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ORIGINAL ARTICLE

Introduction of Somatic Mutation in MED12 Induces Wnt4/β-Catenin and Disrupts Autophagy in Human Uterine Myometrial Cell

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Abstract

Uterine fibroids (UFs) or leiomyoma are frequently associated with somatic mutations in the mediator complex subunit 12 (*MED12*) gene; however, the function of these mutations in human UF biology is yet to be determined. Herein, we determined the functional role of the most common MED12 somatic mutation in the modulation of oncogenic Wnt4/β-catenin and mammalian target of rapamycin (mTOR) signaling pathways. Using an immortalized human uterine myometrial smooth muscle cell line (UtSM), we constitutively overexpressed either MED12-Wild Type or the most common MED12 somatic mutation (c.131G>A), and the effects of this MED12 mutation were compared between these cell lines. This immortalized cell line was used as a model because it expresses wild type MED12 protein and do not possess MED12 somatic mutations. By comparing the effect between MED12-WT and MED12-mutant (mut) stable cell populations, we observed increased levels of protein expression of Wnt4 and β-catenin in MED12-mut cells as compared with MED12-WT cells. MED12-mut cells also expressed increased levels of mTOR protein and oncogenic cyclin D1 which are hallmarks of cell growth and tumorigenicity. This somatic mutation in MED12 showed an effect on cell-cycle progression by induction of S-phase cells. MED12-mut cells also showed inhibition of autophagy as compared with MED12-WT cells. Together, these findings indicate that the MED12 somatic mutation has the potentials for myometrial cell transformation by dysregulating oncogenic Wnt4/β-catenin and its downstream mTOR signaling which might be associated with autophagy abrogation, cell proliferation, and tumorigenicity.

Keywords MED12 · Uterine fibroid · Leiomyoma · Somatic mutations

Introduction

Leiomyoma or uterine fibroids (UFs) are the leading cause of hysterectomy in premenopausal women [1–3]. UFs are associated with significant morbidities including pelvic pain, excessive vaginal bleeding, recurrent miscarriage, pre-term birth, infertility, and abortion [4]. The development of human UFs (hUFs) is a complex process that involves various factors including sex steroid hormones, various growth factors, and inherent/genetic traits. Recent studies have demonstrated that somatic mutations in the mediator complex subunit 12 (*MED12*) gene exon 2 occur at high frequency in UFs in women from diverse racial and ethnic origins and are the major cause of pathogenesis [5–9]. These mutations are present in MED12 exon 1 and 2, and most of these mutations are missense, deletion, and insertions [10]. While these missense mutations are present throughout the coding sequence of exon 2, most of them are clustered in codons 36, 43, and 44. The most common MED12 missense mutations are present in codon 44, which suggests a functional role of these mutations in fibroid transformation. A recent study with transgenic mice that conditionally expressed MED12 mutation showed development of UF-like tumors in the uterus which provides the direct proof of disease causality [11]. A connection between

A Precis: Somatic mutations in MED12 can potentially induce oncogenic Wnt4/β-catenin pathways and inhibit cellular autophagy leading to transformation of human normal uterine myometrial cells.

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MED12 mutations and induction of gene expression of wingless-type MMTV integration site family, member 4 (Wnt4), and β-catenin signaling has been shown previously [7]. Furthermore, upregulation of the mammalian target of the rapamycin (mTOR) signaling pathway has been documented in hUFs, and a critical role of mTOR signaling in UF growth and development has been established [12]. Nevertheless, the impact of MED12 somatic mutations and their tumorigenic potential in hUF development remains to be clarified.

Mediator is a complex of 30 subunits, and its function is to regulate eukaryotic transcription and control diverse physiological processes, including cell growth and homeostasis, development, and differentiation [13, 14]. Mediator interacts directly with transcription factors to facilitate RNA polymerase II (Pol II) recruitment to target genes [15–17]. MED12 is a major functional component of the Mediator complex which is large and conserved, and it interacts with many transcription factors for gene-specific transcription. Oncogenic MED12 mutations might involve the disruption of RNA Polymerase II-dependent gene regulation in hUFs. Core Mediator is assembled into three structurally discrete modules including *head*, *middle*, and *tail* modules which bind to Polymerase II for it biological function [18]. A fourth *kinase* module comprising MED13/MED13L, MED12/MED12L, Cyclin C, and CDK8/CDK19 associates with the core Mediator and is implicated in both activation and repression of transcription [18]. Previously, we showed that MED12 within the kinase module is a functional transducer of Wnt4/β-catenin signaling [19]. More recently, we showed that oncogenic mutations in MED12 disrupt Cdk8/19 kinase activity within Mediator [20, 21]. MED12 mutations are also found in colorectal and prostate cancers [22, 23]. MED12 physically interacts with β-catenin, and this interaction is required for the transactivation of Wnt4/β-catenin signaling [19]. A study with gene knockout experiments showed the importance of MED12 in early mouse embryogenesis and for canonical Wnt and Wnt/PCP signaling [24]. Moreover, MED12 plays a role in expression of estrogen receptor alpha (ER-α) in human breast cancer cells [25]. Upregulation of MED12 has been documented in pancreatic cancer, and suppression of MED12 expression showed reduced cell-cycle progression in pancreatic cancer cells [26].

