Cancer Letters 345 (2014) 100–105

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Mesenchymal stromal cell ciliogenesis is abrogated in response to tumor necrosis factor-α and requires NF-κB signaling

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ARTICLE INFO

Article history:
Received 18 July 2013
Received in revised form 4 November 2013
Accepted 27 November 2013

Keywords:
Cancer biomarker
Inflammation
Mesenchymal stromal cells
NF-κB
Primary cilium

ABSTRACT

The primary cilium is a cell surface-anchored sensory organelle which expression is lost in hypoxic cancer cells and during mesenchymal stromal cells (MSC) adaptation to low oxygen levels. Since pro-inflammatory cues are among the early events which promote tumor angiogenesis, we tested the inflammatory cytokine tumor necrosis factor (TNF)-α and found that it triggered a dose-dependent loss of the primary cilia in MSC. This loss was independent of IFT88 expression, was abrogated by progranulin, an antagonist of the TNF receptor and required the NF-κB signaling intermediates IκB kinase α, β, and γ, as well as NF-κB p65. These findings strengthen the concept that the primary cilium may serve as a biomarker reflecting the tumor-supporting potential of MSC and their capacity to adapt to hypoxic and pro-inflammatory cues.

1. Introduction

The primary cilium is an immotile microtubule-based organelle anchored to the membrane of most human cells [1]. Originally believed to be an artifact of evolution with very little physiologic purpose, it is now clear that the primary cilium bears important signaling and sensory functions [2]. Mechanosensation, photosensation, chemosensation, calcium level regulation, left–right patterning asymmetry in embryonic development and intercellular communication are all crucial physiologic events in which primary cilia are involved [3–6]. The primary cilia also represent an important gateway in cell signaling, providing the extracellular matrix (ECM) components and growth factors with access to several receptors [7]. Bearing such important roles, dysfunction or dysgenesis of the primary cilia leads to ciliopathies such as polycystic kidney disease, nephronophthisis, Joubert syndrome and Meckel–Gruber syndrome [8]. While most of healthy human cells express primary cilia, cancer cells have been reported to express very low or undetectable levels of the cilia [9,10]. How ciliogenesis is regulated by pro-carcinogenic stimulation or within a tumor-associated pro-inflammatory microenvironment remains poorly understood.

A number of cell signaling pathways associated with tumor cell growth, particularly the Hedgehog and Wnt pathway, are modulated following ablation of cilia [11]. Several studies reported that cilia removal activated GLI2, a known effector of the Hedgehog mitogenic pathway, leading to repression of the P16 tumor suppressor [12–14]. Moreover, ciliogenesis seems to depend on cell cycle status, as it has been observed that primary cilium expression only occurs in non-proliferating cells, again supporting the hypothesis that highly proliferative cancer cells do not display this organelle [15]. Several other proliferating and ciliated cell types have been reported, suggesting a complex regulation of ciliary assembly depending on the cell's signaling needs and according to the cell cycle status [16]. Among these cell types, bone marrow-derived mesenchymal stromal cells (MSC) express primary cilia although ciliogenesis was abrogated when MSC were cultured in hypoxic conditions approximating those oxygen levels found within solid tumors [17]. There is growing interest in MSC's immunomodulatory properties and links to cancer [18,19], and MSC were found to migrate and engraft within the tumor hypoxic and inflammatory microenvironment where they express and secrete cytokines that trigger angiogenesis [20–23].

To date, no molecular links have been established between inflammation and ciliogenesis, and it is unknown whether ciliogenesis is regulated by inflammatory cues. As tumor-associated inflammation is, in part, triggered through the pro-inflammatory cytokine tumor necrosis factor (TNF)-α/TNF receptor (TNFR)

Abbreviation: COX-2, Cyclooxygenase-2; ECM, extracellular matrix; IκB, inhibitor of NF-κB; IKK, IκB kinase; IFT88, intraflagellar transport protein 88 homolog; MSC, mesenchymal stromal cell; MMP, matrix metalloprotease; NF-κB, nuclear factor kappa B; TNF, tumor necrosis factor; TNFR, TNF receptor.

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http://dx.doi.org/10.1016/j.canlet.2013.11.021

signaling pathway [24], we therefore questioned whether ciliogenesis is affected by TNF-α treatment and whether this required any TNFR downstream signaling. In the current study, we report that TNF-α triggers the loss of primary cilium expression and that this occurs through a TNFR-mediated NF-κB signaling with an absolute requirement for the IκB kinase (IKK) family.

