found that MC+1 cells were less sensitive to gemcitabine than the MP6.1 cells, significantly at the dose of 1 nM (Figure 6C). These experiments were performed on the MP6.2, MP6.3, MC+2 and MC+3 clones and the above results were confirmed.

In vivo sensitivity to treatment

We injected the six clones MP6.1, MP6.2, MP6.3, MC+1, MC+2 and MC+3 into SCID mice and monitored their *in vivo* progression and tumor formation. The control clones were able to form tumors unlike the MC+1, MC+2 and MC+3 clones that presented lower capacities of growth *in vivo*. When treated with paclitaxel, mice injected with MC+ clones revealed higher sensitivity to the treatment compared to mice injected with MP6 clones (Figure 6D).

Discussion

Tubulin binding cofactor C is a crucial protein for the proper folding of α- and β-tubulins to form heterodimers able to polymerize into microtubules. In this study, the major aim was to investigate the impact of TBCC overexpression on the proliferation, cell cycle distribution and tumorigenesis of MCF7 cells as well as on the microtubule contents and dynamics. Since the antimicrotubule agents are common treatments for breast cancer, we examined the response of our models to these treatments both *in vivo* and *in vitro*. In addition to this, expression levels of α and β tubulins were found to be involved in predicting the response to treatments in many cancers [22,23]. Some posttranslational modifications of α tubulins like detyrosination are of high occurrence in breast cancer [24]. This differential expression level of tubulins in many cancers has made of them targets for treatments.

The overexpression of TBCC in MCF7 cells profoundly altered the distribution of tubulin monomers amongst cellular fractions and diminished the content and dynamicity of their microtubules but did not prevent the cells from completing mitosis and proliferating correctly. We must insist however on the fairly low differences in TBCC content observed between transfected cells and controls, suggesting that higher levels of expression may be incompatible with cell survival. Overexpression of TBCC had a major impact on tubulin fractions, with a large increase in the nonpolymerizable fraction and a consequent decrease in the soluble tubulin dimers fraction. The nonpolymerizable tubulin fraction consists of the pool of tubulins in the α-tubulin/TBCE/TBCC/TBCD/β-tubulin complex, the pool of α-tubulins bound either to TBCB or TBCE or TBCE/TBCC/TBCD/β-tubulin, and the pool of β-tubulins bound either to TBCA or TBCD or α-tubulin/TBCE/TBCC/TBCD. The reduced availability of α/β tubulins to form polymerizable heterodimers may be due to the fact that a large amount of the monomers is included in TBCC-containing complexes. Of note and contrary to a commonly accepted tubulin dogma, MC+ cells appear to have disequilibrium between the contents of total α-tubulin and β-tubulin. It is classically considered that such disequilibrium would be lethal for mammalian cells. However in this model the increased content can be attributed to a specific enrichment in the non-functional fraction of β-tubulin.

During mitosis, especially at the level of anaphase chromosome movement, microtubules disassemble by depolymerization and release free heterodimers. The heterodimers released can be directly recruited by the excess of TBCC in the cytoplasm. Indeed, TBCC appears to be highly present in the cytoplasm during mitosis as observed in cells blocked in mitosis by paclitaxel. We hypothesize that equilibrium exists between the soluble amount of nonpolymerizable and polymerizable tubulins in MC+ cells and the cells' requirement for microtubules. We also observed a strong impact of TBCC content on microtubule dynamics. At the onset of mitosis the interphase microtubule network disassembles while there is simultaneously a decrease in

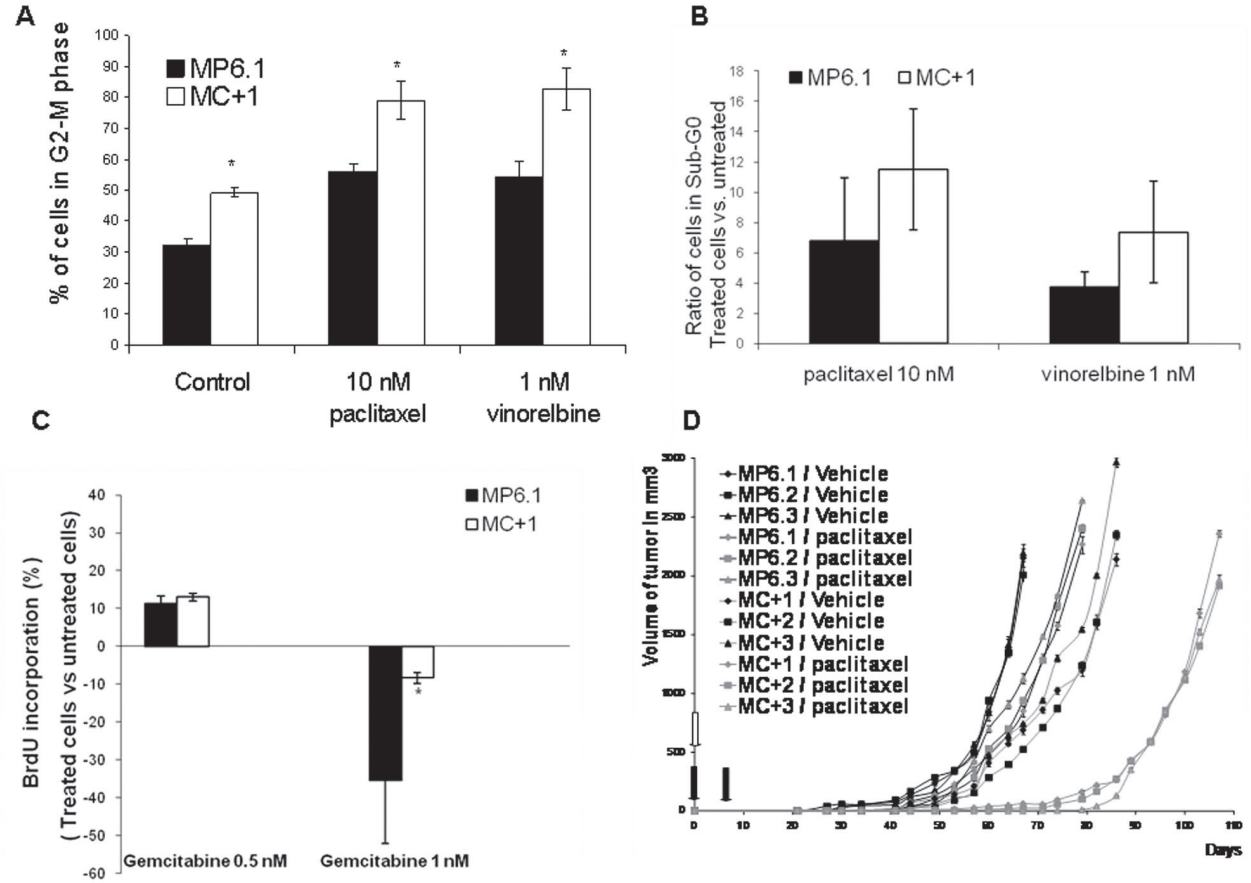

Figure 6 The influence of TBCC protein content on the response to treatment in vitro and in vivo. (A) The Percentage of MC+1 and MP6.1 cells in the G2-M phase of the cell cycle after 24 hours of treatment with 10 nM of paclitaxel and 1 nM of vinorelbine. Results presented are the average values of three experiments. Bars represent standard deviation.*: Values differ significantly from MP6.1 at ≥ 95% confidence level by Student's *t*-test. (B) The ratio of treated vs. untreated MC+1 and MP6.1 cells in the Sub-G0 phase of the cell cycle after 24 hours of treatment with 10 nM of paclitaxel and 1 nM of vinorelbine. The cell cycle was studied after incubation with propidium iodide. Results presented are the average values of three experiments. Bars represent standard deviation. (C) Proliferative response by BrdU labelling (treated versus untreated cells) after one week exposure to 0.5 and 1 nM of gemcitabine in MC+1 and MP6.1 cells. Results presented are the average values of three experiments.*: Values differ significantly from MP6.1 at ≥ 95% confidence level by Student's *t*-test. Bars represent standard deviation. (D) Progression of tumors growth and response to paclitaxel measured at 2-3 days interval after subcutaneous injections of each of MC+1, MC+2, MC+3, MP6.1, MP6.2 and MP6.3 cells at day 0 (empty arrow). At day 0 and day 7, paclitaxel was injected intraperitoneally in a dose of 10 mg/kg (black arrow). Results presented are the mean values of the three mice, untreated or treated. Bars represent standard deviation.

total microtubule polymer mass and an increase in microtubule dynamics [25]. In some cells, the increase in dynamics is due to an increase in catastrophe frequency and a reduction in the rescue frequency rather than changes in growth and shortening rates [26,27]. In our study, we found that the microtubules in MC+1 cells grew and shortened more slowly and for shorter periods of time than those in MP6.1 cells. The dynamicity was significantly decreased in MC+1 cells in comparison to MP6.1 cells. These observations are coherent with the tubulin fraction alterations observed. The relative lack of available polymerizable tubulin dimers could explain the reduced growing and overall dynamicity.

The highly dynamic microtubules in the spindle are required for all stages of mitosis [2]. During prometaphase, the dynamicity of microtubules is very important in order to probe the cytoplasm and attach to chromosomes at their kinetochores [28]. Any single chromosome unable to attach to the spindle is enough to prevent a cell from transitioning to anaphase and therefore be blocked at or before metaphase-anaphase transition and undergo apoptosis later on [29,30]. In our study, the decreased dynamicity in MC+ cells did not affect the prophase and metaphase progression and only affected the anaphase-telophase transition. We observed a significant increase in the duration of anaphase-telophase which is the cause of a slower mitosis in the

MC+ cells. This means that the less dynamic microtubules affected the mitosis without causing blockage or cell death. The distribution of MC+ cells in the cell cycle was different from that of the control cells in that they presented higher percentage of cells in the G2-M phase and lower percentage in the S-phase. By inhibiting TBCC protein through transient transfection of siRNA targeting TBCC, we obtained an increase in the percentage of cells in S-phase and a decrease in the percentage of cells in G2-M, thereby confirming the involvement of TBCC in the cell cycle alteration observed.

Previous publications have suggested that the dynamicity of microtubules depends on microtubule composition and is correlated with post-translational modifications of α-tubulins. Detyrosinated microtubules (Glu microtubules) present enhanced stability against end-mediated depolymerisation however the detyrosination alone is not sufficient to confer this enhanced stability [31]. Tubulin detyrosination occurs frequently in breast cancer and is linked to tumor aggressivity [24]. Reduced abundance of α and α-acetylated tubulin is associated with enhanced apoptosis in leukemia cells [32]. Acetylated α-tubulin is present in microtubules that under depolymerising condition are more stable than the majority of cytoplasmic microtubules [31,33]. The existence of a direct effect of acetylation on microtubule stability and dynamics remains controversial [34]. In our study, we investigated the expression levels of detyrosinated (Glu) α-tubulin, tyrosinated α-tubulin and acetylated α-tubulin. Reproducible results were obtained with the tyrosinated tubulin which was not modified and for the acetylated tubulin that was increased in the MC+ cells with respect to MP6 cells. The results of increased expression level of acetylated α-tubulin in MC + cells can be explained as an attempt of these cells to protect their microtubules. The microtubules in MC+ cells have diminished dynamicity and their reduced growth rate is not enough to compensate for their continuous shortening events. As a means to prevent excessive depolymerisation, the microtubules of MC+ might have incorporated acetylated α tubulin in order to acquire more stability against depolymerisation. It has been previously reported that high expression of class III beta tubulin by tumor cells is associated with resistance to taxane chemotherapy in non-small cell lung cancer [35]. Here we can't do any correlation, since the expression profile of the class III beta tubulin in our models is not determined.

The MCF7 cells (human mammary adenocarcinoma) emerge from an invasive ductal carcinoma type of cancer [36,37]. The *in vivo* invasiveness and metastasis processing of MCF7 cells depend on many factors that influence the cell such as steroid hormones, growth factors, oncogenes and tumor suppressor genes [38]. In our study, MC+ cells presented a limited tumor growth *in vivo* compared to MP6 cells. Since the proliferative activity of MC+ cells *in vitro* was not reduced in comparison to control cells and their volume was not altered (data not shown), we hypothesize that this reduced *in vivo* growth may be at least partially explained by the potential loss of aggressive and invasive capacities of MC+ cells rather than by decreased proliferation rate [39]. The study of expression levels of TBCC, α tubulin and β tubulin in the tumors extracted from the mice revealed that the MC+ cells maintained the same alterations *in vivo* as those reported *in vitro* (data not shown). In the intent to understand the potential involvement of TBCC in the invasiveness phenotype, we have studied by quantitative RT-PCR the levels of expression of the *TBCC* gene in thirteen different human breast cancer cell lines for which *in vitro* invasiveness properties have been reported [40]. We found that the four cell lines that expressed *TBCC* the highest (MCF7, MDA-MB361, MDA-MB453 and UACC812) were the ones with low *in vitro* invasiveness capacity and the other nine cell lines that were highly invasive had low *TBCC* expression (Additional file 1). While this observation does not allow us to conclude a direct role of TBCC it suggests a possible involvement of TBCC in tumor aggressivity whether through microtubules or through other unidentified pathways such as interaction with the Arl2 protein. It is important to note that in the cells overexpressing TBCC we noticed that the expression levels of Arl2 and tumor suppressor p53 were slightly increased. ADP ribosylation factor like 2 (Arl2) protein is a GTPase that belongs to ADP ribosylation factor (ARF) family [41,42] and plays a role in microtubule dynamics [21,42]. Arl2 is known to directly bind to TBCD and can inhibit TBCD from dissociating the α/β tubulin heterodimers [17]. MCF7 cells overexpressing Arl2 were found to have low i*n vivo* growth capacity [43]. However *in vitro*, the behaviour of MC+ cells in terms of cell cycle, microtubule dynamics, response to antimicrotubule agents largely differ from that of cells overexpressing Arl2 [21]. Therefore one possible hypothesis could be that *in vivo* a mechanism involving TBCC and p53 or an interaction between Arl2 and TBCC is involved in the loss of aggressivity of MC+ cells.

Based on the success and efficiency of microtubule-targeted drugs in the treatments of cancer in general and breast cancer in specific, microtubules remain the best cancer target identified to date [44]. Even though paclitaxel and vinorelbine have different mechanisms of action with respect to microtubule, their cellular effects at low but clinically relevant concentrations are reduced microtubule dynamics inducing mitotic arrest. Paclitaxel, from the taxanes family binds to β-tubulin, causes

lateral polymerization and suppresses microtubule dynamics [45]. The cellular effect of paclitaxel at low concentrations (<10 nM) include suppression of microtubule dynamics without affecting microtubule content and mitotic arrest then apoptosis [46]. Vinorelbine, from the vinca alkaloids binds with high affinity to the plus end of the microtubule and with low affinity to the sides of the microtubule and leads to depolymerization [46,47]. However at low concentrations, vinca alkaloids block mitosis with little or no depolymerisation of spindle microtubules [48]. Therefore, compounds that depolymerize microtubules can also stabilize microtubule dynamics at relatively low concentrations [49]. In our study, we have observed an increased sensitivity of the MC+ cells *in vitro* toward the mitosis blockage compared to MP6 cells. The low non toxic concentrations of both paclitaxel (10 nM) and vinorelbine (1 nM) induced a stronger G2-M block in MC+ cells than in MP6 cells. We used subtoxic concentrations of treatments as shown by the absence of significant increase in percentage of sub-G0 cells. The results observed were homogenous in the three clones of each cell line. The enhanced sensitivity of MC+ cells to paclitaxel was confirmed *in vivo*. We explain the increased sensitivity of the MC+ for the antimicrotubule agents by two main hypotheses. One is the basal lower dynamicity of microtubules in these cells compared to MP6 cells. Second is the high percentage of G2-M cells in MC+ cells which means high percentage of target cells for antimicrotubule agents. These results suggest that TBCC content of breast tumor cells significantly influences their sensitivity to tubulin binding agents both *in vitro* and *in vivo*. When we tested the response of MC+1 and MP6.1 cells to gemcitabine, a nucleoside analog and S-phase specific antimetabolite, we showed that MC+1 cells were less sensitive to gemcitabine than the MP6.1 cells, significantly at 1 nM. This lower sensitivity to gemcitabine is due to the fact that MC+1 cells present a lower percentage of cells in the S-phase at the basal level. The differential responses to antimicrotubule agents and gemcitabine reveal that our cell models respond to these treatments based on their distribution in the cell cycle. Therefore we suggest that other anticancer treatments that target the cell cycle can have interesting effects on our cell models.

Conclusion

Our results suggest that the overexpression of TBCC protein in MCF7 cells influences their tubulin pools and microtubule dynamics, with important consequences in terms of mitotic progression, tumor growth and sensitivity to antimicrotubule agents. Moderately increased TBCC content was associated with an increase in the nonpolymerizable tubulin pool and reduced microtubule dynamics. This was associated with prolonged anaphase-telophase and reduced growth *in vivo*. The higher percentage of cells in G2-M phase of the cell cycle made of the MC+ cells better targets for antimicrotubule agents. These results underline the importance of the microtubular network in the tumor cell aggressivity phenotype. While currently available compounds mostly target microtubule dynamics, another possibility could be to alter tubulin pools in tumor cells. Another perspective for these results would be to look for potential partners of TBCC. It would be interesting to investigate if TBCC interacts or binds with microtubule binding proteins and to try to synthesize a ligand that can stabilize this protein inside the cytoplasm so that it would be continuously active.

Additional file 1: Correlation between *TBCC* expression level and *in vitro* invasive capacity of breast cancer cell lines. Values of gene expression are calculated with respect to the *TBCC* expression level in HME cells (human mammary epithelial cells). The cDNA levels were normalized to the expression of the 18S ribosomal gene as previously described by Saussede-Aim et al. 2009. Saussede-Aim J, Matera EL, Herveau S, Rouault JP, Ferlini C, Dumontet C: **Vinorelbine Induces β3-Tubulin Gene Expression through an AP-1 Site**. *Anticancer research* 2009, 29:3003-3009.

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Authors' contributions

RHS and CD conceived and designed the experiments. RHS and CD wrote the manuscript. RHS and SH performed the *in vivo* studies and analyzed the data. RHS, SH, ELM and JFL performed the *in vitro* studies. RHS analyzed the *in vitro* data. CD contributed with reagents/materials/analysis tools. All authors read and approved the final manuscript.

Competing interests

Rouba Hage-Sleiman benefits from financial support from the Lebanese CNRS.

