

# Cullin-5, a ubiquitin ligase scaffold protein, is significantly underexpressed in endometrial adenocarcinomas and is a target of miR-182

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Received October 21, 2015; Accepted November 17, 2015

DOI: 10.3892/or.2016.4605

**Abstract.** Altered expression of cullin-5 (CUL5), a member of the cullin-RING E3 ubiquitin ligase family, has been implicated in a number of types of cancers including breast, cervical and hepatocellular cancers. In the present study, we found that CUL5 expression was significantly decreased in both endometrioid and serous endometrial adenocarcinomas with the more aggressive serous type displaying a higher reduction (-4.3-fold) than the less aggressive endometrioid type (-2.9-fold). Overexpression of CUL5 mRNA and protein in Ishikawa H endometrial cancer cells resulted in decreased cell proliferation and in a reduction in CUL5-RING E3 ligase downstream clients JAK2 and FAS-L. Finally, we demonstrated for the first time that CUL5 is a direct target of miR-182 that we previously showed to be significantly overexpressed in endometrial adenocarcinomas and we provided evidence that increased miR-182 expression is, at least in part, a result of demethylation of its upstream promoter. These data suggest a cascade in which miR-182 expression is epigenetically increased leading to decreased CUL5 expression and increased cellular proliferation. The final step in the cascade may be operating through a decrease in ubiquitination of pro-growth CUL5 ubiquitin ligase clients. This cascade offers a series of potential interventional steps involving epigenetic modification, miRNA and/or gene targeting and ubiquitination.

## Introduction

Cullin-5 (CUL5) is a member of the cullin-RING E3 ubiquitin ligase (CRL) family. CUL5 has been shown to be involved

in numerous important cellular processes including the cell cycle and proliferation (1). Recognition of the role of CUL5 in cancer cell growth and invasiveness began with the demonstration that overexpression of CUL5 in T47D breast cancer cells led to significant suppression of proliferation (2). Subsequent studies involving CUL5 expression in breast cancer as well as in cervical and hepatocellular cancer have confirmed the antiproliferative effect of increased CUL5 expression (3-5).

Endometrial adenocarcinoma is the most common gynecologic cancer and one of the most common cancers in women worldwide (6). The American Cancer Society estimates that there will be nearly 55,000 new cases of endometrial cancer in the US alone in 2015 with more than 10,000 deaths. Indeed, while patient outcomes for most cancers have improved over the past two decades, overall survival among women diagnosed with endometrial cancer has worsened (7). Thus, more effective therapeutic intervention in endometrial cancer is needed.

More than 95% of endometrial cancers are either the less aggressive type I, or endometrioid adenocarcinomas (~80%), or the more aggressive type II, or serous, adenocarcinomas (~10-15%). Using a screening panel composed of primary endometrial endometrioid and endometrial serous tumors we found that CUL5 was significantly underexpressed, -2.92-fold ( $p < 0.001$ ) in the former and -4.33-fold ( $p < 0.001$ ) in the latter, compared with benign endometrium. We also found that overexpression of CUL5 in Ishikawa H endometrial cancer cells significantly slowed growth. Furthermore, we showed that microRNA miR-182, which is significantly overexpressed in this cancer (8) and which is associated with poor outcome in colorectal adenocarcinoma (9), targets CUL5 in both Ishikawa H and Hec50co endometrial cancer cells. Finally, overexpression of wild-type CUL5 in Ishikawa H cells led to diminished cell growth compared with untransfected cells and lower expression of potential downstream client proteins such as JAK2 and FAS-L.

Taken together, miR-182, CUL5 and downstream clients including JAK2 and FAS-L represent a potentially important and useful set of new therapeutic targets in endometrial cancers.

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*Key words:* cullin-5, miR-182, methylation, ubiquitination

## Materials and methods

**Tumor tissue procurement.** Primary tumor tissues from 18 primary endometrial endometrioid adenocarcinomas and 16 primary endometrial serous adenocarcinomas along with 6 benign endometrium tissues were obtained under informed consent through the Gynecologic Malignancies Tissue Repository, housed in the University of Iowa Hospitals and Clinics, Department of Obstetrics and Gynecology (IRB#200209010). All tissues are flash frozen specimens for which a clear histologic diagnosis has been assigned.

