MiR-142-3p May Be Involved in the Development of Solitary and Multiple Uterine Leiomyomasby Interacting with CTNNB1 and AXIN-2 Through Wnt Signaling Pathway

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Research article

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Abstract

Background

The pathogenesis and clinical behaviors between solitary uterine leiomyoma (SUL) and multiple uterine leiomyomas (MUL) vary, which lead to the difference in management for childbearing-aged patients. Herein, we aim to find the potential miRNAs involved in the development of SUL and MUL.

Results

The top 5 differentially expressed miRNAs, Wnt signalling pathway and its two central molecules APC and CTNNB1 were screened out according to microarray analysis and bioinformatics. MiR-142-3p was selected for further exploration. In validation of qRT-PCR, MiR-142-3p was significantly upregulated in SUL, while downregulated in MUL, CTNNB1 and sequencing target AXIN-2 were expressed at higher level in MUL than SUL. Overexpression of MiR-142-3p resulted in lower transcription level of CTNNB1 and AXIN-2, and lower cell proliferation level.

Conclusions

MiR-142-3p may be involved in the development of SUL and MUL by interacting with CTNNB1 and AXIN-2 through Wnt signaling pathway. MiR-142-3p could serve as a potential biomarker for individualized treatment between SUL and MUL in the future.

Background

Uterine leiomyoma (UL) is the most prevalent neoplasm in reproductive-age females. UL is associated with excessive menstrual bleeding, impaired fertility, recurrent abortion, and accounts for the most common cause of hysterectomy among gynaecological disorders(1). However, the understanding of UL pathobiology is unclear, thus alternative treatment options are limited. As hysterectomy is invasive and can result in permanent infertility, myomectomy has become one of the only options to preserve fertility. Several studies have reported that recurrence of multiple uterine leiomyomas (MUL) occurs at higher rates than solitary uterine leiomyoma (SUL) (2–4). Moreover, clinical observation and epidemiology showed that SUL tended to grow in a larger diameter and rarely developed into multiple tumours. Cytogenetic studies have suggested recurrent and mutually exclusive mutations exist in UL (5), including the most frequently mutated gene mediator subcomplex 12 (MED12)(6) and a frequently rearranged allele of HMGA2 (7). Moreover, gene polymorphisms studies showed that carriage of higher CYP17A1 G allele frequency was correlated with MUL, while higher CYP17A1 A allele frequency was correlated with a healthy population and SUL (8). Taken together, these suggested the involvement of molecularly distinct pathobiology between SUL and MUL, encouraging further studies to reveal the differentiated molecular processes between SUL and MUL for novel perspective of UL pathobiology.

MiRNAs are negative regulators of gene expression through post-transcriptional gene silencing (9) and they have been found to take part in disease pathogenesis and embryonic development. Few studies have described miRNA dysregulation between UL versus corresponding myometrium, based on microarray analysis (10), which yielded some dysregulated miRNAs. However, the roles of dysregulated miRNAs in UL pathogenesis is still to be elucidated. Here, aiming to explore individualised therapeutic applications and molecular interventions for UL, we investigated miRNA-target networks between SUL and MUL compared to their corresponding myometrium. Taken together with previous studies, we found that β-catenin signalling was significantly differentially regulated between SUL and MUL, as compared to their corresponding myometrium, as a potential result of post-transcriptional negative regulation by miR-142-3p directly targeting β-catenin.

Results

The top five dysregulated miRNAs between SUL and MUL were filtered by microarray analysis

To determine potentially dysregulated microRNAs between SUL and MUL, we firstly performed a miRNA microarray analysis of 12 samples, including 3 pairs of SUL with corresponding MSUL and 3 pairs of MUL with corresponding MMUL (Fig. 1). The top 5 ranked miRNAs: miR-142-3p, miR-146a-5p, miR-146b-5p, miR-136-3p and miR-608(Fig. 1 and Additional file 1) were filtered for further validation in enlarged samples.

miR-142-3p was the most dysregulated miRNAs between SUL and MUL

Secondly, qRT-PCR was used to validate the top five miRNAs in enlarged UL samples (13 pairs of SUL vs. MSUL and 19 pairs of MUL vs. MMUL, total 64 tissues) (Fig. 2). The expressions of miR-142-3p, miR-146b-5p and miR-136-3p were significantly different (Fig. 2a). MiR-142-3p was significantly upregulated in SUL group, while downregulated in MUL group. Moreover, the baseline expression of miR-142-3p in MSUL was significantly lower than MMUL, and the expression level in SUL was significantly higher than MUL (Fig. 2b). Similarly, miR-146b-5p was upregulated in SUL group, while downregulated in MUL group. Varying from miR142-3p, baseline expression of miR-146b-5p in MSUL and MMUL showed no statistical difference (Fig. 2c). MiR-146a-5p, which harbours the identical seed region with miR-146b-5p, was downregulated in both groups, respectively. However, no significant interaction between groups and sites was found between SUL group and MUL group (Fig. 2d). MiR-136-3p was significantly upregulated between SUL group and MUL group, respectively, due to a lower baseline expression in MSUL and similar upregulated levels in SUL and MUL (Fig. 2e). No statistical difference was found in miR-608 (Fig. 2f). Above all, miR-142-3p was the most dysregulated one between SUL and MUL. **Targets and pathway analysis of miR-142-3p**