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Additional files

Additional file 1

Correlation between *TBCC* expression level and *in vitro* invasive capacity of breast cancer cell lines

Cell line	*TBCC* Expression level	*In vitro* invasive capacity
MCF7	4.10	Low
UACC812	3.56	Low
MDAMB361	3.49	Low
MDAMB453	3.00	Low
MDAMB436	2.79	High
BT20	2.52	High
HS578T	2.31	High
MDAMB157	1.71	High
CAL51	1.55	High
HBL100	1.02	High
T47D	1.00	High
BT474	0.94	High
MDAMB231	0.83	High

Values of gene expression are calculated with respect to the *TBCC* expression level in HME cells (human mammary epithelial cells).
The cDNA levels were normalized to the expression of the 18S ribosomal gene as previously described by Saussede-Aim et al. 2009.

Saussede-Aim J, Matera EL, Herveau S, Rouault JP, Ferlini C, Dumontet C: **Vinorelbine Induces β3-Tubulin Gene Expression through an AP-1 Site.** *Anticancer research* 2009, 29:3003-3009.

PART 3 ARTICLE 2

Inhibition of tubulin binding cofactor C modifies microtubule dynamics and cell cycle distribution and enhances sensitivity to gemcitabine in breast cancer cells.

(Submitted to Cancer Research)

Inhibition of tubulin binding cofactor C modifies microtubule dynamics and cell cycle distribution and enhances sensitivity to gemcitabine in breast cancer cells.

Objective of this study

Since we have explored the effect of overexpression of TBCC in the breast cancer cells, it was important to look in parallel at the potential effects that low expression level of TBCC might have on these cells. Our results show that inhibition of TBCC in MCF7 cells had effects opposite to those induced by overexpression of TBCC. However, the altered cell cycle distribution observed in the low-TBCC level cells, with a high percentage of S-phase, made these cells increasingly sensitive to gemcitabine along with a moderately enhanced sensitivity to antimicrotubule agents. These results underline the importance of modulation in TBCC level in breast cancer cells in order to enhance their response to treatments.

Inhibition of tubulin binding cofactor C modifies microtubule dynamics and cell cycle distribution and enhances sensitivity to gemcitabine in breast cancer cells.

Running Title

Inhibition of TBCC in breast cancer cells

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Keywords

TBCC; Tubulin folding; Microtubule dynamics; Cell cycle; Breast Cancer; Antimicrotubule agents; Gemcitabine

Abstract

Tubulin Binding cofactor C (TBCC) is essential for the proper folding of α and β tubulins into microtubule polymerizable heterodimers. Since microtubules are considered major targets in the treatment of breast cancer, we investigated the influence of inhibition of TBCC on tubulin pools, microtubule dynamics and cell cycle distribution of breast cancer cells by developing a variant MCF7 cells with reduced content of TBCC (MC-). MC- cells displayed decreased content in nonpolymerizable tubulins and increased content of polymerizable/microtubule tubulins when compared to control MP6 cells. Microtubules in MC- cells showed stronger dynamics than those of MP6 cells. MC- cells proliferated faster than MP6 cells and showed an altered cell cycle distribution, with a higher percentage in S-phase of the cell cycle. Consequently, MC- cells presented higher sensitivity to the S-phase targeting agent gemcitabine than MP6 cells *in vitro*. Although the complete duration of mitosis was shorter in MC- cells and their microtubule dynamics was enhanced, the percentage of cells in G2-M phase was not altered nor was there any difference in sensitivity to antimicrotubule targeting agents when compared to MP6 cells. Xenografts derived from TBCC variants displayed significantly enhanced tumor growth *in vivo* as well as increased sensitivity to gemcitabine in comparison to controls. These results are the first to suggest that proteins involved in the proper folding of cytoskeletal components may have an important influence on the cell cycle distribution, proliferation and chemosensitivity of tumor cells.

Introduction

Microtubules (MT) are highly dynamic structures composed of α/β-tubulin heterodimers and are involved in many cellular functions such as cell motility, cell division, intracellular transport and cellular architecture (1). Microtubule dynamics are highly regulated in the cell by a variety of stabilizing and destabilizing molecules (2). Due to their intense dynamicity during mitosis, MT constitute important targets for anticancer drugs (3). MT are necessary for the execution and timing of mitosis as well as for the determination of the overall duration of the cell cycle (4). Microtubule nucleation involves centrosomes that play additional roles such as organization of cytosolic microtubules, formation of spindle microtubules during mitosis and axonemes formation in ciliogenesis (5-7). In the absence of centrosomes, the spindle is assembled but cell cleavage is interrupted (8, 9). Centrosome duplication occurs during S-phase of the cell cycle and is regulated throughout the cell cycle to control their MT nucleation potential (7). In some breast cancer tumor cells, an accumulation of supernumerary centrosomes has been reported (10).

Formation of α/β-tubulin heterodimers requires a folding pathway that involves many protein chaperones and cofactors (11). Cytosolic chaperonins are highly expressed during cell growth especially from G1/S transition to early S phase and are essential for the folding of actin, tubulin and other cytosolic proteins (12). The five Tubulin Binding Cofactors (TBC) A to E act at different levels of this pathway to generate polymerizable heterodimers (13). Many of these cofactors have also been shown to play other roles in regulating microtubule stability and dynamics (14). TBCD present in the cytoplasm functions as a centrosomal protein critical to the recruitment of the γ-TuRC as well as microtubule growth and mitotic spindle formation. TBCD centrosomal binding partners remain unknown and further studies are to be done to elucidate the regulatory mechanisms behind the recruiting role of TBCD during interphase and mitosis (15). Little is known regarding the role of TBCs in cancer. It has been shown that the inhibition of TBCA in MCF7 and HeLa cells modified the structure of

microtubules and caused cell cycle arrest in G1 (16). TBCC is an essential and key player required for the formation of the tubulin heterodimers by promoting the assembly of the α and β-tubulin peptides. A study on POR gene in Arabidopsis thaliana showed that it encodes an ortholog of TBCC and plays an important role in releasing competent polymerizable heterodimers (17, 18). Conversely there are no data in the literature regarding the relationship between TBCC and the cell cycle or response to antimicrotubule agents in breast cancer cells. Here we report the consequences of TBCC inhibition in the breast cancer line MCF7, in terms of tubulin pool distribution and microtubule dynamics, cell cycle distribution as well as tumorigenic and sensitivity to chemotherapeutic agents in vivo.

Materials and Methods

Plasmid construction

Human *TBCC* (NM_003192) was amplified with from hTerT-HME-1 human mammary epithelium cells (American Type Culture Collection, ATCC) and the purified amplicon was cloned into pcDNA6 in the antisense orientation (designated as pcDNA6/C-) using EcoRI (Fermentas, France) as described previously (19).

Cell culture and transfections

MCF7 (human mammary adenocarcinoma, ATCC) were cultured and transfected with pcDNA6/C- or empty pcDNA6 as previously described (19). Selection of stable transfectants was performed with blasticidin (20µg/ml) (KN-1004, Euromedex, France). Three clones representative of each population were obtained: MP6.1, MP6.2 and MP6.3 correspond to control clones and MC-1, MC-2 and MC-3 correspond to low TBCC clones.

Western blot analysis

Protein extraction and western blot analysis were performed as described previously (19). The antibodies used were anti β III-tubulin (clone Tuj1, 1/2500; Covalab, Lyon, France), anti p53

(clone DO7, 1/1000; Dako, Denmark), anti cyclin E (CC05, 1/500, Calbiochem Oncogene), anti cyclin B1 (sc-245, 1/500, Santa Cruz Biotechnology), anti p21 (sc-397, 1/500, Santa Cruz Biotechnology), anti Cdk2 (sc-748, 1/500, Santa Cruz Biotechnology), anti Cdc2 p34 (sc-56261, 1/500, Santa Cruz Biotechnology), anti β-actin (clone AC-15, 1/5000), anti α-tubulin (clone DM1A, 1/1000) and anti β-tubulin (clone 2.1, 1/1000) from Sigma Aldrich (St Quentin Fallavier, France). Polyclonal antibodies against TBCC (1/800) and TBCD (1/3000) were generously provided by N. Cowan (New York University Medial Center, USA) and that against Arl2 (1/1000) was generously provided by R. Kahn (Emory University School of Medicine, Atlanta, USA).

Quantification of soluble unfolded tubulins, polymerizable αβ-tubulin heterodimers and microtubules

Cells (20.10^6) were harvested, lysed in 200 µl of buffer (100 mM Pipes, pH 6.7, 1 mM EGTA, and 1 mM $MgSO_4$) and polymerizable tubulin (PT), nonpolymerizable tubulin (NPT) and microtubule (MT) fractions were obtained as previously described (19).

Time lapse fluorescent microscopy and analysis of microtubule dynamics

Cells (3.10^5) were seeded in 6-well plate with circular glasses of 24 mm in their bottom and transfected with the pAcGFP1-tubulin vector (Clontech) using lipofectin (Invitrogen) following the manufacturer's instructions. Analysis of microtubule dynamics was preformed by time lapse microscope as previously described (19). The experiment was performed twice on 50 microtubules of the two cell lines MP6.1 and MC-1.

Immunofluorescence

Cells were processed for Immunofluorescence as previously described (19). Antibodies used were directed toward β-tubulin 1:100 (clone 2.1, Sigma Aldrich), or TBCC 1:30 (Abnova, Taiwan) or γ-tubulin 1:50 (clone GTU-88, Sigma Aldrich) followed by secondary FITC-

antibody (Dako, Denmark). DNA staining was performed using diaminido-phenyl-indol (DAPI) (Roche, Manheim, Germany).

Short pulse BrdU incubation

Cells were incubated with 10 μM of BrdU (B5002, Sigma Aldrich) for 5, 15, 30 and 60 minutes. Cells were then collected and labelled with anti-BrdU (347583, Becton Dickinson) and propidium iodide for microscopic analysis following the manufacturer's instructions.

Long time-lapse microscopy and analysis of mitosis

Cells (3.10^5) were seeded in 35 mm cell culture dish in culture medium maintained at 37°C in a 5% CO_2 atmosphere, time lapse microscope and mitotic analysis were performed as previously described (19).

Cell proliferation analysis

Cell proliferation was estimated using nonradioactive BrdU–based cell proliferation assay (Roche, Basel, Switzerland) according to the manufacturer's protocol after 48, 72, 96 and 168 hours. Cells (5.10^3 cells) were seeded in 96-well plastic plates and exposed cells were incubated one week with either 0.5 nM or 1 nM or 3 nM of gemcitabine (Lilly, IN, USA).. MC- and MP6 nomenclatures refer to an average value of the three clones of each of the batch populations.

RNA interference assays

Desalted duplex siRNA targeting *TBCC* 5'-CUGAGCAACUGCACGGUCA-3' and scrambled sequences were designed by Sigma-Aldrich (St Quentin Fallavier, France). The siRNAs (200 nM) were transfected into 25.10^4 MCF7 cells using oligofectamine (Invitrogen, Cergy Pontoise, France) according to the manufacturer's protocol on two consecutive days. Western blot and flow cytometry experiment were done on the third day after transfection.

Analysis of cell cycle distribution by flow cytometry

Cells were collected, incubated 1 hour at 4°C with propidium iodide (0.05 mg/ml) solution containing Nonidet- P40 (0.05%) and analyzed using a FACS Calibur flow cytomoter (BD Biosciences Europe, Erembodegem, Belgium) as previously described (19). For the siRNA's transfected clones, the cell cycle was studied 48 h after the first transfection. Synchronization of cells at the beginning of S- phase was done by a double thymidine block as previously described by Whitfield et al. 2000 (20).

In vivo growth analysis

Female CB17/SCID mice purchased from Charles River Laboratories (Arbresle, France) were bred under pathogen-free conditions at the animal facility of our institute and treated as previously described (19). Animals were treated in accordance with the European Union guidelines for laboratory animal care and use and the study was approved by the local animal ethical committee. All mice used were 5 to 6 weeks old at the time of cells injections. Mice were divided into six groups of six mice injected with six clones. 3.10^6 cells were injected subcutaneously in mice with 50% matrigel (BD Biosciences, Belgium). The six mice were divided into two groups of treated and untreated mice. In the treated groups, gemcitabine (120 mg/kg) was injected intraperitoneally on days 1, 4, 7 and 10. Tumor volumes were measured twice per week and computed with the formula 4/3 ($3.14 \times r^3$), r as the mean radius.

Statistical analysis

The statistical significance of the *in vivo* and *in vitro* data was determined with a Student's t-test. $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ indicate a statistically significant, highly significant and extremely significant difference, respectively.

Results

TBCC protein expression in stable clones

Three clones obtained from MCF7 stably transfected with the empty vector were designated MP6.1, MP6.2 and MP6.3 and used as controls. Three stable clones of MCF7 cells with reduced expression levels of the protein TBCC (MC-) were established and named MC-1, MC-2 and MC-3 in increasing order of expression of TBCC (Figure 1A). In some experiments average results of the three overexpressing clones and the three control clones are globally represented in Figures as "MC-" and "MP6". Of note the decrease of TBCC levels in MC- cells remained limited, suggesting that TBCC is a protein essential for cell survival and that a minimal content is required.

Impact of TBCC inhibition on the expression level of microtubule proteins and related proteins

We have investigated the effect of inhibition of TBCC on the protein content of total α and β-tubulins in MC- cells. No difference was observed for the total α- tubulins between the MC- and MP6 cells while the total β- tubulins content was slightly increased in MC- clones (Figure 1A). The βIII-tubulin isotype content proved to be heterogeneous among control clones, precluding definitive conclusions regarding the impact of TBCC inhibition on this protein. We investigated the expression levels of TBCD and ADP-ribosylation factor-like 2 (Arl2) which are also involved in tubulin folding and found that Arl2 was decreased in the three MC- clones while TBCD was not modified (Figure 1A).

Effect of TBCC content on tubulin pools

The different pools of tubulins were investigated in MP6.1 and MC-1cells, the latter having been chosen since they have the lowest content of TBCC among the available clones. MC-1 cells contained a significantly larger pool of polymerizable tubulin (confirmed both for α and β tubulin) while the non-polymerizable pool appeared to be slightly reduced (Figure 1 B).

Localisation of TBCC in the cytoplasm

The distribution of β-tubulin in the cytoplasm was not affected by the modification in expression of TBCC in MC-1 cells, as evidenced by immunofluorescence performed using an antibody directed against β-tubulin (Figure 2A). Immunofluorescence with an antibody directed against TBCC demonstrated diffuse cytoplasmic staining both in MC-1 and MP6.1 cells (Figure 2B). However we found a larger proportion of stained cells in MP6.1 cells than in MC-1 cells. No difference in γ-tubulin staining was observed (Figure 2C).

Inhibition of TBCC increases microtubule dynamics

The effects of inhibition TBCC on microtubule dynamic instability were compared in MP6.1 and MC-1 cells 48 h after transient transfection with pAcGFP1-α tubulin. Dynamic parameters which were significantly modified included the mean rates and lengths of growth and shortening and the mean duration in pause. Mean duration of growth or shortening as the frequencies of rescue and catastrophe were not significantly different between the two types of cells. The overall dynamicity was increased by 44% in MC-1 cells in comparison to control cells (Table 1).

Influence of TBCC protein content on proliferation rate *in vitro* and growth *in vivo* of MCF7 cells

The proliferation rates of MC- and MP6 cells *in vitro* were evaluated using the BrdU incorporation assay up to one week. MC- cells were found to proliferate slightly yet significantly faster than MP6 cells (Figure 3A). To investigate the i*n vivo* growth capacity of MCF7 models, we injected the six clones MP6.1, MP6.2, MP6.3, MC-1, MC-2 and MC-3 into SCID mice and monitored their *in vivo* tumor formation. While all clones generated tumors, the tumors derived from MC- clones grew significantly faster ($p<0.0001$) than those derived from the MP6 clones (Figure 3B).

Influence of TBCC protein content on cell cycle distribution and expression levels of cell cycle related proteins of MCF7 cells *in vitro*

Cell cycle analysis by propidium iodide in the six clones (Figure 4A) showed that MC- cells had a significantly higher percentage of cells in S-phase (20.04 ± 0.68) as compared to MP6 cells (16.7 ± 0.28) ($p<0.05$). This observation was confirmed when MP6.1 cells were transiently exposed to siRNA directed against TBCC, and displayed profound reduction of TBCC content (Fig 4B). In cells exposed to anti-TBCC siRNA we also observed a significant decrease in the G2/M fraction.

We have investigated the effect of inhibition of TBCC on cell cycle related proteins in MC-1 cells at baseline as well as after a 24 h exposure to 1 nM gemcitabine. At the basal level, cyclin dependent kinase 1 (CDK1) and cyclin B, involved in the G2/M transition checkpoint, as well as cyclin dependant kinase 2 (CDK2) and cyclin E, involved in the S-phase checkpoint, were less expressed in MC- than in MP6.1 cells. In addition, P21, an inhibitor of cyclins/CDKs and its activator P53 were also downregulated in MC-1 cells with respect to MP6.1 cells (Figure 4C). After exposure to gemcitabine, MP6.1 cells responded by increasing the expression levels of all checkpoint-related proteins while the gemcitabine sensitive MC-1 cells responded by increasing the levels of CDK1 and cyclin B but not CDK2 or cyclin E. P53 and P21 were not increased in MC-1 cells as in the case of MP6.1 cells (Figure 4D).

Influence of TBCC protein content on mitosis and S-phase durations of MCF7 cells *in vitro*

Analysis by time lapse microscopy showed that the total duration of mitosis was significantly shorter in MC-1 cells than in MP6.1 cells (Figure 5A). This difference was mainly attributable to shorter prophase-metaphase duration, while there was no difference in the duration of anaphase/telophase. Once released from a double thymidine block, synchronized MP6.1 and MC-1 cells progressed differently through the cell cycle. MC-1 cells completed S-phase in 8

hours while MP6.1 cells required 12 hours (Figure 5B). This was also observed with short pulse incubations with BrdU where MC-1 cells presented higher percentages of cells in S-phase starting 5 min till 1 hour (Figure 5C).

Response to gemcitabine *in vitro* and *in vivo*

First, we investigated the response of the cells to gemcitabine *in vitro*, an S-phase specific antimetabolite. We incubated our cells for one week with 0.5, 1 and 3 nM of gemcitabine and found that at these 3 concentrations, MC- cells presented significantly higher sensitivity to gemcitabine compared to the MP6 cells as demonstrated by reduced BrdU incorporation (Figure 6A). Second, to investigate the *in vivo* response of MC- and MP6 clones to gemcitabine, mice injected with the six different clones were divided into control groups and groups exposed to gemcitabine. The growth of the clones in control groups presented the same profile as in the first experiment (Figure 3B). Furthermore the antitumor activity of gemcitabine was markedly stronger in mice injected with MC- clones than in mice injected with MP6 clones (Figure 6B). The results presented as MP6 and MC- are the mean values of the volumes of the tumors formed by the three clones of each group.

