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ESTABLISHMENT OF A MODEL OF MURINE ODONTOBLASTS UNDEREXPRESSING PKD1 USING shRNA

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Abstract

We have previously shown that *PKD1*, the gene encoding Polycystin-1 (or TRPP1) is expressed in human odontoblasts and that this protein is localized at the primary cilium of the cell. Nevertheless, its function remains unclear in this cell even if studies on osteoblasts, osteocytes and chondrocytes give TRPP1 as a promising candidate for mechanotransduction in response to mechanical stress. Consequently, to evaluate the role of TRPP1 in this transduction process, we needed first to generate an *in vitro* murine model down expressing Pkd1. Using lentivirus-mediated shRNA technology, we obtained a 60% suppression of Pkd1 mRNA expression in transfected MO6-G3 cells associated with a decrease of cell proliferation. Thus, establishment of this murine odontoblast model underexpressing *Pkd1* associated with applied mechanical forces (compression or shear stress) will allow us to go further in the determination of TRPP1 involvement in odontoblasts mechanotransduction.

Résumé

Nous avons montré précédemment que *PKD1*, le gène codant pour la polycystine 1 ou TRPP1, est exprimé dans les cultures d'odontoblastes humains avec une localisation préférentielle de la protéine au niveau des cils primaires. Cependant, la fonction de TRPP1 reste à ce jour inconnue malgré un rôle potentiel de mécanotransducteur mis en évidence dans les ostéoblastes, ostéocytes et chondrocytes. Pour évaluer le rôle de cette protéine dans les odontoblastes nous avons choisi de mettre au point un modèle cellulaire sous-exprimant *Pkd1*. Ainsi, à l'aide de shRNA, nous avons obtenu une lignée cellulaire de souris (MO6-G3) sous-exprimant de façon stable *Pkd1* (60% de sous-expression), et présentant une diminution de la prolifération cellulaire. Ce nouveau modèle cellulaire associé à l'application de forces mécaniques (compression ou étirement) devrait nous permettre d'évaluer l'implication de TRPP1 dans les processus de mécanotransduction des odontoblastes.

Key words

odontoblast, *Pkd1* down expression, mechanotransduction

Introduction

Many cell behaviors including at least growth, volume, shape migration, gene expression and tissue development are regulated by mechanical forces (1, 2, 3). It concerns all living organisms and the process by which cells convert mechanical energy into electrical or chemical signals is called mechano-transduction. Mineralized tissues are particularly submitted to mechanical forces during physiological functions (walking, running, mastication…). In tooth, odontoblasts, which are responsible for dentine formation, are best placed to sense both external stimuli and/or transient changes in pulp microcirculation. Indeed, odontoblasts are organized as a single layer of specialized cells at the interface between pulp and dentine. Each cell has an extension

running into a dentinal tubule and bathing in the dentinal fluid, and a cell body included in the soft tissue. Because of this spatial organization, odontoblasts could be able to sense their biomechanical environment and transduce it into cellular signals that are subsequently propagated to the nucleus where gene transcription would be modified. In all cells, many molecules, cellular components, and extracellular structures have been shown to contribute to mechanochemical cell transduction (2). These transduction elements include ECM, cell-ECM and cell-cell adhesions, membrane components, cytoskeletal filaments, nuclear structures and specialized surface processes (as primary cilium). Cells orchestrate all these transduction mechanisms in the context of a living tissue to produce a concerted response to mechanical signals.

