REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad.

1.	Name and designation of ICMR- IF	: **Dr. Rajesh Kumar Jha** Principal Scientist
2.	Address	: Endocrinology Division, CSIR-Central Drug Research Institute, Sector-10, Jankipuram Extension, Lucknow 226031.
3.	Frontline area of research in which training/ research was carried out	: Female reproductive biology
4.	Name & address of Professor and host institute	: Dr. Ramakrishna Kommagani Associate Professor Dept. of Pathology and Immunology Dept. of Molecular Virology and Microbiology Alkek Center for Metagenomics and Microbiome Research T228A, One Baylor Plaza Baylor College of Medicine Houston, TX, 77030 Email: Rama.Kommagani@bcm.edu
5.	Duration of fellowship with exact date	: March 30th 2023- March 25th, 2024 (Excluding the travel time, etc.)
6.	Highlights of work conducted	:
	i) Technique/expertise acquired	: Please refer the attached Annexure-I.
	ii) Research results, including any papers, prepared/submitted for publication	: Please refer the attached Annexure-II.
	iii) Proposed utilization of the experience in India	: Please refer the attached Annexure-III

Signature of ICMR-IF

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ICMR Sanction No. INDO/FRC/452/(Y-42)/2022-23-IH and HRD

Annexure- I

6.i. Highlights of work conducted

During the training period there was mandatory course work for the proposed research project's execution at the bench and >80% marks were essential to pass.

A. Baylor college of Medicine mandatory eight course works for research conduct.

The below course work were undertaken at the Baylor College of Medicine.

1. Bloodborne pathogens
2. BCM Social Media Policy
3. Employee Relation Orientation
4. Building Supportive Communities: Clery Act and Title IC (Calfornia)
5. Confidentiality Orientation
6. Annual Compliance Education-Lerner
7. Mandatory Reporter Training
8. Hazard Communication and Fire Safety
9. Security Orientation
10. OSSA COVID19 Healthcare Emergency Temporary Standard (ETS)
11. Worker's Compensation Orientation
12. J-1 Nonclinical Briefing
13. Laboratory Biosafety Training
14. Lab Safety Training
15. Security Orientation
16. SGA BCM General Compliance (HIPPA)

B. The technique acquaints with during the fellowship period

i) Technique/expertise acquired:

During the fellowship period, I have been exposed to various techniques mentioned below.

Assay/technique in-hand experience. I have performed the *in vitro* decidualization using the human origin endometrial stromal cells. During the *in vitro* decidualization assay, I have been doing the human endometrial epithelial cell culture, propagation, freezing, etc. Further, I have also achieved >80% transient siRNA-based knockdown efficiency, which is a very crucial factor for any *in vitro* gene function analysis. Herein, I have performed the QPCR-based on the TaqMan chemistry; hence, the TaqMan chemistry-based QPCR probed experience is obtained. Further, the metabolic flux was studied during the *in vitro*

decidualization, serve a basic technique to assess the cellular metabolic response during the PCOS, endometriosis and miscarriages.

Exposure to various assays/techniques. Since I have worked on autophagy-associated genes; hence, my understanding of autophagy genes has widened. The basic understanding of uterine tissue-specific gene ablation is understood. Moreover, the Crispr-Cas9 *in vitro* gene modulation assay was studied. I also got work-culture exposure here.

Annexure-II

ii) Research results, including any papers, prepared/submitted for publication.

Proposed small research project title. To investigate autophagic genes, *Atg16L1* and FIP200 mediated glycolytic flux fine tuning via ILK activation during endometrial stromal cells reprograming for decidualization.

Introduction. The Endocrine and metabolic disturbance-associated disorders, polycystic ovarian syndrome (PCOS) and endometriosis disorders are significantly present (~10% each) in childbearing women, posing various complications in routine life along with infertility. The treatment regimen is symptomatic as there are no detailed studies on the associated processes to find the targeted therapy. We filed a patent application in 2020 (202011002962) for PCOS management, and wish to cover more domains by future studies.

Endometrial receptivity coordinated with decidualization is a prerequisite for **e**mbryo implantation for a successful pregnancy establishment(Carson et al., 2000) , which is negatively affected by endometriosis and PCOS (Piltonen et al., 2015; Younas et al., 2019; Rahman et al., 2018; Li et al., 2016a). Pregnant women with PCOS demonstrated a high incidence of gestational diabetes, insulin resistance, HOMA of insulin resistance (HOMA-IR), and HOMA-β at preconception (Sawada et al., 2015), while an impaired glucose metabolism leads to miscarriage (Edugbe et al., 2020; Silva et al., 2021). Recent studies demonstrate that autophagy is essential for successful decidualization, while its impairment results in spontaneous abortion/ implantation failure, making autophagy an attractive therapeutic target (Park et al., 2021; Tan et al., 2020; Wang et al., 2019; Elshewy et al., 2020; Mestre Citrinovitz et al., 2019; Zhang et al., 2021; Lu et al., 2021; Oestreich et al., 2020a) (Lu et al., 2021) and the host lab demonstrated various aspect of the autophagy role in endometrial physiology, decidualization (Oestreich et al., 2020a; Oestreich et al., 2020b; Popli et al., 2023)and integrin-linked kinase (ILK) has been seen associate with autophagy (Gu et al., 2012). ILK, regulated by AMPK, has been found associated with the decidualization of endometrial stromal cells, while the ILK substrate Glycogen synthase kinase (GSK-3) too is associated with decidualization and autophagy (DeBerardinis et al., 2008) (Hackl, 1973; Kommagani et al., 2013a; Metallo and Vander Heiden, 2013; Moulton and Barker, 1974; Surani and Heald, 1971; Vander Heiden et al., 2011). (Hackl, 1973; Kommagani et al., 2013a; Metallo and Vander Heiden, 2013; Moulton and Barker, 1974; Surani and Heald, 1971; Vander Heiden et al., 2011)It has been debated that poor endometrial receptivity in PCOS cases negatively

affects endometrial decidualization and blastocyst implantation, increasing adverse pregnancy outcomes, such as miscarriage and poor embryonic development (Zhao et al., 2022; Luo et al., 2016).