**Cell culture.** Endometrial cancer cells Ishikawa H and Hec50co were grown and maintained under optimal conditions of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin. These cells were chosen since Ishikawa H cells are a model for endometrial endometrioid adenocarcinoma (ER<sup>+</sup>, PR<sup>+</sup>, p53 wt, PTEN mut) and Hec50co cells are a model for endometrial serous adenocarcinoma (ER<sup>-</sup>, PR<sup>-</sup>, p53 mut and PTEN wt) (10).

For the purpose of examining the effects of CUL5 overexpression, we chose the type I cancer cell line Ishikawa H which represents the great majority of endometrial cancers. A CUL5 expression vector, CMV6-CUL5 (OriGene), was purchased and prepared for transfection. A stable and reliable CUL5-overexpressing Ishikawa H subline, CUL5wt35, was produced by selection on G418 (Geneticin), and the identity of the integrated plasmid was verified by direct sequencing of cellular gDNA.

In order to assess CUL5 targeting by miR-182, both Ishikawa H and Hec50co cells were transiently transfected with a miR-182 mimic (Life Technologies). Transfections were carried out three separate times and total cellular RNA was purified from untransfected, mock-transfected and transfected cells from each replicate.

Expression of CUL5 protein as well as other relevant proteins was assessed via standard western blotting hybridizations. Anti-CUL5 antibody was purchased from Abcam (ab97280), antibodies for JAK2 and FAS-L were purchased from Cell Signaling (#3230 and #4273, respectively) and the anti- $\beta$ -actin loading control was purchased from Sigma (A1978).

**RNA purification and qPCR assays.** Whether from primary tissues or cultured cells, total cellular RNA was purified using the mirVana miRNA Isolation kit following the manufacturer's recommendations (Life Technologies). RNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

Quantitative PCR CUL5 expression assays were performed on both primary tissue RNAs, and cultured cells following SuperScript III (Invitrogen) reverse transcription of fixed total RNA mass (200 ng for tissues and 100 ng for cells). Resulting cDNAs were amplified using primer sets: CUL5for, 5'-CTCAAGTCAACTCACCAAGA-3' and CUL5rev, 5'-GCTGCAACGTCCAATCAAG-3'; and 18S rRNAfor, 5'-AACTTTCGATGGTAGTCGCCG-3' and 18S rRNArev, 5'-CCTTGGATGTGGTAGCCGTTT-3'. Amplicons were detected in a Power SYBR-Green qPCR assay carried out on an Applied Biosystems 7900HT Real-Time PCR System in the Genomics Division of the Iowa Institute of Human Genetics (IIHG).

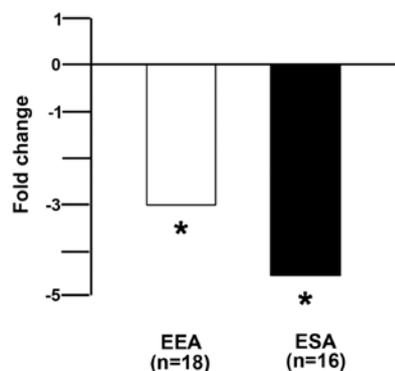


Figure 1. CUL5 mRNA expression in endometrial endometrioid adenocarcinomas (EEA) and endometrial serous adenocarcinomas (ESA) relative to benign endometrium (BE). In both cases underexpression in the tumors was statistically significant. \* $p < 0.001$  EEA or ESA vs. BE.

Quantitative PCR miR-182 expression assays were performed on both primary tissue RNAs and cultured cells following MultiScribe MuLV reverse transcription (TaqMan MicroRNA Reverse Transcription kit) of fixed total RNA mass (200 ng for tissues and 100 ng for cells) in the presence of miRNA-specific RT primers (both from Life Technologies). Resulting cDNAs were amplified using miRNA-specific TaqMan primers (Life Technologies) also on an Applied Biosystems 7900HT Real-Time PCR system in the Genomics Division of the IIHG.

