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Update article

Towards an understanding of the isotype-specific functions of tubulin in neurons: Technical advances in tubulin expression and purification

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ABSTRACT

Microtubules are cytoskeletal filaments critical for determining the complex morphology of neurons, as well as the basic architecture and organization of mitosis in all eukaryotic cells. Microtubules in humans are composed of 8 α- and 9 β-tubulin isotypes, each of which is encoded by different members of a multigene family. The expression pattern of tubulin isotypes, in addition to isotype-specific post-translational modifications, is thought to be critical for the morphogenesis of axons and dendrites. Recent studies revealed that several neurodevelopmental disorders are caused by mutations of specific tubulin isotypes, suggesting that each tubulin isotype has distinct functions. Therefore, *in vitro* and *in vivo* functional analyses of tubulin isotypes are important to understand the pathogenesis of developmental disorders. Likewise, analysis of developmental disorders may clarify the function of different tubulin isotypes. In this respect, both the preparation of specific tubulin isotypes and of specific mutant tubulin proteins is critical to understanding the function of tubulin. In the last 20 years, various methods have been developed to study functional differences between tubulin isotypes and the functional defects caused by tubulin mutations. These technical achievements have been discussed in this review. The function of tubulin/microtubules in neuronal morphogenesis as revealed through these techniques has also been described.

1. Introduction

Microtubules are important structural components of the cytoskeleton that are critical for regulating cell shape, motility, cell division and intracellular transport. They are highly dynamic tubular polymers that assemble from heterodimers of α- and β-tubulin. Lower eukaryotes have a small number of tubulin genes than vertebrates. For example, budding yeast (*Saccharomyces cerevisiae*) has two α-tubulin genes and one β-tubulin gene (Schatz et al., 1986; Neff et al., 1983). Both the fungus, *Aspergillus nidulans*, and a unicellular algae, *Chlamydomonas reinhardtii*, have two α- and two β-tubulin genes (Silflow and Rosenbaum, 1981). A small number of tubulin genes are therefore sufficient to build the microtubules capable of establishing the cellular architecture of these unicellular organisms. It is therefore interesting that higher organisms, including vertebrates, typically have six or more genes that encode α- and β-tubulin. For example, humans have eight α- and nine β-tubulin genes (Breuss and Keays, 2014). The presence of multiple gene copies is likely related to the presence of more complex and sophisticated body structures. Each gene encodes a specific tubulin isotype and each has a distinct role. This idea is called the "multitubulin hypothesis," which was originally proposed to explain distinctive tubulin expression during flagellar regeneration (Fulton and Simpson, 1976; Cleveland, 1987; Wilson and Borisy, 1997). This hypothesis is widely accepted because of the isotype-specific patterns of tubulin expression in various organs. For example, human TUBB3 (β_{III}) and TUBB4 (β_{IVa}) are highly expressed in neurons, while human TUBB (β_I) is ubiquitously expressed (Cleveland, 1987; Leandro-Garcia et al., 2010).

The multitubulin hypothesis is also supported by recent clinical studies. In the last 10 years, various mutations in human tubulin genes that cause congenital disorders have been reported. Interestingly, most defects caused by tubulin mutations affect the nervous system. For example, mutations in *TUBA1A*, which encodes α_{IA}-tubulin, causes lissencephaly, pachygyria, polymicrogyria, and other neurodevelopmental disorders involving abnormal morphogenesis of the cortex (Keays et al., 2007; Poirier et al., 2007;

Abbreviations: CFEOM3, congenital fibrosis of extraocular muscles type 3; PTM, posttranslational modification; CTT, C-terminal tail.

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Table 1
A list of pathogenic mutations in human tubulin genes.

tubulin gene	Mutations	disease	References
TUBA1A (α1A)	E55K, L70S, L92V, V137D, I188L, C200Y, Y210C, D218Y, I238V, P263T, R264C, A270T, L286F, V303G, N329S, G366R, L397P, R402H/C/L, S419L, R422C/H, S419L, M425K, G436R	Lissencephaly (agyria and pachygyria)	Keays et al. (2007), Poirier et al. (2007), Fallet-Bianco et al. (2008), Morris-Rosendahl et al. (2008), Kumar et al. (2010), Lecourtois et al. (2010), Jansen et al. (2011), Mokanszki et al. (2012), Okumura et al. (2013), Cushion et al. (2013), Poirier et al. (2013), Zanni et al. (2013), Hikita et al. (2014), Bamba et al. (2016)
TUBA1A (α1A)	I5L, Y161H, V235L, A333V, R390C/H	Polymicrogyria	Jansen et al. (2011), Poirier et al. (2013), Cushion et al. (2013), Zanni et al. (2013)
TUBA4A (α4A)	G43V, T145P, R215C, R320C/H, A383T, K430N, W407X	Amyotrophic lateral sclerosis (ALS)	Smith et al. (2014)
TUBB (β1)	N15K, Y222F	Circumferential skin creases Kunze type (CSC-KT)	Isrie et al. (2015)
TUBB2B (β2)	G98R, L117P, G140A, S172P, L207P, I210T, L228P, A248V, N256S, F265L, T312M, R380C/S/L, D417N	Polymicrogyria, agyria	Jaglin et al. (2009), Cushion et al. (2013), Guerrini et al. (2012), Amrom et al. (2014)
TUBB2B (β2)	E421K	Polymicrogyria, congenital fibrosis of extraocular muscle	Cederquist et al. (2012)
TUBB3 (β3)	R62Q, R262C/H, A302T, R380C, E410K, D417H/N	Congenital fibrosis of extraocular muscle type 3 (CFEOM3)	Tischfield et al. (2010)
TUBB3 (β3)	G82R, T178M, E205K, A302V, M323V, M388V	Malformation of cortical cell development (MCD)	Poirier et al. (2010)
TUBB4A	R2G, A271T	Dystonia	Lohmann et al. (2013), Hersheson et al. (2013)
TUBB4A	D249N	Leukoencephalopathy	Simons et al. (2013)
TUBB5 (β5)	M299V, V353I, E401K	Microcephaly	Breuss et al. (2012)
TUBB8 (β8)	R2K, S176L, V229A, R262Q, M300I, M363T	Infertility	Feng et al. (2016)