After microarray analysis and qRT-PCR validation, we focused on researching miR-142-3p including its target genes and pathway analysis. Based on target gene prediction and functional annotation clustering

analysis and a previous miR-142-3p target gene signalling prediction conducted through miRTar by Carraro et al (11), the Wnt signalling pathway was found the first significantly enriched signalling pathway. Interestingly, activation of Wnt signalling was reported to resulted in myometrium cell hyperplasia and development of leiomyoma-like tumours in a Cre-recombinase mouse model (12). Further literature retrieval found that the regulation of canonical Wnt signalling by miR-142-3p was explored in several studies. However, involvement of different target genes showed contrary regulation results. Isobe et al.(13) and Carraro et al.(11) reported that miR-142-3p activated Wnt signalling by targeting APC, a member of the β-catenin (CTNNB1) destruction complex. Hu et al.(14) reported that miR-142-3p inactivated Wnt signalling by directly targeting CTNNB1. Further, Shrestha et al (15) reported that β-catenin protein levels increased, while the CTNNB1 gene was not altered in miR-142 knockout mice. APC and β-catenin were both validated as miR-142-3p targets by luciferase assay (11, 13, 14). Several studies have suggested that the intracellular β-catenin pathway of Wnt signalling is constitutively active in the majority of LM cells (12, 16). Therefore, APC and CTNNB1, two central molecular of Wnt signalling and validated gene targets of miR-142-3p, were selected for further validation.

Contrary to the expression of miR-142-3p, CTNNB1 and AXIN2 were significantly upregulated in MUL

APC and CTNNB1, potential dysregulated target genes of miR-142-3p (Fig. 3a) and central members of the canonical Wnt/β-catenin signalling pathway, and AXIN2, a β-catenin signalling target gene, were further validated in UL samples (10 pairs of SUL and 10 pairs of MUL, a total of 40 tissues included in previous miRNA validation). The results showed that CTNNB1 was upregulated in both SUL group and MUL group, but with a significantly higher FC value of 12.327 in MUL group (Fig. 3a). Furthermore, the baseline expression of CTNNB1 between MSUL and MMUL, and upregulated levels between SUL and MUL, were significantly different (Fig. 3c), consistent with the miR-142-3p expression profile. Conversely, the expression of APC showed no differences in any statistical comparisons (Fig. 3b, e). AXIN2, representing the activated level of β-catenin signalling, was significantly upregulated at 2.303 FC value in MUL group. The detailed expression profile was consistent with CTNNB1 (Fig. 3b, d).

MiR-142-3p overexpression downregulated CTNNB1 and AXIN2 and inhibited cell proliferation in vitro

To further investigate the function of miR-142-3p on β-catenin signalling, we detected the expression changes of the predictive target genes and subsequent downstream genes of β-catenin signalling after overexpressed miR-142-3p in Ishikawa cells (Fig. 4a). The results revealed that CTNNB1 and AXIN-2 were significantly downregulated (Fig. 4c, d), while APC showed no difference (Fig. 4b), consistent with the expression in human leiomyoma samples. Furthermore, the inhibition rate of cell proliferation was lower (Fig. 5).

Discussion

Canonical Wnt/β-catenin signalling pathway has been reported to be involved in UL pathobiology. Selective overexpression of constitutively activated β-catenin in embryonic uterine mesenchyme and in the uterus of adult mice leads to growth of leiomyoma-like tumours, while selective deletion of β-catenin

in embryonic uterine mesenchyme significantly reduces uterine size and replaces normal myometrium smooth muscle cells with adipocytes(12). More recently, Ono and colleagues demonstrated that Wnt/β-catenin signalling is involved in proliferation of leiomyoma stem cells (representing 1% of tumour cells) through Wnt paracrine behaviour. The co-treatment of oestrogen and progesterone could induce expression of Wnt11 and Wnt16 in mature leiomyoma cells. Sequentially, through paracrine effects, Wnt11 and Wnt16 bind to leiomyoma stem cell-surface FZD family receptors (FZD1 and FZD7), leading to activation and nuclear tranSULocation of β-catenin in leiomyoma stem cells with subsequent AXIN2 transcription and stem cell proliferation (Fig. 6). Interestingly, this study validated that AXIN2 transcription was constitutively activate in mature leiomyoma cells, which was not dependent on Wnt secretion. Based on our results, we found that β-catenin signalling was upregulated in UL, and at a notable higher level in MUL vs. MMUL than SUL vs. MSUL. Further, miR-142-3p, the validated negative regulator of β-catenin, was found negatively correlated to β-catenin expression. Moreover, APC, another potential target gene of miR-142-3p, showed no difference in our results, thus was not regulated by miR-142-3p in leiomyomas and myometrium. However, APC as a member of the β-catenin destruction complex might not be involved in β-catenin activation in UL pathobiology. Taken together with previous studies, we propose that β-catenin is initially expressed at high transcriptional level in both SUL and MUL. However, in SUL, the highly expressed β-catenin transcripts are silenced partially by upregulated miR-142-3p, as the biological function of miRNAs is post-transcriptional negative regulation of target genes. Conversely, the lower level of miR-142-3p in MUL than MMUL enhances β-catenin upregulation in MUL, resulting in upregulation of β-catenin in MUL vs. MMUL at a notable higher level than SUL vs. MSUL. Further, MED12 gene, which is reported to regulate canonical Wnt signalling through direct binding to β-catenin,(17) is altered in about 70% of leiomyomas(6) and is correlated to higher possibility of MUL.(18) However, it is unknown whether the mutated MED12 in leiomyoma cells leads to β-catenin signalling alternation. However, the difference between MSUL and MMUL is hardly explained by MED12 alternation in leiomyomas. In addition, Markowski et al. reported that Wnt4b was expressed at higher level in MED12 altered UL cells,(19) leading to the speculation that MED12 mutation in UL is more likely to be involved in paracrine signalling of stem cell activation. Taken together, evidence suggests that β-catenin activation in leiomyomas is regulated at least partially by miR-142-3p negatively regulation during post-transcriptional process. Further studies to elucidate the regulatory mechanism of miR-142-3p targeting CTNNB1 and APC are expected in the future.