Primary cilium is an ubiquitous organite described as a non-motile antenna emerging from the cell and extending into the extracellular space (4). It forms a single organelle consisting of a membrane-bound cylinder surrounding the axoneme, made of 9 microtubule doublets. Underneath the ciliary membrane, the intraflagellar transport machinery assembles and maintains the cilium structure, allowing the protein complexes to move up and down using the microtubule doublets as a track (5). A striated cytoskeleton structure (rootlet) extends from the basal body toward the cell nucleus. In many tissues, primary cilia are essential for sensing mechanical and biochemical signals (6,7). These processes involve $Ca2^{+}$ channels localized on cilia (8). Consequently, the primary cilium may control fundamental aspects of cellular physiology and development, via its involvement in different signaling pathways, such as hedgehog and Wnt (9). Therefore, mutations in genes encoding cilium components can generate major human genetic diseases, called ciliopathies (5), with sometimes dental abnormalities (OFD1 syndrome, BBS syndrome). Concerning odontoblast primary cilium, *in vivo*, cilia are aligned parallel to the dentin walls, with the top part oriented toward the pulp core (10). Close relationships between cilium and dental nerve fibers are evidenced. Calcium channels are concentrated in the vicinity of the basal body. Nevertheless, the role of the primary cilium in the control of odontoblast behavior remains to be elucidated.

TRPP1 is a mechanosensitive protein currently activated by a wide range of stimuli (11) and located at the base of the primary cilium and in cell junctions of odontoblasts. It is a large integral membrane glycoprotein with 11 transmembrane domains and an extensive aminoterminal extracellular domain involved in cell-cell, cell-matrix interactions and signaling pathways. It is proposed to function as a G protein-coupled receptor (GPCR) when expressed alone. TRPP1 has been suggested to act as a mechanical sensor regulating the opening of the associated calcium-permeable channel TRPP2, a six transmembrane domain protein of the TRP ion channel family (12). Both are involved in one of the most frequent inherited human ciliopathies, the autosomal dominant polycystic kidney disease. The biological role of TRPP1-TRPP2 has been mainly documented in the kidney epithelium. These proteins are proposed to transduce luminal shear stress into a calcium signal (8, 13). However, mutant mice for *Pkd1*, the gene encoding TRPP1, not only display multiple cysts in the kidney and liver and cardiovascular abnormalities but also show a severely compromised skeletal development. *Pkd1*-targeted mutant mice exhibit osteochondrodysplasia and delayed endochondral and intramembranous bone formation (14, 15). The long bones are shorter and smaller in diameter. The function of TRPP1 in bone appears to mirror its function in kidney: it is required for chondrocyte differentiation and maturation in the bone, as it is needed for epithelial differentiation and maturation in kidney. Xiao's group, using the heterozygous *Pkd1*m1Bei mouse, which has an inactivating mutation of *Pkd1* and survives to adulthood without polycystic kidney disease, has recently shown that these mice develop osteopenia and impaired osteoblastic differentiation (16, 17). These results indicate that *Pkd1* has a direct role to regulate osteoblast function and skeletal homeostasis. They demonstrated that TRPP1 regulates osteoblast function through intracellular calcium-dependent control of the bone specific transcription factor Runx2-II leading to the down-regulation of other osteoblast specific genes such as osteocalcin and osterix (18). Olsen's group, working on cartilage, has shown that TRPP1 regulates proliferation in chondrocytes and osteochondroprogenitor cells in midpalatal suture (19). Moreover, this group has shown that TRPP1 plays a critical role in the response of craniofacial cells to mechanical tissue stress. Using the mouse midpalatal suture expansion model, they

provide evidence that *Pkd1*-deficient mice exhibit a significantly reduced osteogenic response to tensile stress across the suture. This is due to reduced proliferation, delayed differentiation and increased apoptosis of osteochondroprogenitor cells. Furthermore, nasal cartilage of *Pkd1*-deficient mice undergoes unusual postnatal endochondral ossification and this process is accelerated upon application of expansive forces across the midpalatal suture (20). Therefore, it has long been recognized that osteoblasts, osteocytes, chondrocytes and odontoblasts can all respond to mechanical stress, and TRPP1 is a promising candidate for mechanotransduction in these cells. In this way, we decided to generate a *Pkd1* down expressing odontoblast cell line using lentivirus-mediated shRNA technology to study in the future the effects of TRPP1 on odontoblast function.