2. Methods

2.1. Materials

Murine progranulin and murine tumor necrosis factor (TNF)-α were from EMD Millipore (Gibbstown, NJ), sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The enhanced chemiluminescence (ECL) reagents were from Perkin Elmer (Waltham, MA). Microbicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The polyclonal antibody against IκB was purchased from Cell Signaling (Danvers, MA). The goat anti-IFT88/Polaris antibody was from MyBioSources (San Diego, CA). Dilutions of 1:1,000 was used for immunoblotting and for immunofluorescence. The Alexa Fluor 488 donkey anti-goat IgG antibody was from Molecular Probes (Grand Island, NY). The mouse anti-acetylated tubulin antibody (clone 6-11B-1) and the anti-mouse Red-X secondary antibody for immunofluorescence were purchased from Invitrogen (Carlsbad, NM). The monoclonal antibody against GAPDH was from Advanced Immunochemical Inc. (Long Beach, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse IgG secondary antibodies were from Jackson Immuno-Research Laboratories (West Grove, PA). All other reagents were from Sigma–Aldrich Canada.

2.2. Cell culture

Bone marrow-derived MSC were isolated from the whole femur and tibia bone marrow of C57BL/6 female mice; cells were cultured and characterized as previously described [25]. Analysis by flow cytometry, performed at passage 14, revealed that MSC expressed CD44 yet were negative for CD45, CD31, KDR/flk1 (VEGF-R2), flt-4 (VEGF-R3) and Tie2 (angiopoietin receptor) (data not shown). Wild-type (Wt) or mutant murine embryonic fibroblasts (MEF) IKKα$^{-/-}$ [26], IKKβ$^{-/-}$ [27], and IKKγ/Nemo$^{-/-}$ [28] were described previously and were kindly provided by Dr. Terence Dermody (Vanderbilt University, USA).

2.3. Immunofluorescence

MSC were grown on coverslips to 60% confluence and then treated for 18 h. Cells were then incubated in 10% formalin phosphate buffer for 20 min, followed by incubation in permeabilization buffer (0.5% triton X-100 in PBS) for 5 min and then blocked in 1% BSA, 0, 1% NaN_3, 1% CS in PBS for 1h. The coverslips were incubated 1 h with a goat polyclonal anti-IFT88/Polaris 1:200 in 1% BSA, 0, 1% NaN_3, in PBS. After three PBS washes, the cells were incubated for 1 h with Alexa Fluor 488 donkey anti-goat secondary antibody in 1% BSA, 0, 1% NaN_3, 1% CS. After three PBS washes, the cells were further incubated for 1 h with 10 mM DAPI and anti-mouse Red-X 1:200 in antibody solution. The nucleus were stained with 10 mM DAPI for 5 min. The coverslipes were then washed 3 times in PBS and laid down on microscope slides using ProLong Gold Antifade Reagent from Invitrogen (Carlsbad, NM). The samples were viewed under a fluorescence microscope using Northern Eclipse software (Empix Imaging Inc.) with labeled acetylated tubulin in red and DAPI-stained nucleus in blue. Three random fields were acquired for each condition and analyzed with the Image J software (NIH). The expression ratio of acetylated tubulin antibody-labeled cells (primary cilium) over DAPI-stained nucleus (total cells) was calculated.

2.4. Immunoblotting procedures

Proteins from control and treated cells were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked for 1 h at room temperature with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1000 dilution) in TBST containing 3% bovine serum albumin, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-goat or anti-mouse IgG (1/2500 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfé, QC).

2.5. Transfection method and RNA interference

MSC were transiently transfected with 20 nM murine siRNA (Qiagen) against NF-κB p50 (Mm_Nfkb_2 Flexitube siRNA, SI01326990) or NF-κB p65 (Mm_Rela_2 Flexitube siRNA, SI01399622) or scrambled sequences (AllStar Negative Control siRNA, 1027281) using Hiperfect (Qiagen). Specific gene knockdown was evaluated by qRT-PCR as described below. Small interfering RNA and mismatch siRNA were synthesized by Qiagen and annealed to form duplexes.