Raw cycle threshold (Ct) values for both CUL5 and miR-182 were normalized ( $\Delta$ Ct) against standard normalizers; 18S rRNA for CUL5 and RNU48 for miR-182. Fold-change differences for all analyses were calculated as  $2^{-\Delta\Delta Ct}$  where  $\Delta\Delta Ct = (\text{Mean } \Delta Ct_{\text{experiment}} - \text{Mean } \Delta Ct_{\text{control}})$  (11,12). Statistical significance of fold-changes was assessed via a standard t-test with unequal variances (13).

**Cell proliferation assay.** Cell proliferation was determined by measuring (<sup>3</sup>H)-thymidine incorporation into Ishikawa H cells mock-transfected or overexpressing Cul5 (CUL5wt35) as previously described (14).

**Methylation-specific PCR.** In order to assess the methylation state of the miR-182 promoter, bisulfite conversion of genomic DNA from five endometrial cancer cell lines (Ishikawa H, Hec50co, ECC-1, RL95-2 and KLE) was carried out using the EZ DNA Methylation-Direct kit (Zymo Research) according to the manufacturer's recommendations. Methylation-specific PCR primer sets for the miR-182 promoter were designed using the MethPrimer package (15). A well-documented hypermethylated miRNA promoter (miR-181c) was chosen as a positive control (16). Previously validated methylation-specific PCR primers for miR-181c were used following MethPrimer *in silico* validation. PCR reactions were carried out using ZymoTaq™ PreMix (Zymo Research).

## Results

**CUL5 is significantly underexpressed in endometrial cancers.** Our primary tumor screening panel was composed of 18 endometrial endometrioid adenocarcinomas, 16 endometrial