Discussion

Tubulin binding cofactor C is a crucial protein for the proper folding of α and β tubulins to form heterodimers able to polymerize into microtubules. The importance of TBCC in the cell was underlined by the moderate inhibition obtained in transfected cells with respect to controls, suggesting that lower levels of expression may be incompatible with cell survival. We showed that the inhibition of TBCC in MCF7 cells altered the distribution of tubulin peptides in the various tubulin pools, with a significant increase in the polymerizable fraction and increased microtubule dynamics. Cells with reduced TBCC content also displayed a larger fraction of cells in S phase as well as shorter duration of mitosis. These cells showed

increased proliferation *in vitro* and enhanced tumorigenicity *in vivo*, as well as enhanced sensitivity to gemcitabine, an S-phase specific agent.

Inhibition of TBCC had a major impact on tubulin fractions, with a large increase in the polymerizable fraction (consisting of soluble tubulin dimers) and a decrease in the non-polymerizable pools. The nonpolymerizable tubulin fraction consists of the pool of tubulins in the α-tubulin/TBCE/TBCC/TBCD/β-tubulin complex, the pool of α-tubulins bound either to TBCB or TBCE or TBCE/TBCC/TBCD/β-tubulin, and the pool of β-tubulins bound either to TBCA or TBCD or α-tubulin/TBCE/TBCC/TBCD. The inhibition of TBCC is likely to decreases the amount of tubulin trapped in TBCC-containing complexes and thus increases the availability of α/β tubulins to form polymerizable heterodimers. Of note and contrary to tubulin dogma, MC- cells appear to have a disequilibrium between the contents of total α-tubulin and β-tubulin. It is classically considered that such disequilibrium would be lethal for mammalian cells and that the cell controls this by regulating the synthesis and folding of these proteins. However in this model the increased content can be attributed to a specific enrichment in the non-functional fraction of β-tubulin, contained in the non-polymerizable pool. We also observed a strong impact of TBCC content on microtubule dynamics. At the onset of mitosis, the interphase microtubule network disassembles while there is simultaneously a decrease in total microtubule polymer mass and an increase in microtubule dynamics (21). In some cells, the increase in dynamics is due to an increase in catastrophe frequency and a reduction in the rescue frequency rather than changes in growth and shortening rates (22, 23). In our study, we found that the microtubules in MC-1 cells grew and shortened faster and for longer distances than those in MP6.1 cells, with increased dynamicity in MC-1 cells. These observations are in keeping with the alterations of tubulin fractions observed since the increased availability of polymerizable tubulin dimers could explain the enhanced dynamicity.

Cells with reduced TBCC content proliferate at a significantly higher rate and their cell cycle distribution was deeply altered. We have shown that the MC- cells presented a higher percentage of cells in the S-phase compared to the MP6 cells. The increase in the percentage of S-phase cells was also observed when we transiently inhibited TBCC in MP6.1 cells using siRNA. Under these conditions we also observed a significantly lower percentage of cells in the G2/M phase which was not the case in the stable MC- clones. This could be explained by the fact that transient transfections in some cases induce stronger effects than those which are compatible with the survival of stable clones. During prometaphase, the dynamicity of microtubules is very important in order to probe the cytoplasm and attach to chromosomes at their kinetochores (24). Any single chromosome unable to attach to the spindle is enough to prevent a cell from transitioning to anaphase and therefore be blocked at or before metaphase-anaphase transition and undergo apoptosis later on (25, 26). In our study, the increased microtubules dynamics in MC- cells highly reduced the overall duration of mitosis by reducing the duration of prophase-metaphase. The enhanced microtubule dynamics of interphase microtubules may also have influenced the S-phase of the cell cycle in MC- cells. During early S-phase, newly synthesized tubulin polypeptides are transferred by the protein prefoldin to the cytosolic chaperonins (12). Centrosomes are also duplicated during S-phase. Highly dynamic microtubules could provide faster transport of the proteins synthesized, the duplicated organelles, the centrosomes and the enzymes needed for DNA replication, as suggested by the shorter S-phase which we observed by BrdU-short pulse in MC-1 cells.

It is generally believed that when a checkpoint is dysregulated, the progression into the cell cycle is influenced. However, it was also shown that inhibition of the checkpoint CDK2 in colon cancer cells didn't stop cell proliferation (27). Cancerous cells are example of cells that can override defects in checkpoints and proceed normally into the cell cycle. MC- cells are a model of cancer cells that have acquired a high proliferative activity, a more aggressive phenotype *in vivo* as well as an increased capacity of tumor growth. We have

observed that the checkpoints of G2/M and S-phase transitions were downregulated in the MC-1 cells. It is known that checkpoints monitor the integrity and assembly of microtubules during the cell cycle (4, 28). To our knowledge, it is not clear yet if enhanced microtubule dynamics is correlated or can be behind the downregulation of cell cycle checkpoints. It was previously described that cells that can't go through the spindle assembly checkpoint (SAC) can exit mitosis by progressively degrading cyclin B by proteosome-mediated proteolysis (29). In our study, the high content in polymerizable tubulins in MC- models increased the dynamics of microtubules and potentially enhanced speed of assembly and disassembly of the spindle and influenced the binding affinity of checkpoints to microtubules. In addition, the enhanced microtubule dynamics might have rendered our models nonresponsive to checkpoints such as SAC during many successive cycles and consequently have caused a progressive degradation of the checkpoints including CDK1, CDK2, cyclin E and cyclin B. In general, cells exposed to gemcitabine overexpress CDK1 and cyclin B checkpoint as a secure way to prevent cells with defective DNA to proceed into mitosis. This was observed in both MP6.1 and MC-1 cells in response to gemcitabine. In MP6.1 cells, the expression levels of other checkpoints and cell cycle related proteins were increased such as CDK2, cyclin E, p21 and p53, preventing the cells to proceed into a defective cell cycle. However in MC-1 cells, S-phase checkpoints CDK2, cyclin E as well as p21 and p53 that were downregulated at the basal level, remained inhibited after exposure to gemcitabine. The consequent lack of cell cycle arrest is likely to be correlated with the enhanced sensitivity to gemcitabine.

The MCF7 cells (human mammary adenocarcinoma) emerge from an invasive ductal carcinoma type of cancer (30, 31). In our study, clones with reduced content presented an enhanced tumor growth *in vivo* compared to control cells. This is in keeping with the observation that MC- cells proliferate more rapidly *in vitro* and have a larger percentage of cells in S phase. In a previous study, we found that nine of thirteen different human breast cancer cell lines reported to be highly invasive *in vitro* also presented low *TBCC* expression level (19, 32). This observation strengthens the hypothesis of a possible involvement of

TBCC in tumor aggressivity whether through its impact on microtubule dynamics or through other unidentified pathways such as interaction with the Arl2 protein. It is important to note that in the MC- cells, the expression level of Arl2 is decreased. ADP ribosylation factor like 2 (Arl2) protein is a GTPase that belongs to ADP ribosylation factor (ARF) family and also plays an important role in microtubule dynamics (33, 34). Arl2 is known to directly bind to TBCD and consequently can inhibit TBCD from dissociating the α/β tubulin heterodimers (35). MCF7 cells with low Arl2 expression level were found to have high i*n vivo* growth capacity (36). However *in vitro*, the behaviour of the MC- cells in terms of cell cycle distribution, microtubule dynamics, and response to antimicrotubule agents largely differ from that of cells with low content of Arl2, suggesting that the phenotype of MC- cells is not simply explained by reduced Arl2 content (37).

Reduction of TBCC content was associated with enhanced sensitivity to gemcitabine, an S-phase specific compound, but not to microtubule-targeted agents, both *in vivo* and *in vitro*. Increased sensitivity to gemcitabine could be explained by significantly higher percentage of cells in S-phase in cells with low TBCC expression level. In literature it was shown that normal cells are treated with paclitaxel or any other cell cycle targeting drug, the cell cycle is arrested. When the drug is removed, the block is removed and the cell proceeds in the cycle. However in cancerous cells with defects in any checkpoints, the unsynchronized cells will proceed in an abnormal cycle and they will realize that they have overridden a checkpoint so they will undergo apoptosis (38). Although, MC- cells acquired high microtubule dynamics, they still presented slight sensitivity to antimicrotubule agents both *in vivo* and *in vitro* (Supplemental figure 1). Even though MC- cells progress faster through mitosis than MP6 cells, their distribution in the G2-M phase of the cell cycle was not altered with respect to control cells which might explain the lack of different sensitivity to antimicrotubule agents.

In conclusion, our results suggest that reduction of TBCC protein content in MCF7 cells has profound consequences on tubulin pools, microtubule dynamics, cell cycle distribution and duration as well as proliferation and tumorigenicity. Remarkably cells with reduced TBCC content proved to be exquisitely sensitive to gemcitabine, both *in vitro* and *in vivo*. This observation raises the interesting hypothesis that TBCC content in tumors may influence sensitivity to gemcitabine and will require additional studies both in breast cancer and in other diseases. Moreover this work suggests that the modulation of tubulin pools in tumor cells could represent a therapeutic target *per se*.

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Table 1. Parameters of microtubule dynamics in MC-1 and MP6.1 cells

Parameters		MP6.1	MC-1	Change
Mean rate (µm/min ± SE)	Growth	14.74 ± 3.23	19.08 ± 2.27 *	29%
	Shortening	13.33 ± 1.67	15.50 ± 0.81 *	17%
Mean length (µm ± SE)	Growth	2.16 ± 0.38	2.82 ± 0.26 *	31%
	Shortening	2.23 ± 0.41	2.77 ± 0.35 *	24%
Mean duration (min ± SE)	Growth	0.15 ± 0.01	0.15 ± 0.01	
	Shortening	0.17 ± 0.01	0.18 ± 0.03	
	Pause	0.25 ± 0.01	0.15 ± 0.01 *	-40%
Mean frequency (min^{-1}± SE)	Rescue	9.88 ± 0.53	11.52 ± 1.50	
	Catastrophe	4.56 ± 0.75	6.13 ± 0.27	
Dynamicity (µm/min ± SE)		8.69 ± 0.75	12.52 ± 1.32 *	44%

The percentage of change represents the percentage difference of the MC-1 values with respect to MP6.1 values. Values differ significantly from MP6 at $p<0.05$ (*). Values are represented as mean ± SD

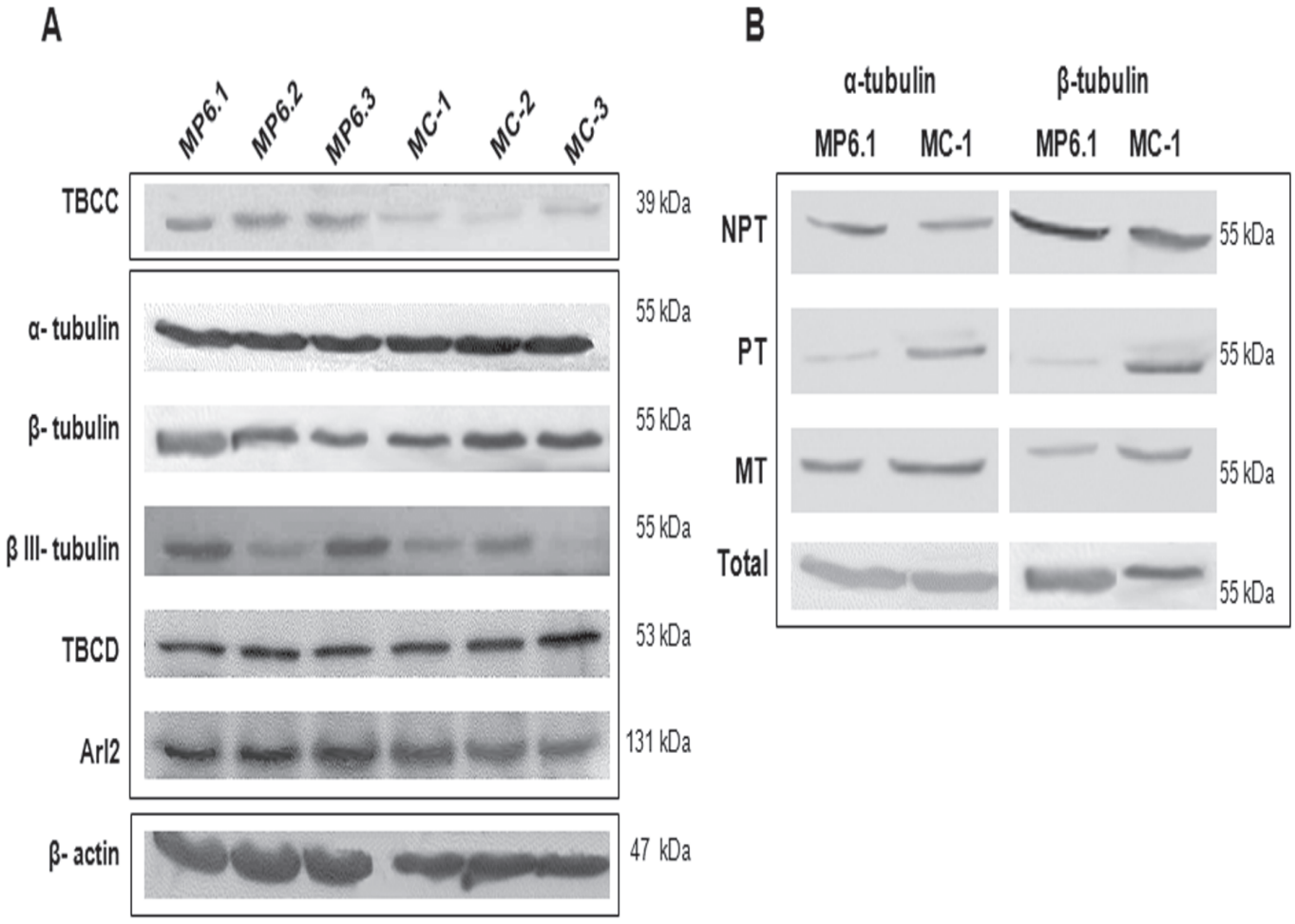


Figure 1. Expression levels of different proteins in MCF7 cells with low TBCC levels

(A) Representative blots corresponding to TBCC, α-tubulin, β-tubulin, β III-tubulin, TBCD, Arl2 and β-actin in MC-1, MC-2, MC-3, MP6.1, MP6.2 and MP6.3 cells (B) Representative blots corresponding to α- tubulin and β- tubulin in nonpolymerizable tubulin heterodimers (NPT), polymerizable tubulin (PT) heterodimers, microtubule heterodimers (MT) and total pool of tubulins in MC-1 and MP6.1 cells

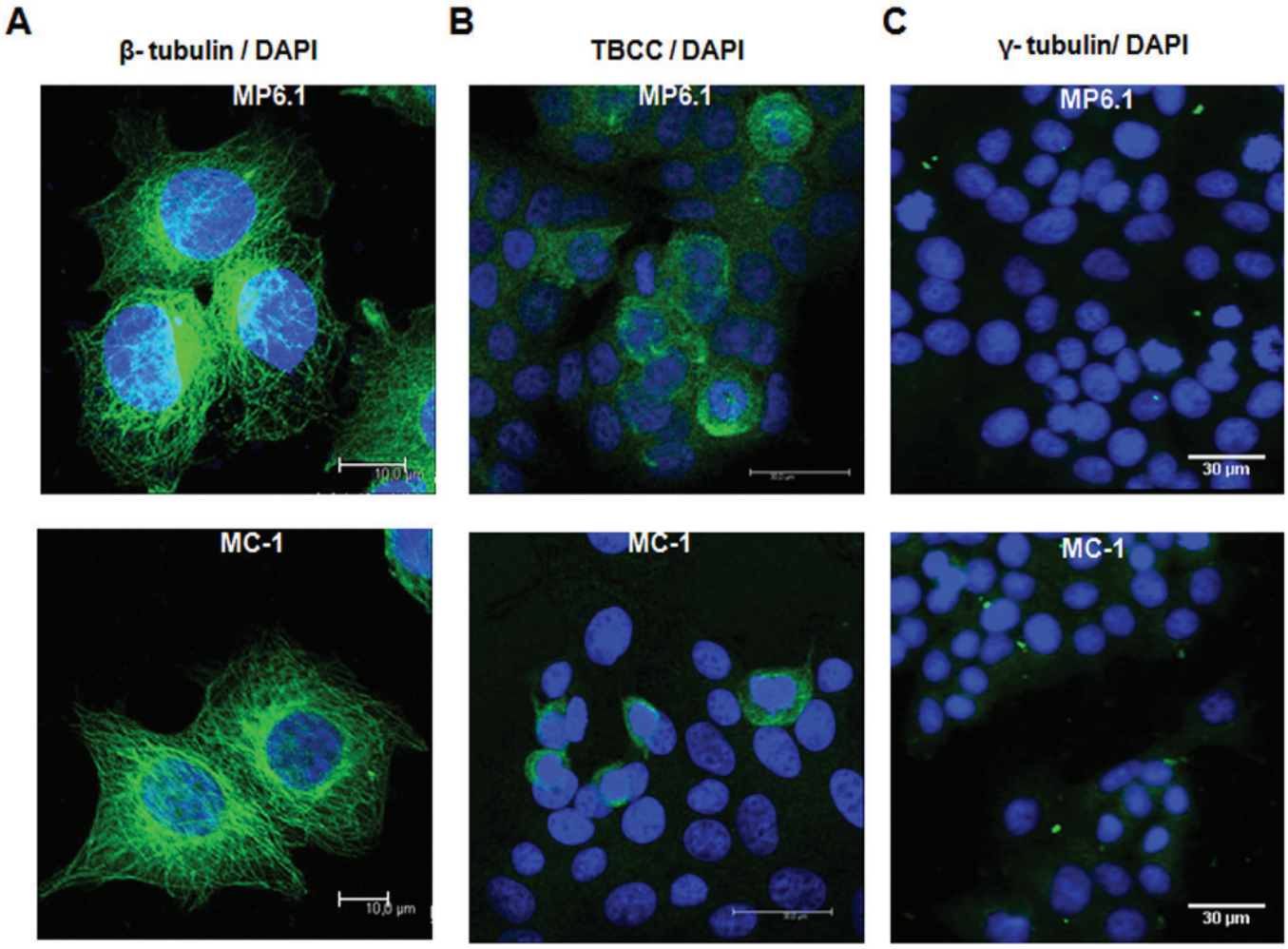


Figure 2. Immunocytochemistry of β tubulin, TBCC and γ tubulin

Representative images of MC-1 and MP6.1 cells after DNA (DAPI, blue), (A) β-tubulin (FITC, green; scale 10 µm), (B) TBCC (FITC, green; scale 30 µm) and (C) γ- tubulin (FITC, green; scale 30 µm) staining