Although evidence indicates an association between MED12 with the canonical Wnt4/β-catenin pathway, it has not been yet established whether mutations in MED12 can directly modulate Wnt4/β-catenin signaling in uterine myometrial cells. The aim of this study was to examine whether MED12 somatic mutation can affect uterine myometrial cell proliferation and transformation, and to establish a direct link between MED12 somatic mutation and oncogenic Wnt4/β-catenin and its associated downstream mTOR signaling pathways. Herein, we established the tumorigenic potential of the most common MED12 somatic mutation (c.131G>A) by ectopic expression of MED12-WT and a MED12-mut protein in an immortalized human uterine myometrial smooth muscle cell line. The functional impact of the MED12 somatic mutation was examined by comparative analysis of MED12-WT and MED12-mut stable cell lines using various methods, including FACScan analyses for cell-cycle progression, western blots for induction of oncogenic cyclin D1, Wnt4/β-catenin, and mTOR signaling proteins. As autophagy plays an important role in regulating cell proliferation and tumorigenicity [27], we also investigated the link between the MED12 somatic mutation and the disruption of cellular autophagy that may play a critical role in hUF pathophysiology.

Materials and Methods

Cell Lines and Cultures

The immortalized human uterine myometrial smooth muscle (UtSM) cell line was a generous gift from Dr. Darlene Dixon, as we have described in our previous study [28]. These cells were grown in smooth muscle cell medium (SmBm) with 5% fetal bovine serum (FBS) and several growth factors at 37 °C in a humidified atmosphere of 5% CO_2 as previously described [29].

Reagents and Antibodies

The pCMV6-AC-GFP (Empty-vector), Myc-DDK-tagged MED12-Wild Type (WT), and Myc-DDK-tagged MED12-mutant (mut) (c.131G>A) constructs, and transfection reagents were purchased from ORIGENE (Northampton, MA). Anti-Flag antibody that recognizes recombinant tagged protein was purchased from Origen. Rabbit polyclonal anti-MED12 antibody was purchased from Bethyl Laboratories (Montgomery, Texas). Lipofectamin LTX transfection reagent was purchased from Invitrogen (Waltham, MA). Anti-β-actin antibody was purchased from Sigma Biochemicals (St. Louis, MO). Nuclear and cytoplasmic extraction reagents were purchased from Pierce Biotechnology (Rockford, IL). Monoclonal anti-β-catenin antibody was purchased from BD Biosciences (San Jose, CA). Polyclonal anti-Wnt4 antibody was purchased from Abcam (Cambridge, MA). Anti-cyclin D1, anti-PARP, and anti-RhoGDI antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Pierce enhance chemiluminescence (ECL) western blotting substrate was purchased from ThermoFisher Scientific (Grand Island, NY).

Plasmid Transfection into 293T Human Cells

To confirm expression of recombinant protein, empty-vector and MED12-WT vector constructs were transiently transfected into human embryonic kidney cells (293T) cells

purchased from ATCC. These plasmid constructs express a Green Fluorescence Protein (GFP), so that transfection efficiency can be monitored by the expression of GFP. Plasmids were transiently transfected into 293T cells using lipofectamin LTX according to the manufacturer's instruction (Invitrogen). Twenty hours after transfection, fresh medium was added and cultured for another 48 h. Transfection efficiency was verified by fluorescence microscopy, and then those transfected cells were lysed and the expression of MED12-WT recombinant protein was verified by western blotting using anti-Flag antibody.

Generation of Stable MED12-WT and MED12-Mut UtSM Cell Lines

To examine the role of MED12 mutation, MED12-WT and a MED12-mut (c.131G>A) Flag-Tagged constructs were stably transfected into an immortalized UtSM cell line. UtSM cell line was chosen because these cells were derived from human normal myometrial smooth muscle cells and they do not possess MED12 somatic mutations, and maintained normal characteristic of uterine myometrial phenotype, and thus provide a proper model to verify the role of the MED12 somatic mutation in human fibroid transformation. These plasmid constructs contains a puromycin selection marker gene and can express Green Fluorescence Protein (GFP). The immortalized UtSM cell line was cultured in 60-mm dish the day before transfections. Plasmid constructs were transiently transfected into UtSM cells using lipofectamin LTX transfection reagent as mentioned above. Twenty hours after the transfection, fresh medium was added to the plates and continue culture for another 48 h. Untransfected cells were killed by culturing these cells in media containing puromycin (1.0 μg/ml) for another 2 weeks to establish stable cell populations. In order to generate stable clones from these polyclonal populations, cells were diluted and platted (100, 200, or 300 cells/100 mm dishes) and cultured in puromycin containing medium for approximately 3 weeks to develop colonies. Individual colonies were isolated, cultured, and further characterized. Two stable MED12-WT clones and three MED12-mut clones were analyses for Wnt4/β-catenin and mTOR signaling pathways.

Western Blot Analyses

Western blot analyses were performed using protein lysates prepared from MED12-WT and MED12-mut cell populations and also from stable clones. 1.5×10^6 cells from either MED12-WT or MED12-mut cell populations or from stable clones were platted into 10-cm tissue culture dishes and cultured for 48 h. Cell lysate preparation and western blot analyses were performed according to our previously publications [30–32]. For detection of antigen-antibody complexes, we used Pierce enhance chemiluminescence (ECL) western blotting substrate that was purchased from ThermoFisher Scientific (Grand Island, NY). Target protein bands were visualized after the exposure to X-ray films, and thereafter by automatic x-ray developer. The intensity of each protein band was quantified and normalized against corresponding β-actin, as described in the respective figure legends.