During decidualization, a high rate of cellular proliferation requires a substantial glucose uptake for bioenergy and metabolic intermediates (Hackl, 1973; Kommagani et al., 2013a; Metallo and Vander Heiden, 2013; Moulton and Barker, 1974; Surani and Heald, 1971; Vander Heiden et al., 2011)(von et al., 2003). Pregnant women with PCOS demonstrated a high incidence of gestational diabetes, severe insulin resistance, HOMA of insulin resistance (HOMA-IR), and HOMA-β at preconception (Sawada et al., 2015) and the impairment in glucose metabolism leads to miscarriage (Edugbe et al., 2020; Silva et al., 2021). Recent studies demonstrated that autophagy is essential for successful decidualization (Oestreich et al., 2020a; Lu et al., 2021; Zhang et al., 2021; Mestre Citrinovitz et al., 2019; Elshewy et al., 2020). Interestingly, spontaneous abortion and implantation failure are associated with autophagy impairment as well (Lu et al., 2021; Wang et al., 2019; Tan et al., 2020) and autophagy is suggested as one of the therapeutic targets(Park et al., 2021; Park et al., 2021).

Glycogen synthase kinase (GSK-3) is a known regulator of glycolytic flux and an ILK substrate(Crawford et al., 2020; Yen et al., 2017a), which is associated with decidualization (Du et al., 2018)(Khaliq et al., 2020). ILK has been found as regulators of the decidualization of stromal cells and at the same time GSK-3beta phosphorylation regulator (Yen et al., 2017b), which are associated with autophagy (Du et al., 2018) (Sosa et al., 2018). Indirectly, ILK seems to act as a co-sensor in the glucose metabolism pathway, AMPK regulation (Xiao et al., 2020; Tang et al., 2015). .

However, to capture the gamut of stromal cell reprogramming, we have utilized human-origin endometrial cells and endometrial tissue- specific transgenic animals for autophagy genes (ATg16l and FIP200). Therefore, in the host lab, using the available endometrial tissue specific autophagic genes, *Atg16L1* and *FIP200* (retinoblastoma 1–inducible coiled-coil 1, *R*B1CC) transgenic mice,(Oestreich et al., 2020a; Oestreich et al., 2020b) we have elucidated the *Atg16L1* and *FIP200* mediated glycolytic flux through ILK activation in the endometrial stromal cells reprogramming for the decidualization to capture the gamut of said cellular process.

Aims: The overall aim of the proposed research project was to determine the role of autophagy genes; *Atg16L1* and *FIP200* mediates-ILK activation, leading to glycogen reserve status change in the glycolytic flux during reprogramming of endometrial stromal cells for decidualization, an event of embryo implantation during pregnancy establishment.

Hypothesis: Whether the autophagy genes, *Atg16L1* and *FIP200* activate ILK signaling to regulate the glycogen reserve and fine tune the glycolytic flux in the reprogramming of endometrial stromal cells for decidualization.

The specific objectives of this proposal

i. To investigate autophagy genes, *Atg16L1* and FIP200 triggered glycolytic flux in the reprogramming of endometrial stromal cells during decidualization.
ii. To elucidate autophagy genes, *Atg16L1* and FIP200 mediated activation of ILK signaling to modulate the glycolytic flux in cellular differentiation during decidualization
iii. To determine autophagy mediated ILK activation role in the glycogen reserve in the glycolytic flux during decidualization process. Hands on training of the endometrial tissue specific gene transgenic biology, endometrial stromal cells autophagy and glycolytic biology to utilize back in India to explore the management strategies of PCOS and endometriosis.

Experimental design

***In vitro* assay**

Human endometrial stromal cells (THESC) **revival and propagation.**

THESCs revival. We begin our study with human-origin hTERT-immortalized cells from non-carcinoma uterus-derived endometrial stromal cell line (THESC)available with the host lab. First, I needed to establish the cell line for my planned experiments. Therefore, the THESCs was revived in the DMEM/F-12 media containing 10% FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin, insulin-transferrin-salenium medium (0.5% mg/ml) and Sodium bicarbonate (07.5 %). The medium was prepared in a 100mm dish and incubated for equilibration with 5% O_2/10% CO /85% N_2 for nearly 15 mins before the cell revival. The frozen cells were thawed in the dry water bath until the media was nearly 90% liquified, which was mixed with the prewarmed media, 1-2 ml and the entire 3-4 ml cell suspension was poured/layered onto the prewarmed media in the 100 mm dish and the cells suspension was gently spreading by gentle gyratory motion and the floating cells were observed under the microscope followed by incubation inside the incubator, having temperature 37 and 5% O_2/10% CO /85% N_2 for 6 hrs. Thereafter, the media was replaced with fresh media and incubated in the incubator at 37°C and 5% O_2/10% CO /85% N_2 for 2 days.

THESC propagation. The 100 mm dish was trypsinized (3 ml) for ~2 mins inside the laminar hood followed by incubation inside at temperature 37 ° C and 5% O_2/10% CO /85% N_2 and the cells

detachment was confirmed under the microscope. The action of trypsin was stopped by adding the fresh media (5-6ml) under the laminar hood and transferred to a 15 ml centrifuge tube and the 15 ml tube containing cells was spun at 2000 rpm for 2 mins at room temperature. Inside the laminar hood, the trypsin-containing medium was removed gently, and 2 ml a fresh media was added. Quickly cell counting was performed using 10 µl cell suspension using the hemacytometer and the tally counter under the phase contrast inverted microscope (4 x magnification) and the cell count per ml was ~85000 and total cells were ~1.5 x 10^5. The cell was distributed equally in three flasks, T-75/ 25 which media was already equilibrated with 5% O_2/10% CO /85% N_2 for nearly 15 mins for propagation purposes, the media was changed every two days until the cell's confluency to 70-80%. This process was followed for passage 27 and 29 culture dish to expand the cells for the transient knockdown of *FIP200* , *ATG16L*, autophagy genes and *ILK* followed by the activation analysis of ILK, decidualization and metabolic flux.