2.6. Total RNA isolation, cDNA synthesis and real-time quantitative RT-PCR

Total RNA was extracted from MSC monolayers using TriZol reagent (Life Technologies, Gaithersburg, MD). For cDNA synthesis, 2 μg of total RNA were reverse-transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was stored at −80 °C prior to PCR. Gene expression was quantified by real-time quantitative PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). DNA amplification was carried out using a CFX Connect Real-Time System (Bio-Rad, Hercules, CA) and product detection was performed by measuring binding of the fluorescent dye SsoFast EvaGreen to double-stranded DNA. The following QuantiTect murine primer sets were obtained from Qiagen (Valencia, CA): NF-κB p50$^{NF\text{-}\kappa B1}$ (QT00154091) and NF-κB p65^{RelA} (QT00149415). The relative quantities of target gene mRNA were compared against murine GAPDH and β-Actin internal mRNA controls, and were measured by following a ΔC_T method employing an amplification plot (fluorescence signal *vs.* cycle number). The difference (ΔC_T) between the mean values in the triplicate samples of the target gene and those of GAPDH and β-actin mRNAs were calculated by (Bio-Rad, Hercules, CA) and the relative quantified value (RQV) was expressed as $2_T^{-\Delta C}$.

2.7. Analysis of cell cycle by flow cytometry

Distribution of MSC throughout the cell cycle was assessed by flow cytometry [29]. Serum-starving preparation was performed prior to analysis and therefore the cell populations were synchronous prior to TNF-α treatment. Cells were harvested by gentle trypsinization, pelleted by centrifugation, washed with ice-cold PBS/EDTA (5 mM), then resuspended in 1 volume PBS/EDTA and fixed with 100% ethanol overnight. Three volumes of staining solution, containing propidium iodine (PI, 50 μg/ml) and DNAse-free RNAse (20 μg/ml), were added. The fraction of the population in each phase of the cell cycle was determined as a function of the DNA content using a Becton Dickinson FACS Calibur flow cytometer equipped with CellQuest Pro software.

2.8. Statistical data analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test Probability values of less than 0.05 were considered significant and an asterisk (*) identifies such significance in the figures.

3. Results

3.1. TNF-α abrogates primary cilia expression in mesenchymal stromal cells

The pro-inflammatory cytokine TNF-α was first evaluated for its capacity to affect primary cilium expression. MSC were cultured on coverslips for immunofluorescence and serum-starved in the presence of increasing concentrations of TNF-α for 18 h. The resulting slides were incubated with the anti-acetylated tubulin antibody and nuclei were stained with DAPI. The number of cells expressing a primary cilium was visualised using fluorescent microscopy (Fig. 1A) and assessed in three different random fields for each condition. We observed that the primary cilium was expressed in more than 80% of MSC (Fig. 1B, control) and that TNF-α dose-dependently repressed ciliogenesis reaching ∼90% inhibition at 10 ng/ml (Fig. 1B). This effect of TNF-α was accompanied by a decrease in IκB expression (Fig. 1C) and similarly reached a maximal effect at 10 ng/ml (Fig. 1D).

3.2. TNF-α-mediated cilia loss does not alter cell cycle phases and IFT88 nuclear expression

Loss of primary cilia function is thought to be linked to proliferative diseases and to cell cycle [30]. Among components of mammalian intraflagellar components, the intraflagellar transport protein 88 homolog (IFT88) is essential for ciliogenesis and has been ascribed regulating functions in G1/S transition [31]. We next assessed the effect of TNF on cell cycle phases, and whether the TNF-mediated

