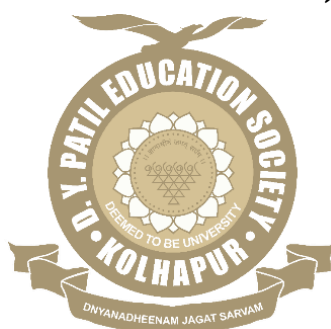


**ROLE OF CD146 IN ENDOMETRIAL STEM CELLS AND
ANGIOGENESIS UNDER NORMAL AND DISEASED
CONDITIONS**

**THESIS SUBMITTED TO
D. Y. PATIL EDUCATION SOCIETY
(DEEMED TO BE UNIVERSITY), KOLHAPUR**



**FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
STEM CELL AND REGENERATIVE MEDICINE
BY**

Ms. Priyanka Pramodkumar Hilage M.Sc. SCRM

UNDER THE SUPERVISION OF

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and

Dr. Meghnad Ganesh Joshi M.Sc. Ph.D.

**DEPARTMENT OF STEM CELL AND REGENERATIVE MEDICINE
CENTRE FOR INTERDISCIPLINARY RESEARCH
D. Y. PATIL EDUCATION SOCIETY (DEEMED TO BE UNIVERSITY),
KOLHAPUR, 416006.**

2025

DECLARATION

I hereby declare that the thesis entitled **“Role of CD146 in endometrial stem cells and angiogenesis under normal and diseased conditions”** is original and carried out by me, in D. Y. Patil Education Society (Deemed to be University), Kolhapur.

It has not been submitted by me for any Degree or Diploma before this to any other University.

Place: Kolhapur

Date: 05.04.2025



Ms. Priyanka Pramodkumar Hilage

(Research Student)

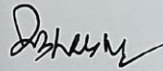
Certificate

This is to certify that the thesis entitled "**Role of CD146 in endometrial stem cells and angiogenesis under normal and diseased conditions**" which is being submitted herewith for the award of the degree of **Doctor of Philosophy (Ph.D.) in Stem Cell and Regenerative Medicine** of **D. Y. Patil Education Society (Deemed to be University)**, Kolhapur, is the result of the original research work completed by **Ms. Priyanka Pramodkumar Hilage** under my supervision and guidance. To the best of my knowledge and belief, the work embodied in this thesis has not formed earlier the basis for the award of any degree or similar title of this or any other university or examining body.

Place: Kolhapur

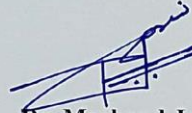
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LIST OF PUBLICATIONS

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LIST OF CONFERENCES AND WORKSHOPS

Conferences:

1. Presented paper in **International conference on Nanotechnology addressing the convergence of material science, biotechnology and medical science- 2024 (IC-NACMBM 2024)**. organized by Centre for interdisciplinary research, D. Y. Patil Education Society (Deemed to be University) Kolhapur on 12-14 February 2024.
2. Participated in **International conference on ‘Cancer Biology: Basic Science to Translational Research’ (CBTR-2020)**. organized by Dept. of Stem Cell and Regenerative Medicine, Centre for interdisciplinary research, D. Y. Patil Education Society (Deemed to be University) Kolhapur on 17-18 January 2020.
3. Attended **International E-Conference on “Strategies & Challenges in Higher Education during COVID-19 Lockdown Period in India with reference to the World”** organized by Government Vidarbha Institute of Science & Humanities, Amravati, India on 15th - 17th May, 2020.
4. **Presented a poster in 2nd National Conference on Regenerative Medicine and Stem Cell Research**, held at Perambalur, Tamilnadu on 6-7th April 2018.
5. **Presented poster in National Research Scholars Meet (NRSM) 2018**, ACTREC, Navi Mumbai. 3-4 December 2018.
6. **Attended International conference on Nanotechnology addressing the convergence of material science, biotechnology and medical science- 2017 (IC-NACMBM 2017)** organized by Centre for interdisciplinary research, D. Y. Patil Education Society (Deemed to be University) Kolhapur on 9-11th November 2017.

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1. Successfully completed the **workshop on ISO 13485:2016 Medical Devices Internal Auditor & Transition Training Course** Onsite by SGS academy held at Yashraj Biotech Ltd, Navi Mumbai on 20-21 July 2022.
2. Attended **workshop on Basics, Experimental design, Data analysis and presentation of Flowcytometry**, conducted by Flowcytometry Solution Pvt Ltd on 22-23 April 2020.

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Chapter IV: Prognostic significance of CD146 in Endometrial Carcinoma

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Fig. 5.7: Growth factor secretion profile of CD146⁺ EC cells with and without M2J-1 treatment. A significant reduction in the levels of key angiogenic factors, including VEGF, ANGPT2, PDGF, and others, is observed upon M2J-1 treatment, indicating its inhibitory effect on pro-angiogenic signaling. Data are presented as mean $\pm$ SEM.