Materials and methods

Cell culture

Murine odontoblast-like cells (MO6-G3) were a gift from Pr Mary MacDougall (University of Alabama, Birmingham, USA). MO6-G3 cells were cultured in MEM (Eagle's basal medium, Life Technologies, Saint Aubin, France) containing 15% fetal bovine serum (FBS) (Abcys, France) supplemented with 50 μg/ml ascorbic acid (Sigma-Aldrich, St. Quentin Fallavier, France), and 100 IU/ml penicillin-50μg/ml streptomycin (Biomerieux, France), 10mM b-glycerophosphate (Sigma-Aldrich, St. Quentin Fallavier, France) at 33° C in 5% CO2 humidified air. The MO6-G3 cells stably expressing *Pkd1*-shRNA were maintained in growth medium with puromycine (3 μg/ml).

shRNA expression vector transfection

Mission®shRNA Lentiviral Transduction Particles were used for the transfection of the MO6-G3 cells (Sigma-Aldrich, St. Quentin Fallavier, France). Three validated shRNA sequences targeted for mouse *Pkd1* were chosen: MISSION® TRC shRNA TRCN0000302260 (*Pkd1*-shRNA1: 5'-CCGGGCTTCACTACTCTTCCTGCTTCTCGAGAAGCAGGAAGAGTAGTGAAGCTTTTTG-3') TRCN0000304612 (*Pkd1*shRN$_A$2:5'CCGGA CACAATACCACGCAT ATTTACTCGAGTAAATATGCGTGGTATTGTGTTTTTG- 3') and TRCN0000304664 (*Pkd1*-shRNA$_3$: 5' CCGGGGTGGACACCACTCAGTATTACTCG AGTAATACTGAGTGGTGTCCACCTTT TTG-3'). The shRNA sequence targeting the *tGFP* *(SHC204V)*, which has no homology to any mouse mRNA sequence in the NCBI Reference Sequence Database, was used as a negative control (Ctr-shRNA$_2$: 5'-CCGGCGTGATCTTCACCGACAAGATCTCGAGATCTTGTCGGTGAAGATCACGTTTTT-3') as well as the MISSION TRC2 pLKO.5-puro Empty Vector Control (SHC201V) (Ctr-shRNA$_1$).

The MO6-G3 cells were plated at 50-60% confluence and transfected with appropriate dilutions of lentivirus supernatants to obtain a MOI of 5 in presence of hexadimethrine bromide (8μg/mL). Forty-eight hours after transfection, the cells were cultured in cell growth medium containing puromycine (25 μg/ml) to obtain the stable transfected MO6-G3 cells.

Real-Time PCR

Total RNA was extracted with a Nucleospin RNA kit (Macherey –Nagel, Düren, Germany) according to the manufacturer's instructions. Ribonucleic acid samples were then converted to first-strand cDNAs using 500ng oligo(dT)15 primers (Roche Diagnostics, Meylan, France) and superscript III Reverse Transcriptase (Invitrogen Life Technologies, Grand Island, NY, USA). Real-time PCR was performed in a CFX96 Real-TimePCR Detection System

	Primers sets	Annealing Temperature	Amplicon Size
Pkd1	F 5' GTG TCC CTG TGG CCT AAT AAC 3' R 5' AGA GGG CAG AAG GTA AT 3'	67°C	150 bp
Ppia	F 5' CGC CAC CAC TCA CTA CCA CA 3' R 5' ACC ATT GGG AAC TGA TAG GAT G 3'	62°C	144 bp

Table 1: primer sets, annealing temperatures and amplicon sizes

(Bio-Rad Laboratories, Hercules, CA, USA) with the iQ SYBR Green Supermix kit (Bio-Rad) according to the manufacturer's specifications. All runs were performed in duplicate. Cyclophilin A housekeeping gene (Ppia) was used for sample normalization. Primer sets, annealing temperatures and amplicon sizes for Pkd1and Ppia genes are listed in table 1.