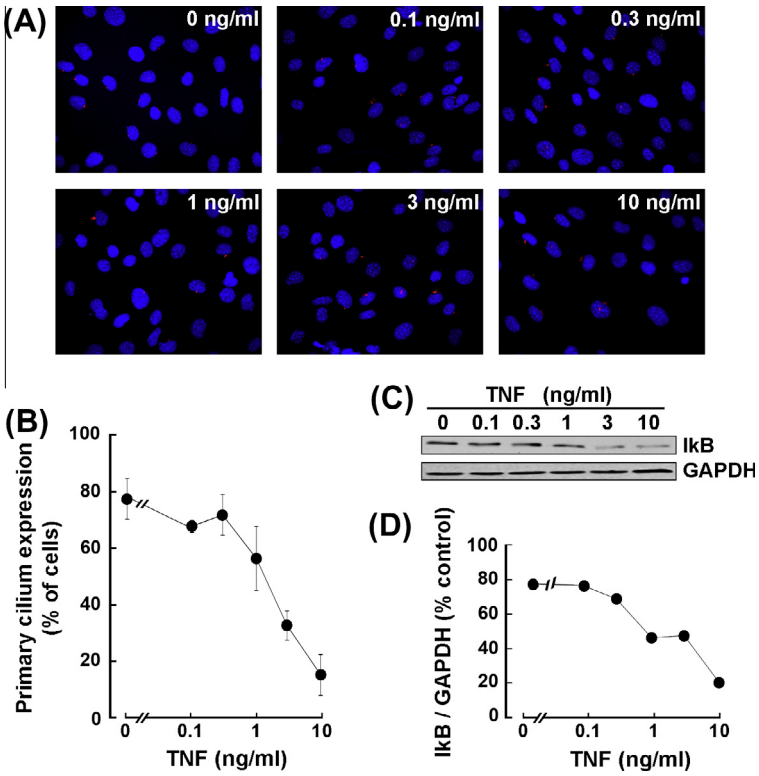

Fig. 1. TNF-α abrogates primary cilia expression in mesenchymal stromal cells. (A) MSC were seeded on coverslips for immunofluorescence and serum-starved in the presence of various concentrations of TNF-α for 18 h. The coverslipes were then fixed and incubated with anti-acetylated tubulin antibody, DAPI-stained, then visualised using a fluorescence microscope. (B) The number of primary cilia (in red) was counted in three different random locations for each condition and the cilia expression ratio determined as a proportion of the number of cells (in blue). Typically, 100–150 cells were evaluated. (C) Lysates of samples which underwent the various treatments were isolated, electrophoresed via SDS–PAGE and immunodetection was performed as described in the Methods section. (D) Quantification was performed by scanning densitometry of the autoradiograms for GAPDH and IκB.

loss of acetylated tubulin expression also altered IFT88 expression. We found that increasing concentrations of TNF-α did not alter cell cycle phases as no significant difference was observed between control and TNF-treated cells in G0/G1, G2/M, and S phases (Fig. 2A). While COX-2 increased in response to TNF-α treatment in cell lysates, that of IFT88 and of GAPDH remained unchanged (Fig. 2B). IFT88 nuclear expression also remained unaffected, and

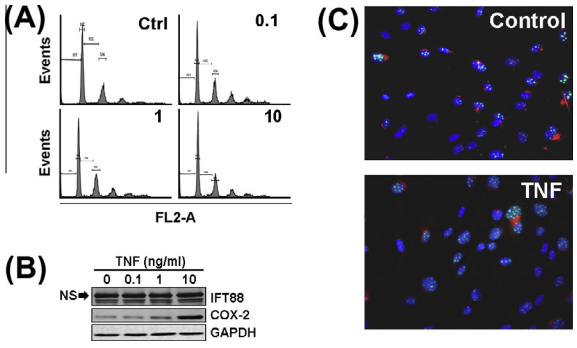

Fig. 2. TNF-α-mediated cilia loss does not alter cell cycle phases and IFT88 nuclear expression. MSC were serum-starved in the presence of various concentrations of TNF-α for 18 h, then either (A) labeled for G0/G1, G2/M, and S cell cycle evaluation as described in the Methods section, or (B) total lysates isolated for immunoblotting detection of IFT88, COX-2, or GAPDH protein expression. (C) MSC were seeded on coverslips for immunofluorescence and serum-starved in the presence of vehicle (Control) or 10 ng/ml TNF-α for 18 h. The slides were then fixed and incubated with anti-acetylated tubulin antibody (red), anti-IFT88 (green), DAPI-stained (blue), then visualized using a fluorescence microscope.). Typically, 100–150 cells were evaluated.

no co-localization of IFT88 with acetylated tubulin was observed within our cell model and under our experimental settings (Fig. 2C) suggesting that TNF-mediated loss of the primary cilium is independent of IFT88 expression in our cellular model.

3.3. Progranulin reverses TNF-α-mediated cilia loss

In order to address whether this primary cilia loss required TNF-α binding to its receptor TNFR, we next treated MSC with TNF-α, the TNFR antagonist progranulin, or a combination of both and visualized primary cilium expression (Fig. 3A). While progranulin itself was ineffective, TNF-α significantly decreased ciliogenesis, and this effect was reversed by progranulin (Fig. 3B). This indicates that TNF-α required interaction with its receptor TNFR in order to transduce the downstream signaling that ultimately led to ciliogenesis disruption.