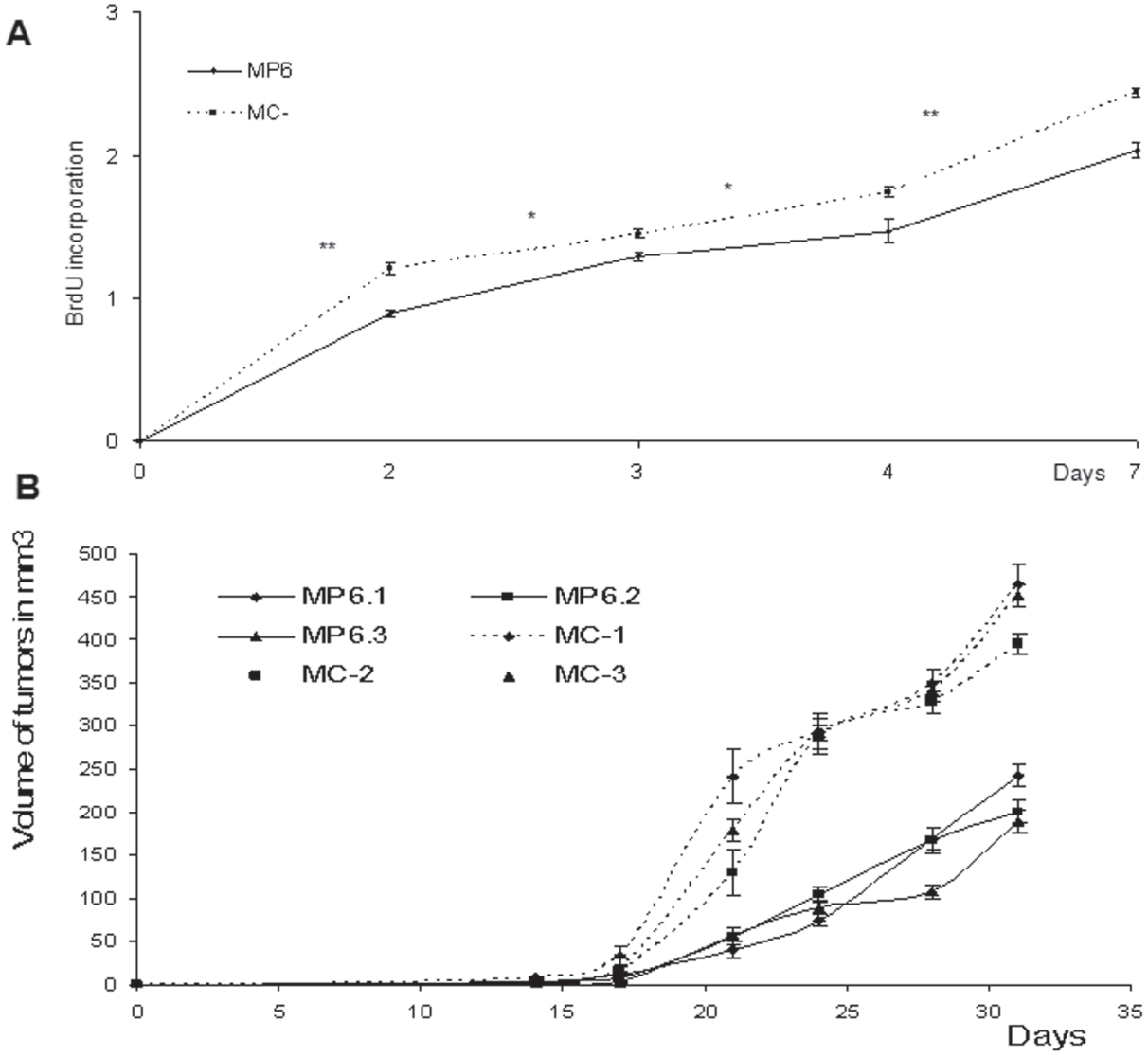


Figure 3. Proliferation rate *in vitro* and growth *in vivo* of cells

(A) Proliferative capacity (BrdU labelling) of MC- and MP6 cells for one week.

MC- and MP6 represent the average value of three clones of MC- or MP6, respectively. Values differ significantly from MP6 at $p<0.05$ (*) and $p<0.001$ (**), respectively. Bars represent standard deviation. (B) Progression of tumors growth measured after subcutaneous injections in mice of each of MC-1, MC-2, MC-3, MP6.1, MP6.2 and MP6.3 cells. Bars represent standard deviations.

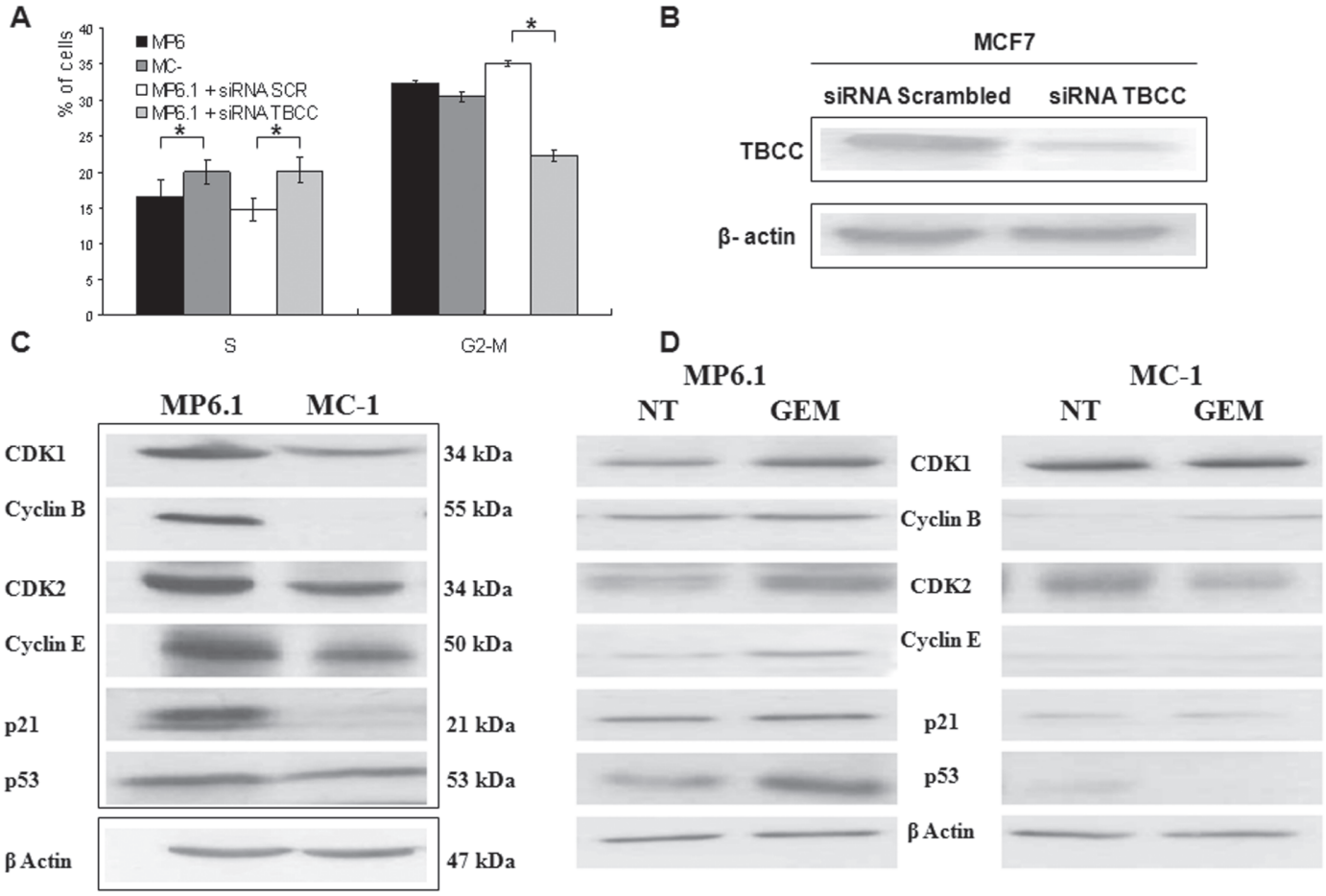


Figure 4. Cell cycle distribution and expression level of related proteins

(A) Percentages of cells in the S- and G2-M phase of the cell cycle. MC- and MP6 represent the average value of three clones of MC- or MP6, respectively. MP6.1 + siRNA SCR and TBCC correspond to MP6.1 cells transfected with siRNA scrambled and siRNA targeting TBCC, respectively. Values differ significantly at $p<0.05$ (*). Bars represent standard deviation. (B) Representative blots showing TBCC content in MCF7 cells 48 hours after double transient transfection with siRNA targeting TBCC and scrambled siRNA. (C and D) Representative blots corresponding to the expression levels of CDK1, cyclin B, CDK2, cyclin E, p21 and p53 at basal levels (C) and after 24 hours exposure to gemcitabine (1 nm) in MC-1 and MP6.1 cells (D). In panel D, the protein contents in the control and the exposed cells are only comparative within either MP6.1 and or MC-1 cells.

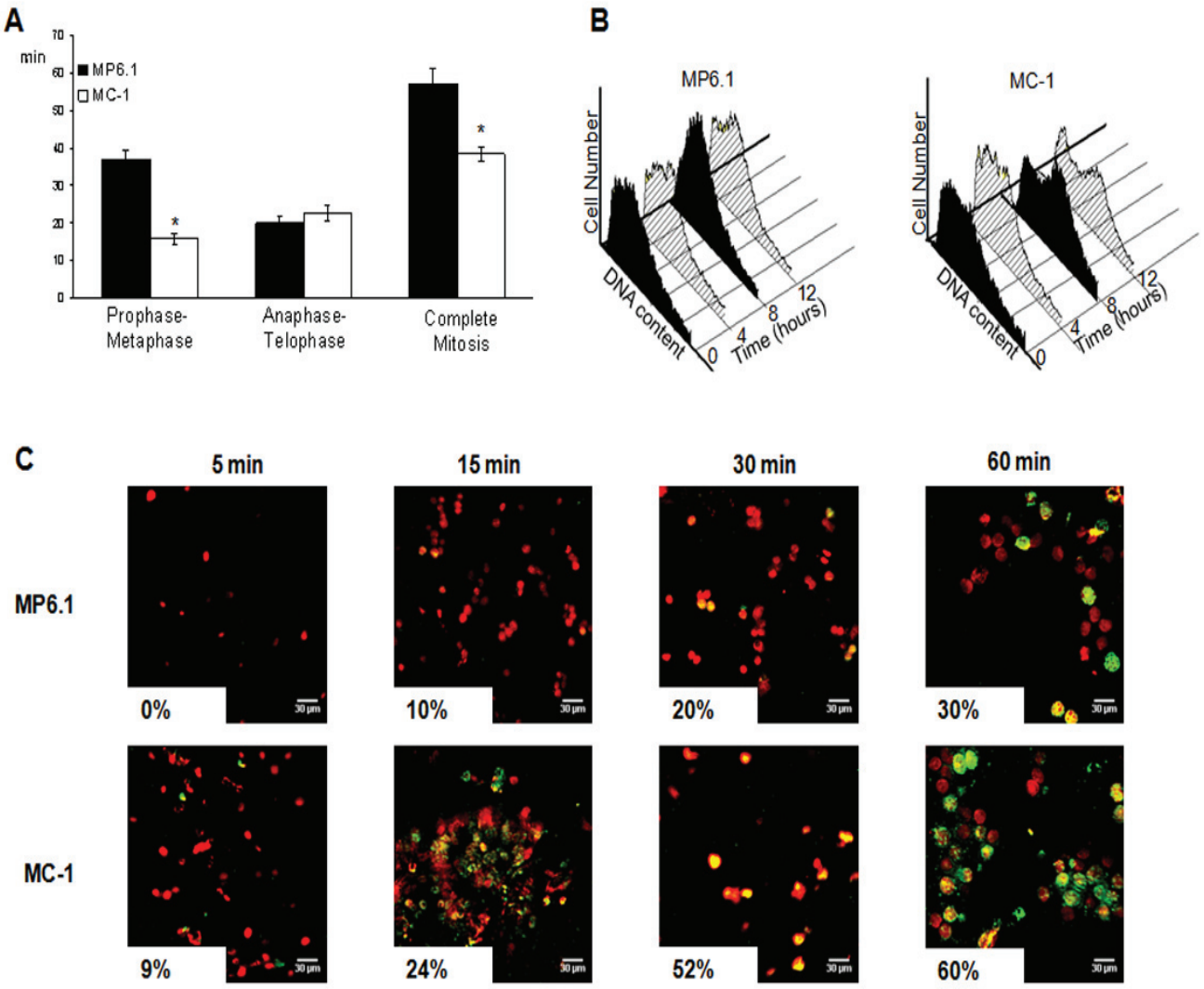


Figure 5. Durations of mitosis and S- phase of the cell cycle

(A) Durations of prophase-metaphase, anaphase-telophase and mitosis in MC-1 and MP6.1 cells in minutes. Values differ significantly from MP6 at $p<0.05$ (*). Bars represent standard deviation. (B) Flow cytometry data of the progression into S-phase of the cell cycle after release from a double thymidine block. The number of cells is plotted against the DNA content (propidium iodide staining) for time points at 4 hours intervals. (C) Short pulse staining of cells incubated with 10 μM of BrdU at 37 °C for 5, 15, 30 and 60 minutes. Red cells (propidium iodide) represent cells in the cell cycle and green cells (anti- BrdU-FITC) are cells in the S-phase of the cell cycle. The percentage shown in each image is the percentage of cells in S-phase.

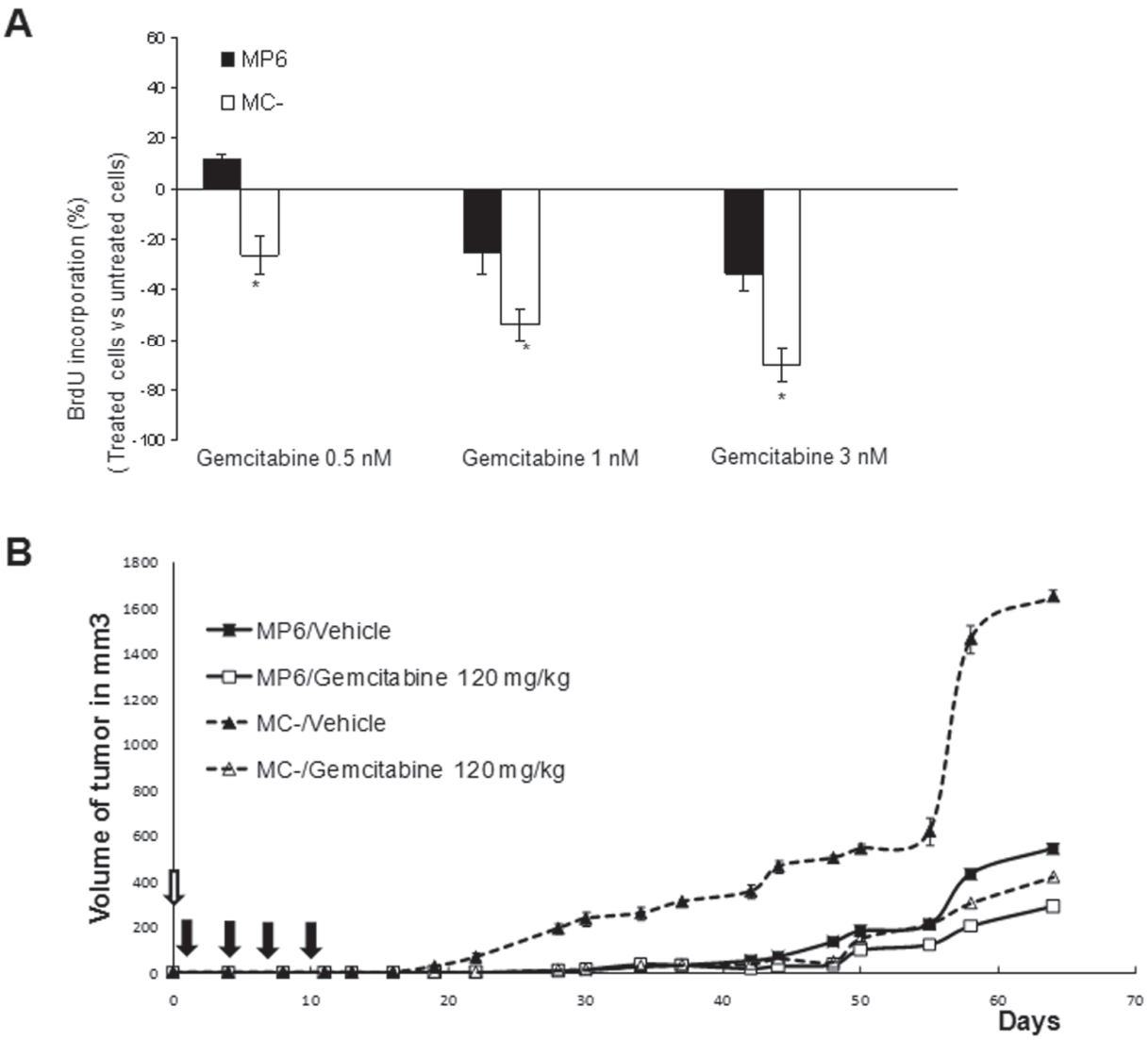
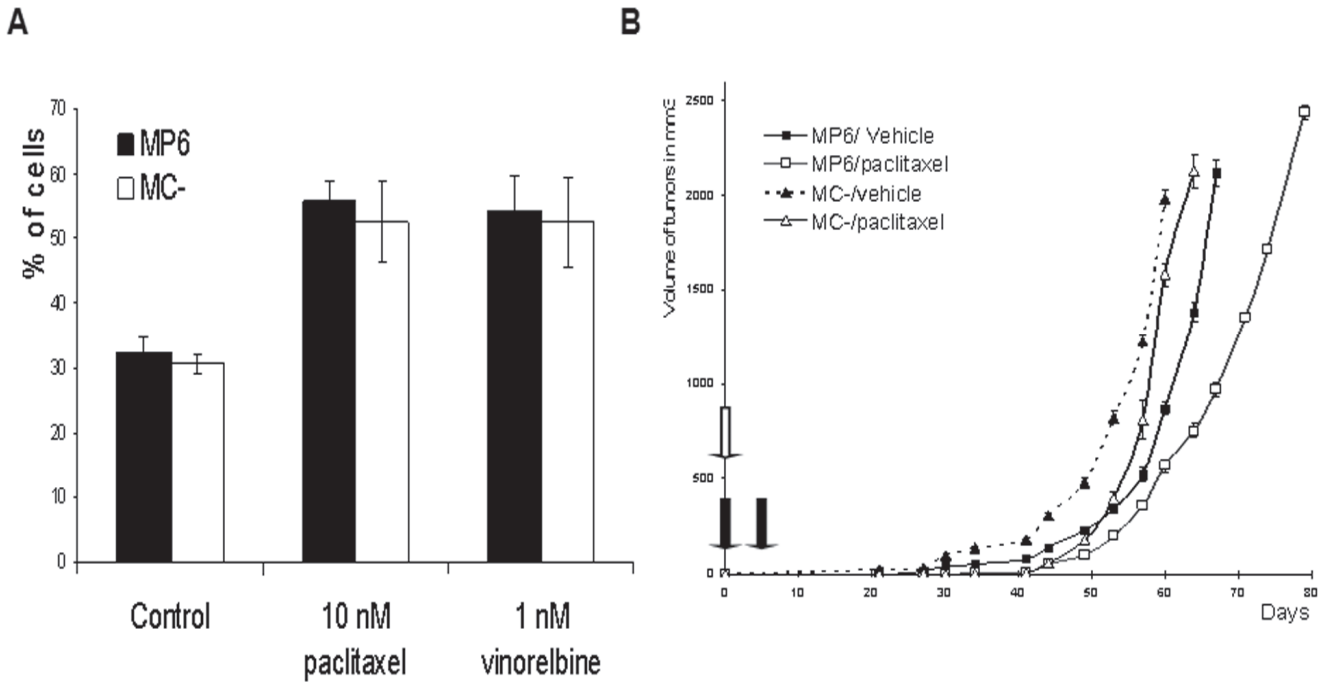


Figure 6. Response to gemcitabine *in vitro* and *in vivo*

(A) Proliferative response by BrdU labelling (treated versus untreated cells) after one week exposure to 0.5, 1 and 3 nM of gemcitabine in MC- and MP6 cells. Values differ significantly from MP6 at $p<0.05$ (*). Bars represent standard deviations. (B) Response to gemcitabine measured after subcutaneous injections of each of MC-1, MC-2, MC-3, MP6.1, MP6.2 and MP6.3 cells at day 0 (empty arrow). At days 1, 4, 7 and 10, gemcitabine was injected intraperitoneally at a dose of 120 mg/kg (black arrows). Results presented are the mean values of the three clones of MC- or MP6, untreated or treated. Bars represent standard deviations.



