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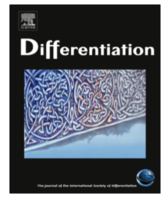

The ciliary proteins Meckelin and Jouberin are required for retinoic acid-dependent neural differentiation of mouse embryonic stem cells

Sveva Romani [a,b,1], Barbara Illi [a,*,1,2], Roberta De Mori [a], Mauro Savino [c], Joseph G. Gleeson [d], Enza Maria Valente [a,e]

[a] *Neurogenetics Unit, Mendel Laboratory, IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, FG, Italy*
[b] *Department of Molecular Medicine,"Sapienza" University, Rome, Italy*
[c] *Nucleic Acids Laboratory, Institute of Molecular Biology and Pathology, National Council of Research (IBPM-CNR), Rome, Italy*
[d] *Department of Neuroscience and Pediatrics, Neurogenetics Laboratory, Howard Hughes Medical Institute, University of California, La Jolla, San Diego, CA, USA*
[e] *Department of Medicine and Surgery, University of Salerno, Salerno, Italy*

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A B S T R A C T

The dysfunction of the primary cilium, a complex, evolutionarily conserved, organelle playing an important role in sensing and transducing cell signals, is the unifying pathogenetic mechanism of a growing number of diseases collectively termed "ciliopathies", typically characterized by multiorgan involvement. Developmental defects of the central nervous system (CNS) characterize a subset of ciliopathies showing clinical and genetic overlap, such as Joubert syndrome (JS) and Meckel syndrome (MS). Although several knock-out mice lacking a variety of ciliary proteins have shown the importance of primary cilia in the development of the brain and CNS-derived structures, developmental *in vitro* studies, extremely useful to unravel the role of primary cilia along the course of neural differentiation, are still missing.

Mouse embryonic stem cells (mESCs) have been recently proven to mimic brain development, giving the unique opportunity to dissect the CNS differentiation process along its sequential steps. In the present study we show that mESCs express the ciliary proteins Meckelin and Jouberin in a developmentally-regulated manner, and that these proteins co-localize with acetylated tubulin labeled cilia located at the outer embryonic layer. Further, mESCs differentiating along the neuronal lineage activate the cilia-dependent sonic hedgehog signaling machinery, which is impaired in Meckelin knock-out cells but results unaffected in Jouberin-deficient mESCs. However, both lose the ability to acquire a neuronal phenotype. Altogether, these results demonstrate a pivotal role of Meckelin and Jouberin during embryonic neural specification and indicate mESCs as a suitable tool to investigate the developmental impact of ciliary proteins dysfunction.

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1. Introduction

Primary cilia are non-motile microtubule-based, dynamic, elongated structures extending from the membrane of non-proliferating and G1 phase cycling cells, that play a central role in sensing and transducing cell signals, in regulating developmental pathways and in maintaining tissue homeostasis (Marshall and Nonaka, 2006). A variety of signal transduction pathways depend on the primary cilium, especially during the embryonic development. In particular, the sonic hedgehog (shh), the non-canonical wnt/Planar Cell Polarity (PCP), and the Platelet Derived Growth Factor Receptor (PDGFR) pathways are all known to be regulated by primary cilia (Lancaster and Gleeson, 2009). Both positive and negative effects on the canonical wnt pathway have been reported (Lancaster et al., 2009; Ocbina et al., 2009) and it has recently been shown that primary cilia down-regulate the wnt/β-catenin signaling through a spatial mechanism involving compartimentalization of specific downstream signaling components (Lancaster et al., 2011a).

Abbreviations: JS, Joubert syndrome; MS, Meckel syndrome; Jbn, Jouberin; NB, Neurobasal medium

* Correspondence to: Neurogenetics Unit, CSS-Mendel Laboratory, Viale Regina Margherita 261, 00198 Rome, Italy. Phone: +39 0644160537; fax: +39 0644160548.
E-mail address: b.illi@css-mendel.it (B. Illi).

[1] These two authors equally contributed to this work.

[2] Permanent address: Institute of Molecular Biology and Pathology, National Council of Research (IBPM-CNR), P.le Aldo Moro 5, 00185 Rome, Italy. Phone.: +39 0649912227; fax: +39 0649912500.

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Mutations in several ciliary genes have been found to cause an expanding number of human disorders now grouped under the term "ciliopathies". The wide genetic heterogeneity of these diseases is the consequence of the high complexity of the primary cilium, whose central structure is constituted by more than 1000 polypeptides (Gherman et al., 2006 and http://www.ciliaproteome.org); on the other hand, the broad phenotypic spectrum of ciliopathies, often involving distinct organs and tissues, well reflects the extensive distribution of cilia in all sorts of cell types, including renal podocytes, endothelial and smooth muscle cells, fibroblasts, retinal photoreceptors and neurons.

The involvement of the central nervous system (CNS) is found in a subset of ciliopathies, including Joubert syndrome (JS; MIM 213300) and Meckel syndrome (MS; MIM 249000), and is mainly characterized by an abnormal development of the mid-hindbrain structures, leading to cerebellar vermis hypoplasia and other posterior fossa abnormalities. The pathogenetic mechanism underlying these neurological phenotypes has not been fully elucidated. A perturbed shh signaling has been evoked as a major cause of primary cilia-dependent CNS developmental defects (Han et al., 2008; Louie and Gleeson, 2005); more recently, decreased wnt activity was found in the developing cerebellum of mice knock out for *Ahi1*, the first gene found mutated in JS patients, which encodes for the ciliary protein Jouberin (Jbn) (Lancaster et al., 2011b). The vast majority of these studies are based on the use of knock out animals, and related functional analyses have been largely performed in recipient cells overexpressing the ciliary protein(s) of interest, beyond any developmental context.