3.4. IKKs are required for TNF-α-mediated cilia loss

In an attempt to elucidate the NF-κB signaling axis required to transduce TNF-α-mediated inhibition of ciliogenesis, wild-type mouse embryonic fibroblasts (MEF) and MEF mutants for IKKα$^{-/-}$, IKKβ$^{-/-}$, and IKKγ$^{-/-}$ were used. Cells were cultured on coverslips for immunofluorescence and serum-starved in the presence of TNF-α for 18 h. The resulting slides were immunolabeled for acetylated tubulin, nucleus-stained with DAPI and visualised with a fluorescence microscope (Fig. 4A). The number of primary cilia was counted in three different random fields for each condition. Similar to what was found in MSCs, TNF triggered cilia disappearance in up to 40% of the cells in the wild type condition, suggesting

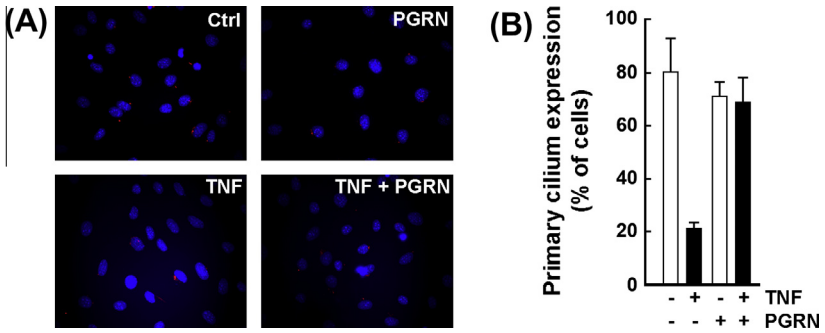

Fig. 3. Progranulin reverses TNF-α-mediated cilia loss. (A) MSC were seeded on coverslips for immunofluorescence and serum-starved in the presence of 10 ng/ml TNF-α and/or 10 nM progranulin for 18 h. The slides were then fixed and incubated with anti-acetylated tubulin antibody, DAPI-stained, then visualized using a fluorescence microscope. Typically, 100–150 cells were evaluated. (B) The number of primary cilia (in red) was counted in three different random locations for each condition and the cilia expression ratio determined as a proportion of the number of cells (in blue).

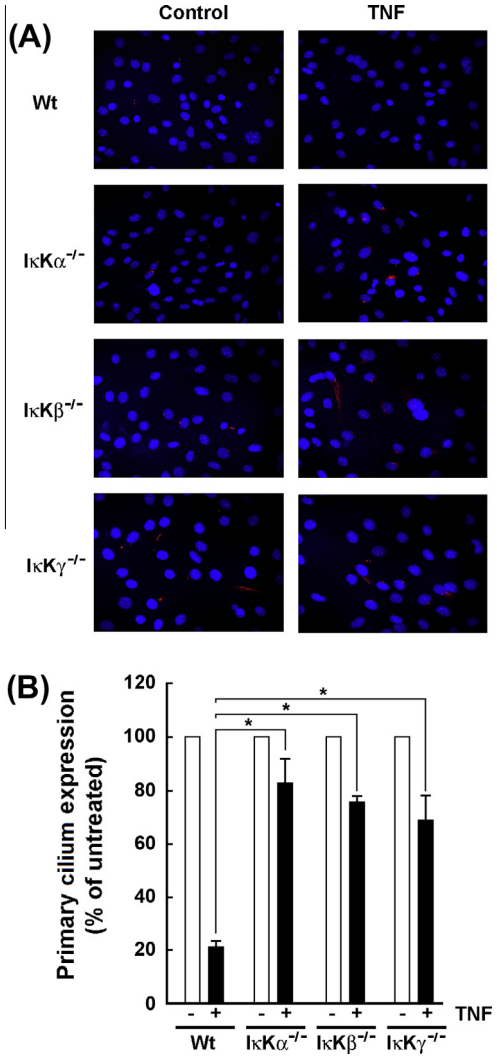

Fig. 4. IKKs are required for TNF-α-mediated cilia loss. (A) Four subtypes of mouse embryonic fibroblast cells (MEFs): Wild Type (WT), IκKα$^{-/-}$, IκKβ$^{-/-}$, and IκKγ$^{-/-}$ were seeded on coverslips for immunofluorescence and serum-starved in the presence of 10 ng/ml TNF-α for 18 h. The slides were then fixed and incubated with anti-acetylated tubulin antibody, DAPI-stained, then visualized using a fluorescence microscope. Typically, 100–150 cells were evaluated. (B) The number of primary cilia (in red) was counted in three different random locations for each condition and the cilia expression ratio determined as a proportion of the number of cells (in blue).