Additional figure 1. Response to antimicrotubule agents *in vitro* and *in vivo* in MCF7 clones

(A) Percentage of MC- and MP6 cells in the G2-M phase of the cell cycle after 24 hours of treatment with 10 nM of paclitaxel and 1 nM of vinorelbine MC- and MP6 represent the average value of three clones of MC- and MP6, respectively. Values differ significantly from MP6 at $p<0.05$. Bars represent standard deviation. (B) Response to paclitaxel measured after subcutaneous injections of each of MC-1, MC-2, MC-3, MP6.1, MP6.2 and MP6.3 cells at day 0 (empty arrow). At day 0 and day 7, paclitaxel was injected intraperitoneally in a dose of 10 mg/kg (black arrow). Results presented are the mean values of the three clones of MC- or MP6, untreated or treated. Bars represent standard deviations.

PART 4 Supplemental results

1. Expression level of proteins in tumors extracted from mice

2. Apoptosis in MC-1, MP6.1 and MC+1 cells

3. Contributions to the study done on Arl2 (ADP Ribosylation Factor Like 2)

Supplemental results

4.1. Expression level of proteins in tumors extracted from mice

The expression levels of TBCC, α tubulin, β tubulin, Arl2, p53 and TBCD in the tumors extracted from the mice revealed that the MC+ cells maintained the same alterations *in vivo* as those reported *in vitro* (Figure 1). TBCC was still inhibited in the tumors issued from MC- cells with respect to the tumors issued from MP6 cells. TBCC was overexpressed in tumors issued from MC+ cells with respect to MP6 tumors. We observed no significant difference in the expression of total α-tubulin, β-tubulin and TBCD between the different tumors. Arl2 expression was increased in the MC+ and decreased in the MC- tumors with respect to the Mp6 tumors. p53 expression was slightly higher in MC+ tumors and significantly lower in MC- tumors with respect to MP6 tumors.

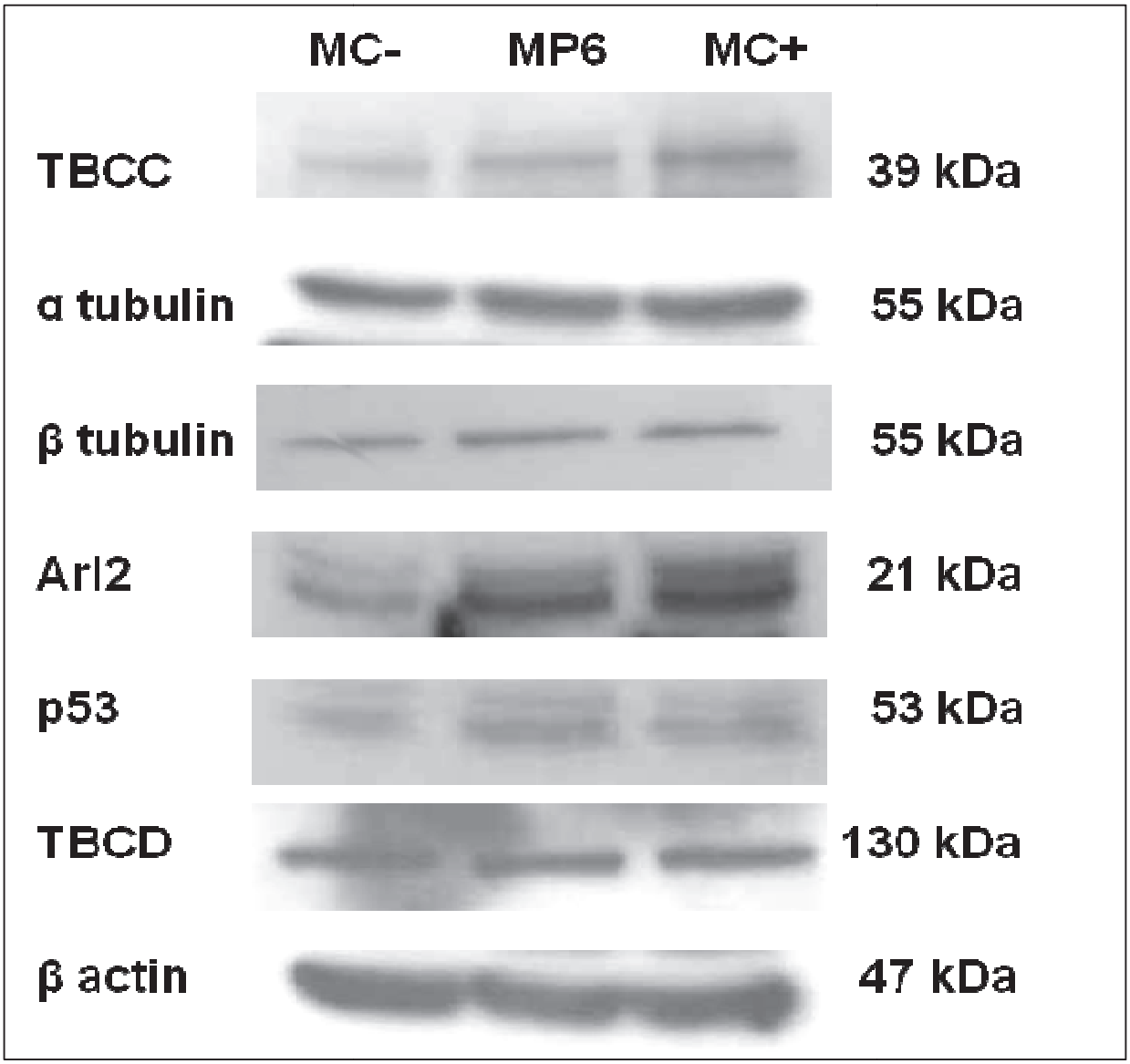


Figure 1. Expression levels of proteins in tumors extracted from mice

Western blot analysis on the tumors of MP6, MC- and MC+ clones showing the effect of modification of TBCC on the expression level of TBCC, α-tubulin, β-tubulin , Arl2, p53 and TBCD. The profile of expression of the clones was retained *in vivo*. MP6, MC- and MC+ are the pool of tumors formed from MP6.1/MP6.2/MP6.3, MC-1/MC-2/MC-3 and MC+1/MC+2/MC+3, respectively.

4.2. Apoptosis in MC-1, MP6.1 and MC+1 cells

The modification of TBCC expression level in MCF7 cells had no effect on the content in apoptotic cells since similar percentages were observed for MC-1 (3%), MP6.1 (4%) and MC+1 (2%) cells. For the early necrotic cells, slight difference with no statistical significance was observed between the MC-1 (8%) and the MP6.1 (14%) cells.

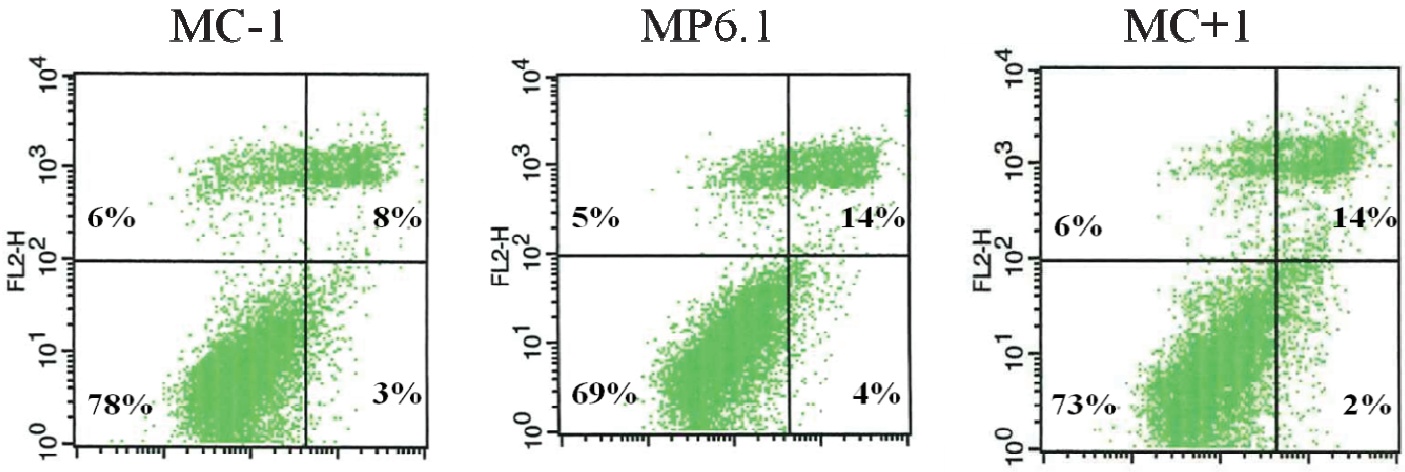


Figure 2. Apoptotic cells in MC-1, MP6.1 and MC+1 cells

Apoptotic cells were detected using FITC-Annexin-V and analyzed by flow cytometry.

4.3. Contributions to the study done on Arl2 (ADP Ribosylation Factor Like 2)

I have contributed to the article by Anne Béghin on the implication of Arl2 in breast tumor aggressivity in immunodeficient mice. I have conceived, designed, performed and analyzed the experiment for the activity of PP2A and the tumor growth *in vivo*. It was shown that the cells overexpressing Arl2 displayed stronger phosphatase activity against phosphoserine substrate than the control or the cells with low Arl2 expression level. In addition, siRNA sequences targeting each of *Arl2* and *PP2A* were designed and injected in mice after injection of MDA-MB 231 cells. This experiment showed the involvement of both Arl2 and PP2A in the tumor cell aggressivity *in vivo*. Inhibition of both Arl2 and PP2A enhanced the tumor growth in comparison with mice injected with scrambled siRNA. The modulation of expression of the target genes in vivo was confirmed by RT-PCR on tumors and livers extracted from mice (Beghin et al. 2009).

For the results, please refer to Annexe, Part 1 Article.

GENERAL DISCUSSION

Tubulin binding cofactor C is a crucial protein for the proper folding of α and β tubulins to form heterodimers polymerizable into microtubules. The indispensable presence of this protein in the cell was highlighted by the moderate inhibition obtained in cells transfected with the antisens of *TBCC* with respect to the control cells. In addition, it was also pointed out that the presence of TBCC in the cell is also controlled since the overexpression of this protein was also moderate with respect to control cells. These results suggest that strong inhibition and high overexpression of TBCC are not compatible with the survival of MCF7 cells.

The alterations of TBCC expression level had a strong impact on the microtubules of the MCF7 cells. Since TBCC is an important player in the folding pathway of the tubulins into polymerization-competent tubulins, it can be found in different complexes in the cytoplasm. TBCC forms a complex with TBCE and TBCD and can bind to α and β tubulins to form the polymerizable heterodimers or to the heterodimers to prevent their polymerization into microtubules. We have observed that by modulating the expression levels of TBCC in the cell, the content in polymerizable heterodimers was influenced. A possible explanation for this result is that when we inhibit TBCC in the cell, the remaining TBCC proteins preferentially form the complex α tubulin/TBCE/TBCC/TBCD/β tubulin to release polymerizable heterodimers and consequently the content in microtubules increases. In this case, the content of nonpolymerizable tubulins, which is the pool of tubulins in the α tubulin/TBCE/TBCC/TBCC/TBCD/β tubulin complex, the pool of α tubulins bound either to TBCB or TBCE or TBCE/TBCC/TBCD/β tubulin and the pool of β tubulins bound either to TBCA or TBCD or α tubulin/TBCE/TBCC/TBCC/TBCD, is decreased. In addition, when TBCC was overexpressed in the MCF7 cells, the contents in polymerizable and microtubule tubulins were diminished along with an increase in the content in nonpolymerizable tubulins. All these observations support our hypothesis that when TBCC is overexpressed, the TBCC-containing complexes are more preferentially formed with the tubulins and the availability of polymerizable heterodimers is diminished. The impact of TBCC on the different fractions of

tubulins influenced the microtubule dynamics, specifically the dynamic instability. The overexpression of TBCC decreased the amount of polymerizable tubulins and decreased both the growth and shortening rates of microtubules in MCF7 cells. The growth and shortening durations were also decreased and consequently the overall dynamicity of microtubules was reduced in cells overexpressing TBCC. The opposite effects were observed in the cells with reduced expression level of TBCC. The increased content in polymerizable tubulins increased the rates and distances of growth and shortening as well as the overall dynamicity in MCF7 cells with low-TBCC level.

Of note and contrary to tubulin dogma, both the cells with low and high-TBCC level appear to have disequilibrium between the contents of total α and β tubulins. In general, this disequilibrium is lethal for mammalian cells and the cell controls the synthesis and folding of both tubulins by an autoregulatory mechanism. However, in our models, the increased content of β tubulins can be attributed to a specific enrichment in the non-functional fraction of β tubulin and is independent from the content of TBCC in the cell. It was previously described that microtubule dynamics and response to stabilizing / destabilizing treatments depend on the microtubule composition in tubulin isotypes as well as in post-translational modifications of α tubulins. For this reason, we were interested to look at the detyrosinated (Glu), tyrosinated and acetylated α tubulins and class III β tubulin in our models. The only two results that came out were the acetylated α tubulin and the class III β tubulin. The increased level of acetylated α tubulin in cells overexpressing TBCC can be seen as an attempt of these cells to protect their microtubules from depolymerization since it was described that microtubules rich in acetylated tubulins are more stable under depolymerizing conditions (Piperno, LeDizet et al. 1987). We correlated the decreased level of class III β tubulin in cells with low-TBCC level to the response of these cells to stabilizing and destabilizing treatments that target microtubules. It has been reported that high expression of class III β tubulin by tumor cell is associated with resistance to taxane chemotherapy in non-small cell lung cancer (Dumontet, Isaac et al. 2005). Low-TBCC expressing cells that present high microtubule dynamics were supposed to be less

sensitive to antimicrotubule agents but it is not the case in our models. Therefore, even though our models presented enhanced microtubule dynamics they still responded to antimicrotubule agents similarly to control cells and this might be due to the low level of class III β tubulin in these cells as compared to control cells.

The modifications in microtubule dynamics in our models influenced the distribution into the cell cycle as well as the durations of both mitosis and S-phase. The cell with high levels of TBCC presented longer anaphase-telophase duration and overall longer mitosis. They also presented higher percentage of cells in the G2-M and lower percentage of cells in the S-phase of the cell cycle. The cells with low-TBCC level presented higher percentage of cells in the S-phase, a shorter S-phase and shorter prophase-metaphase and overall mitosis. This new cell cycle distribution influenced the response of our models to cell cycle targeting agents such as antimicrotubule agents and S-phase targeting agent, gemcitabine. Cells overexpressing TBCC presented enhanced sensitivity to antimicrotubule agents that block cells in mitosis and lower sensitivity to gemcitabine that arrests cells in S-phase. However, cells with reduced-TBCC level were more sensitive to gemcitabine. Therefore we expect that our models can respond differentially to cell cycle targeting agents depending on their cell cycle distribution. An interesting approach would be to co-treat our low-TBCC models with both antimicrotubule agents and gemcitabine to get a synergic cytotoxic effect.

Finally, our models presented different proliferation rates *in vitro* and tumor growth capacities *in vivo*. The MCF7 cells (human mammary adenocarcinoma) emerge from an invasive ductal carcinoma type of cancer (Engel and Young 1978; Lacroix and Leclercq 2004). MCF7 cells with reduced TBCC level presented enhanced proliferation rate *in vitro* which is consistent with the durations of their S-phase and mitosis. The overall cell cycle of these latter cells was shortened and their checkpoints were found to be downregulated or in comparison to control cells. These variant cells also presented an enhanced tumor growth *in vivo* compared to control cells. For the cells overexpressing TBCC, the *in vitro* proliferation was not modified while the *in vivo* growth was decreased. The tumor growth capacity in our

models depends on the potential gain or loss of aggressive and invasive capacities of the cells after the modifications in their microtubule dynamics and their cell cycle progression however we can't conclude a direct role of TBCC in the aggressivity of the cells. This however underlines a possible involvement of TBCC in tumor aggressivity whether through microtubules or through other unidentified pathways such as interaction with the Arl2 protein. It is important to note that the expression levels of Arl2 and tumor suppressor p53 were modified in our models. They were decreased with decreased TBCC levels and increased with increased TBCC level. This finding is interesting since it reminds us of a study done on Arl2 that showed that MCF7 cells with low Arl2 expression level were found to have high *in vivo* growth capacity (Beghin, Belin et al. 2009). However in this latter model, the behaviors of the low-Arl2 cells as for the cell cycle, microtubule dynamics, response to antimicrotubule agents largely differ from the behaviors observed in our cells with low expression level of TBCC (Beghin, Honore et al. 2007). Therefore one possible hypothesis could be that *in vivo* a common mechanism involving TBCC and p53 or TBCC and Arl2 is behind the gain in aggressivity observed in MC- cells.