Characterization of Cell-Cycle by Flow Cytometric Analyses

Cell-cycle analyses were performed using FACScan. 1×10^6 cells from either MED12-WT or MED12-mut stable cell populations were platted onto 10-cm tissue culture dishes and cultured for 48 h in serum free medium. Cells were cultured in regular growth medium for another 48 h. Cells were then harvested, fixed in cold 70% ethanol for 30 min, washed in phosphate buffer saline (PBS), and then treated with RNase. Cells were then treated with propodeum iodide (PI) for 24 h at 4 °C. Stained cells were analyzed for cell-cycle status by BD FACSCalibur flow cytometer system (Beckman-Coulter, CA, USA) at Augusta University Flow Cytometry Core Facility. Each data point is the mean ± standard deviation (SD) of triplicate wells ($n = 3$).

Nuclear and Cytoplasmic Fractions

2.5×10^6 cells from either MED12-WT or MED12-mut stable cell populations were seeded into 10-cm tissue culture dishes, and then incubated for overnight. Nuclear and cytoplasmic fractions were prepared from pelleted cells using NE-PER nuclear and cytoplasmic extraction reagents. Fifteen micrograms of either cytoplasmic or nuclear fractions were resolved in 10% SDS PAGE and then subjected to western blot analyses as indicated in figure legends.

Immunoprecipitation Analyses

Immunoprecipitation analyses were performed as described in our previous publications [30, 33]. Briefly, 2.5×10^6 cells from MED12-WT or MED12-mut stable cell populations were seeded into 10-cm tissue culture dishes and incubated for overnight. Cell lysates were prepared in non-reducing condition in the absence of dithiothreitol (DDT) and β-mercaptoethanol as described previously [33]. Each cell lysate (1 mg) were incubated with 2 μg of anti-MED12 antibody for 2.5 h at 4 °C, followed by 1 h incubation with 25 μl of protein G-Sepharose from Sigma Biochemicals (St. Louis, MO). The antigen-antibody complexes were precipitated by centrifugation, washed four times with incubation buffer. A 20 μl of 4× SDS sample loading buffer was added to the beads, heat denatured at 95 °C for 5 min, and centrifuged for collection of eluted proteins. Collected protein samples were analyzed for

recombinant MED12 protein expression by western blots using anti-Flag antibody.

RNA Isolation and Real-Time PCR

RNA was extracted from either MED12-WT clone or MED12-mut clone using TRIzol reagent as described previously [27]. One to 5 μg of total RNA was reverse transcribed to prepare cDNA. Real-time PCR was performed using iTaq Universal SYBR Green Supermix from Bio-Rad (Raleigh, NC, USA). The primers sequences of the specific genes and the conditions of cDNA amplifications were described previously [27]. mRNA expression of each gene is normalized with actin and the results are shown as relative expression.

Transmission Electron Microscopy Analysis

Cells from either MED12-WT clone or MED12-mut clone were fixed and prepared for Transmission Electron Microscopy as described previously [27]. Cells were stained with uranyl acetate and lead citrate which were purchased from TED PELLA as described previously [27]. Cells were then examined using a Hitachi H7600 electron microscope at an accelerating voltage of 100 kV using standard techniques, at Augusta University histology core facilities.

Statistical Analysis

Student's paired *t* test was used to assess any significant differences between MED12-WT and MED12-mut data points using GraphPad Software. Values were considered statistically significant when p is less than 0.05 ($p < 0.05$) at a 95% confidence. Data were presented as the mean ± standard deviation (SD) of three measurements.

Results

Generation of Stable MED12-WT and MED12-Mut Stable Cell Populations

A transfection strategy was used to first verify whether exogenous MED12-WT is efficiently expressed in mammalian cells. Empty vector and MED12-WT constructs were transiently transfected into 293T cells and fluorescent microscopy showed that the majority of cells were transfected as indicated by GFP expression (Fig. 1a). Expression of WT recombinant MED12 protein was verified by western blot analyses using anti-Flag antibody. MED12-WT Flag-Tagged protein was detected in 293T cells, while empty-vector control cells or untransfected 293T cells did not show recombinant protein as expected (Fig. 1b), suggesting that the MED12-WT construct is efficient to produce recombinant protein in mammalian 293T cells. To assess the functional impact of the most common MED12 somatic mutation in myometrial cell line, Flag-Tagged MED12-WT and MED12-mut (c.131G>A) derivatives were stably transfected into the immortalized UtSM cell line. Using a lipofectamin LTX transfection method and puromycin in culture medium, we successfully generated stable cell populations that expressed recombinant MED12-WT or MED12-mut proteins. Comparable expression of recombinant MED12-WT and MED12-mut proteins in these cells was confirmed by western blot analyses (Fig. 1c). Furthermore, western blot analysis was performed to measure the endogenous MED12 protein in above stable UtSM cell populations. We observed that both cell populations expressed similar levels of endogenous MED12 protein (Fig. 1d). These results indicate the presence of endogenous MED12 protein in UtSM cell line.