Conclusion

In the uterus of MUL patients, it is impossible to thoroughly remove undetected leiomyomas during myomectomy, possibly contributing to recurrence. However, SUL rarely spreads into multiple areas during the course of the disease. More interestingly, differential expression of miRNAs (miR142-3p and miR-136-3p) and β-catenin signalling in the myometrium of SUL and MUL, together with exclusive gene subgroup correlations with SUL and MUL, indicates that MUL is more similar to “myometrial disease” than simple leiomyoma disease. Thus, with the gradually deepening understanding of UL pathobiology, individualised clinical decision of myomectomy or hysterectomy could be guided by evidence of specific disease grading biomarkers, and more importantly, molecular intervention for leiomyoma could become possible.

Methods

Study samples: Tissues of UL and corresponding myometrium were collected from women with symptomatic leiomyomas, who underwent operations at West China Second University Hospital (Chengdu, P.R. China). Written informed consents were obtained from all patients, and the Institutional Review Board of West China Second University Hospital approved the study protocol. During tissue collection, eligible subjects and tissue specimens were defined as: 1) no GnRH-a or oral contraception used before surgery; 2) pathological diagnosis of leiomyoma; 3) no accompanying adenomyosis or endometriosis; 4) minimum number of leiomyomas involved in multiple leiomyomas was 5; and 5) leiomyomas intraoperatively revealed as multiple leiomyomas fused as one were excluded from solitary leiomyoma group. 6) SUL was defined as one leiomyomas identified by both ultrasound and surgery findings.

MiRNA microarray analysis

MiRNA microarray analysis was performed on three pairs of SUL with corresponding myometrium(MSUL), and three pairs of MUL and myometrium(MMUL). The frozen tissues were cryopulverised to fine powder with the BioPulverizer (BioSpe, America). The powdered tissue was homogenised with TRIzol Reagent (Invitrogen) using the Mini-beater-16 (BioSpec), and total RNA was purified using the RNeasy Mini Kit (Qiagen). The quality of the total RNA was verified by spectrophotometry using NanoDropND-1000 (ND-1000, Nanodrop Technologies). The integrity of RNA was evaluated by denaturing agarose gel electrophoresis. Total RNA (1 μg) was labelled with Hy5 fluorescent and Hy3 fluorescent probes using the miRCURY Array Power Labeling kit (Exiqon). The miRCURY LNA Array version 7th generation (Exiqon) was used to hybridise the labelled RNA. Subsequently, the hybridised SULides were scanned using the Axon GenePix 4000B microarray scanner (Axon), and image reading was performed using GenePix pro V6.0 (Axon).

Microarray analysis: With a cut-off fold-change (FC) value of 2, miRNAs expression profiles were yielded. Dysregulated miRNAs were filtered by taking intersection. The validated functional information of each filtered miRNA was conducted using miRTarBase 6.0 and search of published studies in PubMed. Candidate miRNAs were rated in four aspects: 1) The false discovery rate (FDR); 2) expression condition in another group; 3) expression level (normalised signal intensity (NSI)). 4). Validated function potentially related to leiomyoma genesis (Wnt signalling/fibrogenesis/TGF/hormone synthesis or metabolism(20)).

MiRNA gene targets prediction and pathway analysis

Target gene prediction of miRNAs was conducted using intersections of results yielded from four databases (miRWalk 2.0, miRanda, TargetScan, and RNA22). Yielded genes were uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 to perform functional annotation clustering an KEGG PATHWAY. We further searched miRTarBase 7.0 and PubMed for validated functional information of miR-142-3p.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed on 13 pairs of SUL and MSUL and 19 pairs of MUL and MMUL to quantify miRNAs (miR-142-3p, miR-146b-5p, miR-146a-5p, miR-136-3p, and miR-608). Further, 10 pairs of SUL and MSUL and 10 pairs of MUL and MMUL (all included in those tissues used for miRNA quantification) were measured to quantify gene transcripts (CTNNB1, APC, and AXIN2). Total RNA was extracted and verified as before. Reverse transcription (RT) to complementary DNA (cDNA) was conducted using the miScript II RT Kit with miScript HiSpec Buffer (Qiagen) on the GeneAmp® PCR System 9700 (Applied Biosystems, USA). U6 was used as the housekeeping primer for miRNAs detection and ACTB for gene transcripts. The primer sequences were designed and synthesised by Generay Biotech (Generay, PRC) based on the miRNA sequences obtained from the miRBase database. Reactions were carried out in an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) using the miScript SYBR Green PCR Kit (Qiagen). Each tissue was run in independent experiments in triplicate. After PCR amplification, the relative expression of miRNAs and mRNAs was calculated based on the $2^{-\Delta Ct}$ methods.(21) The relative fold-change of miRNAs and mRNAs between SUL vs MSUL and MUL and MMUL was calculated based on the $2^{-\Delta\Delta Ct}$ methods.(22)

Cell culture, transfection of miRNA mimics, signalling detection, and cell proliferation assay

Ishikawa cells (acquired from Key Laboratory of Birth Defects and Related Diseases of Women and Children, Sichuan university, Chengdu, China) were used instead of primary uterine leiomyoma cells due to growth failure. Ishikawa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA) mixed with 10% foetal bovine serum (FBS, Gibco) and 1% penicillin and streptomycin (Gibco). All cells were cultured in a 37°Cincubator containing 5% CO_2. Ishikawa cells were seeded at a density of 10^6/well in a 24-well dish and cultured for 12 h. Following, transfection of miR-142-3p mimics (25 nM and 250 nM) and its negative control (RiboBio, Guangzhou, China) was performed using Lipofectamine 3000 (Invitrogen). Cells were treated with 30M LiCl 12 h after transfection and incubated for another 2 h. Further, the transfection effects were examined by qRT-PCR. The inhibition rate of cell proliferation was measured by CCK8 (Jikai Gene, China) at 12, 24, 36, and 48 h after transfection according to the manufacturer's instructions. Optical density (OD) was measured by Varioskan Flash (Thermo Scientific) at a wavelength of 450 nm.