Transient and efficient knockdown of autophagic genes *ATG16L* and *FIP200* in THESC. 12 / 8 well cell culture dish was seeded with a cell density of ~22656/~55000 cells per well for ~48 hrs-72 hrs. For the transient and efficient knockdown of *ATG16L, FIP200* and *ILK* genes, siRNA-mediated transfection of autophagic genes, *ATG16L* (L-021033-01-0005) and *FIP200* (L-021117-00-0005) and *ILK* (L-004499-00-0005) from GE Healthcare Dharmacon Inc., USA was done. The control siRNA was from Dharmacon Inc. (D-0018-10-10-05) as reported earlier (Oestreich et al., 2020a; Oestreich et al., 2020b). The cells were transfected with Lipofectamine RNAi Max -siRNA (30nmol) complex in reduced serum Opti-MEM for 5-6 hrs in the CO2 incubator followed by the media change at 5-6 hrs with complete THESC media. After, 48 hrs, the decidualization was initiated**.**

Decidualization induction. The decidualization was induced in the respective siRNA transfected (Control, *ATG16L, FIP200* and *ILK*) groups with a decidual media (Opti-MEM, 2% Charcoal stripped Fetal Bovine Serum, 10nM estradiol (E2), 1µM medroxyprogesterone 17 -acetate (MPA) and 50µM 8-Bromoadenosine 3′,5′-cyclic monophosphate sodium salt (cAMP) as reported earlier(Oestreich et al., 2020a; Oestreich et al., 2020b). On day 6 post-EPC treatment, the cells were imaged and processed for RNA isolation to confirm the decidualization by PRL expression determination.

RNA isolation, quantitation and cDNA synthesis for the real-time PCR for *ATG16 L, FIP200* and *ILK* knockdown confirmation. The RNA was isolated using the Purelink RNA mini kit (Cat no. 12183018A, Invitrogen, Thermo Fisher Scientific) and the RNA quantitation was performed using the NanoDrop One spectrophotometer (Thermo Fisher Scientific) and later, processed for the cDNA synthesis using the reverse transcriptase kit (Reverse Transcriptase, 4319983, dNTP 10X, 362271, Hexamer primer /RP, 4319979, RT buffer 10X 4319981, Thermo Fisher Scientific) (High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor 4374967)(Oestreich et al., 2020a; Oestreich et al., 2020b).

Transcript expression analysis of ATG16L, FIP200, ILK , SDHA , GLUT1 PRL and 18S RNA by Q-PCR of in after the ATG16L, FIP200 and ILK transient knockdown from THESC during decidualization at day 0 and day6. We examined the gene expression using the TaqMan 2× master mix (Applied Biosystems/Life Technologies, Grand Island, NY) and gene-specific assays as described earlier. We used the Taqman probes (FAM) for the *ATG16L* (Hs01003142_m1, Cat. No.4331182), *FIP200* (Hs01089002_m1, Cat.no.4331182), PRL (Hs00168730_m1, Cat no.4331182, PN4351370), ILK (Hs00177914, PN4453320, Cat . 4331182,), *Slc2a1* (Hs00892681_m1, cat no. 4453320), *SDHA* (Hs00188166_m1 cat. no. 4453320) and *18S rRNA* (4318829/ Eukaryotic 18S rRNA Endogenous Control (VIC™/TAMRA™ probe, primer limited, Cat. no. 4310893E) from the Thermo Fisher Scientific. The cycling conditions were as reported earlier using the Quant Studio RT PCR system (Thermo Fisher Scientific). Cycling parameters were as reported earlier), which were 50°C for 2 mins and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The delta-delta cycle threshold method was used to normalize expression to the reference gene 18S (Oestreich et al., 2020a; Oestreich et al., 2020b).

***In vivo* assay**

Experimental mice. All animal studies protocols were according to the Animal Care and Use Committee at Baylor College of Medicine, Houston, TX. All mice were on a C57/BL6 genetic background (The Jackson Laboratory, Bar Harbor, ME).

ATG16L Atg16L1flox/flox PRcre/+ mice. In the Atg16L1flox/flox mice, the ATG16L exons 3 is flanked by loxp sites, which were bred to progesterone receptor cre (PRcre/+) mice to obtain

(Atg16L1flox/flox PRcre/+ mice) and referred to as Atg16L1 cKO as reported earlier(Oestreich et al., 2020b). Mice were genotyped using the gene specific primers as reported earlier.

FIP200$^{flox/flox}$ PR$^{cre/+}$ mice. FIP200$^{flox/flox}$ mice possess FIP200 gene's exons 4 and 5 flanked by loxp sites as described earlier (Oestreich et al., 2020a), and the progesterone receptor cre (PR$^{cre/+}$) mice were generated as shown previously (Soyal et al., 2005). To generate *FIP200$^{flox/flox}$ PR$^{cre/+}$ mice* FIP200$^{flox/flox}$ and PR$^{cre/+}$ mice were mated and referred to as FIP200 cKO (FIP200$^{flox/flox}$ PR$^{cre/+}$). Genotyping was performed using the gene-specific primers as described previously(Oestreich et al., 2020a).

ATG16L $^{flox/flox}$ PR$^{cre/+}$ mice. The details of PR Cre $^{-/-}$ and Atg16L1 cKO is mentioned elsewhere(Oestreich et al., 2020b). In the Atg16L1$^{flox/flox}$ mice (C57/BL6 genetic background), exons 3 is flanked by loxp sites, which were bred to progesterone receptor cre (PR$^{cre/+}$) mice (C57/BL6 genetic background) (27) to generate (Atg16L1$^{flox/flox}$ PR$^{cre/+}$ mice) and confirmed by the RT PCR.

Artificial decidualization. We induced the decidualization in mice model by intraluminal oil infusion (50 µl of sesame oil) in the ovariectomized mice, 6- to 8-week-old, and hormones-primed mice as demonstrated earlier. The ovariectomized mice received 100 ng of estrogen (E2) for two consecutive days. After 2 days of rest, injections of 10 ng E2 plus 1 mg P4 were given for the next 2 day. Thereafter, ~6 h following the last hormone injection, intra-luminally 50 µL of sesame oil was infused in one uterine horn. Mice were injected daily with 1 mg P4 for four additional days and uterine horns were collected on 5 days after oil stimulation (Oestreich et al., 2020a; Oestreich et al., 2020b).

Analytical techniques

SDS-PAGE and Western blotting. The protein extract, 30 µg (per well) was loaded on a 4% to 15% SDS-polyacrylamide gel (Bio-Rad), and separated with 1xTris-Glycine Running Buffer (Bio-Rad), followed by transfer to polyvinylidene difluoride (PVDF) membranes in a wet electroblotting system (Bio-Rad) as described earlier (Oestreich et al., 2020a; Oestreich et al., 2020b). PVDF membranes were incubated with a blocker (5% nonfat milk) for 1 hour in TBS-T (Bio-Rad). The primary antibodies, FIP200, ILK (3856, Cell Signaling Technology), ILK (S246) (AB1076, Merck) and actin-beta (Sigma-Aldrich) were incubated overnight at 4ºC in TBS-T containing 5% milk blocker in 1:500, 1:2000 and 1:50000 , respectively.