Ectopic Expression of a MED12-Mut Protein Disrupts Wnt4/β-Catenin Signaling, and Promotes Myometrial Cell-Cycle Progression in UtSM Cell Line

To determine whether the most common MED12 somatic mutation functionally impact Wnt4/β-catenin signaling and promote cell-cycle progression, we used stable cell populations for protein expression and cell-cycle analyses by FACScan. The induction of Wnt4/β-catenin signaling was verified by western blots using lysates from MED12-WT and MED12-mut stable cell populations. MED12-mut stable cells showed induction of Wnt4 and β-catenin proteins as compared with MED12-WT stable cells (Fig. 2a). To further determine the distribution of Wnt4 and β-catenin proteins, nuclear and cytosolic extracts were prepared from these stable cell populations and those extracts were used for western blot analyses. The levels of both cytosolic and nuclear Wnt4 and β-catenin proteins were considerably higher in MED12-mut cells as compared with MED12-WT cells (Fig. 2b). To further determine whether the MED12 somatic mutation can affect cell-cycle progression, FACScan analyses were performed using the above cell populations. Data from FACScan analyses showed a higher percentage of S-phase cells in MED12-mut stable cell populations as compared with MED12-WT stable cell populations (Fig. 2c). In contrast, lower percentage of G_1 phase cells was observed in MED12-mut stable cell populations as compared with MED12-WT stable cell populations (Fig. 2c). Cells that were in S-phase having more DNA and thereby stained more than cells that was in G_1 phase. These data suggest that ectopic expression of the mutant MED12 recombinant protein induces Wnt4/β-catenin protein expression and promotes cell-cycle progression to S-phase that may support growth and proliferation of UtSM cell line.

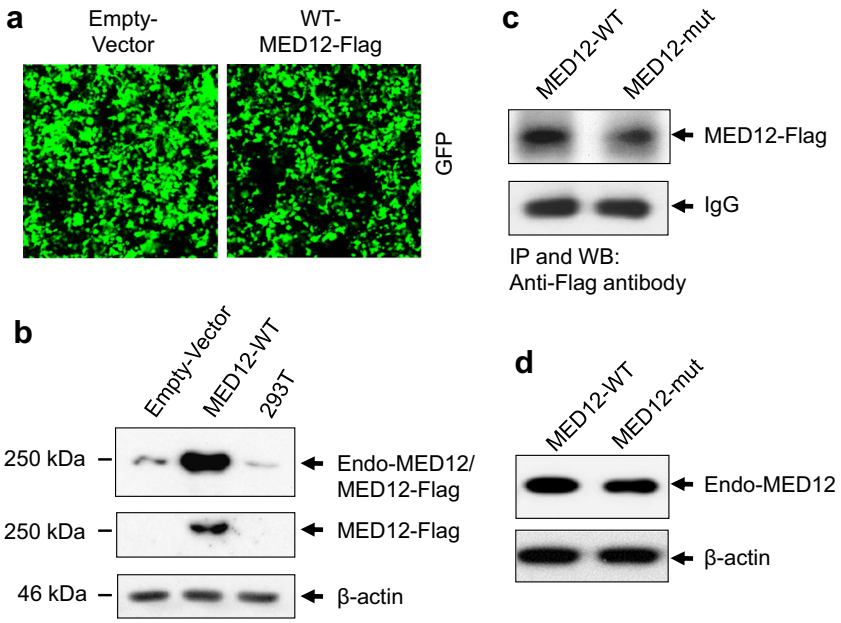

Fig. 1 Generation of MED12-WT and MED12-mut stable UtSM cell lines. **a** Plasmid constructs are well-transfected to mammalian cells. 293T cells were transiently transfected with lipofectamin reagent, and 48 h after transfection GFP expression was monitored using confocal pictures. **b** Lysates from above transfected cells were used to perform western blots using anti-MED12 antibody and anti-Flag antibody. Anti-β-actin antibody was used as a loading control. **c** MED12-WT and MED12-mut UtSM cell populations were generated by stable transfections as described in the "Materials and Methods" section. Immunoprecipitation assay using lysates from MED12-WT and MED12-mut stable UtSM cell populations showing similar expression of Flag-Tagged MED12-WT and MED12-mut proteins. Similar levels of IgG indicate the equal precipitation. **d** Equal amounts of protein lysates from MED12-WT and MED12-mut UtSM cell populations were analyzed for endogenous protein expression using anti-MED12 antibody. Similar levels of endogenous MED12 protein were detected in both cell populations. GFP, green fluorescence protein; IP immunoprecipitation; WB Western blot

Ectopic Expression of a MED12-Mut Protein Disrupts the Oncogenic Cyclin D1 Protein in UtSM Cell Line

To examine whether the MED12 somatic mutation is connected to cell proliferation-associated protein expression, western blots were performed using lysates from MED12-WT and MED12-mut stable clones. Cell lysates were analyzed for protein expression of cell growth–associated Wnt4/β-catenin and cyclin D1. Induced levels of protein expression of Wnt4, β-catenin, and cyclin D1 were detected in all three MED12-mut stable clones as compared with MED12-WT clones (Fig. 3), indicating a role for the mutant MED12 in cell proliferation and tumorigenicity. However, the expression of oncogenic Wisp1 was unchanged. These results suggest that the somatic mutation in MED12 can disrupt Wnt4/β-catenin signaling and cyclin D1 protein expression to support cell-cycle progression in UtSM cell line.