similar mechanisms of action are found in both murine cell lines. However, mutations in any of the IKK family members significantly reversed the TNF effect (Fig. 4B). These results suggest that TNF-mediated NF-κB activation is required for cilia loss and that the IκB kinases are essential in this mechanism.

3.5. Gene silencing of NF-κB p65, but not of NF-κB p50, abrogates the TNF-α-mediated decrease in ciliogenesis

TNF-α-mediated activation of the IKK complex leads to the phosphorylation of IκB and subsequent degradation via the proteasome pathway as observed in Fig. 1D. This process is known to release the heterodimer p50/p65, allowing it to translocate to the nucleus where it binds to specific κB sites [24]. We therefore used gene silencing strategies to reduce either NF-κB p50 or NF-κB p65 murine genes in MSC, then treated the cells with or without TNF-α. Initially, total RNA was extracted and gene silencing efficacy found to typically range between 65% and 82% (Fig. 5B). When expression of the primary cilium was evaluated (Fig. 5A), only the NF-κB p65 silencing prevented TNF-α from decreasing ciliogenesis (Fig. 5C). This observation suggests that NF-κB p65 acts as the primary ciliogenesis repressor in the TNF-mediated signaling axis.

4. Discussion

The link between inflammation and cancer is well recognized, but current research still continues to reveal new intricate mechanisms by which they cooperate [32,33]. In fact, the tumor inflammatory and hypoxic microenvironment results in conditions that trigger carcinogenic processes where the NF-κB signaling axis is thought to play a critical role [24]. In support, the oncogenic-related hypoxia condition has been shown to downregulate ciliogenesis in MSC, in part through the Wnt/HIF-1 signaling axis [17]. In the current study, we further provide evidence that cells exposed to TNF-α-mediated inflammatory stimulation also tend to lose their primary cilia through NF-κB-mediated signaling with crucial contributions from the IKK family members and NF-κB p65. Altogether, the above combined hypoxic and inflammatory cues provide molecular evidence that support MSC oncogenic transformation capacity as reflected by their loss of the primary cilium biomarker.

Considering that most neoplastic tissues lack expression of the primary cilium and that MSC possess the capacity to migrate and transform within hypoxic and inflammation-prone sites of injury, it is tempting to consider monitoring ciliogenesis in pathologic conditions. In addition to primary cilium expression, several MSC biomarkers have been linked to the tumour-initiating phenotype