Cell proliferation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) assay was performed to compare the capacity for cellular proliferation of MO6-G3 cells (non- infected cells), MO6-G3 cells with empty vector (Ctr-shRNA$_1$), MO6-G3 with TurboGFP-shRNA (Ctr-shRNA$_2$), and MO6-G3 with *Pkd1*-shRNA$_2$. Each kind of cells was plated at a density of 10^4cells/well in 12 wells of a 96-well plate. Then, the cells were incubated with MTT (5mg/mL) for 3 h to 4 h at 33°C and dimethyl sulfoxide (DMSO)/ethanol mix was added to solubilize the crystals at room temperature. A spectrophotometer (Multiskan FC, Thermo Scientific, France) was used to measure the optical density (OD) at a wavelength of 620 nm. All experiments were performed three times.

Results

Knockdown efficiency of lentivirus-mediated *Pkd1* shRNA in MO6-G3 cells

Pkd1-shRNA construct was delivered into human MO6-G3 cells by means of lentiviral vector TRC2-pLKO-puro. The relative abundance of *Pkd1* mRNA in transfected control shRNA (Ctr-shRNA$_1$ and Ctr-shRNA$_2$) and *Pkd1*-shRNA MO6-G3 cells was analyzed by quantitative real-time RT-PCR. Compared to Ctr-shRNA$_1$ (empty vector), there was no difference of *Pkd1* mRNA expression in non-infected MO6-G3 cells and in transfected Ctr-shRNA$_2$ MO6-

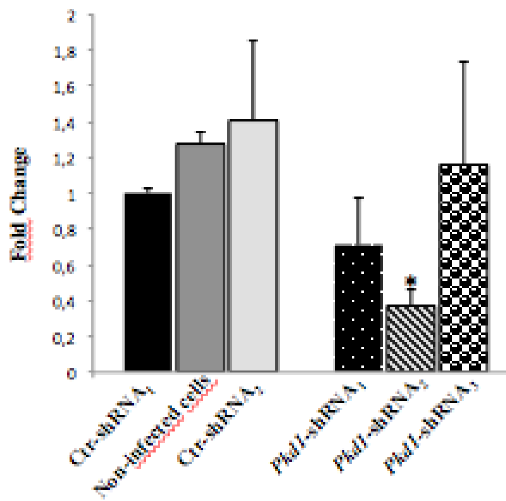

Fig.1 : Quantitative real-time PCR analysis of Pkd1 transcripts from mouse MO6-G3.
(Ctr-shRNA$_1$: empty vector, Ctr-shRNA$_2$: shRNA sequence targeting turboGFP)
Compared to Ctr-shRNA$_1$, there was no significant difference of Pkd1 mRNA
expression in non-infected cells and Ctr-shRNA$_2$. However, Pkd1-shRNA$_1$ and
Pkd1-shRNA$_2$ exhibited respectively a 30% and 60% suppression of Pkd1 mRNA
expression. Pkd1 mRNA was normalized by housekeeping gene cyclophilin.