Ectopic Expression of a MED12-Mut Protein Disrupts mTOR in Stable UtSM Cell Line

mTOR is the master regulator of cell growth and proliferation [34]. To determine if the MED12 somatic mutation affects oncogenic mTOR signaling, lysates from MED12-WT and MED12-mut stable clones were analyzed for protein expression of phospho-specific mTOR and total mTOR. Mutation in MED12 induced the levels of phosphorylation of mTOR protein in all three MED12-mut stable clones as compared with MED12-WT stable clones (Fig. 4). Higher levels of total mTOR protein were also detected in all MED12-mut stable clones when compared with MED12-WT stable clones. These results suggest that the somatic mutation in MED12 has the potential to induce mTOR signaling leading to proliferation of human uterine myometrial smooth muscle cell line.

MED12 Somatic Mutation Disrupt Cellular Autophagy in MED12-Mut Stable UtSM Cell Line

Prior work has revealed that β-catenin deficiency induces autophagy in multiple myeloma cells [35]. To test whether the above MED12 somatic mutation is associated with the defects in the expression of autophagy associated factors, including ATG3, ATG4, ATG5, ATG7, ATG10, ATG12, and ATG16, we performed quantitative RT-PCR and western blot analyses (Fig. 5a–c). We observed upregulation of mRNAs for ATG3, ATG4, ATG10, and ATG12 in MED12-mut stable clone as compared with MED12-WT stable

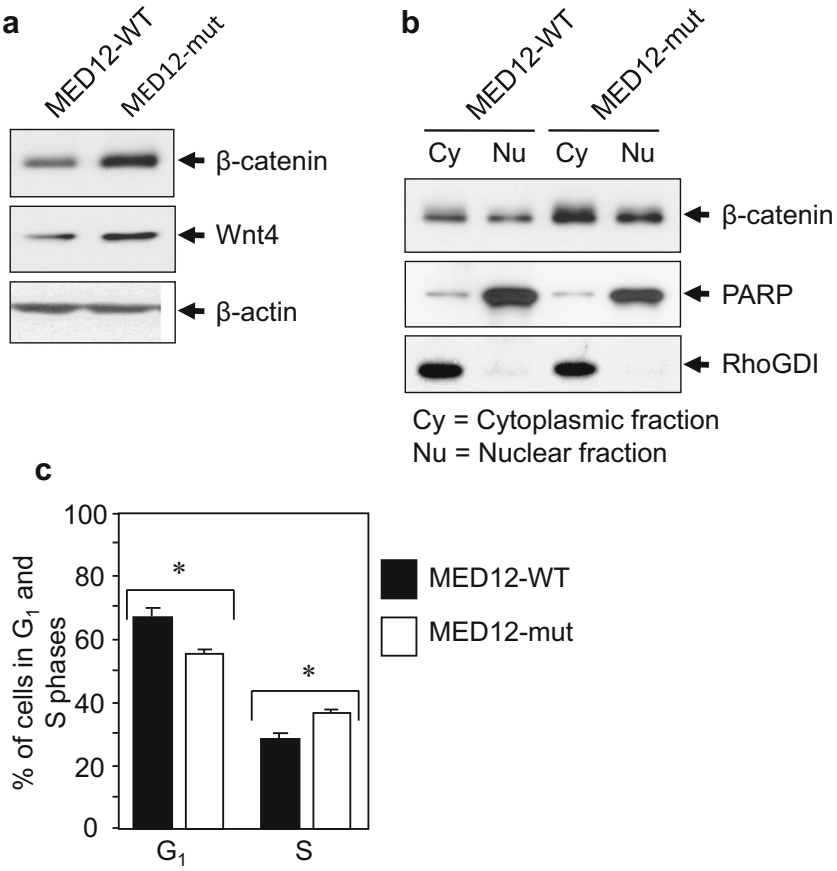

Fig. 2 Ectopic expression of a MED12-mut Flag-Tagged protein induced Wnt4/β-catenin and promotes cell-cycle progression. **a** Western blot analyses using lysates from above stable UtSM cell populations showing induced expression of Wnt4 and β-catenin in MED12-mut stable cell populations as compared to MED12-WT stable cell populations. **b** Cytoplasmic (Cy) and nuclear (Nu) fractions were isolated from cultured MED12-WT and MED12-mut UtSM stable cell populations, and then used for western blot analyses. PARP (nuclear) and RhoGDI (cytoplasmic) western blots are showing the purity of the separation. **c** FACScan analyses are indicating higher percentage of S-phase cells in MED12-mut stable cell populations as compared with MED12-WT stable cell populations. *$P < 0.05$ as compared with corresponding control

clone, while the mRNA levels of ATG7 and ATG16 were downregulated (Fig. 5a). Western blot data showed down-regulation of protein expression of ATG5 and ATG7 in MED12-mut clone as compared with MED12-WT clone (Fig. 5b, d). These findings suggest that the mutation in MED12 affects the expression of specific ATGs in stable UtSM clone. Analysis of classical markers of autophagy-flux reveals a significant decrease in the accumulation of autophagosome-bound long chain 3 (LC3II) and accumulation of p62 in MED12-mut clone as compared with MED12-WT clone (Fig. 5c, d), indicating a possible defect in the autophagy-flux in MED12-mut clone. Moreover, the expression of GSK-3β was considerably inhibited in MED12-mut clone as compared with MED12-WT clone (Fig. 5c, d). These results indicate a possible correlation between GSK-3β inhibition and upregulation of β-catenin to disrupt autophagy which can be demonstrated by transmission electron microscopy (TEM) images (Fig. 5e). In TEM images, MED12-WT clone showed autophagic vacuoles representing different steps of autophagy-flux as compared with MED12-mut clone where autophagy is quasi absent (Fig. 5e). Together, these results suggest that the somatic mutation in MED12 has the potential to disrupt cellular autophagy that may lead to cell growth and proliferation.