After washing the membranes with TBST, an incubation with Anti-rabbit IgG, HRP-linked Antibody (1:5000, cat. no. #7074 Cell Signaling Technology) in TBS-T for 1 hour at room temperature. The blots were developed with HRP Chemiluminescent Substrates (Merck) and imaged on an Invitrogen ChemiDoc imaging system. The actin-beta was used as an internal loading control (Oestreich et al., 2020a; Oestreich et al., 2020b).

ILK Immunolocalization on the uterine tissues. The immunolocalization of ILK on the uterine tissues was done as described earlier(Oestreich et al., 2020a; Oestreich et al., 2020b). Formalin-fixed and paraffin-embedded uterine tissue sections were deparaffinized in xylene, and rehydrated in an ethanol gradient followed by antigen unmasking using the boiled citrate buffer (Vector Laboratories Inc., CA, USA). Tissue sections were encircled by the hydrophobic pen and blocked with 2.5% goat serum in PBS (Vector laboratories) for 1 h at room temperature. Primary antibody ILK diluted in 2.5% normal goat serum was incubated with sections (1:200, 1:500 and 1:1000 dilutions) overnight at 4 °C with . After washing with PBS, tissue sections were incubated with Alexa Fluor 488-conjugated secondary antibodies (Life Technologies) for 1 h at room temperature. The washing was followed by mounting with ProLong Gold Antifade Mountant with DAPI (Thermo Scientific) (Oestreich et al., 2020a; Oestreich et al., 2020b; Kommagani et al., 2013b).

Results.

Decidualization in the THESC was reduced in response to ATG16L and FIP200 transient silencing . First, we confirmed the transient knockdown of *ATG16L* and *FIP200* followed by decidualization response. The transient knockdown (Fig. 1) was confirmed where the mRNA level of *ATG16L1* was reduced to ~60% (Figure-1A) and FIP200 transcript to ~80% (Fig. 1B) in the THESCs. Protein expression of FIP200 was also confirmed and found it downregulated in response to *FIP200 siRNA* transfection (Fig. 1C).

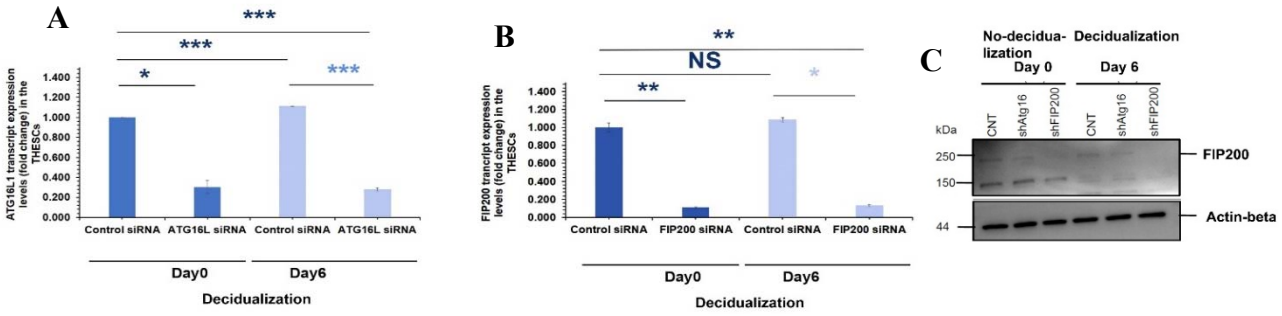

Figure-1. *ATG16L* and *FIP200* mRNA and protein expression analysis during their transient knockdown in THESCs during decidualization stimulation.

Later, we examined the decidualization response by *PRL* transcript analysis and found that *ATG16L* transient knockdown reduced the transcript level of *PRL* ~50% during decidualization stimulation in response to E2, MPA and cAMP treatment (Fig. 2 A and B). The *PRL* transcript levels were not altered by *FIP200* knockdown (Fig. 2A and B). The morphology of the THESCs also resembled epithelioid during E2, MPA and cAMP-mediated in vitro decidualization (Fig. 3).

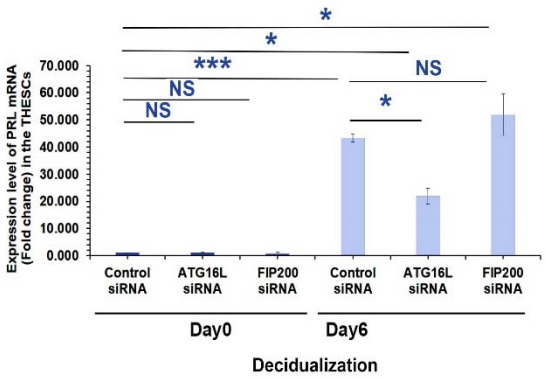

Figure-2. *PRL* mRNA expression analysis during the transient knockdown of *ATG16L* and *FIP200* in THESCs during decidualization stimulation.

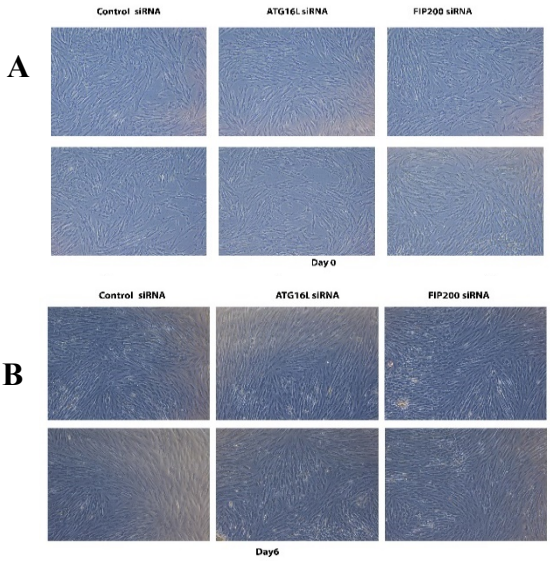

Figure-3. THESCs during decidualization stimulation (six days) by E2, MPA and cAMP.