Statistical analysis

Quantitative data of each miRNA and mRNA were recorded as mean ± S.E.M. A two-way ANOVA with repeated measures was used for integral data analysis as experimental group was one factor (two groups based on number of leiomyomas) and site was a repeated measure (paired leiomyoma with corresponding myometrium). Two-tailed paired Student's t-tests were performed for RNA dysregulated level between paired leiomyomas and myometrium. Two-tailed independent Student's t-tests were performed between SUL and MUL, MSUL and MMUL. Detailed statistics are shown in Supplementary file

1–2. SPSS 23.0 (SPSS) was used for analyses. GraphPad Prism 6.0 (GraphPad Software, Inc) was used for figure drawing. Statistical significance was defined as $P < 0.05$.

alysis. Signalling pathway enrichment was carried out based on

Abbreviations

UL
uterine leiomyoma
SUL
solitary leiomyoma
MUL
multiple leiomyomas
FC
fold-change
MSUL
myometrium of solitary leiomyomas
MMUL
myometrium of multiple leiomyomas

Declarations

Ethics approval and consent to participate

Written informed consents were obtained from all patients, and the study protocol was approved by Institutional Review Board of West China Second University Hospital.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

L.X. contributed to the conception and design of the study, sample collection and preparation, interpretation of data, manuscript drafting and final approval. E.Y. contributed to the sample collection and preparation and manuscript drafting. J.G. contributed to figures design and data analysis. D.N. contributed to sample collection and data analysis. T.Y. contributed to experiment guidance. W.M. contributed to manuscript editing and language polish. Z.L. contributed to conception of the study and design, manuscript revising and critical discussion.

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Figures

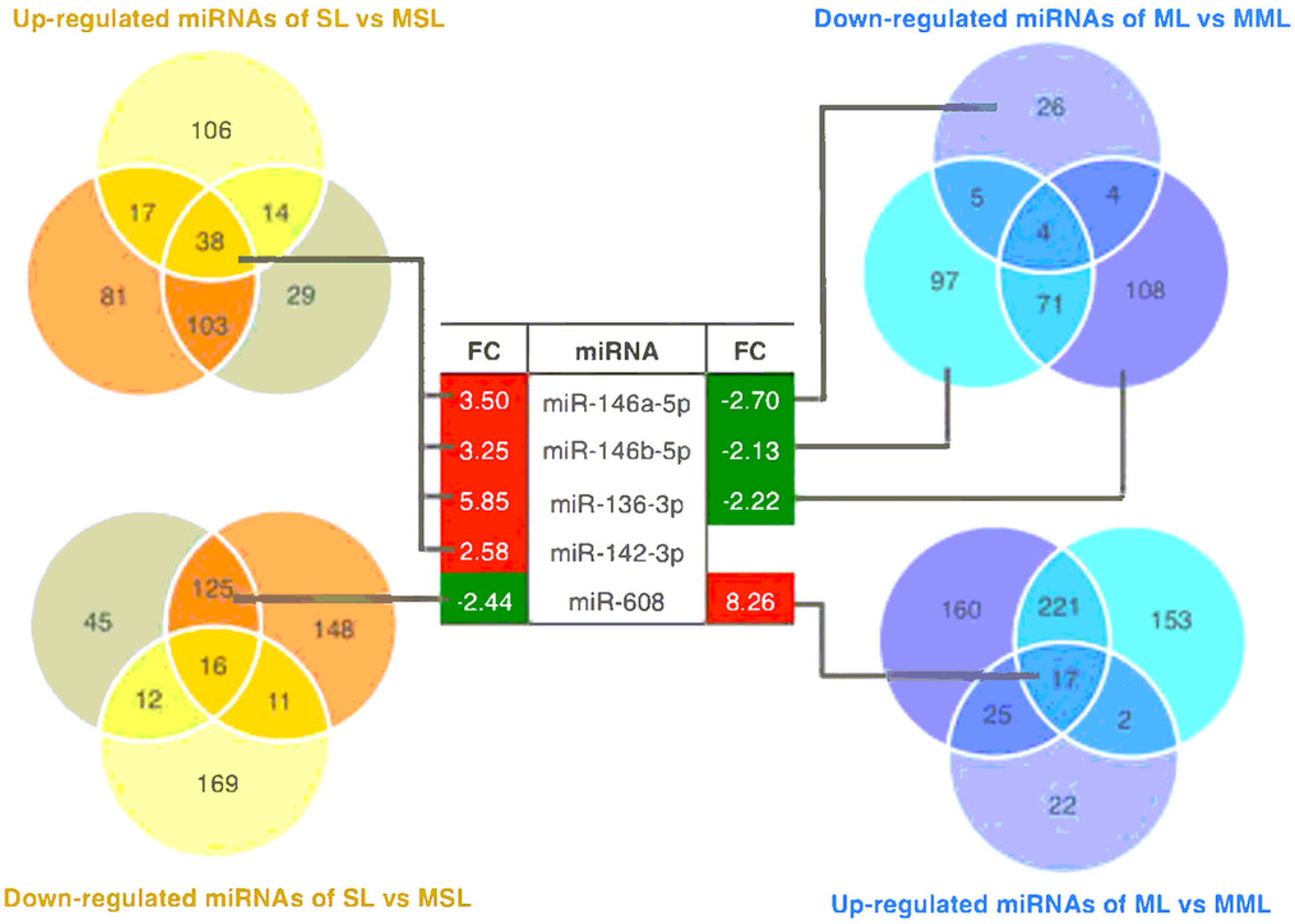

Figure 1

Potentially dysregulated miRNAs between SUL and MUL in microarray analysis. SUL: solitary leiomyoma, MUL: multiple leiomyoma.