Discussion

UFs are the primary indication for hysterectomy and are the major health problems in women [36]. The health care cost associated with hUFs is estimated to be approximately $34 billion annually, and it represents a significant public health problem [37]. Although several non-surgical treatment methods including uterine artery embolization (UAE), magnetic resonance-guided focused ultrasound (MRgFUS), and hormonal therapies are available, none of these methods are safe for long-term treatment [38]. Thus, profound understanding the mechanisms of fibroid etiology will help to develop new therapies for hUFs.

MED12 somatic mutations are frequently associated with hUFs, and those mutations were found up to 85% of fibroid cases [5–9, 39, 40]. It has been demonstrated that 50% of those identified mutations are linked to mutations of the 44th codon of *MED12* gene exon 2, which indicates their possible functional role [8]. Although a high prevalence of MED12 somatic mutations occurs in hUFs, very little is known about the role of these mutations in the pathogenesis of hUFs. Despite the fact that a recent study supports a causality of a most common MED12 somatic mutation in the development of UFs in transgenic mice [11], but their finding is difficult to apply in human without performing experiments in human systems. The aim of this project is to evaluate the role of the most common MED12

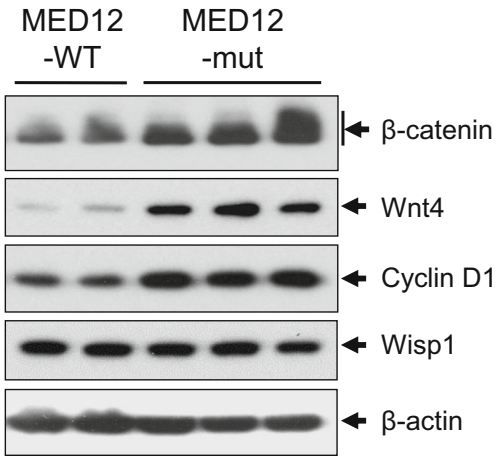

Fig. 3 Ectopic expression of the MED12-mut Flag-tagged protein in UtSM stable clone induced Wnt4/β-catenin signaling and oncogenic Cyclin D1. Stable clones were generated from MED12-WT and MED12-mut cells populations. Western blot analyses were performed using lysates from these stable clones showing induced expression of β-catenin, Wnt4, and cyclin D1 in MED12-mut stable clone as compared with MED12-WT stable clone. β-actin was used as loading control

somatic mutation (c. 131G>A) in the modulation of Wnt4/β-catenin and mTOR signaling that may support the growth and proliferation leading to the transformation of an immortalized human myometrial smooth muscle cell line. Clarification of the role of the MED12 somatic mutation in the development of hUFs might help to develop therapeutic options for a safe non-surgical treatment of hUFs.

MED12 somatic mutations have been linked with the activation of Wnt4/β-catenin signaling in human UFs [7, 19]. However, those studies have not established a direct role for MED12 somatic mutations in the induction of Wnt4/β-catenin signaling, and thus the functional impact of these mutations is still unclear in humans. Previously, by knockdown of MED12 expression, we identified a link between MED12 expression and Wnt4/β-catenin signaling in hUF-derived cell line [41]. The

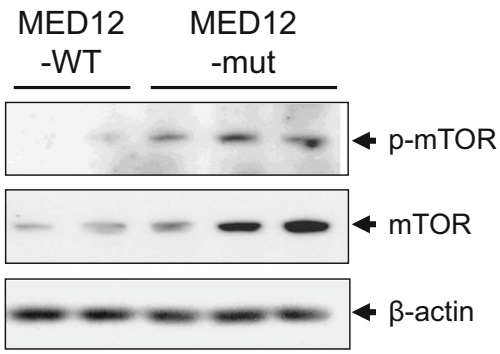

Fig. 4 Ectopic expression of a MED12-mut Flag-Tagged protein in UtSM stable clone induced mTOR signaling. Western blot analyses using lysates from MED12-WT and MED12-mut stable clones showing induced expression of phospho-specific p-mTOR and total mTOR in MED12-mut stable clone as compared with MED12-WT stable clone. β-actin was used as loading control

current study was undertaken to test if there is any functional role associated with the most common MED12 somatic mutation (c. 131G>A) in the modulation of Wnt4/β-catenin and its associated mTOR signaling pathway. mTOR is known to function downstream of Wnt4/β-catenin signaling, and it plays an important role in fibroid cell proliferation [41]. This study was performed by establishing UtSM stable cell lines that ectopically expressed MED12-WT (as control) or MED12-mut Flag-Tagged proteins.