CONCLUSION

To conclude, we have shown that the modifications in TBCC expression level influenced tubulin fraction distribution and microtubule dynamics. Cell cycle distribution and the durations of mitosis and S-phase were altered. The proliferation rate *in vitro* was slightly modified whereas *in vivo* the TBCC variants presented major differences in tumor growth capacity. Chemosensitivity to antimicrotubule agents (paclitaxel and vinorelbine) as well as to gemcitabine was observed to be dependent on the cell cycle distribution of the TBCC variants. We have shown that the overexpression of TBCC in breast cancer cells decreased their aggressivity and enhanced their response to antimicrotubule agents. However, another approach would be to inhibit TBCC in cancerous cells to accelerate their cell cycle and increase their sensitivity to cell cycle targeting agents. These results underline the essential role of fine tuned regulation of tubulin content in tumor cells and the major impact of dysregulation of tubulin dimers content on tumor cell phenotype, cell cycle progression and response to chemotherapy. A better understanding of how the microtubule cytoskeleton is dysregulated in cancer cells would greatly contribute to a better understanding of tumor cell biology and characterization of resistant phenotypes.

PERSPECTIVES

This study raises several interesting questions that need to be addressed in the future. Since the checkpoints were found to be inhibited in the MCF7 cells with low TBCC expression level, we suggest that it would be interesting to explore the underlying mechanism for this observation. We suggest that TBCC might play other roles in the cell and regulate the functions of checkpoints by being regulated through phosphorylation by the cyclin dependent kinases. Using the NetPhos 2.0 server which gives predictions for serine, threonine and tyrosine phosphorylation sites in eukaryotic proteins, we have looked for phosphorylation sites on the protein TBCC. We found that it contains 13 serine sites and 2 threonine sites and the most probable kinase involved is the Protein kinase C (http://www.cbs.dtu.dk/services/NetPhos/). From this preliminary data, it would be interesting to work on a recombinant TBCC protein and understand more its structure.

In addition, it would be interesting to search for potential partners of TBCC in the cell, using immunoprecipitation. This will help us know if TBCC can bind to the microtubules under certain conditions and if it is implicated with other proteins of the cell cycle. For this study to be possible, a better monoclonal TBCC antibody than the one used in this study must be developed. This new antibody, if developed, can also be used in immunohistological studies to study the localization and distribution of TBCC in tumor samples from patients under treatments.

Finally, it would be interesting to synthesize a ligand that can stabilize TBCC inside the cytoplasm so that it would be continuously active and thereby enhances the response of cells to antimicrotubule agents.

ANNEXES

PART 1 ARTICLE

ADP ribosylation factor like 2 (Arl2) regulates breast tumor aggressivity in immunodeficient mice

Beghin A, Belin S, Hage-Sleiman R, Brunet Manquat S, Goddard S, Tabone E, Jordheim LP, Treilleux I, Poupon MF, Diaz JJ, Dumontet C.

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ADP Ribosylation Factor Like 2 (Arl2) Regulates Breast Tumor Aggressivity in Immunodeficient Mice

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Abstract

We have previously reported that ADP ribosylation factor like 2 (Arl2), a small GTPase, content influences microtubule dynamics and cell cycle distribution in breast tumor cells, as well as the degree and distribution of phosphorylated P53. Here we show, in two different human breast adenocarcinoma models, that Arl2 content has a major impact on breast tumor cell aggressivity both *in vitro* and *in vivo*. Cells with reduced content of Arl2 displayed reduced contact inhibition, increased clonogenic or cluster formation as well as a proliferative advantage over control cells in an *in vitro* competition assay. These cells also caused larger tumors in SCID mice, a phenotype which was mimicked by the *in vivo* administration of siRNA directed against Arl2. Cells with increased Arl2 content displayed reduced aggressivity, both *in vitro* and *in vivo*, with enhanced necrosis and were also found to contain increased PP2A phosphatase activity. A rt-PCR analysis of fresh human tumor breast samples suggested that low Arl2 expression was associated with larger tumor size and greater risk of lymph node involvement at diagnosis. These data underline the role of Arl2, a small GTPase, as an important regulator of breast tumor cell aggressivity, both *in vitro* and *in vivo*.

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Introduction

Arl2 is a GTPase belonging to the ADP ribosylation factor (ARF) family [1,2]. Several genetic studies suggest that Arl2 plays a role in MT dynamics [3–5]. In mammalian cells, Bhamidipati *et al.* have demonstrated direct binding between Arl2 and tubulin binding cofactor D (TBC-D), a protein involved in tubulin folding [6]. TBC-D interacts with beta tubulin and contributes to the production of polymerizable tubulin heterodimers. TBC-D can also induce the dissociation of soluble alpha/beta heterodimers [7–10]. Binding of Arl2 to TBC-D inhibits the heterodimer-dissociating activity of TBC-D [6]. More recently, it has been reported that Arl2 inhibited TBC-D-dependent cell dissociation from the monolayer and disassembly of the apical complex [11].

The entire cytosolic pool of Arl2 is complexed with TBC-D and with the heterotrimeric protein phosphatase 2A (PP2A) [12]. PP2A is one of the major serine/threonine phosphatases of mammalian cells. It is composed of three subunits: a regulatory A subunit (PP2Aa), various types of regulatory B subunits (PP2Ab) and a catalytic C subunit (PP2Ac). By its involvement in different signal transduction pathways, PP2A plays a crucial role in the regulation of several fundamental cell processes such as cell cycle progression [13], DNA replication [14], apoptosis [15] and protein synthesis. PP2A is considered to behave as a tumor suppressor protein [14]. Mutations affecting PP2A genes have been found in lung, breast and colon carcinoma [16,17], as well as in cervical, ovarian, gastric and nasopharyngeal carcinomas. These mutations frequently consist in deletions in the 11q23 region that encodes the A subunit [16,17]. Furthermore, inhibitors of PP2A such as okadaic acid or microcystin-LR are known to promote cell proliferation and tumorigenesis [12,18,19].

Using breast adenocarcinoma derived MCF7 and MDA-MB 231 cells expressing different levels of Arl2, we have shown that alterations of cellular Arl2 protein content were associated with modifications of polymerization-competent −alpha/beta tubulin heterodimer levels resulting in altered MT dynamic properties and with modifications of mitotic progression as well as with modifications of the content, localization and activity of PP2Ac with no significant changes in PP2Ac mRNA levels [20]. We also showed that Arl2 content was associated with the degree and distribution of phosphorylated P53, in particular phosphor-ser15-P53, a form which was preferentially bound to microtubules [21].

In this study, we show that Arl2 content is a major determinant of aggressive phenotype and tumorigenicity in two different breast cancer cell models, and low Arl2 expression levels appear to be correlated with enhanced aggressivity in the clinic.

Results

Contact inhibition of cancer cells is dependent on Arl2 expression levels

To study the contact inhibition of the cell lines expressing different levels of Arl2 we have evaluated their respective proliferation rates at confluence using phase contrast microscopy, MTT assays and flow cytometry analyses. For these experiments the cellular seeding concentrations were adjusted according to morphological differences of these cell lines previously described [20]. The biggest cells (MA+ and MdaA+) were seeded at a 30% lower concentration whereas the smallest cells (MA- and MdaA-) were seeded at a 30% higher concentration than the respective control cells (MP and MdaP). Phase contrast microscopy observations were used to analyse the cell distribution in Petri dishes (Figure 1a). MA- cells presented a heterogeneous surface occupation with some unoccupied spaces along with high cellular density regions (Figure 1a). Conversely MP, and most notably MA+ cells, occupied available space more evenly with a homogeneous distribution. In addition MA- cells were able to form multi-layers while MP and MA+ grew only in mono-layers, suggesting that MA- cells had lost contact inhibition (Figure 1a).

The proliferation rates were evaluated using MTT assays at 3, 5 and 6 days post seeding. Three days of culture corresponded to the confluence conditions for the three cell lines (Figure 1b). The proliferation rates of both MA- and MP were higher than that of MA+ whatever the time of culture ($p<0.05$). The proliferation rate of MA- and MP cells were very similar until 5 days of culture whereas that of MA- cells was significantly increased ($p<0.05$) compared to that of MP cells after 5 days of culture. Finally flow cytometry analysis (Figure 1b) showed that the ratio of cells in S-G2/M phase at confluence versus cells in exponential growth was

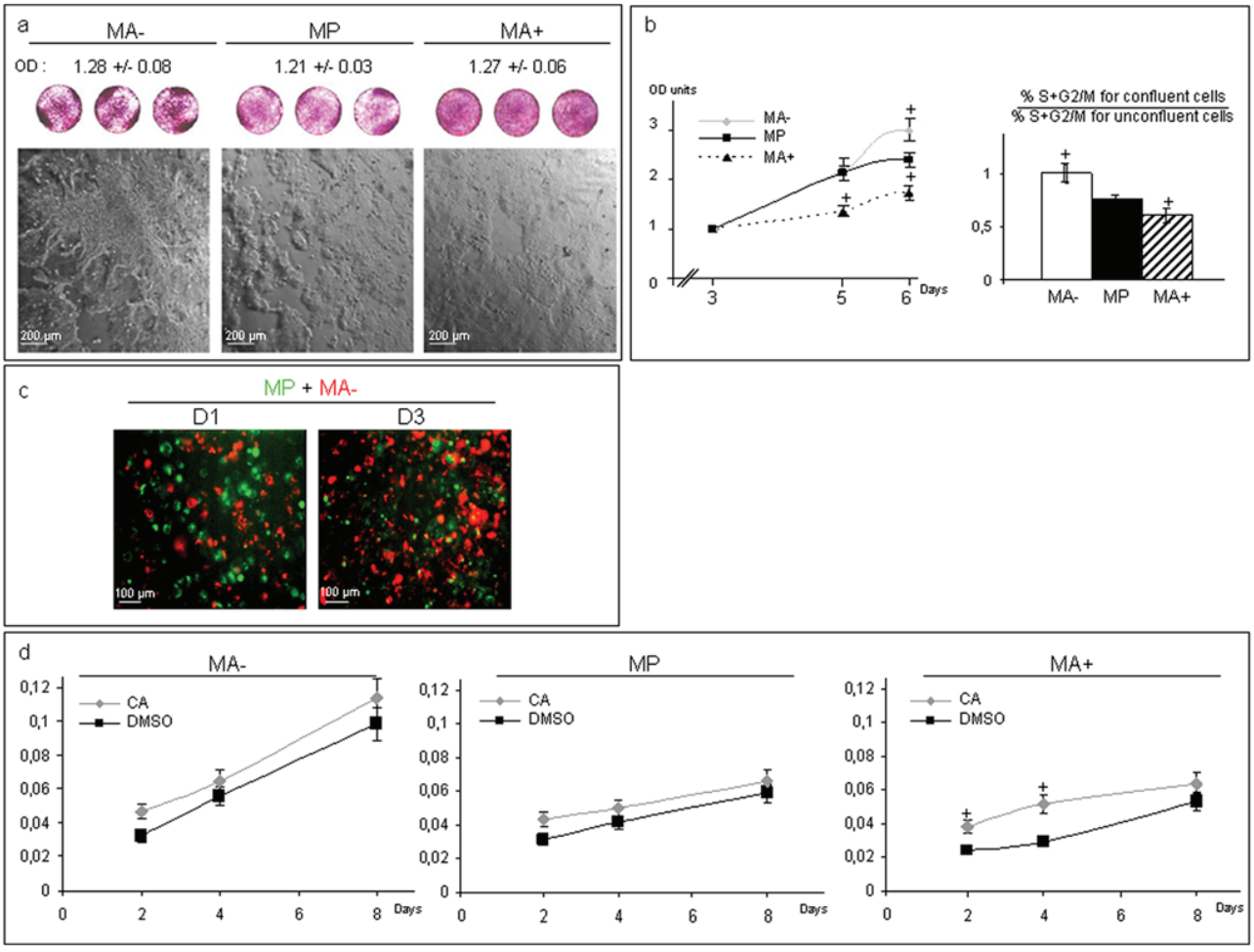

Figure 1. Arl2 content alterations induce modification of proliferation and contact inhibition behavior of cancer cells. A: Upper: MA-, MP and MA+ cells were visualized after MTT incubation and before dissolvation of formazan crystals (purple dishes) to observe cell surface occupation. Formazan crystals were then dissolved and optic density (OD) measured to obtain the control of cells seeding (in arbitrary units). Lower: Phase contrast microscopy observations of MA-, MP and MA+ cells. B: Left: Means of proliferation rates of MA−, MP and MA+ cells evaluated by MTT assay expressed in optic density units per day. Right: Means of percentages of confluent cells versus non-confluent cells in S+G2-M phases evaluated by flow cytometry. Bars represent standard deviations. Statistical significance was determined using Student's t test ($p<0.05$). C: Coculture cell competition assay using different fluorescent vital cell linker dyes, PKH 67 (green) for MP cells and PKH 26 (red) for MA-. Distributions of each cellular population were followed using a fluorescent microscope from day 1 to day 3 after cell seeding (representative images). D: Means of proliferation rates of MA−, MP and MA+ cells incubated with vehicle (DMSO) or with cantharidic acid (CA), evaluated by MTT assay expressed in optic density units (OD units) per day. Bars represented standard deviations. Statistical significance was determined using Student's t test.
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significantly lower (−14%, $p<0.05$) in MA+ cells and higher (+22%, $p<0.05$) in MA- cells in comparison to that of control MP cells. Similar results were obtained for MDA-MB 231 derived models (data not shown). These data confirmed that cells with reduced Arl2 content continued to grow at confluence whereas cells with increased Arl2 content were particularly sensitive to contact inhibition.

Co-culture experiments with MP and MA- cells were performed using specific fluorescent linker dyes to follow each cell population (Figure 1c). After 3 days of co-culture, MA- cells (red fluorescent dye) have proliferated more quickly than MP cells (green fluorescent dye) under confluent conditions, confirming the strong proliferative behavior of MA- cells, and its relative growth advantage over control cells. Similar results were obtained using a green fluorescent dye for MA- cells and a red dye for MP cells (dye swap experiment, data not shown).

Altogether these results showed that, at confluence, cells expressing high levels of Arl2 (MA+ and MdaA+) displayed a stronger growth arrest with increased contact inhibition whereas cells with reduced Arl2 content (MA- and MdaA-) did not seem to undergo growth arrest and maintained growth ability even at confluence.

Since Arl2 interacts with PP2A and the content of Arl2 influences PP2A activity [20], we sought to determine whether the proliferation rates of the different cell lines could be related to PP2A activity. Growth rates of each cell line were measured in the presence of cantharidic acid, a well characterized inhibitor of PP2A. In the presence of cantharidic acid at a dose selectively inhibiting PP2A (IC50 for PP2A activity = 40 nM and for PP1 = 473 nM, manufacturer's indications) (Figure 1d), the growth rate of MA+ cells was significantly enhanced (+30–70%, $p<0,05$) whereas those of MA- and MP cells were not significantly modified. This result suggests that the proliferation rate is related to PP2A activity, since cells with the highest Arl2 and PP2A contents were the most prone to proliferate in the presence of a PP2A inhibitor.

Effect of Arl2 content on three-dimensional cell growth

Experiments using soft agar and soft agar complemented with 30% Matrigel (extracellular matrix) were performed in order to distinguish between anchorage-free growth ability (soft agar) and clonogenic behavior (soft agar+Matrigel) (Figure 2a). No significant differences were observed between MA-, MP and MA+ cells grown in soft agar alone. However, in the soft agar complemented with 30% of Matrigel, MA+ cells developed very few (less than 3 per well) and smaller colonies after 26 days of growth in comparison with MP cells, whereas MA- cells developed significantly more (>40 per well, $p<0.05$) and bigger (+50%

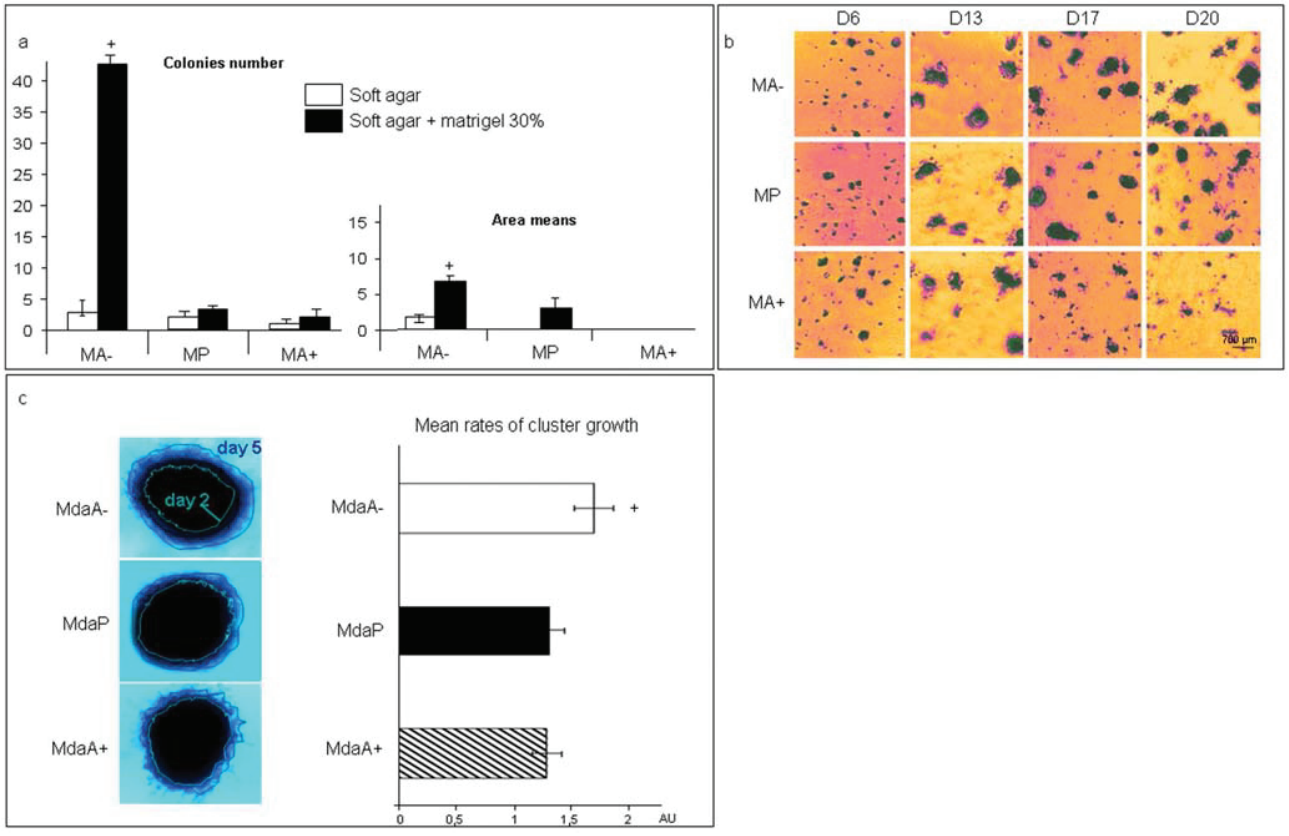

Figure 2. Effect of Arl2 content on three-dimensional cell growth. A: Soft agar and soft agar complemented with matrigel (30% v/v) assay for MA-, MP and MA+ cells expressed in colonies number (left) and in area means (right). Bars represented standard deviations. Statistical significance was determined using Student's t test. B: Follow up of MA-, MP and MA+ cells overlayed on matrigel. Representative images displayed cellular colonies growth from day 6 to Day 20 after cells seeding. C: Matrigel cluster assay performed for MdaA-, MdaP and MdaA+ cells. Light blue lines represent the contour of cells clusters at day 2 and dark blue lines at day 5 after cells seeding (left, representative images). Mean rates of cluster growth were evaluated by daily measurements of cell clusters areas from day 2 to day 5. Bars represented standard deviations. Statistical significance was determined using Student's t test.
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$p<0.05$) colonies than MP cells (Figure 2a). There results suggested that MA- cells have a stronger, and MA+ cells a weaker, clonogenic potential than MP cells.