Mouse embryonic stem cells (mESCs) represent the prototype of pluripotent stem cells and are a useful tool to investigate developmental pathways *in vitro*. They maintain their self-renewal properties when cultured in the presence of the Leukemia Inhibitory Factor (LIF), ensuring the constant expression of a transcriptional network, whose members (mainly Oct4, Nanog and Sox2) are responsible for the epigenetic maintenance of mESC stemness (Boheler, 2009). Upon LIF deprivation, the expression of these factors begins to "oscillate" to decline when differentiation occurs Boheler, 2009; Spallotta et al., 2010). Under proper culture conditions and addition of specific morphogens, mESCs may give origin to virtually any cell type. A great advantage is represented by the opportunity to culture these cells both as adherent cultures and three-dimensional embryoid bodies (EBs), which mimic at the best the early stages of *in vivo* embryonic differentiation, as soon as they form the three embryonic germ layers (ectoderm, mesoderm and endoderm) (Kurosawa, 2007). Currently, at least 200 somatic cell types have been obtained by using mESCs, including those belonging to the neuronal lineage. Indeed, using differentiation protocols based on the administration of defined cocktails of growth factors and/or morphogens within precise temporal windows, neuronal population from forebrain, mid-hindbrain and spinal cord have been obtained together with glial cells, following sequential differentiation steps that closely resemble those occurring *in vivo* (Gaspard and Vanderhaeghen, 2010). These results have given the unique opportunity to study complex developmental pathways in a relatively simple *in vitro* cell model.

Intriguingly, human ESCs possess primary cilia and cilia-dependent signaling machinery, and it has been recently demonstrated that also mESCs are provided with primary cilia (Hunkapiller et al., 2011; Kiprilov et al., 2008). Further, primary cilia are required for the formation of neural progenitors (Spassky et al., 2008). In the present study, we demonstrate that two ciliary proteins implicated in the pathogenesis of MS and JS, Meckelin (Baala et al., 2007; Brancati et al., 2010; Smith et al., 2006) and Jouberin (Louie et al., 2010), influence mESCs neuronal differentiation. Specifically, we found that Meckelin and Jouberin localized to cilia found at the outer ectodermal embryonic layer when mESCs were cultured as EBs, in a neural differentiation medium allowing the generation of mESC-derived neurons and, to a lesser extent, astrocytes. Moreover, Meckelin and Jouberin silencing (siMeckelin and siJbn) severely impaired the onset of specific early neural markers in mESC, but showed a differential effect on shh and wnt ligands production. In fact, siMeckelin blocked retinoic acid-dependent expression of shh (Chang et al., 1997), and consistently, the production of wnt3a was enhanced in siMeckelin mESCs with respect to control cells (Corbit et al., 2008). On the contrary, siJbn cells showed a marked decrease in wnt3a protein, while shh expression levels remained comparable in siJbn and scrambled transfected mESCs.

Altogether, these results provide new insights about the role of primary cilia and ciliary proteins during embryonic neurogenesis and, more importantly, suggest mESCs as a suitable model to study primary cilia-related molecular mechanisms, that may be implicated in embryonic development and in the pathogenesis of human ciliopathies.

2. Materials and methods

2.1. Cell culture, treatments and embryoid bodies formation

mESCs (ESD3, LGC Promochem, London, UK) were cultured as described (Illi et al., 2005). Briefly, mESCs were adapted in culture without feeder layer and grown in Dulbecco's modified eagle medium (DMEM, Life Technologies, Carlsbad, CA, USA) supplemented with 20 ng/ml Leukemia Inhibitory Factor (LIF) (Euroclone, Milan, Italy), 0.1 mM β-mercaptoethanol, 10% ES-tested Fetal Bovine Serum (FBS, Euroclone, Milan, Italy), 20 mM glutamine and penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA). EBs were obtained in hanging drops as described (Kurosawa, 2007). mESCs were dissociated with trypsin and diluted to a concentration of 2.5×10^4 cells/ml. 20 μl of the cell suspension were used to obtained drops on the cover plate of a Petri dish (about 20 drops/dish for at least 80–100 total drops to be analyzed later) previously filled with sterile PBS. Cells were allowed to form EBs for 48 h at 37 °C/5% CO_2. Thereafter, only well formed EBs were used for experimental procedures. For neural induction, mESCs, either as EBs or as adherent cells, were plated in culture medium without LIF onto 0.1% gelatin-coated dishes and treated, immediately after plating, with 10 nM RA or control DMSO for 7 days. Fresh RA was added every 2 days. After 1 week, mESCs were shifted to Neurobasal medium supplemented with N2 cocktail (100 μg/ml human transferrin, 5 μg/ml insulin recombinant full chain, 6 ng/ml progesterone, 16 μg/ml putrescine, 5.2 ng/ml selenite) (Life Technologies, Carlsbad, CA, USA), glutamine and penicillin/streptomycin (hereafter named NB+N2) for another week. Cell density at plating was as follow: for earlier time points (*i.e.* 5 and 7 days) cells were at 60% confluence; for the latest time point (*i.e.* 14 days) cells were at 30% confluence. Control cells were maintained in mESCs culture medium (Illi et al., 2005) deprived of LIF. HEK293T cells were cultured in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS, Life Technologies, Carlsbad, CA, USA), 20 mM glutamine and penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA).

2.2. Reverse transcription and real time PCR

Total RNA was extracted with TRIZOL reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was produced by using SuperScript III (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. mRNA levels were analyzed using the QuantiTect

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