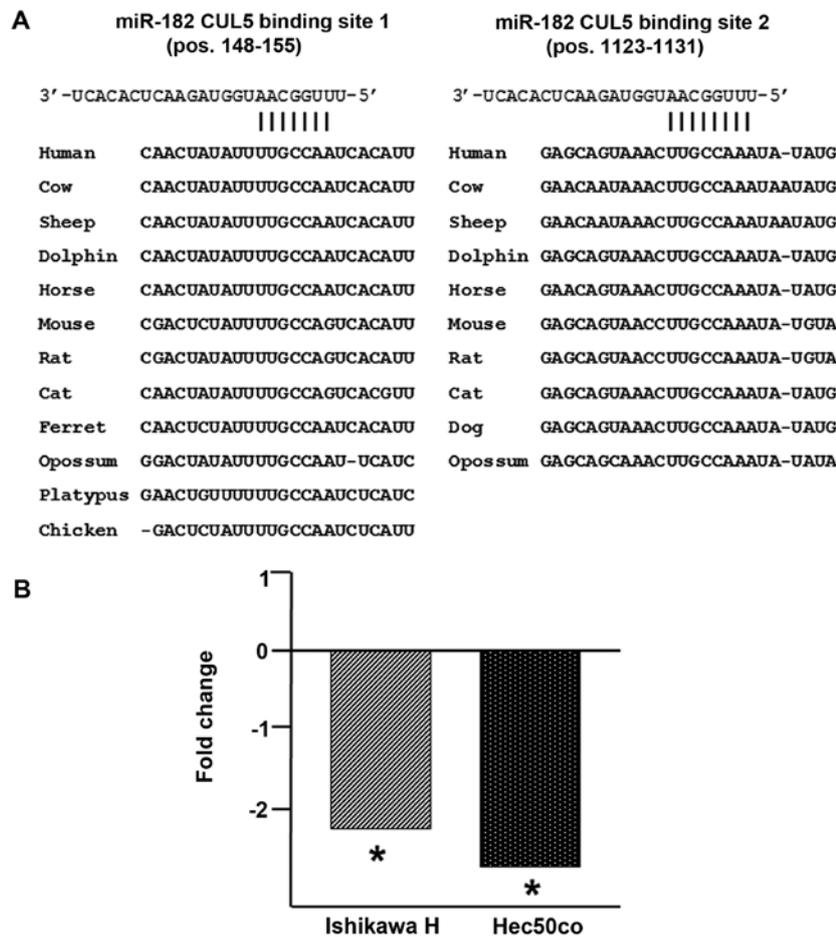


Figure 2. miR-182 targets CUL5 in endometrial cancer cells. (A) Evolutionary conservation of the miR-182 binding sites in the 3'-UTR of CUL5. Position 1 conservation covers >350,000,000 years while position 2 conservation covers 180,000,000 years. (B) Relative CUL5 mRNA in Ishikawa H and Hec50co endometrial cancer cells transiently transfected with a miR-182 mimic compared with mock-transfected Ishikawa H and Hec50co cells; \*p<0.01.

serous adenocarcinomas and 6 benign endometrium. The CUL5 qPCR assay results presented in Fig. 1 showed that both endometrial endometrioid and endometrial serous adenocarcinomas displayed significant underexpression (-2.9-fold, p<0.01; and -4.3-fold, p<0.01, respectively) compared with the benign endometrium.

**miR-182 targets CUL5.** Two prior cancer studies have shown that CUL5 is a validated target of microRNAs miR-19a/b and miR-7, the former in cervical and the latter in hepatocellular cancer (4,5). Our previous study of miRNA expression in endometrial cancers showed that, while both miR-19a/b and miR-7 display increased expression relative to benign endometrium, neither miRNA achieves statistical significance (8). In addition, both TargetScan 5.2 and PicTar gave high confidence predictions that CUL5 would be a target of miR-182. This prediction was supported by an evolutionarily deep conservation of two miR-182 binding sites in the CUL5 3'-UTR (Fig. 2A). Furthermore, our previous study (8) showed that miR-182 was significantly overexpressed in both endometrioid and endometrial serous adenocarcinomas compared with benign endometrium (27.2-fold, p<0.01; and 11.5-fold, p<0.01, respectively) which is consistent with the significant underexpression of CUL5 in these cancers. We transiently transfected miR-182 into the type I and II endo-

metrial cell lines Ishikawa H and Hec50co (10) and observed that CUL5 was significantly underexpressed (-2.2-fold, p<0.01; and -2.6-fold, p<0.01, respectively) in these cells when compared with the mock-transfected cells (Fig. 2B). Thus, at least in these two cancers, CUL5 is a target of miR-182 such that CUL5 downregulation is achieved, in part, through modulation of miR-182 expression.

**Overexpression of CUL5 inhibits cell growth.** In order to assess the effect of CUL5 on endometrial cancer cell behavior, we created a stable CUL5-overexpressing Ishikawa H subcell line, CUL5wt35, using a CMV6 entry vector containing the CUL5 coding region. Cells were selected on G418. We measured cell proliferation with a tritiated (<sup>3</sup>H) thymidine uptake assay. Results, as presented in Fig. 3A, showed that overexpression of CUL5 significantly reduced proliferation compared with the untransformed Ishikawa H cells.

**Overexpression of CUL5 affects known downstream 'client' proteins.** In order to confirm that both CUL5 mRNA and protein are overexpressed in CUL5wt35 cells, mRNA levels in the CUL5wt35 and mock-transfected Ishikawa H cells were assayed by SYBR-Green qPCR and CUL5 protein levels were assessed via western blotting. The SYBR-Green CUL5 mRNA expression assay showed that there was a 32-fold increase in