***ILK* expression is under the regulation of *FIP200* in the THESCs.** Subsequently, we examined the association of *ILK* with the autophagic genes, *ATG16L* and *FIP200* in the THESCs during decidualization stimulation for six days (Fig. 4). In response to *ATG16L* and *FIP200* transient knockdown in the THESCs, we found that *FIP200* transient silencing led to down-regulation of *ILK* transcri

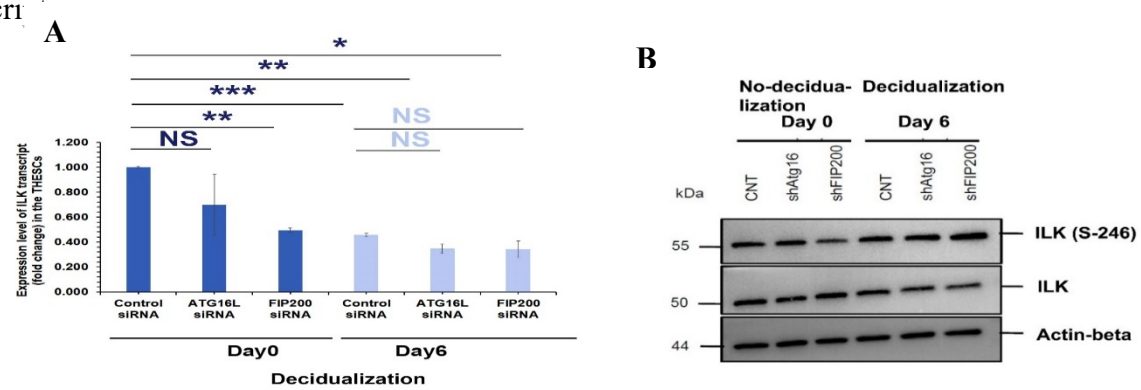

Figure-4. mRNA and protein expression analysis of ILK during the transient knockdown of *ATG16L* and *FIP200* in THESCs during decidualization stimulation.

However, the protein expression of ILK was negatively affected by *ATG16* and *FIP200* transient silencing during the day6 of decidualization (Fig. 4B). The phosphorylation of ILK at S246 was downregulated during day0 in response to FIP200 knockdown in the THESCs , but seems to be enhanced during day6 of the decidualization (Fig. 4 B).

ILK is involved in the *in vitro* decidualization. Subsequently, we examined the role of ILK in the decidualization by transient knockdown of it in the THESCs and stimulation of the decidualization by E2, MPA and cAMP during the six-day period. The *ILK* siRNA transfection led to the knockdown of its mRNA of nearly 80 % in the THESCs (Fig. 5A). Next, we determined the role of it in the decidualization and we found that the decidualization biomarker, *PRL* is downregulated in the transient knockdown of *ILK* (Fig. 5B), indicating involvement of *ILK* in the *in vitro* decidualization process.

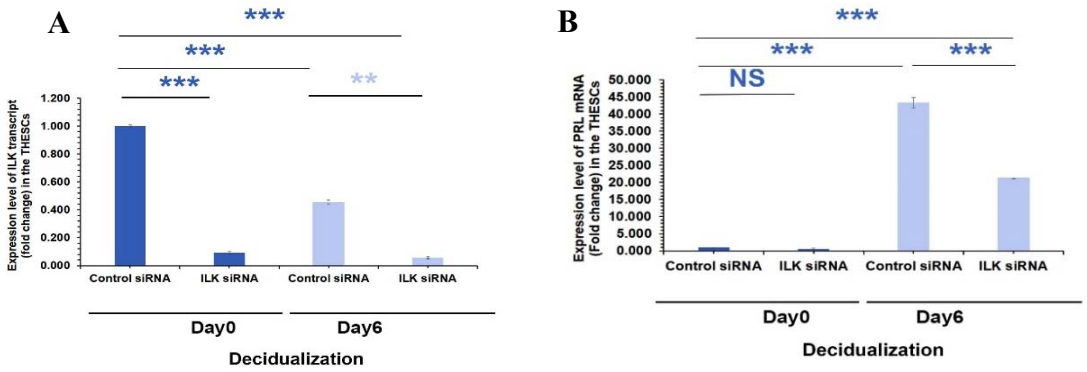

Figure-5. mRNA expression analysis of *ILK* and *PRL* during the transient knockdown of *ILK* in THESCs during decidualization stimulation.

During the decidualization stimulation, the morphological appearance was like epithelioid as reported earlier (Fig. 6).

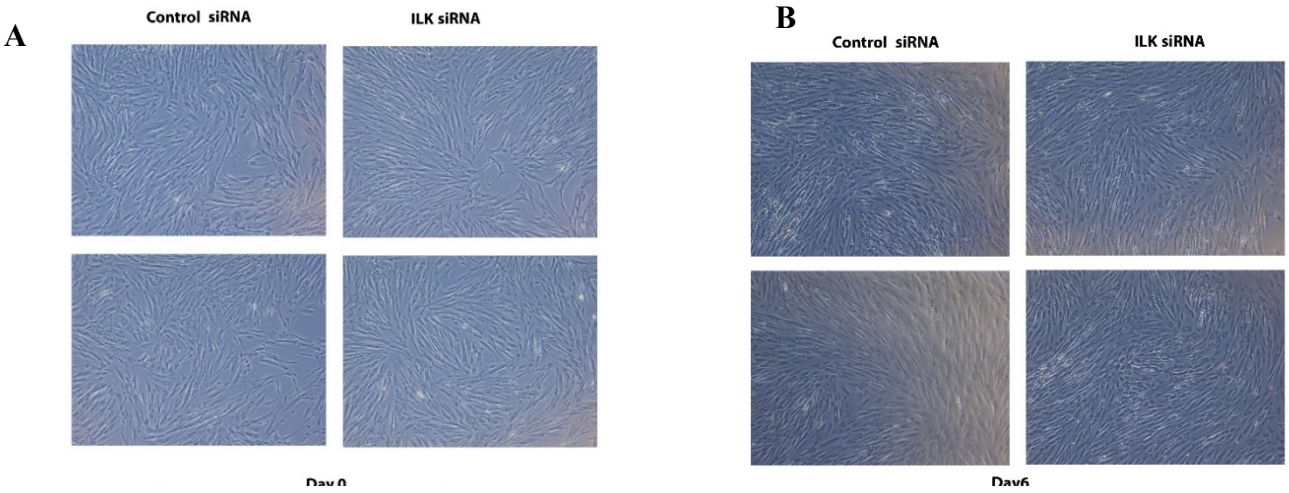

Figure-6. THESCs during decidualization stimulation (six days) by E2, MPA and cAMP.