Fig. 5.8 In-ovo angiogenic assay using CAM model to evaluate the angiogenic nature of CD146⁺ EC cells and also after the treatment of M2J-1. In the group CD146⁺ EC high vasculature was observed compared to normal indicates the angiogenic potential of CD146⁺ cells. While in the treatment group, the vasculature on CAM reduced significantly after the treatment of M2J-1.

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Table 2: ANOVA results for CD146 expression.

Table 3: Correlation matrix of key variables

Table 4: Model performance comparison across classifiers

Table 6: Confusion matrix for the Random Forest classifier

Table. 7: Decision tree classification

LIST OF ABBRIVATION

Aldehyde dehydrogenase 1A3	ALDH1A3
Alpha Smooth Muscle Actin	α -SMA
Angiopoietin-2	Ang-2
Angiopoietin-2	ANGPT2
Basic fibroblast growth factor	FGF-basic
Cadherin 5	CDH5
Cellular- myelocytomatosis	c-Myc
Chemokine (C-C motif) ligand 20	CCL20
Chemokine (C-C motif) ligand 3	CCL3
Chorioallantoic membrane	CAM
Cluster of differentiation 133	CD133
Cluster of differentiation 14	CD14
Cluster of Differentiation 140 b	CD140b
Cluster of Differentiation 146	CD146
Cluster of Differentiation 150	CD105
Cluster of differentiation 19	CD19
Cluster of Differentiation 31	CD31
Cluster of Differentiation 34	CD34
Cluster of differentiation 44	CD44
Cluster of differentiation 45	CD45
Cluster of Differentiation 73	CD73
Cluster of Differentiation 90	CD90
Coagulation factor III	F3
CXC chemokine ligand 5	CXCL5
CXC chemokine ligand 8	CXCL8
Cysteine-rich angiogenic inducer 61	CYR61
Dulbecco's Modified Eagle Medium	DMEM

Dulbecco's Modified Eagle Medium	DMEM-LG
Dulbecco's Phosphate Buffered Saline	DPBS
Endometrial carcinoma	EC
Endometrial hyperplasia	EIN
Endometrial regenerative cells	ERC
Endometrial stem cells	eSCs
Epidermal growth factor	EGF
Epidermal growth factor	EGF
Epithelioid trophoblastic tumors	ETTs
Erythropoietin	EPO
Extracellular signal-regulated kinase ½	ERK ½
Fetal bovine serum	FBS
Fibroblast growth factor 1	FGF1
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Granulocyte colony-stimulating factor	G-CSF
Granulocyte macrophage colony-stimulating factor	GM-CSF
Hepatocyte Growth Factor	HGF
Human umbilical vein endothelial cells	HUVEC
Hypoxia-inducible factor 1-alpha	HIF-1 α
Insulin-Transferrin-Selenium	ITS
Insulin-Transferrin-Selenium	ITS
Ethanolamine	
Intercellular adhesion molecule-1	ICAM-1
Interleukin-1 beta	IL-1 β
Interlukin-6	IL-6
International Federation of Gynecology and Obstetrics	FIGO
Krüppel-like factor 4	KLF4
Leukocyte cell-derived chemotaxin 1	LECT1
macrophage colony-stimulating factor	M-CSF
Matrix metalloproteinase-9	MMP-9

Melanoma cellular adhesion molecule	MUC18
Mesenchymal stem cells	MSC
Octamer-binding transcription factor 4	Oct4
P38 mitogen-activated protein kinases	AKT/p38
	MAPK
Peripheral artery disease	PAD
Peroxisome Proliferator–Activated Receptor γ	PPAR γ
Platelet-derived growth factor subunit A	PDGF-AA
Platelet-derived growth factor subunit B	PDGF-BB
Runt-related transcription factor 2	RUNX2
Serine protease inhibitor E	SERPINE
Sex determining region Y-box 2	SOX2
SRY-Box Transcription Factor 7	SOX9
Stem Cell Factor	SCF
Support Vector Machine	SVM
Thrombospondin-1	THBS2
Tissue inhibitor of metalloproteinase 3	TIMP3
Transforming growth factor-alpha	TGF- α
Vascular Associated protein	VAP
Vascular endothelial growth factor	VEGF
vascular smooth muscle cells	vSMCs
Von Willebrand factor	vWF

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Professional Summary

Highly motivated and dedicated scientist with expertise in induced pluripotent stem cells (iPSCs), various iPSC derivatives, as well as a strong background in the in endometrial stem cells and angiogenesis. Seeking a challenging position as a Scientist to contribute to cutting-edge research and development in the field of regenerative medicine.