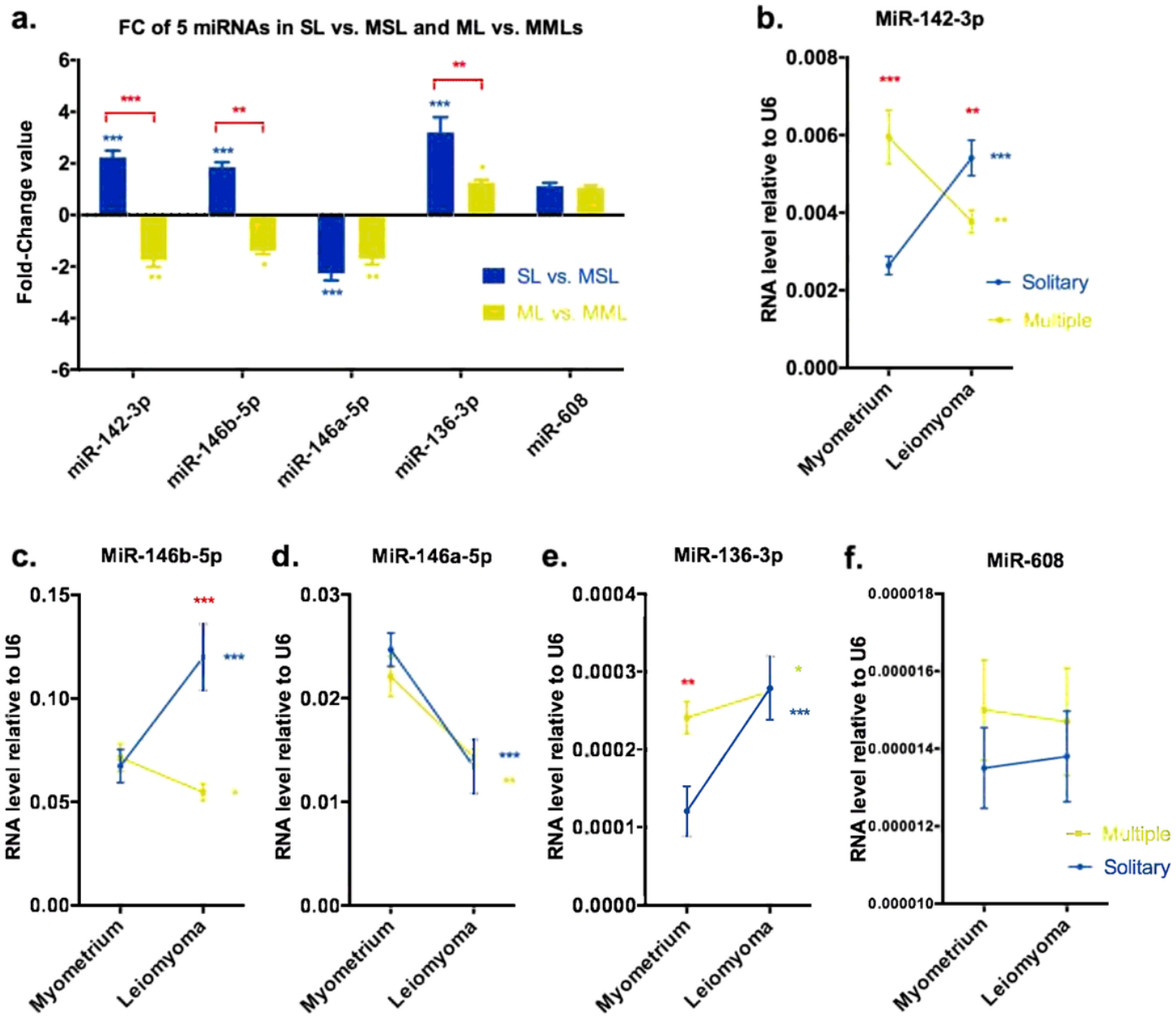

Figure 2

Dysregulated miRNAs between SUL and MUL in qRT-PCR validation. a, Expressions of 5 miRNAs in 13 pairs of SUL and 19 pairs of MUL samples; FC: Fold-change value = mean ± standard error, 2-ΔΔCt methods. b,c,d,e,f: The relative expressions of miR-142-3p, miR-146b-5p, miR-146a-5p, miR-136-3p and miR-608 in SUL, MSUL, MUL, and MMUL. * P<0.05, ** P<0.005, *** P<0.001.