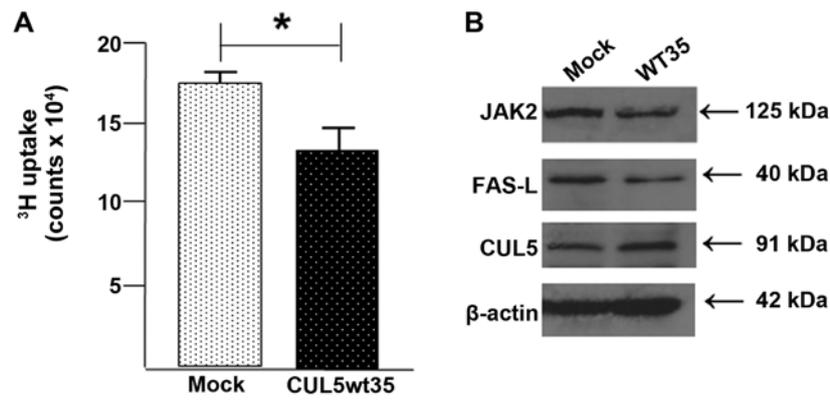


Figure 3. The effect of CUL5 protein overexpression on cell proliferation and on other proteins. (A) A  $^3\text{H}$ -thymidine uptake assay comparing mock-transfected Ishikawa H cells with the stable CUL5-overexpressing Ishikawa H subcell line CUL5wt35. (B) Western blot analyses of CUL5 and possible client proteins in the mock-transfected Ishikawa H cells and the stable CUL5-overexpressing Ishikawa H subcell line CUL5wt35; \* $p < 0.05$ .

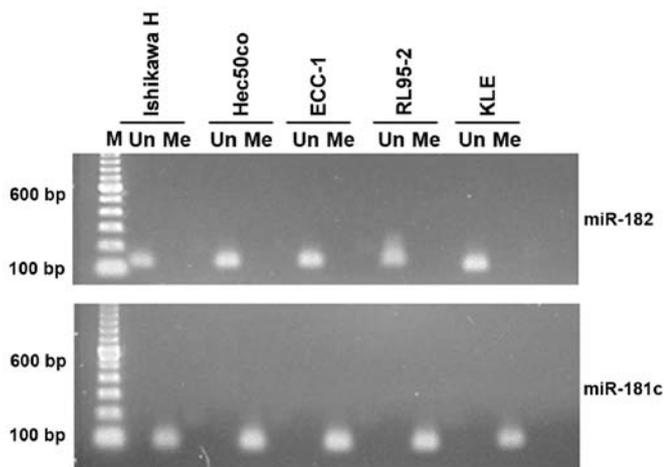


Figure 4. Methylation-specific PCR (MSP) of the miR-182 promoter in five endometrial cancer (EC) cell lines. MSP of the miR-182 promoter (top) showed that all five EC cell lines lacked methylation in the promoter while all five cell lines were methylated in the control miR-181c promoter (bottom). In both amplifications MSP primers were designed (miR-182) or validated (miR-181c) (16,24) using MethPrimer (15). Un, unmethylated; Me, methylated; M, 100-bp ladder (TrackIt<sup>®</sup>; Thermo Fisher).

CUL5 mRNA in the CUL5wt35 cells as compared with the mock-transfected cells (data not shown). CUL5 protein expression was also increased in the CUL5wt35 cells compared with the mock-transfected cells although the increase was more modest (Fig. 3B). Also shown in Fig. 3B are expression levels of three proteins proposed to be affected by CUL5 expression.

## Discussion

The cullin-RING ligase (CRL) ubiquitin family, first reported in the late 1990s (1), has taken a central position in regulatory biology. One of seven members of the human cullin gene family, the ubiquitin ligase scaffold protein cullin-5 (CUL5) has been shown to be an important element in cardiovascular biology (17) and has been implicated in several types of cancers including breast cancer (2,3). We examined CUL5 expression in two histologic forms of endometrial (uterine) cancers; the less aggressive and better prognosis endometrioid

adenocarcinoma and the more aggressive, poorer prognosis serous adenocarcinoma. We reported in the present study, that CUL5 expression was significantly less in both histologic types and that the loss of CUL5 expression was greater in the serous histologic type than that in the endometrioid histologic type. This finding suggests that an increased loss of CUL5 expression in endometrial cancers could be a marker for prognosis. Such a connection will obviously require study of more endometrial tumors than the 34 tumors we had available for the present study but it is a question worth pursuing.