To study the role of the above MED12 somatic mutation in myometrial cell transformation, we have successfully generated stable cell populations that ectopically expressed MED12-WT and MED12-mut recombinant proteins in an immortalized UtSM cell line. Our limitation is that we were unable to generate stable cell population from primary myometrial cells because these cells were difficult to transfect and they were not survived after antibiotic selections, which may indicate the poor transfection efficiency and limited expression of high molecular weight MED12 exogenous proteins. Our major focus was only looking at the impact of mutant MED12 on myometrial cells to draw any possible conclusions. Therefore, we compared wt/mut versus wt/wt cell populations to verify the expression levels of exogenous-WT-MED12 (control) and mutant-MED12 proteins. We have not focused on knocking out WT-MED12 or performing various transfections concentrations to highlight a dose response relationship with mutant MED12. We were unable to capture the exogenous-tagged proteins by western blot analysis. However, using western blot analysis, we have confirmed that this immortalized cell line expressed endogenous MED12 protein (Fig. 1d). Although the immortalized UtSM cells maintained myometrial cell phenotype, human primary myometrial cell cultures will be utilized in our future study to confirm whether any impact on immortalization. Our results showed similar levels of exogenous protein expression in MED12-WT and MED12-mut stable cell populations (Fig. 1c). These results motivated us to further characterize these stable cell populations. By performing western blot analyses, we confirmed that the MED12 somatic mutation can induce Wnt4/β-catenin signaling (Fig. 2a). While multiple signaling pathways including Wnt4/β-catenin, TGF-β, and estrogen signaling are well-associated with hUF development, we focused specifically on canonical Wnt4/β-catenin and mTOR signaling because of their strong association with hUF growth and proliferation, as well as the fact that MED12-mutant fibroid tumors are known to support elevated levels of Wnt4 protein expression [7]. By knockdown of MED12 expression in human UF-derived cells, we previously showed that MED12 is a direct transducer of Wnt4/β-catenin signaling [41]. Herein, we found that the most common somatic mutation in MED12 induces Wnt4/β-catenin protein expression in both the cytosol and nucleus, and increases the fraction of proliferating S-phase cells (Fig. 2b, c), thus indicating a possible role of the MED12 somatic mutation in promoting cell

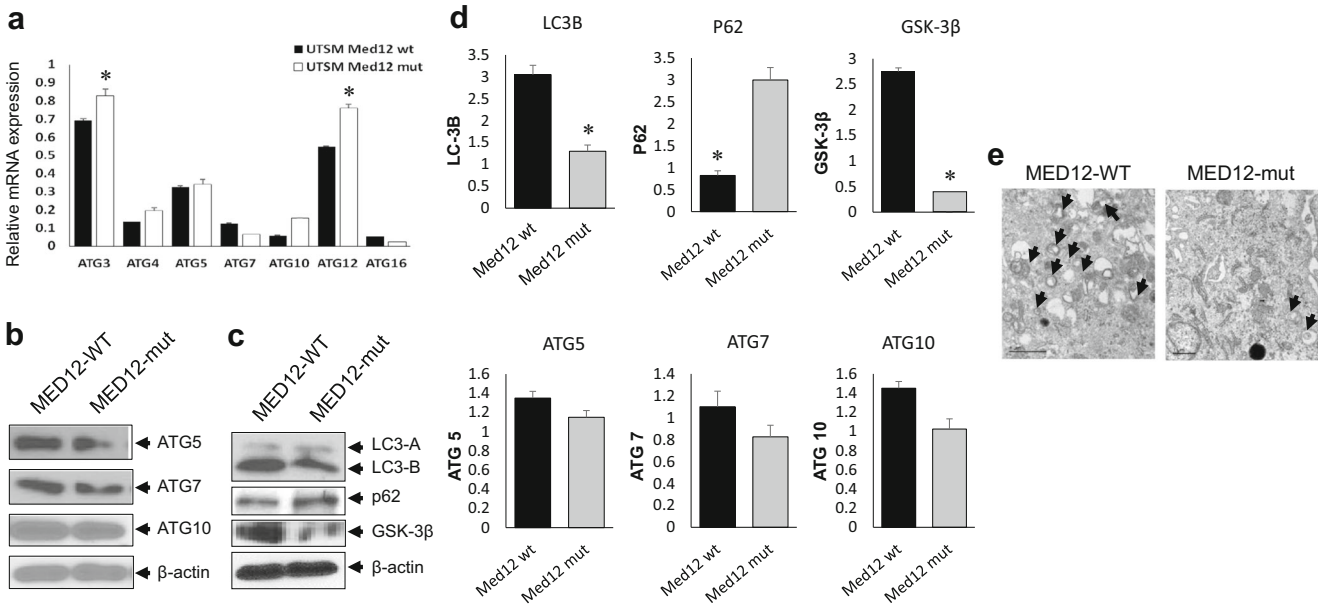

Fig. 5 Altered expression of autophagy-related proteins in MED12-mut UtSM stable clone. **a** Total RNA was extracted from MED12-WT and MED12-mut stable clones and real-time PCR analyses of mRNA levels of several ATGs were performed as described in the "Materials and Methods" section. Higher expression of ATG3, ATGD4, ATG10, and ATG12 are showing in MED12-mut stable clone as compared with MED12-WT stable clone. Reduced expressions of ATG7 and ATG16 are showing in MED12-mut stable clone as compared with MED12-WT stable clone. **b**, **c** Cell lysates from above stable clones were analyzed for protein expression as indicated by each blot. β-actin was used as loading control. **d** Quantitation of expression of autophagy-related proteins. The intensity of each protein band in **b** and **c** was quantified and then the values were normalized with corresponding β-actin. The normalized data graphs were presented as relative protein expression. $*p < 0.05$ when compared with corresponding control. **e** Transmission electron microscopy (TME) was used to examine the autophagy status in MED12-WT and MED12-mut stable clones. TME picture shows the blockade of autophagy in MED12-mut stable clone, while it is unaffected in MED12-WT stable clone. Images were taken at × 200 magnification. Black arrows indicate autophagy vacuoles