Fallet-Bianco et al., 2008; Morris-Rosendahl et al., 2008; Kumar et al., 2010; Lecourtois et al., 2010; Jansen et al., 2011; Mokanszki et al., 2012; Okumura et al., 2013; Cushion et al., 2013; Poirier et al., 2013; Zanni et al., 2013; Hikita et al., 2014; Bamba et al., 2016). Mutations in the gene encoding β_I-tubulin, *TUBB,* cause circumferential skin creases Kunze type. While skin creases of the limbs are the most remarkable phenotype of this mutation, there are also intellectual disabilities that are likely due to neuronal defects (Isrie et al., 2015). Other diseases caused by tubulin gene mutations (tubulinopathies) are listed in Table 1. Mutations in different tubulin genes cause different phenotypes, indicating that tubulins have distinctive roles in neurons and other tissues. However, the molecular mechanisms of how each tubulin isotype mutation is related to the associated phenotype are not well understood.

For the study of tubulinopathies and molecular-level functions of specific tubulin isotypes, it is important to express and purify each specific isotype and its mutant protein. Advancement of the techniques and the results obtained through the techniques are presented in this review.

2. Functional analyses of tubulin isotypes purified by immunoaffinity chromatography

In vitro biochemical and biophysical analyses have been used to study functional differences between tubulin isotypes purified from mammalian brains. The most widely used tubulin protein sample, mammalian brain-derived tubulin is a mixture of isotypes (Fig. 1A). In this section, methods to prepare the brain tubulin sample, and a method to separate isotypes from the mixture, have been described. Using the method, isotype-specific function of tubulin can be analyzed to some extent. Further, analyzing the expression and purification of recombinant tubulin described in the later section are required.

Mammalian brain tubulin can be easily obtained from clarified homogenate of brain, *via* temperature-dependent cycles of polymerization and depolymerization (Shelanski et al., 1973). Bovine, pig and sheep brain obtained from the slaughterhouse are often used. Thus obtained, crude tubulin also contains microtubule-associated proteins (MAPs), which can be removed through ion-exchange chromatography using a phosphocellulose column. Typically, 10–30 mg of pure αβ-tubulin is obtained from 100 g of brain tissue. This final tubulin product is usually called PC tubulin, which is named after "phosphocellulose column" and is widely used for biochemical and biophysical analyses (Gell et al., 2011). In an alternative to this commonly used purification method, a second polymerization cycle can be performed in a 0.5 M PIPES buffer containing 10% dimethyl sulfoxide (DMSO). Through tubulin assembly in this high-molarity PIPES buffer, MAPs can be removed without ion-exchange column purification (Himes et al., 1977). This method enables more rapid purification with a better yield (typically 50–70 mg tubulin from 100 g of brain, see Castoldi and Popov, 2003). Mammalian brain-derived tubulin is also commercially available as a lyophilized powder.

Some tubulin isotypes can be separated from brain tubulin using isotype-specific antibodies (Fig. 1A). Antibodies against the C-terminal region, which is a polypeptide of ~15 amino acid residues extending from the tubulin/microtubule surface, can distinguish β-tubulin isotypes, because the region is the most variable among the tubulin isotypes (Lopata and Cleveland, 1987; Lewis et al., 1987). Immunoaffinity chromatography was used to separate β_{III}-tubulin, which is expressed in neurons, and thus the functional differences between β_{III}-tubulin and other isotypes were identified. Removal of β_{III}-tubulin from a mixture of tubulin isotypes increased the assembly rate, taxol sensitivity, and the stability of microtubules in cold temperatures (Lu and Luduena, 1993). These results indicated that β_{III}-tubulin forms cold-sensitive and taxol-resistant microtubules. Dynamic instability analysis of purified β_{II}-, β_{III}-, and β_{IV}-tubulin also indicated that β_{III}-tubulin forms dynamic microtubules (Panda et al., 1994). In contrast, an isotype-specific antibody for α-tubulin is not available, however, α-tubulin isotypes may also have distinctive functions (Miller et al., 1987). The lack of antibodies limits the study of functional differences between α-tubulin isotypes.