In addition to the important mechanistic studies of CUL5 that have been reported from the laboratory of Dr Burnatowska-Hledin since their initial discovery of CUL5 in 2000 (18-20), two other studies have demonstrated a regulatory role for two miRNAs, one, for miR-19a and miR-19b, in cervical cancer and another, for miR-7, in hepatocellular cancer (4,5). We searched miRNA target prediction algorithms for CUL5 and discovered that several, including TargetScan and PicTar, indicated that miR-182 was an even higher confidence prediction than either of the others. We confirmed that there are two highly conserved miR-182 binding sites in the 3'-UTR of the CUL5 gene and demonstrated in the present study via quantitative PCR assay that miR-182 was overexpressed in the two model endometrial cancer cell lines Ishikawa H and Hec50co (10) and that CUL5 is indeed a target of this miRNA at least in endometrial cancers. This conclusion is supported by our previously reported finding that miR-182 is significantly overexpressed in endometrial adenocarcinomas (8). In addition, it has been demonstrated in colorectal adenocarcinomas that high miR-182 expression is a reliable predictor of a poor prognosis (9,21), and possibly in prostate cancer as well (22). Notably, the opposite relationship has been implied in cervical cancers (23).

Given the proliferative effects of low CUL5 expression reported elsewhere (4,19), we overexpressed CUL5 in the Ishikawa H endometrial cancer cell line by creating a stable subcell line, CUL5wt35. We validated the increased mRNA and protein levels in the CUL5wt35 cells relative to the mock-transfected Ishikawa H cells and showed that CUL5 overexpression significantly slowed cell proliferation by measuring  $^3\text{H}$ -thymidine uptake. This confirmed that CUL5 downregulation, achieved at least in part by miR-182 upregulation, is a

circumstance favorable to endometrial cancer proliferation. As to the mechanism of miR-182 downregulation, it has been shown in highly metastatic C8161.9 and WM266-4 melanoma cells that demethylation using 5-aza-2'-deoxycytidine (DAC) and trichostatin A resulted in significant miR-182 expression increases (24). We examined the methylation status of the miR-182 promoter in 5 endometrial cancer cell lines (Ishikawa H, Hec50co, ECC-1, RL95-2 and KLE) by methylation-specific PCR. All 5 cell lines indicate that the miR-182 promoter is in fact unmethylated (Fig. 4). While this mechanism obviously requires additional exploration, it does suggest that epigenetic modulation to lower miR-182 expression could be an avenue through which to promote higher CUL5 presence in endometrial cancers.

Finally, we observed that overexpression of CUL5 was coupled with modestly lowered expression of two potential CUL5 ubiquitin ligase client proteins, JAK2 and FAS-L. The exact relationships among these and other possible client proteins are as yet undetermined but, clearly, loss of CUL5 ubiquitin ligase would be beneficial in that pro-growth clients would escape targeting. Several studies have concluded that dysregulation of the ubiquitination process could be carcinogenic (25). Although CUL5-RING-ligases may not have a large number of clients, the fact that JAK2 is a client does implicate the highly pro-growth JAK-STAT pathway (26) in the cascade that starts with miR-182 promoter demethylation and ends with highly proliferative endometrial cancer cells. These results present a series of potential interventional modalities that should be explored, particularly in more aggressive endometrial cancers.

### Acknowledgements

The present study was supported in part by NIH Grant RO1CA99908 to K.-K.L., NIH Grant K12HD000849 to H.-D.R., the University of Iowa Carver, College of Medicine, Department of Obstetrics and Gynecology Research Development Fund, and DOD Collaborative Undergraduate HBCU Student Summer training program Awards W81XWH-12-1-0117 and W81XWH-13-1-0178 to B.L. We acknowledge the Department of Obstetrics and Gynecology, Women's Health Tissue Repository and Gynecologic Malignancy Repository (Dr Donna Santillan, Director) and the continued invaluable assistance of the University of Iowa Institute of Human Genetics, Genomics Facility (in particular Garry Hauser and Mary Boes).

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