growth and proliferation. To avoid heterogeneity of recombinant protein expression, we further established individual clones from these polyclonal cell populations, and those clones were further analyzed for Wnt4/β-catenin, oncogenic cyclin D1, and mTOR signaling (Figs. 3 and 4). Results from western blot analyses confirmed the role of the MED12 somatic mutation in the induction of these pathways leading to the promotion of cell-cycle that supports cell growth, proliferation, and tumorigenicity of human UtSM cell line.

We further focused on autophagy-flux in those stable UtSM cell lines. Our results showed that the above somatic mutation in MED12 can virtually prevent the development of autophagic vacuoles that arise in MED12-WT clone (Fig. 5e). We also found a dramatic reduction of GSK-3β in MED12-mut stable clone as compared with MED12-WT stable clone (Fig. 5c, d), suggesting a possible mechanism of decreased autophagy due to the MED12 somatic mutation. In this regard, β-catenin is an established target of GSK-3β, and GSK-3β-mediated phosphorylation of β-catenin marks it for ubiquitination and degradation [42]. Reduced levels of GSK-3β in MED12-mut stable clone could lead to accumulation of β-catenin. Thus, our results suggest a possible role for the MED12 somatic mutation in disruption of automatic-reflex formation by modulating the expression of GSK-3β leading to accumulation of β-catenin which eventually can disrupt or block autophagy (see model Fig. 6). Studies have demonstrated that AKT and mTOR are inhibitors of autophagy, and overexpression of these genes inhibits tumor cells to undergo autophagic transformation [43–45]. Reduced levels of AKT/mTOR signaling pathway proteins such as phospho-AKT and mTOR are also shown in autophagy [46]. Autophagy plays a protective role for

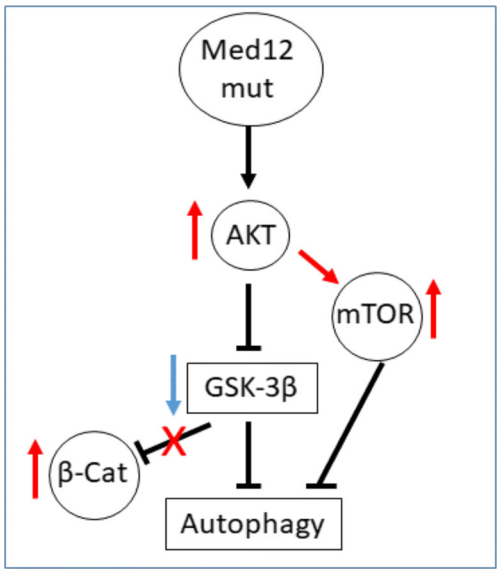

Fig. 6 Schematic diagram for blockade of autophagy in stable MED12-mut UtSM clone. MED12 somatic mutation can lead to activation of AKT/mTOR signaling pathway that inhibits autophagy. Alternatively, the activation of AKT can inhibit GSK-3β expression that leads to β-catenin upregulation

alternative cell death mechanism, and due to lack of autophagy tumor cells became active leading to loss of cellular apoptosis [47]. Thus, the balance between the Wnt4/β-catenin signaling and autophagy may play important roles in the regulation of tumorigenesis. Wnt4/β-catenin signaling is a negative regulator of autophagy [48]. Therefore, the activation of Wnt4/β-catenin pathway and lowering of autophagy levels may be helpful to divide cell rapidly leading to tumorigenicity. The aberrant Wnt4/β-catenin signaling and the disruption of autophagy may influence normal cells to be transformed to tumorigenic cells [49]. Thus, our findings suggest that the somatic mutation in MED12 has the potential to disrupt Wnt4/β-catenin, and its associated downstream mTOR signaling and inhibit normal cellular autophagy in the immortalized UtSM cell line that may eventually lead to higher rate of cell proliferation and tumorigenesis.

In conclusion, the present study demonstrates the effects of the most common MED12 somatic mutation (c. 131G>A) on an induction of cell-cycle progression of the immortalized human myometrial smooth muscle cell line. The above MED12 somatic mutation has the potential to disrupt the normal expression of Wnt4/β-catenin and its downstream mTOR proteins in the immortalized myometrial smooth muscle cell line which may be associated with cell growth, proliferation, and tumorigenicity. Moreover, this somatic mutation in MED12 can disrupt normal cellular autophagy by modulating the expressions of AKT/GSK-3β signaling pathway that promotes β-catenin expression leading to the tumorigenic cell phenotype. Together, our findings suggest that the somatic mutation in MED12 has the potential to induce cell-cycle progression of the immortalized myometrial smooth muscle cell line by modulating Wnt4/β-catenin and mTOR signaling pathways and by disrupting the protective cellular autophagy/cell death mechanism. Further understanding of the effects of other common MED12 somatic mutations and the key cellular process that influence Wnt4/β-catenin signaling and autophagy will help to provide new therapeutics for the treatment of human uterine fibroids.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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