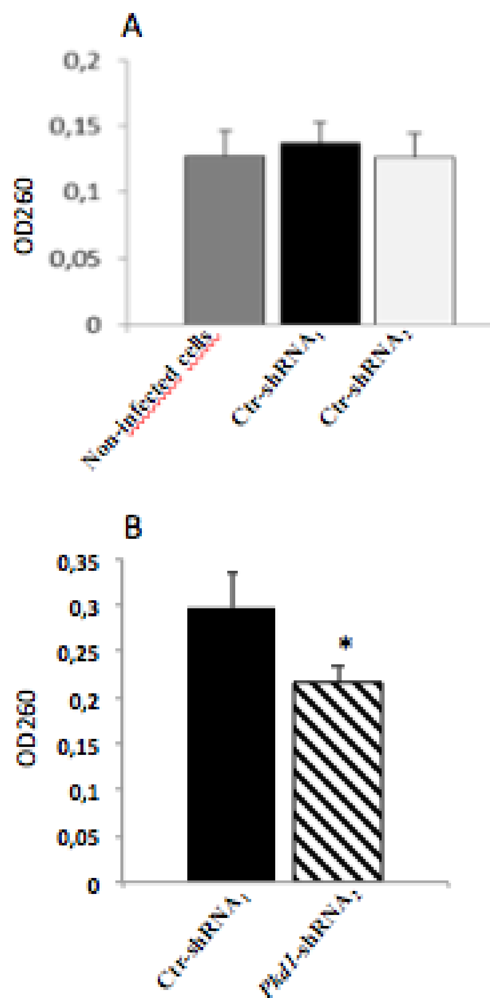

Fig.2: Effects of shRNA knockdown of Pkd1 mRNA on proliferation (MTT test)
Empty vector and shRNA sequence targeting turboGFP have no effect on cell proliferation (A).
But, shRNA knockdown of Pkd1 mRNA resulted in a significant decrease of cell proliferation (B).

G3 cells. Unlikely, transfected *Pkd1*-shRNA$_2$ MO6-G3 cells exhibited a 60% suppression of *Pkd1* mRNA expression (fig.1). *Pkd1*-shRNA$_1$ and *Pkd1*-shRNA$_3$ did not knockdown significantly *Pkd1* expression.

shRNA knockdown of Pkd1 mRNA on proliferation

The effect of *Pkd1* on cell proliferation was investigated by MTT assay. Compared to Ctr-shRNA$_1$, OD values were not significantly different for non-infected MO6-G3 cells and for transfected Ctr-shRNA$_2$ MO6-G3 cells (fig2a.). However OD value was significantly decreased in transfected Pkd1-shRNA$_2$ MO6-G3 cells (fig.2b).

Discussion

This is a preliminary report on the establishment of a *Pkd1* down expressing odontoblast cell line. In the current study, to determine whether TRPP1 has an important role in odontoblasts, we used lentivirus-mediated shRNA technology to stably silence *Pkd1* mRNA messages and examine the effects of TRPP1 on odontoblast function in the murine odontoblastic M06-G3 cell line. Lentiviral-based particles allow efficient infection and integration of the specific shRNA construct into both differentiated and dividing cells.

Three *Pkd1*-shRNA were used, two of them targeting the CDS (*Pkd1*-shRNA$_{1,2}$) and the last one targeting the 3'UTR (*Pkd1*-shRNA$_3$). In this first round of experiment only *Pkd1*-shRNA$_2$ was efficient to knockdown *Pkd1* expression by 60%. The two others were unable to down regulate *Pkd1* expression in MO6-G3 cells. Even if *Pkd1*-shRNA$_1$ seems to down regulate *Pkd1* expression of about 40%, this down expression was not significant compared to the control. To further examine the role of TRPP1 in odontoblast physiology, we choose to work on the stably *Pkd1*-shRNA$_2$ transfected cell line. We first evaluated the effect of *Pkd1* down regulation on cell proliferation. We observed a decrease of proliferation in the cells transfected by the *Pkd1*-shRNA$_2$ compared to the control. This result is contrary to what happens in osteoblasts as the *Col1a1(3.6)-Cre*-mediated conditional deletion of *Pkd1* increases osteoblastic proliferation in vitro and impairs osteoblastic maturation (16, 17). However, a decrease of proliferation of mesenchymal osteochondroprogenitor cells in the cranial base was observed in the *Dermo-Cre1*; *Pkd1* conditional knockout mice (19).

This preliminary result indicates an effect of *Pkd1*-shRNA on the physiology of odontoblasts and will allow us to go further in the characterization of the effects of the down regulation of *Pkd1* in odontoblasts. We will determine if the expression of mineralizing and matrix protein genes is regulated by the down expression of *Pkd1*. In a second time, we will subject these *Pkd1*-down expressing cells to mechanical forces (compression or shear stress) to determine if TRPP1 is involved in the mechanotransduction of odontoblasts.

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