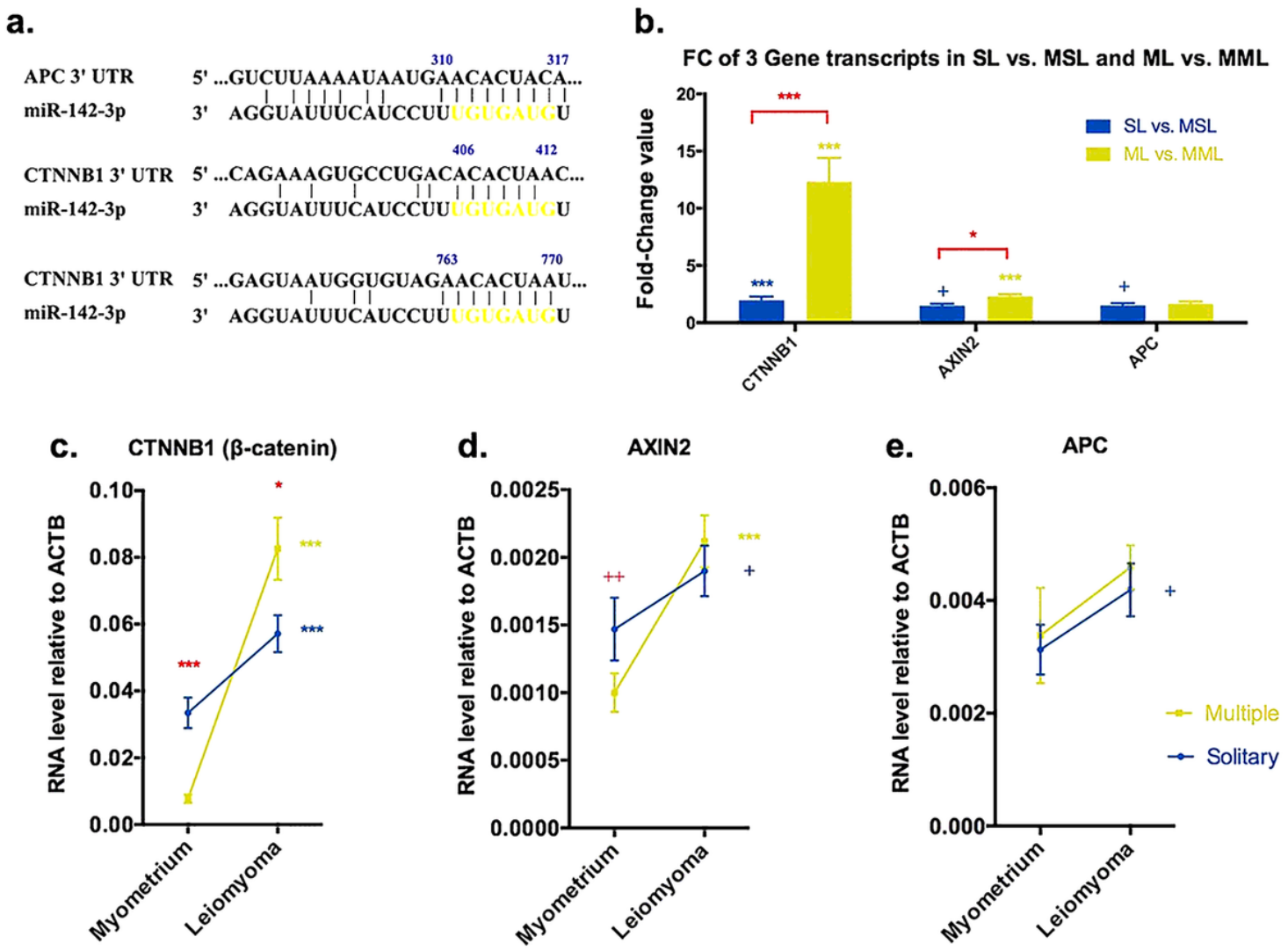

Figure 3

Target genes of miR-142-3p involved in β-catenin signaling between paired SUL and paired MUL samples. a. The binding sites between miR-142-3p and its target genes validated by dual-luciferase reporter assay (seed region of miR-142-3p showed in yellow). b. Regulation profile of 3 mRNAs in β-catenin signaling (CTNNB1 (β-catenin) and APC as gene targets of miR-142-3p, AXIN2 as gene targets of β-catenin signaling) in 10 pairs of SUL and 10 pairs of MUL samples. CTNNB1: FCSUL vs. MSUL = 1.952 ± 0.333, FCMUL vs. MMUL = 12.327 ± 2.077; AXIN2: FCSUL vs. MSUL = 1.497 ± 0.161, FCMUL vs. MMUL = 2.303 ± 0.188; APC: not significant; (Fold-change value (FC) = mean ± standard error, 2-ΔΔCt methods). c, d, e: Expressions of 3 mRNAs in SUL, MSUL, MUL, MMUL. * P<0.05, ** P<0.005, *** P<0.001, + P<0.1, ++ P<0.11.