ILK-mediated regulation of glucose importer, *GLUT1* and Tricarboxylic acid cycle (Krebs cycle) rate-limiting enzyme, *succinate dehydrogenase A (SDHA)* during decidualization. Subsequently, we determined the *ILK*-mediated glycolytic flux as during decidualization a high rate of cellular proliferation requires a substantial glucose uptake for bioenergy and metabolic intermediates(Kommagani et al., 2013b)(DeBerardinis et al., 2008; Hackl, 1973; Kommagani et al., 2013a; Metallo and Vander Heiden, 2013; Moulton and Barker, 1974; Surani and Heald, 1971; Vander Heiden et al., 2011; von et al., 2003). GSK3beta is a known regulator of glycolytic flux and an ILK substrate(Crawford et al., 2020; Yen et al., 2017a), which is associated with decidualization (Du et al., 2018; Sosa et al., 2018). ILK has been found as a regulator of the decidualization of stromal cells and at the same time GSK-3beta phosphorylation regulator (Yen et al., 2017a), which are associated with autophagy (Du et al., 2018; Xiao et al., 2020) . Indirectly, ILK seems to act as a co-sensor in the glucose metabolism pathway, AMPK regulation (Tang et al., 2015; Soyal et al., 2005).

Recently, Insulin regulation of solute carrier family 2 member 1 (*Slc2a1*, glucose transporter 1, GLUT1) – a glucose importer to cells, and SDHA have been shown associated with decidualization in THESCs (Tamura et al., 2021; Citrinovitz et al., 2022) . Thus, we examined the GLUT1 and SDHA association with ILK signaling during decidualization, *in vitro*, using THESCs. Interestingly, we observed that *GLUT1* transcript expression level was increased during decidualization in response to *ATG16L* transient knockdown, but reduced by *FIP200* and *ILK* transient knockdown in the THESCs during decidualization stimulation, day 6 (Fig.7A).

Likewise, we observed the effect of *ATG16L* ,*FIP200* and *ILK* on *SDHA* expression(Fig. 7B) . Interestingly, *ATG16L, FIP200* and *ILK* transient knockdown increased the expression level of *SDHA* in THESCs (Fig. 7B). Surprisingly, neither *ATG16L* nor *FIP200* silencing affected the expression level of *SDHA* during decidualization stimulation at day6. Interestingly, *ILK* knockdown led to the downregulation of *SDHA* in the THESCs during decidualization, day 6 (Fig. 7B).

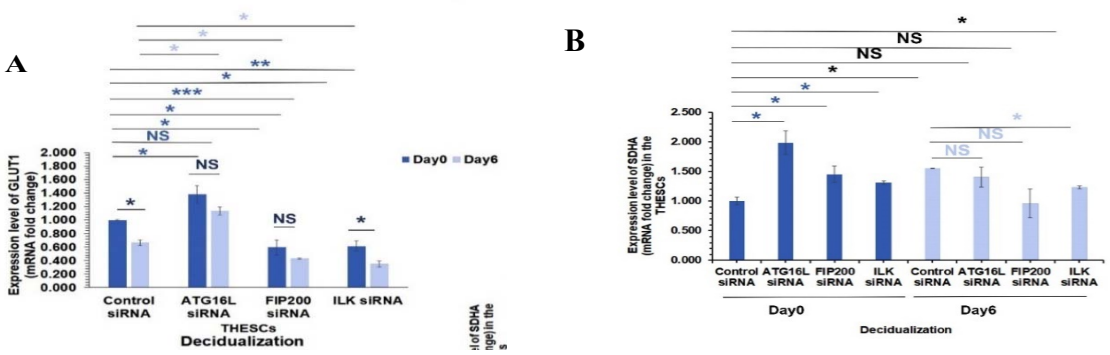

Figure-7. mRNA expression level of *GLUT1* and *SDHA* in THESCs in response to *ATG16L, FIP200* and *ILK* transient knockdown during decidualization stimulation (six days) by E2, MPA and cAMP.

Activation analysis of ILK during decidual stimulation in the FIP200cKO (FIP 200 $^{flox/flox}$ PR$^{cre/+}$ **) mouse.** Next, we examined the expression and activation of ILK during decidual stimulation in response to uterine tissue-specific FIP200 knockout (KO) (FIP 200 $^{flox/flox}$ PR$^{cre/+}$). The expression level of ILK was increased during the decidualization in FIP 200 $^{flox/flox}$; however, the FIP 200 $^{flox/flox}$ PR$^{cre/+}$ mice uterine tissue showed a basal level of expression of ILK as seen in the non-stimulated condition (Fig. 8). Further, we have determined, the ILK phosphorylation at S-246 and found the decreased level of it in the decidual stimulus case and neither unstimulated FIP 200 $^{flox/flox}$ and FIP 200 $^{flox/flox}$ PR$^{cre/+}$ nor FIP 200 $^{flox/flox}$ PR$^{cre/+}$ showed any change and maintained the basal level of expression (Fig. 8).