MA-, MP and MA+ were also seeded on a Matrigel coat to study their proliferative behavior in a three-dimensional extracellular matrix (Figure 2b). After 6 to 13 days of growth, the three cell lines displayed similarly growth properties with formation of small colonies. After 17 days of growth, the MA+ colonies began to disaggregate. After 20 days of growth, MP colonies also began to disaggregate whereas MA- colonies were still present, exhibiting a compact mass (Figure 2b).

These assays were not applicable to the MDA-MB 231 derived cell lines because these cells did not develop isolated cellular colonies in Matrigel or soft agar. We therefore studied the three-dimensional growth capacity of MDA-MB 231 derived cells with the Matrigel cluster assay (Figure 2c). This assay consists in loading a compact cell cluster in a thick layer of Matrigel in order to follow the growth capacity of a "tumor-like" cluster in a three dimensional environment. For the first two days, no significant differences between the three cell lines were observed. After day 2, MdaA- cell clusters had a significant higher (+30%, $p<0.05$) growth rate than MdaP and MdaA+ cell clusters.

Arl2 content influences tumor growth *in vivo*

We evaluated the tumorigenicity of the different MDA-MB 231 and MCF7-derived cell lines in SCID mice. MdaA-, MdaP, MdaA+ cells were transfected with a plasmid encoding firefly luciferase as described in the materials and methods section. Resulting bioluminescent cell lines (MdaA-.luc, MdaP.luc, MdaA+.luc) with bioluminescence *in vitro* were injected into the mammary fat pad of SCID mice and monitored for tumor growth.

Bioluminescent measurements showed that the MdaA-.luc derived tumors were significantly larger (+65% to 80%, $p<0.05$) than those developed by MdaP.luc cells (Figure 3a). Conversely, MdaA+.luc derived tumors were significantly smaller (−45% to 55%, $p<0.05$) than those formed by MdaP.luc cells, resulting in a large difference between MdaA-.luc and MdaA+.luc derived tumors (+100–200%, $p<0.01$). In addition, the mean rate of tumor growth (R-growth) was evaluated between days 17 and 36 (Figure 3b). Tumors derived from MdaA-.luc cells presented a two-fold higher R-growth than MdaP.luc tumors which displayed a 35% higher R-growth than MdaA+.luc tumors. Similar results were obtained in MCF7 and non-bioluminescent MDA-MB 231 derived cells (data not shown).

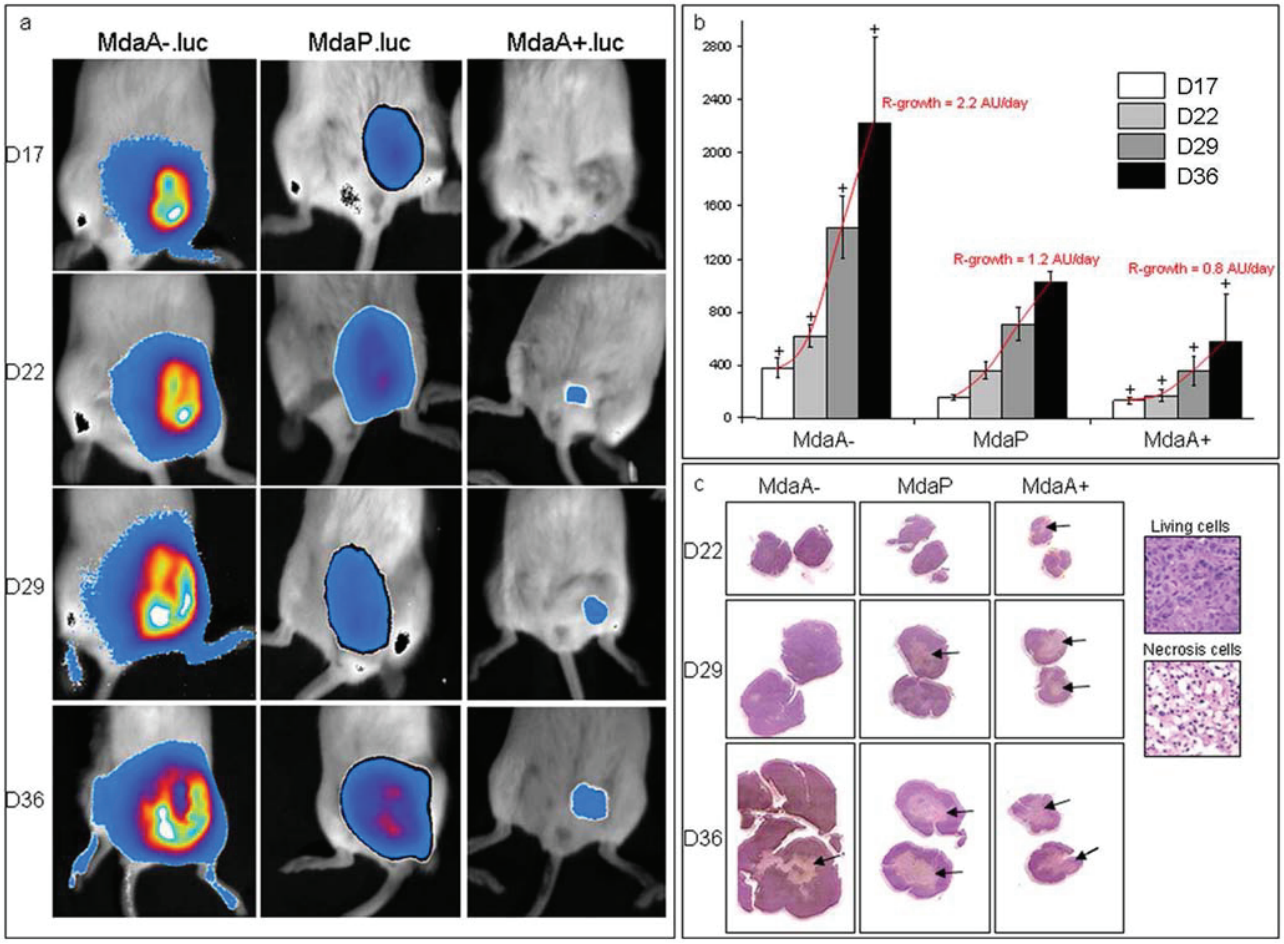

Figure 3. Arl2 content influences tumorigenesis. A: Representative images of non invasive bioluminescent follow up (from day 17 to day 36) for a same tumor of each cell lines (MdaA-.luc, MdaP.luc, MdaA+.luc) developed from mice mammary fat pad injected cells. B: Mean rates of tumoral bioluminescence in mice expressed in photons/sec for each day and sublines. Tumor growths (R-growths) were evaluated using a linear projection of the growth curve (red) and expressed in arbitrary units per day (AU/day). Bars represent standard deviations. Statistical significance was determined using Student's t test. C: Representative images of histochemistry of MdaA-, MdaP, MdaA+ cells tumors slides at day 22, 29 and 36. Black arrows show necrotic regions (in light pink) which are particularly important in MdaA+ cells tumors.
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Tumors were excised from the mice after euthanasia (Figure 3c). Histopathology analyses showed that all MdaA+.luc tumors presented a large necrotic area as early as day 22 post-injection. These areas encompass 15% of the total tumor volume on day 22 and more than 70% on day 36. Conversely, MdaA-.luc tumors, although much larger, contained much less necrotic areas, representing a maximum of 25% of total tumor volume on day 36. MdaP.luc tumors displayed intermediate size necrosis. Similar results were obtained with MA-, MP and MA+ cells (data not shown). These differences in spontaneous necrosis were likely to account at least in part for the differences observed in tumor growth.

To determine whether PP2A activity was significantly different according to Arl2 status during tumor growth *in vivo*, we measured PP2A activity in tumors. As shown in Table 1, MA+ and MdaA+ cells displayed stronger phosphatase activity against the phosphoserine substrate than their corresponding variants, MP and MdaP. In MA- and MdaA- cells, PP2A activity was similar to that of the control cells. These results suggest that Arl2 status modulates PP2A activity *in vivo*.

Inhibition of Arl2 *in vivo* enhances tumor growth and development of human breast cancer xenografts

We assessed the effect of Arl2 and PP2A inhibition *in vivo* on the tumor growth of MDA-MB 231 cells. For this, we administered duplex siRNAs directed against each of these targets intraperitoneally five days a week for four weeks. Scrambled siRNA was used as a control. Both siRNA against Arl2 and against PP2A were found to enhance tumor growth in comparison to controls injected with scrambled siRNA (Figure 4). These results strongly suggest that Arl2 profoundly influences tumor cell aggressivity *in vivo*. As previously shown, inhibition of PP2A was also associated with enhanced tumor aggressivity.

To confirm *in vivo* modulation of expression of target genes, tumor and liver samples were obtained while mice were receiving siRNA therapy. As shown in Table 2, gene expression of PP2A and Arl2 was significantly and specifically repressed by the corresponding siRNA both in liver and in tumors during therapy.

Expression levels of Arl2 in human primary breast tumors

Thirty eight primary breast tumors were available for analysis. Arl2 mRNA content was determined after normalization with 18S ribosomal RNA. Five primary tumors were associated with lymph node involvement and four tumors were of larger size (T3 or T4 of the TNM classification). When classifying tumors according to Arl2 expression in primary tumors (lower or greater than median

Table 1. PP2A activity measured using a phosphoserine substrate in Arl2 variant lines.

	PP2A activity
MA-	1,021±266
MP	984±204
MA+	1,352±160
MdaA-	1,303±82
MdaP	1,466±35
MdaA+	2,006±67

Values represent the picomoles of phosphate issued after the dephosphorylation of the above substrates and are denoted as mean ± standard deviation.
doi:10.1371/journal.pone.0007478.t001

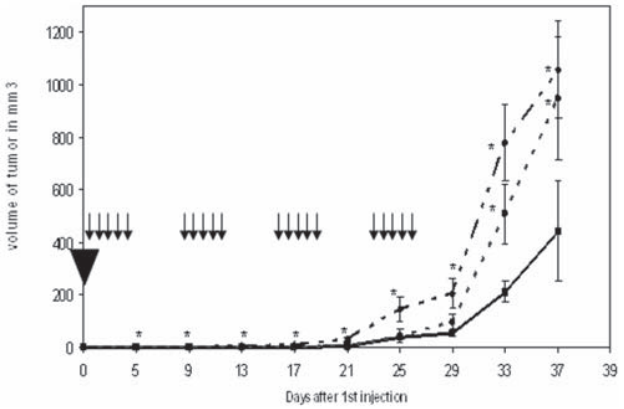

Figure 4. Effect of *in vivo* siRNA directed against Arl2 or PP2A on human MDA-MB-231 xenografts. SCID mice were injected subcutaneously on day 1 (black triangle) with MDA-MB 231 cells then treated daily, 5 days a week for 4 weeks with siRNA (scrambled continous line), directed against Arl2 (dotted line), directed against PP2A (dashed line). *Significant differences were observed between the groups receiving scrambled and Arl2 or PP2A directed siRNA ($p<0.05$). Arrows represent siRNA injections.
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in the entire group) we found that all of the large tumors ($p = 0.05$, Chi-square test) and all of the tumors with lymph node metastases ($p = 0.03$, Chi-square test) were found in samples with low Arl2 expression in the primary tumor (Table 3). Although these observations should be confirmed on larger series, these data suggest that tumors with low Arl2 content are more aggressive clinically.

Discussion

Our results suggest that Arl2 content influences breast tumor growth and aggressivity through a PP2A mediated pathway. In a previous study we have demonstrated that Arl2 content influences the content and the activity of the catalytic subunit of PP2A (PP2Ac) [20]. Given the reported tumor suppressor properties of PP2A [14], we have determined the impact of Arl2 on tumor cell aggressivity and tumorigenic capacity in two breast cancer cell models, MCF7 and MDA-MB-231. We observed that modifications of Arl2 expression levels could induce modifications of contact inhibition, clonogenic potential, and tumor growth, including spontaneous apoptosis of tumor cells. Preliminary data also suggest that cells with reduced Arl2 content possess enhanced metastatic potential when injected orthotopically in SCID mice

Table 2. Expression levels of Arl2 and PP2A of tumor and liver samples in mice exposed to control siRNA, anti PP2A siRNA or anti Arl2 siRNA.

	PP2A (rt-PCRunits)	PP2A (rt-PCRunits)	Arl2 (rt-PCR units)	Arl2 (rt-PCR units)
	Tumor	Liver	Tumor	Liver
Control siRNA	0.37±0.22	0.47±0.16	5.98±0.66	0.80±0.11
Arl2 siRNA	0.38±0.06	0.30±0.02	0.23±0.11*	0.03±0.02*
PP2A siRNA	0.06±0.03*	0.06±0.01*	3.94±4.15	0.61±0.11

*: significantly different from control.
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Table 3. Tumor size and lymph node involvement in primary human breast tumors according to Arl2 expression levels.

	Tumor size	Tumor size	Node involvement	Node involvement
	T1/T2	T3/T4	Negative	Positive
Low Arl2	12	4	11	5
High Arl2	13	0	13	0

Arl2 levels were determined by quantitative rt-PCR on frozen primary breast tumors.

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(data not shown). We have thus shown for the first time, in two different breast cancer cell models, that a reduced Arl2 expression level is associated with a more aggressive neoplastic phenotype. These results were supported by the observation that low Arl2 content was associated with greater aggressivity in primary human breast tumors.

Cells with reduced Arl2 content were found to behave more aggressively, with loss of contact inhibition, ability to grow in multilayer *in vitro*, enhanced clonogenic or cluster formation potential, gain of survival advantage vis-à-vis their normal counterparts in an *in vitro* competition assay as well as enhanced tumor growth and reduced spontaneous necrosis tumors *in vivo*. The role of Arl2 downregulation in the acquisition of an aggressive phenotype is supported by similar results observed when mice injected with parental cells were treated with siRNA against Arl2. This treatment effectively modulated Arl2 gene expression in tumors and was associated with increased tumor growth. Conversely increased expression yielded the opposite phenotype, both *in vitro* and *in vivo*, with reduced clonogenic potential and reduced tumor growth with massive spontaneous tumor necrosis. These results therefore strongly suggest that Arl2 content is correlated with *in vitro* and *in vivo* aggressivity in the two breast cancer models studied.

PP2A has been shown to play an important inhibitory effect on cell proliferation [22,23]. We have previously shown that the content and the activity of the catalytic subunit of PP2A (PP2Ac) is modified in cells with altered Arl2 content [20]. Here we confirm that inhibition of PP2A in SCID mice is associated with increased tumor aggressivity. Analysis of enzymatic activity in Arl2 modified tumors showed that PP2A activity was increased in cells with increased Arl2 content, a result in keeping with our observation that these cells proliferated more actively in the presence of a PP2A inhibitor. PP2A is known to interact directly with many proteins and modify their activity by either activating or inhibiting them. The anti-apoptotic proteins Bcl2 and Bcl-xl and the pro-apoptotic proteins such as Bax are targets of PP2A. In these proteins, the dephosphorylation occurs at the serine residues and shifts the signaling pathway toward apoptosis by activating and deactivating the pro- and anti-apoptotic proteins, respectively [24]. The increased PP2A activity of cells with high Arl2 content is thus likely to explain the massive apoptosis observed in tumors derived from these cells. Conversely the enhanced aggressivity observed in cells with reduced Arl2 content could not be attributed to a reduction in PP2A enzyme activity.

It remains to be established which are the key regulators involved in Arl2/PP2A regulation of tumor aggressivity. PP2A dephosphorylates a large variety of substrates involved in cell cycle regulation. Our previous study found that alterations in Arl2 content profoundly modified microtubule dynamics as well as the duration of the various phases of mitosis, in particular anaphase and telophase [20]. We have also reported that Arl2 content influences the content and distribution of phosphor-ser15-P53 [21]. Moreover, other authors have recently demonstrated the involvement of MT dynamics and of PP2A in the regulation of concentration of E-cadherin at cell-cell contacts. Stehbens *et al.* identified a pool of MTs that extend radially into cell-cell contacts, blocking MT dynamics and thereby altering the ability of cells to concentrate and accumulate E-cadherin at cell-cell contacts [25]. Takahashi *et al.* have shown that treatment of the cells with inhibitors of PP2A caused disruption of cell-cell adhesion and proposed that PP2A could play a crucial role in the maintenance of cell-cell adhesion [26].

In conclusion these data suggest that Arl2, a small GTP protein whose role is yet largely unknown, appears to be a significant regulator of PP2A content and activity in breast cancer cells, both *in vitro* and *in vivo*. In preclinical models, reduced Arl2 content is associated with enhanced tumor aggressivity while increased Arl2 content is associated with reduced aggressivity and enhanced spontaneous necrosis. In primary human breast tumors, low Arl2 mRNA content is associated with larger tumor size and greater risk of lymph node involvement at diagnosis. These data suggest that Arl2, possibly through a PP2A-mediated pathway, is a key regulator of breast tumor aggressivity.

Materials and Methods

Ethics Statement

All research involving animals have been conducted according to relevant national and international guidelines. The protocol was approved by the Lyon Animal Experimental Committee and animals were treated in accordance with European Union guidelines for laboratory animal care and use.