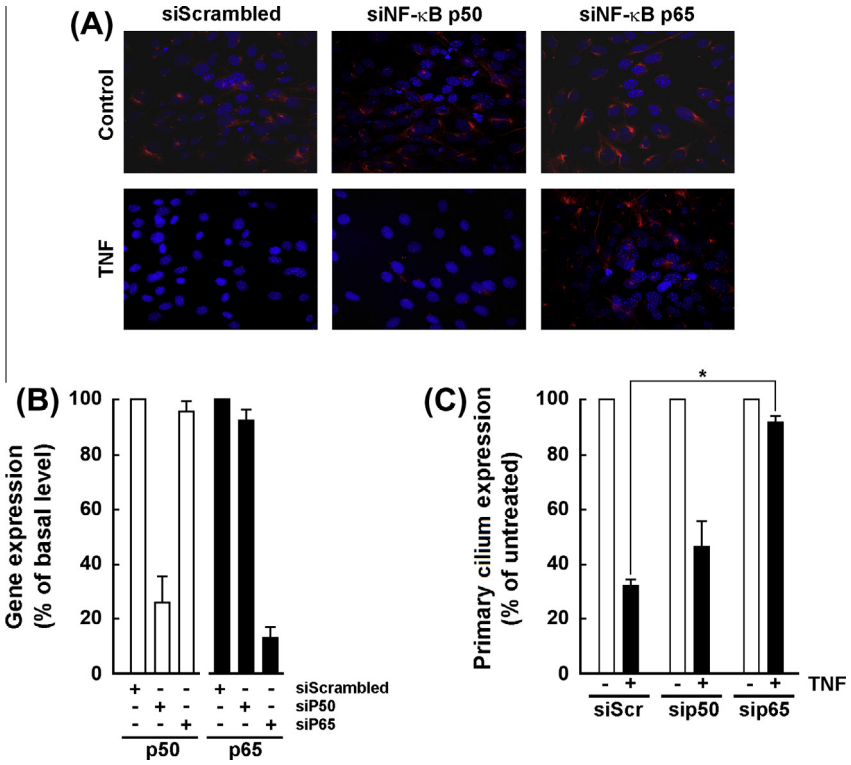

Fig. 5. NF-κB P65, but not NF-κB P50, gene silencing abrogates TNF-α-mediated decrease in ciliogenesis. MSC were seeded on coverslips and transiently transfected with siRNA against NF-κB p50 (sip50), NF-κB p65 (sip65) or with a scrambled sequence (si Scr). Cells were then serum-starved in the presence of vehicle (Control) or 10 ng/ml TNF-α for 18 h. (A) The slides were then fixed and incubated with anti-acetylated tubulin antibody, DAPI-stained, then visualized using a fluorescence microscope. Typically, 100–150 cells were evaluated. (B) Total RNA was isolated from conditions described above, cDNA synthesis, and qPCR performed to assess the extent of p50 and p65 gene silencing. (C) The number of primary cilia (in red) was counted in three different random locations for each condition and the cilia expression ratio determined as a proportion of the number of cells (in blue).

of primary glioblastomas which express mesenchymal stem-like properties [34]. Interestingly, downregulation of the primary cilium in MSC was found to specifically inhibit their differentiation along the osteogenic and adipogenic, but not chondrogenic lineage [35]. Whether such cell differentiating processes, consequent to the loss of the primary cilia, are also differentially regulated in MSC transformation upon hypoxic and inflammatory cues remains to be investigated given IFT88 expression was found unaffected by TNF-α in our cell system model. In support to our current study, neuroinflammation consequent to massive infiltration and activation of macrophages triggered IKK/NF-κB-mediated signaling and was found to impair ependymal ciliogenesis [36]. Recent evidence further supports the view that loss of the primary cilium occurs upon carcinogenic stimulation. As such, human renal proximal tubular epithelial cells treated with carcinogens such as ochratoxin A and potassium bromate resulted in the loss of the primary cilium [37]. In addition, cilia were found to be predominantly lost in renal cell carcinomas compared to tissue of the tumor parenchyma, suggesting that ciliary loss is a common process in renal tumorigenesis [38].

How a decreased ciliogenesis phenotype is involved with cancer initiation or neuroinflammation is currently unknown and will require better understanding of the roles of ciliogenesis in cancer cell biology. Furthermore, how decreases in cilia expression dictate tumor growth, angiogenesis, immune suppression, or cancer cell proliferation also remain open questions. Cilia loss may possibly contribute to the sequence of cellular events leading to malignant tumor development. Interestingly, restoration of primary cilia in tumor cells was recently found to decrease tumor growth and suggests that such targeting may be a potential therapeutic approach for cholangiocarcinoma [39]. Ciliogenesis was recently linked to the centrosome which templates the assembly of the primary cilium. It is hypothesized that dysregulation of centrosome number, a process known to induce genome instability and tumor formation, triggers changes in cilium number and affects cilia-related disease phenotypes [40].

In conclusion, our study highlights the possibility that tumor-associated inflammation may trigger primary cilia disappearance through NF-κB signaling. Given that the tumour cells which proliferate within hypoxic and pro-inflammatory conditions also lack this sensory organelle, it is tempting to suggest that intrinsic tumor-promoting signaling mechanisms triggered by inflammation also inhibit ciliogenesis. Future therapies aimed at restoring or circumventing cilia signaling might therefore aid in preventing the oncogenic processes involved in MSC transformation.

5. Conflict of Interest

The authors declare they have no conflict of interests.

Acknowledgements

BA holds a Canada Research Chair in Molecular and Metabolic Oncology from the Canadian Institutes of Health Research (CIHR). This study was funded by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC).

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