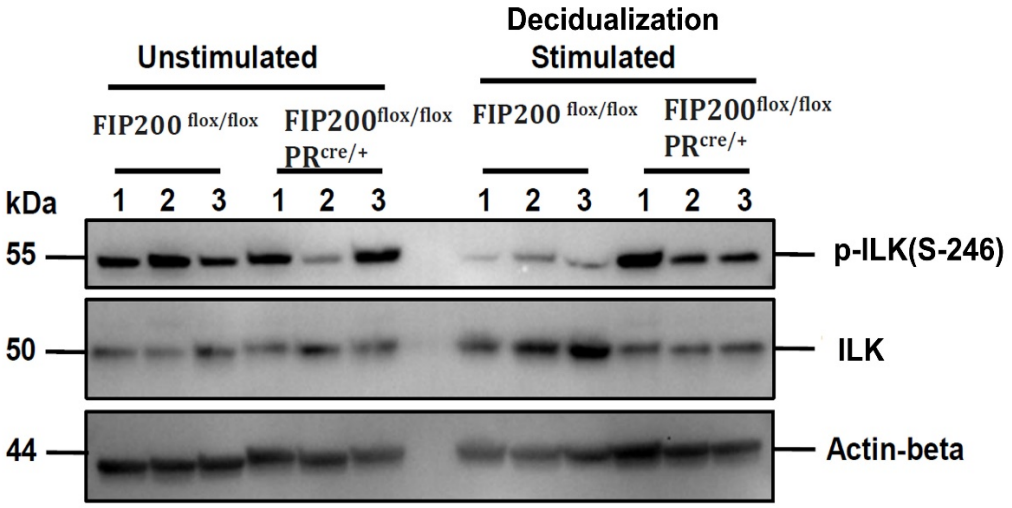

Figure-8

Expression analysis of ILK by immunolocalization of ILK in the uterus during decidual stimulation in the ATG16L cKO (Atg16L1$^{flox/flox}$ PR$^{cre/+}$ **) mouse.** The immunolocalization of ILK was optimized in the uterine tissues using the ILK antibody (rabbit raised) and goat anti-rabbit conjugated with Alexa 488 fluorophore (Fig.9). The entire procedure was done as per the optimized lab protocol(Popli et al., 2023).

First, we optimized ILK (1:200 dil., 1:250 dil and 1:500 dil) and goat anti-rabbit Alexa 488 antibodies dilutions (1:200 dil., 1:500 dil and 1:1000 dil) using 2.5% non-immunized rabbit serum (25 µl volume for each section spot). The 1:100 dilution of ILK antibody and 1:200 dilution of GAR Alexa 488 antibody was selected because of prominent fluorescence for immunolocalization studies in the ATG16L cKO(Oestreich et al., 2020a) mice uterine tissues (Fig. 9).

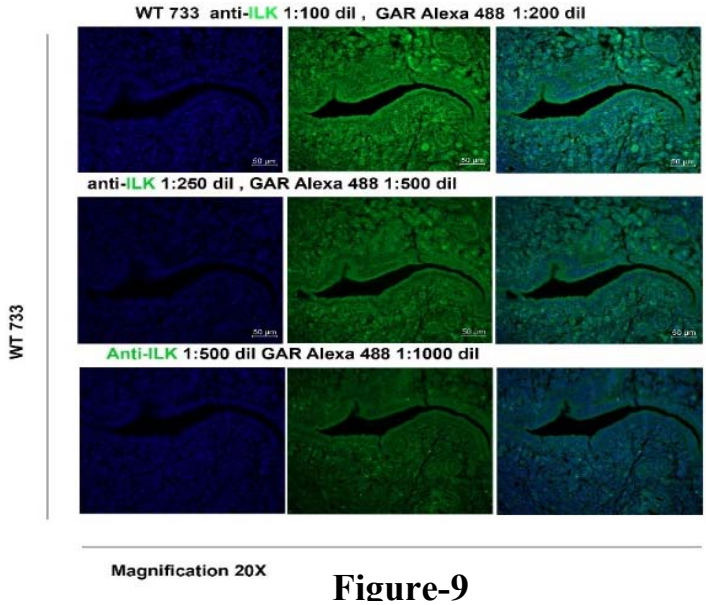

Figure-9

ILK localization by immunofluorescence on PR Cre +/+ (stimulated and non-stimulated 750, 759 and 771) and cKO ATG 16 (stimulated and non-stimulated: 752, 770 and 774 and) showed the presence on endometrial epithelial (perimetrium, endometrium and glandular), myometrial and stromal cells (Fig. 10). The fluorescence intensity of ILK was visually seen intense in particular to the perimetrium, myometrium and endometrial (luminal and glandular) regions in the uterus from PR $^{cre/+}$ mice unstimulated group, without decidualization (Fig. 10). However, decidualized group of animal uterus showed the fluorescence staining comparatively dull. In the cKO ATG16L mice uterine tissue, the ILK fluorescence signal was comparatively weaker than the wild-type animal tissues, unstimulated and stimulated groups (Fig.10).

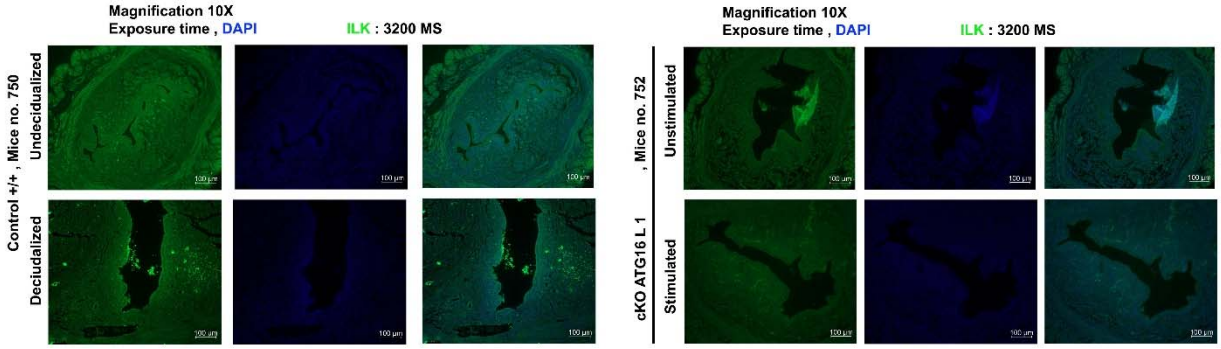

Figure-10

To know the cell-specific change in the ILK fluorescence signal in the PR $^{Cre\ +/+}$ and cKO ATG 16L group of mice, we imaged the tissue section at the higher magnification, 40X and analyzed the fluorescence pattern. In the unstimulated, non-decidualized PR Cre $^{+/+}$ mice, we observed the ILK fluorescence signal in the luminal epithelial cells and certain stromal cells and the signal was mostly seen in the cytoplasmic region of these cells. Further, in comparison to the stimulated or decidualized uterine tissue horn, the fluorescence intensity of ILK in the unstimulated or non-decidualized uterine horn of the same animal was low; however, there was no fluorescence quantitation (Fig. 11). Likewise, the fluorescence localization pattern of ILK was seen in the cKO ATG 16L mice group (Fig. 11). Nonetheless, in comparison to the unstimulated or non-decidualized uterine tissue horn, the fluorescence intensity of ILK in the stimulated or decidualized uterine horn of the same animal was notice predominant in the luminal epithelial apical region (Fig. 11).