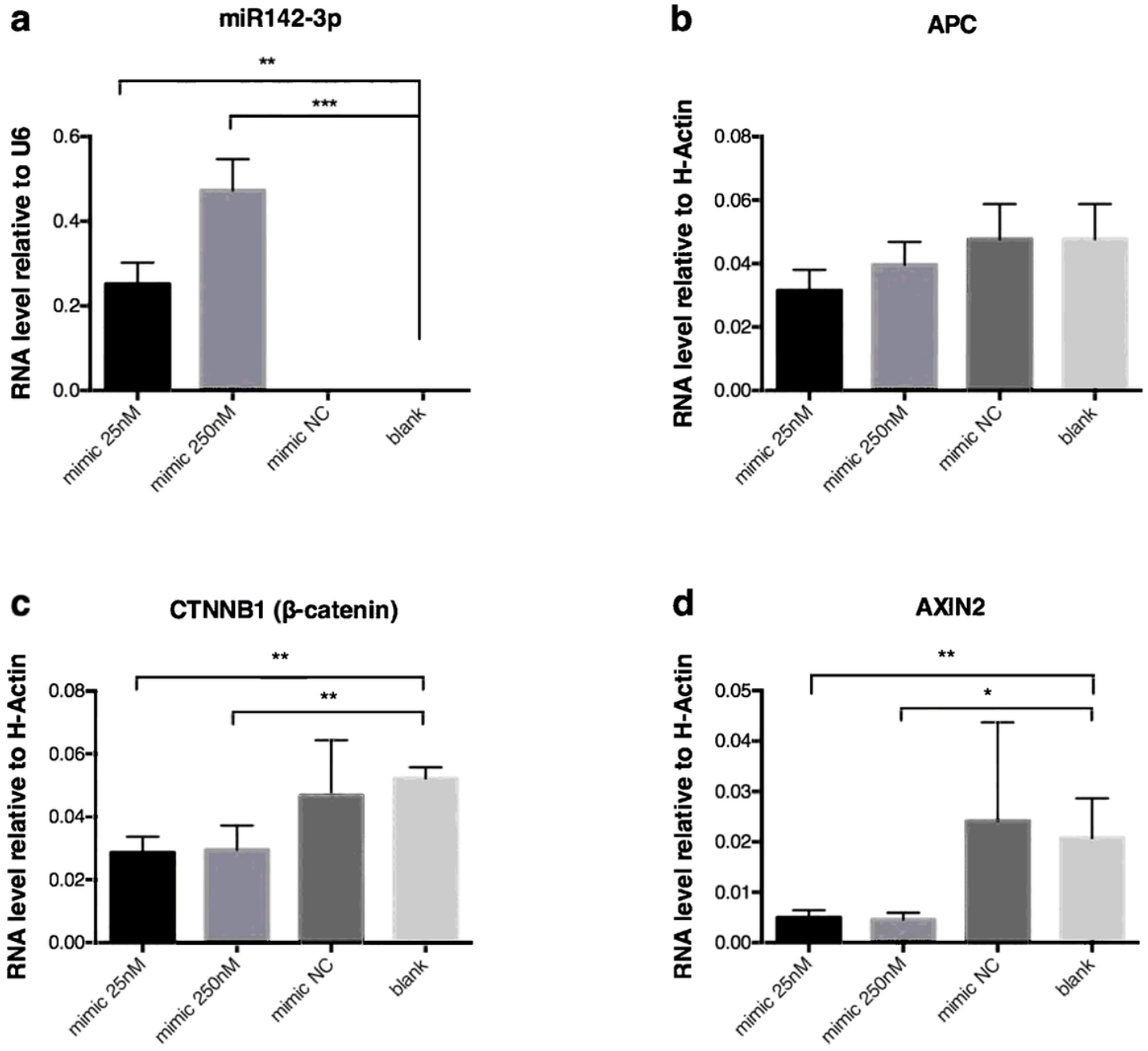

Figure 4

Over-expression of miR-142-3p resulted in CTNNB1(β-catenin) and β-catenin signaling down-regulation in vitro. Transfection of miR-142-3p mimics (25nM or 250nM) and its negative control (NC) was carried out in Ishikawa cells, then β-catenin signaling was activated by treating with 30mM LiCl, and the transfection effects of miR-142-3p mimics and β-catenin signaling expression were examined by qRT-PCR. a, MiR-142-3p was validated to be over-expressed after mimic transcription. b, APC mRNA level had no significant differences after over-expression of miR-142-3p. c, CTNNB1 mRNA level was significantly lower after over-expression of miR-142-3p. d, AXIN-2 mRNA level, the down-stream gene of β-catenin signaling, was significantly lower after over-expression of miR-142-3p. Unpaired t test. * P<0.05, ** P<0.005, *** P<0.001.