Cell culture and transfection

All cell lines were grown in DMEM containing L-glutamine, penicillin (200 IU/ml), streptomycin (200 µg/ml), and fetal bovine serum (10%) at 37°C in the presence of 5% CO2. MCF-7 cells were stably transfected with empty pcDNA3 (MP cells) or pcDNA3 containing sense Arl2 (MA+ cells) or antisense Arl2 (MA- cells) as describe elsewhere [20]. The same experimental strategy was used to obtain stable transfectants from MDA-MB 231 cells (commercial cell lines ATCC number HTB26), yielding MdaP, MdaA+, and MdaA- cells. MdaP, MdaA+ and MdaA-, cells were then co-transfected with plasmids expressing the firefly luciferase gene (pGL3-basic, Promega) and the puromycin resistance gene (pSuper/Puro; Oligoengine) using lipofectamine (Invitrogen) according to the manufacturer's recommendations, and bioluminescent puromycin resistant cells were then selected. The three bioluminescent derivatives cells named, MdaP.luc, MdaA+.luc and MdaA-.luc were used for *in vivo* studies.

Determination of confluent cell proliferation rates

Cell proliferation was estimated using the methylthiazoletetrazolium (MTT) assay. Twenty thousand cells were seeded per well of a 24-well plate and incubated at 37°C. Every 24 h up to 6 days, MTT (500 µg/per well) was added to 3 wells of each plate. After 2 h of incubation at 37°C, supernatants were removed and absorbance measured as described previously [27].

Cell cycle distribution by flow cytometry analysis

Cells were collected and incubated at 4°C during 2 h with 1 ml of propidium iodide solution (0.05 mg/ml) containing Nonidet-P40 (0.05%). Cells were analyzed using a FACS Calibur flow

cytometer (BD Biosciences Europe, Erembodegem, Belgium) and Modfit LT 2.0^{TM} software (VeritySoftware Inc, Topsham, United States).

In vitro cell growth assays

All microscopic analyses were performed in the Centre Commun de Quantimétrie (Université de Lyon I, France), unless otherwise stated.

Coculture cell competition assay. MP and MA- cells were separately incubated with different fluorescent cell linker dyes (respectively PKH 67 green and PKH 26 red (Sigma)) following the manufacturer's recommendations and were then placed in coculture at a 1:1 ratio. A similar dye-swap experiment was performed with PKH 67 green for MA- cells and PKH 26 red for MP cells. Fluorescent images were taken at day 1 and 3 after cell.

Growth in soft-agar and soft-agar containing matrigel. At the time of plating in soft-agar in 6-well plates, 2×10^4 total cells were mixed with 1.5 ml of 0.45% low melting point (LMP) agarose-DMEM (top layer) and then poured on top of 1.5 ml of solidified 0.75% LMP agarose-DMEM (bottom layer) completed with 30% (v/v) matrigel (BD Biosciences) for the matrigel-containing soft-agar condition. Colonies were counted and photographed after 26 days in triplicate.

Overlay three-dimensional culture on matrigel. Overlay three-dimensional cultures on matrigel (BD Biosciences) were performed as described previously in triplicate [28]. 100 µl of matrigel were added in each well of an 8-well glass chamber slide. For each cell line, a cellular suspension containing 12,500 cells/ml in a medium containing 4% (v/v) matrigel was prepared. 400 µl of this mixture was plated per well on top of the solidified matrigel. This corresponds to a final overlay solution of 5000 cells/well in medium containing 2% matrigel. Cell colony formation was monitored using microscopic observation and image acquisition up to 20 days after incubation.

Matrigel cluster assay. For each of the MDA-MB 231 derived cell lines, a cellular suspension containing 1×10^6 cells/µl in DMEM medium was prepared. Matrigel (BDBiosciences) was added to the wells of an 8-well glass chamber slide in a volume of 300 µl. Before matrigel polymerization, 1 µl containing 1×10^6 cells was carefully loaded in the middle of the matrigel coating. Then, matrigel containing preparations were allowed to solidify at 37°C and cells loading were formed a compact crowd of cells. 150 µl of complete medium were then added on wells containing matrigel. Area of each cells cluster was determined for up to 5 days using microscopic observation and quantified using ImageJ software. Measurements were performed in triplicate.

Orthotopic tumorigenicity assays

For each cell line, five female SCID mice aged 4–6 weeks were anesthetized and injected with 50 µl of 2×10^6 cells suspended in 50% DPBS/50% matrigel into the abdominal mammary fat pad. The size of the tumor was measured twice a week by external measurement or *in vivo* bioluminescent imaging. Mice were killed when the tumors reached a greatest diameter of 1,2 cm. The protocol was approved by the Lyon Animal Experimental Committee and animals were treated in accordance with European Union guidelines for laboratory animal care and use.

In vivo bioluminescent imaging was performed by administering the substrate D-luciferin by intraperitoneal injection at 150 mg/kg in D-PBS (Invitrogen), and anesthetized (1–3% isoflurane). Mice were then placed inside a light-tight box under a photon counting camera (NightOWL II LB 983, Berthold Technologies, Germany) with continuous exposure to 1–2% isoflurane + 4% oxygene. Regions of interest from displayed images were identified around the tumor sites and were quantified as photons/s using WinLight[32] software (Berthold Technologies, Germany) with background bioluminescence substraction. Tumor growth (R-growth) was evaluated using a linear projection of the growth curve.

Pathological analysis of tumor clusters

After fixation in Bouin's solution the tumor or cell cluster was dehydrated with alcohol, immersed in xylene and embedded in paraffin. Four µm-thick slides were dried at 58°C for 30 minutes in an incubator. After deparaffination and rehydration, the paraffin sections were stained with hematoxylin and eosin. The slides were mounted with a xylene mounting medium.

siRNA administration *in vivo*

The siRNA were purchased from Sigma-Aldrich as duplex desalted and deprotected non-modified sequences of 21 base pairs. The sequences were the following: Arl2, 5′-AACCCUCCUCAUCUUUGCUAA-TT; PP2Ac, 5′- GAGGUUCGAUGUCCAGUUATT-TT, scrambled (SCR), 5′- GCUGAUAGCAUGGUCUGAUTT-TT.

In this experiment, three groups of ten SCID mice were studied. The groups I, II and III correspond respectively to the scrambled siRNA, Arl2 siRNA and PP2Ac siRNA the injected mice. The first day, 3 millions of MDA-MB 231 cells were implanted subcutaneously in all mice followed by intraperitoneal injections of SCR, PP2A and Arl2 siRNAs (4 µ in 50 µl of PBS). The injections of siRNAs were continued daily for a period of four weeks and the volumes of tumors were monitored twice per week till two weeks after the end of injections.

Quantitative real-time RT-PCR

The level of inhibition exhibited by the Arl2 and PP2A siRNA on their respective targets was assessed by real-time quantitative PCR on tumors and livers extracted 24 hours after the last siRNA injection. Total mRNA extraction, reverse transcription and real-time quantitative RT-PCR were performed using a lightcylcer thermal cycler (Roche, Manheim, Germany). Forward and reverse primer sequences used for Arl2 were respectively 5′-GGCTCCTGACCATTCTGAAG and 3′- TGTAGTTCTGGGACCTCGTG; for PP2Ac: 5′- CCCTGGATCGTTTACAGGAA and 3′- ATGGTAAAAGTCACGTGGGT. Results were analyzed with RelQuant software (Roche, Manheim, Germany).

PP2A enzyme activity assay

The specific activity of the phosphatase PP2A was assessed using a system based on the immunoprecipitation of PP2Ac (Upstate-Millipore, USA) as recommended by the manufacturer by using 300 µg of total protein of each of the cell lines MA-, MP, MA+, MdaA-, MdaP, MdaA+. The peptide containing a phosphorylated threonine residue (K-R-pT-I-R-R) provided with the system was replaced by a similar peptide containing a phophoserine residue (K-R-pS-I-R-R), synthesized by Dr. Ficheux (IBCP Université Lyon 1) in order to better mimick the physiological situation. The optical density of the products issued from the dephosphorylation was read as an absorbance at 620 nm. The samples were compared to a standard curve corresponding to a range of 0 to 2000 pmol of free phosphates. The values obtained for the cells MP and Mda P were used as reference.

PCR analysis of primary breast tumors

Primary breast tumors were obtained from the Centre Léon Bérard anti Cancer Tissue Bank. Patients gave written informed consent for these analyses. Total mRNA was extracted with Trizol

reagent (Invitrogen, Cergy Pontoise, France) and two micrograms were converted into cDNA by Moloney leukaemia virus reverse transcriptase (Invitrogen) as described in the manufacturer's manual. cDNA levels were normalized to the expression of 18S ribosomal gene using the pre-developed TaqMan assay reagents control kit (Applied biosystem, Foster City, Canada) and assayed for Arl2 by real time PCR performed in a LightCycler thermal cycler (Roche, Meylan, France) as previously described [20].

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Author Contributions

Conceived and designed the experiments: AB RHS ET LPJ MFP JJD CMD. Performed the experiments: AB SB RHS SBM SG ET LPJ IT MFP JJD. Analyzed the data: AB SB RHS SBM SG ET LPJ IT MFP JJD CMD. Contributed reagents/materials/analysis tools: AB SB RHS SBM SG ET LPJ IT MFP JJD. Wrote the paper: AB SB SG JJD CMD.

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PART 2 ABSTRACTS

1. 3èmes Journées Scientifiques du Cancéropôle Rhône-Alpes Auvergne (CLARA), Lyon, March 2008

siRNA-mediated Inhibition of PP2A and Arl2 by intraperitoneal injection in mice enhances the tumor growth and development of human breast cancer cells xenografts

Authors: **Rouba Hage-Sleiman**, Stéphanie Brunet-Manquat, Anne Béghin, Charles Dumontet

Previous studies done by our laboratory team showed that two proteins are involved in the mammary tumor growth. The first agent is a serine/threonine phosphatase known as PP2A and essential for the exit from mitosis. The second one is an ADP ribosylation factor like 2 known as Arl2 and involved in the maturation of tubulin peptides in microtubules by forming a complex with TBCD (tubulin binding cofactor D) and inhibiting by that the degradation of tubulin heterodimers by this latter. It was found that a modification in cellular content of Arl2 and PP2A might play an important role in controlling the growth and tumoral development. Based on this finding and after having tested two sequences of siRNA targeting both of Arl2 and PP2A in vitro on human mammary adenocarcinoma MDA , these were studied in vivo by subcutaneous xenografting of MDA cells in SCID mice. The mice were divided as ten mice per group and an overall of four groups was used. The four groups correspond to a nontreated group NT, a group SCR where mice were injected by a scrambled siRNA and two others Arl2 and PP2A where mice were injected respectively by siRNA targeting Arl2 and PP2A. The day 1, 3 millions MDA cells were injected subcutaneously in the mice. Starting day 1, 4 µg of each siRNAs were injected intraperitoneally and the injections of siRNA were continued for 20 days with no stop and every 2-3 days the volume of the tumors was monitored. It was obtained that the inhibition of PP2A in vivo increased significantly the development and growth of the tumor compared to both the NT and SCR mice all over the treatment period and two weeks after stopping treatment. For Arl2, the increase in development of the tumor was

significant in the beginning of the treatment and in the two weeks after stopping the treatment. These results showed an involvement of both Arl2 and PP2A in the tumor growth even if at different levels and were found to correlate with the previous studies. A first and preliminary result of MTT test on the tumors put in culture in vitro showed an enhanced proliferation speed of the cells extracted from Arl2 and PP2A groups with respect to that of SCR and NT groups. In order to find out what is happening at the molecular level, the proteins and RNA levels will be studied in all the tumors extracted from all mice as well as the activity of PP2A. Parts of the tumors were fixed in formaldehyde to be studied in immunohistochemistry. Based on the results that will be obtained, further studies will be done to understand better the interaction of both molecules and the mechanism behind their implication in the mammary tumor growth.

2. The EMBO meeting 2009, Amsterdam, August-September 2009

Alteration of tubulin binding cofactor C expression modifies the cell cycle distribution and the response to antimicrotubule agents in breast cancer cells

Authors: **Rouba Hage-Sleiman**, Stéphanie Herveau, Eva-Laure Matera, Jean-Fabien Laurier, Charles Dumontet

Microtubules were identified as major therapeutic targets for the breast cancer. The commonly known treatments include the vinca alkaloids and the taxanes that destabilize and stabilize the microtubules, respectively. Tubulin binding cofactor C (TBCC) is one of the five cofactors (A, B, C, D and E) necessary for the proper folding pathway of the α and β tubulins into heterodimers that polymerize into microtubules. After being captured by cofactor B and A, the α-tubulin and β-tubulin peptides form complexes with cofactors E (TBCE) and D (TBCD), respectively. Tubulin binding cofactor C (TBCC) interacts with these complexes leading to α/β-tubulin heterodimers. In this study, we generated stable transfectants of human mammary adenocarcinoma MCF7 where the protein TBCC was upregulated. The morphology of the variant MC+ cells was not different from that of the reference cells MP6. The distribution in cell cycle was modified in the MC+ cells and these cells presented a significant increase in the percentage of cells in the G2-M phase and a significant decrease in the percentage of cells in the S-phase with respect to the MP6 cells. No difference was observed in the G0-G1 phase. When the MC+ and MP6 were treated with 10 nM and 1 nM of paclitaxel and vinorelbine, respectively, the MC+ showed an increased sensitivity to the treatments compared to MP6, with a significant higher blockage in the G2-M phase. At the basal level, the cells over expressing TBCC presented a decreased content in soluble polymerizable tubulins and microtubules tubulins compared to the control cells. The contents of nonpolymerizable tubulins and total α and β tubulins were not modified in the new variants. The excess level of TBCC in the MC+ cells affects directly the availability of the α/β heterodimers ready to polymerize into microtubules. The formation of the complex TBCC/TBCD-β /TBCE-α

becomes more frequent and diminishes the amount of free soluble polymerizable heterodimers. These cells with lower content in microtubules will have difficulty in cell division which means a slower mitosis and a higher level of cells in G2-M. This last idea will be developed by studying the durations of mitosis in MC+ and MP6 cells. In conclusion, by modifying the TBCC expression level, we were able to sensitize the breast cancer cells to microtubule targeting agents.

3. 5èmes Journées Scientifiques du Cancéropôle Rhône-Alpes Auvergne (CLARA), Lyon, March 2010

Reduced content of Tubulin binding cofactor C (TBCC) in breast cancer cells modifies the progression into the cell cycle and influences the response to treatments

Authors: **Rouba Hage-Sleiman**, Stéphanie Herveau, Eva-Laure Matera, Jean-Fabien Laurier, Charles Dumontet

Tubulin Binding cofactor C (TBCC) is a crucial protein involved in the mechanism of proper folding of α and β tubulins into microtubules polymerizable heterodimers. In our study, we are investigating the impact of inhibition of TBCC on the phenotype of breast cancer cells by developing a variant of breast cancer cells with low content of TBCC (MC-). Since microtubules play many essential biological roles and are considered as major targets in the treatment of breast cancer, we investigated the influence of inhibition of TBCC on the different tubulin fractions as well as on the microtubule dynamics. The MC- cells presented low content in nonpolymerizable tubulins and increased contents in both the polymerizable and microtubule tubulins. The microtubule dynamics was also influenced and the microtubules in MC- cells showed higher growth rate and dynamicity. MC- cells proliferated faster than the control cells and showed an altered cell cycle distribution. TBCC variants were present in a higher percentage in S-phase of the cell cycle compared to the control cells and showed higher sensitivity to S-phase targeting treatment gemcitabine. Although the complete mitosis duration was shorter in the MC- cells and their microtubules dynamics was enhanced, they didn't show a lower percentage of cells in G2-M phase or any remarkable resistance to antimicrotubule targeting agents when compared to the control cells. Furthermore xenografts derived from TBCC variants displayed significant aggressivity *in vivo* in comparison to control but still showed important sensitivity to chemotherapy. The results underline the fact that by enhancing proliferation in our models of cancerous cells we could increase their sensitivity to treatments that target S-phase without acquiring resistance to antimicrotubule

agents. We suggest that since our MC- cells were both sensitive to Gemcitabine and antimicrotubule agents, a possible hypothesis would be to co-treat our variants with both drugs to enhance response, especially *in vivo*.

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SUMMARY

The proper folding pathway of α and β-tubulin into the α/β-tubulin heterodimers involve five **T**ubulin **B**inding **C**ofactors (**TBC**A to **TBC**E). TBCC plays a crucial role in the formation of polymerization-competent the α/β-tubulin heterodimers. To evaluate the impact of microtubule mass and dynamics on the phenotype and chemosensitivity of breast cancer cells, we targeted TBCC in human breast adenocarcinoma and developed variants of breast cancer cells with modified content of TBCC. We have shown that the modifications in TBCC expression level influenced tubulin fraction distribution and microtubule dynamics. Cell cycle distribution and the durations of mitosis and S-phase were altered. The proliferation rate *in vitro* was slightly modified whereas *in vivo* the TBCC variants presented major differences in tumor growth capacity. Chemosensitivity to antimicrotubule agents (paclitaxel and vinorelbine) as well as to gemcitabine was observed to be dependent on the cell cycle distribution of the TBCC variants. These results underline the essential role of fine tuned regulation of tubulin content in tumor cells and the major impact of dysregulation of tubulin dimer content on tumor cell phenotype, cell cycle progression and response to chemotherapy. A better understanding of how the microtubule cytoskeleton is dysregulated in cancer cells would greatly contribute to a better understanding of tumor cell biology and characterization of resistant phenotypes.

TITRE

Effects de la protéine tubulin binding cofactor C (TBCC) sur la masse et la dynamique microtubulaire, le cycle cellulaire, la croissance tumorale et la réponse à la chimiothérapie dans le cancer du sein.

RESUME

La mise en conformation de l'α et β tubulines en hétérodimères polymérisables nécessite l'intervention de cinq protéines « Tubulin Binding Cofactors » (TBCA à TBCE) dont TBCC qui joue un rôle indispensable. Dans des cellules humaines d'adénocarcinome mammaire, nous avons modifié le niveau d'expression de TBCC et nous avons montré que ceci avait un impact sur le contenu des fractions de tubuline, la dynamique des microtubules ainsi que sur le phénotype et chimiosensibilité des cellules. La distribution en cycle cellulaire et les durées de la mitose et de la phase S ont été altérées. La modification de TBCC avait un faible effet sur la vitesse de prolifération *in vitro* par contre les cellules présentaient des différences significatives de croissance tumorale *in vivo*. Les réponses aux agents antimicrotubulaires et à la gemcitabine ont montrées une chimiosensibilité dépendante de la distribution en cycle cellulaire. Tous ces résultats montrent l'importance de la régulation du contenu en tubulines et l'impact de ceci sur le comportement de la cellule en général et vis-à-vis des traitements.

DISCIPLINE: Cancérologie

MOTS-CLES: TBCC; tubulin folding pathway; microtubule; microtubule dynamics; cell cycle; breast cancer ; antimicrotubule agents

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