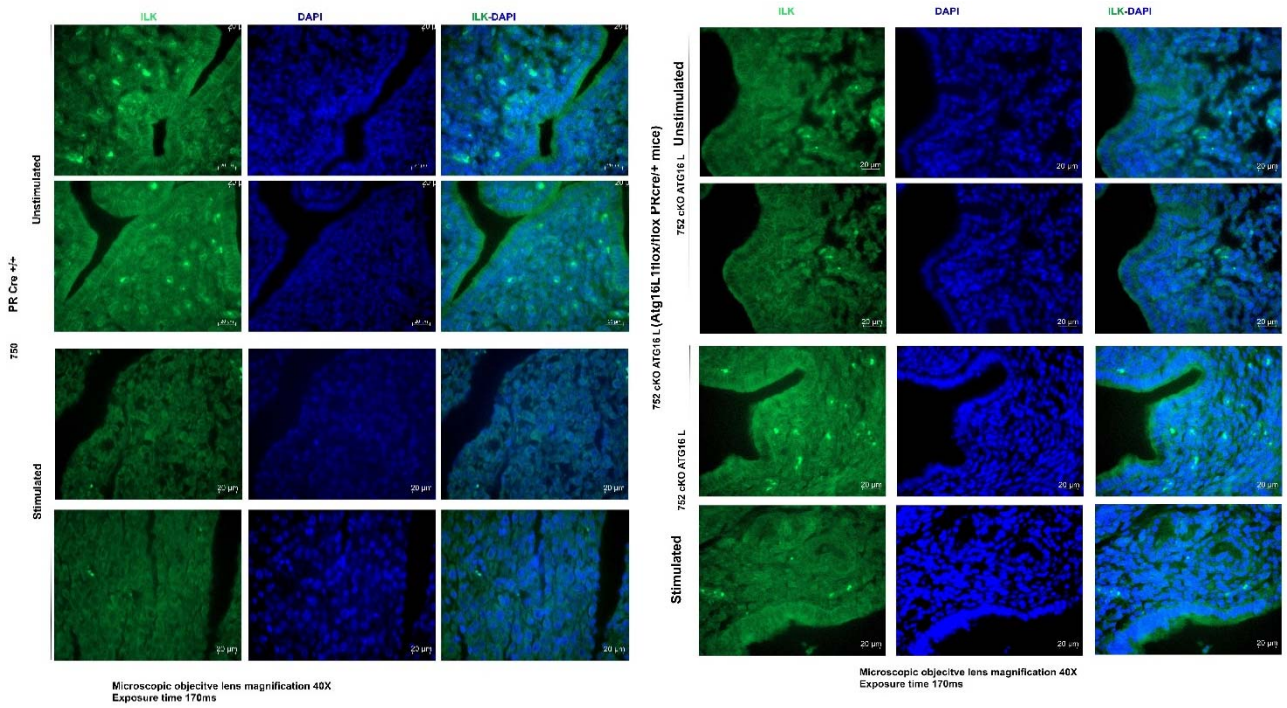

Figure-10

Summary : Collectively, w ediated *ILK* regulation during *in vitro* decidualization in the THESCs and *ILK* involvement with TCA associated gene *SDHA* and *glucose transporter* , *GLUT1* in the THESCs during the decidualization process. Using the uterine specific FIP200 ablation (FIP200 $^{flox/flox}$ PR$^{cre/+}$) in mice model of decidualization, we show that ILK expression is down regulated, but its ILK(S-246) form was down regulated during decidualization process in the FIP200 $^{flox/flox}$ mice. Collectively, we find ILK association with *in vitro* decidualization, which is under the regulation of *FIP200* and in turn controls the *GLUT1* and TCA gene, *SDHA.*

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ii) Proposed utilization of the experience in India:

At present, we are working on the molecular pathways of PCOS, endometriosis, and miscarriage. Further, we are heavily engaged in the therapeutic for PCOS and endometriosis. Our group has been working on understanding endometriosis/PCOS for therapeutic interventions, having established *in vivo*/*in vitro* endometriosis models (Ubba et al., 2017; Soni et al., 2019; Kumari et al., 2023; Kumari et al., 2023; Rashid et al., 2023), leading to patent filing (9202011002962).

PCOS is an endocrine and metabolic disorder and pregnant women with PCOS demonstrated a high incidence of gestational diabetes, insulin resistance, HOMA of insulin resistance (HOMA-IR), and HOMA-β at preconception (Sawada et al., 2015), while an impaired glucose metabolism leads to miscarriage (Edugbe et al., 2020; Silva et al., 2021). It has been debated that poor endometrial receptivity in PCOS cases negatively affects endometrial decidualization and blastocyst implantation, increasing adverse pregnancy outcomes, such as miscarriage and poor embryonic development (Zhao et al., 2022) (Li et al., 2016)

Likewise, another endocrine disorder, endometriosis affects ovarian function with an abnormally prolonged follicular phase (Cahill and Hull, 2000), leading to dysfunctional folliculogenesis and granulosa cell cycle kinetics (Garrido et al., 2000)(Cahill et al., 1997). The normal function of the eutopic endometrium is also compromised in patients with endometriosis due to progesterone resistance (Lessey et al., 1994), (Aghajanova et al., 2009) in consequence the deficient progesterone signaling pathways lead to impaired decidualization in patients with endometriosis (Marquardt et al., 2019) during the natural conceptions (Racca et al., 2024).

Integrin Linked Kinase (ILK), regulated by AMPK, has been found associated with the decidualization of endometrial stromal cells, while the ILK substrate Glycogen synthase kinase (GSK-3) is also associated with decidualization (DeBerardinis et al., 2008; Hackl, 1973; Kommagani et al., 2013; Metallo and Vander Heiden, 2013; Moulton and Barker, 1974; Surani and Heald, 1971; Vander Heiden et al., 2011).

Upon return, I will explore the ILK-mediated cellular metabolic status correction during PCOS and endometriosis and submit the research proposal to ICMR or DHR, New Delhi for funding consideration. Briefly, we will orient our research directions towards the endometrial endocrine regulation with energy expenditure, metabolic flux. At the same time, we utilize the PCOS and endometriosis *in vitro* models to study the ILK-associated downstream gene functions by efficient and transient or stable knockdown of ILK to explore the therapeutic target for PCOS and endometriosis.

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