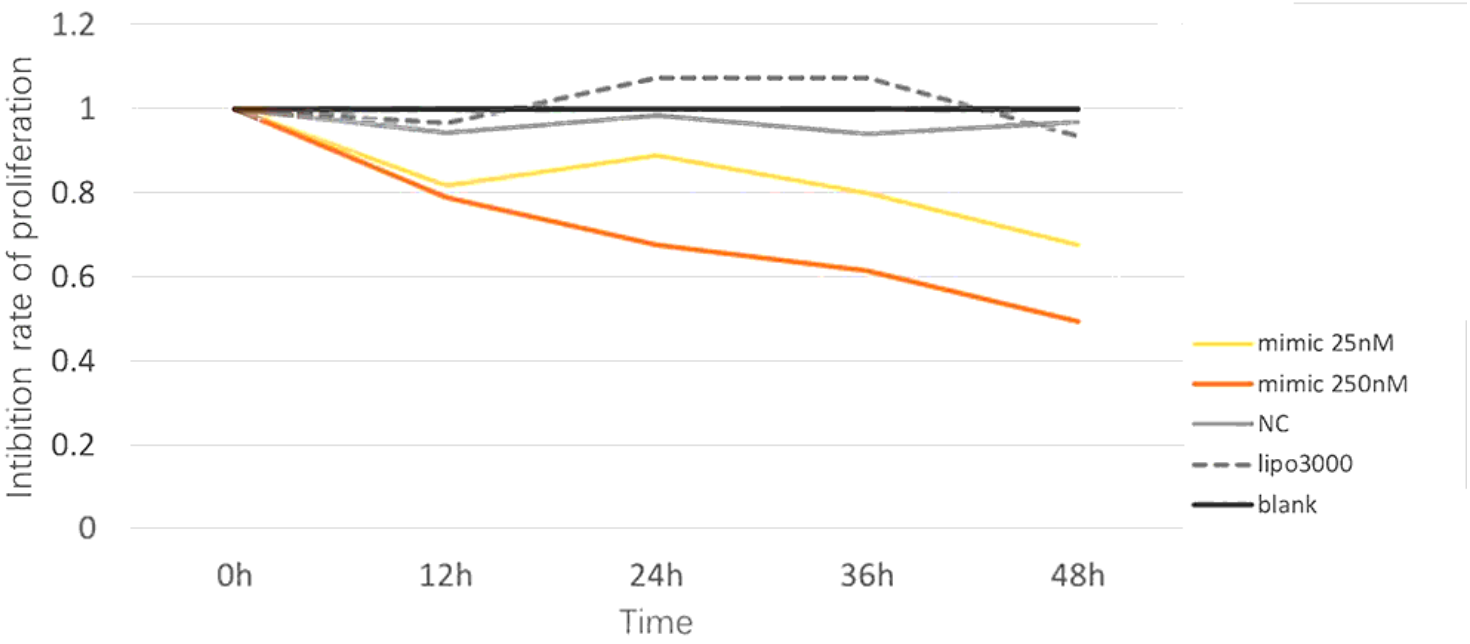

Figure 5

MiR-142-3p over-expression resulted in inhibition of cell proliferation in vitro. The inhibiton rate of cell proliferation was measured by at 12h, 24h, 36h and 48h after transfection of miR-142-3p mimics.

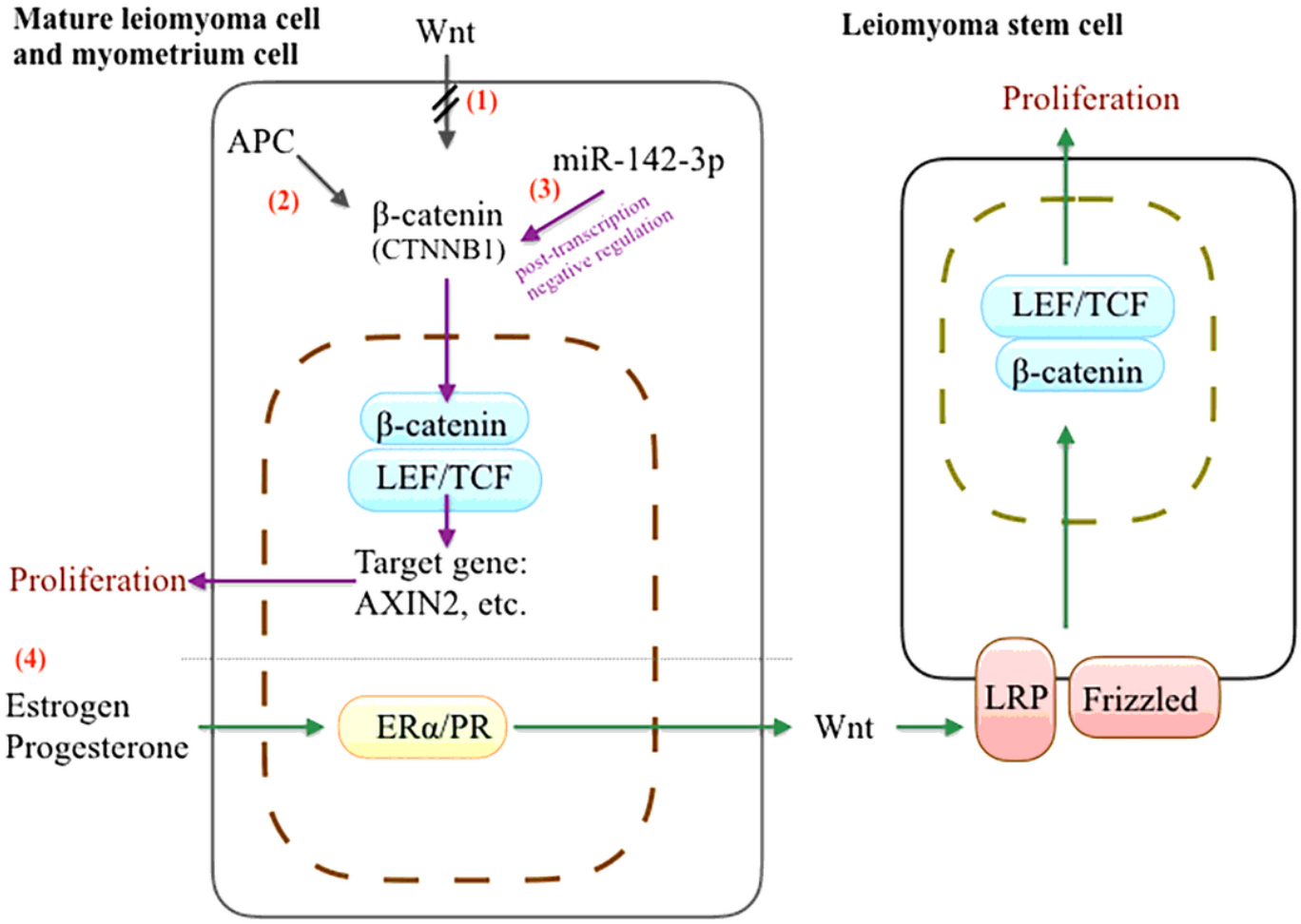

Figure 6

Wnt/β-catenin signaling in UL pathogenesis: (1) β-catenin activity is independent ofWnt expression in mature leiomyoma cells and myometrial cells, demonstrated by Ono and colleagues. (2) APC, a critical member of β-catenin destruction complex and potential targets of miR142-3p, showed no difference in any comparison, thus it could be told that APC was not targeted by miR-142-3p and was not involved in β-catenin activation in UL pathobiology.(3) Potential post-transcriptional negative regulation by miR-142-3p directly targeting β-catenin.(4) Wnt/β-catenin signaling is involved in proliferation of leiomyoma stem cells in aWnt paracrine behavior, raised and demonstrated by Ono and colleagues.

Supplementary Files

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