Work History

RESEARCH SCIENTIST, 07/2021 - 12/2023
Yashraj Biotechnology Limited, Navi Mumbai

- Conducted research on iPSCs and their differentiation into hepatocytes, cardiomyocytes, 3D pancreas as well as Mid Brain organoids and also on Neural Progenitor Spheroids
- Performed cellular toxicity on Neural Progenitor spheroids and Mid Brain Organoids
- Designed and executed experiments to investigate molecular mechanisms of iPSC differentiation
- Developed and optimized protocols for generating and characterizing iPSC-derived cells
- Utilized techniques such as cell culture, immunocytochemistry, qPCR, Western blotting, and flow cytometry for analysis
- Collaborated with multidisciplinary teams to integrate iPSC-derived cells into disease models for drug screening and toxicity testing
- Analyzed and interpreted data, prepared scientific reports
- Trained and mentored team members, participated in internal audits, and contributed to SOP development
- Skilled in documentation management, inventory control through TCSion, and QMS activities
- Attended ISO 13485:2016 workshop on medical devices quality management systems
- Demonstrated ability to multitask and contribute to overall efficiency and compliance of the organization

Publications

- Birajdar A, Sharma R, Hilage P. Stem cells of the endometrium: a leap towards regenerative medicine. *MOJ Women's Health*. 2017;4(6):142-4.
- Somasundaram I, Birajdar A, Hilage P, Sharma RK. Anti-Angiogenic Potential of Itraconazole and Its Reversal by Endometrial Stem Cells Using Chick Embryo Model. *Journal of Stem Cells*. 2019 Jan 1;14(1).

- Hilage P, Birajdar A, Marsale T, Patil D, Patil AM, Telang G, Somasundaram I, Sharma RK, Joshi MG. Characterization and angiogenic potential of CD146+ endometrial stem cells. Stem Cell Research & Therapy. 2024 Sep 27;15(1):330.
- Hilage P, Damle MN, Sharma RK, Joshi MG. Melanoma Cell Adhesion Molecule. Cell Biology and Translational Medicine, Volume 23: Stem Cell in Organoids, Signaling, Regeneration and Cancer.:131.

Education

Ph.D.: Stem cell and Regenerative Medicine
D. Y. Patil Education Society - Kolhapur, Maharashtra Current

Thesis Title: Role of CD146 in Endometrial Stem Cells and Angiogenesis under Normal and Diseased Conditions

Master of Science (M.S.): Stem Cell and Regenerative Medicine
D. Y. Patil Education Society - Kolhapur, Maharashtra, 2014 - 2016

- GPA: First class
- Project Title: Effect of cobalt ferrite nanoparticles hyperthermia treatment on chemically induced cancer in chick model

Bachelor's: Biotechnology
Shivaji University - Kolhapur, Maharashtra, 2012 - 02014

- GPA: First class
- Project Title: Optimizing the protocol for DNA isolation from blood and Amplification of DNA by RAPD and Restriction fragmentation

Skills

Tissue Culture Techniques	Bioinstrumentation
Molecular Biology Techniques	Strong understanding of stem cell biology
regenerative medicine	Strong problem-solving and critical-thinking abilities
keen attention to detail	

Introduction:

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a critical process for tissue growth, repair and homeostasis. dysregulation of this process leads to various pathological conditions such as cancer. Recent studies have highlighted the angiogenic efficacy of several stem cells which includes umbilical cord, Wharton's jelly, dental pulp, bone marrow as well as endometrium. Among all these types, endometrium derived stem cells (eSCs) stand out due to its unique characteristic. eSCs are sourced from the endometrium, tissue that undergoes many cyclic remodelling and regeneration. This dynamic environment equips eSCs with distinct angiogenesis ability. CD146 is a cell surface glycoprotein and a pericyte marker expressed on endothelial cells plays a pivotal role in angiogenesis. It facilitates adhesion, migration and signal transduction which mediates endothelial cell interaction, vessel sprouting and pericyte recruitment. Emerging research suggests, eSCs exhibit pericyte like properties, contributing to vascular remodelling and stability.

Endometrial carcinoma is the most common gynaecological malignancy. Angiogenesis plays a critical role in tumor progression. CD146 is over expressed in many tumors, contributing tumor growth and metastasis. It also plays a crucial role in pathological angiogenesis of endometrium carcinoma. Studies suggest that inhibiting CD146 using specific monoclonal antibody disrupts endothelial pericyte interaction, reduce vascular stability resulting limiting pro-angiogenic effect. Thus, we would determine the function of CD146 in eSCs and the process of angiogenesis under both normal and disease condition.

1.1 Angiogenesis

Angiogenesis refers to the formation of new blood vessels from already existing one. This involves intricate molecular activation and release of growth factors and cells like pericytes that help in forming the new capillaries. It is a normal and vital process in growth and development, as well as in wound healing and in the formation of granulation tissue. Angiogenesis occurs throughout life in both health and disease, beginning in utero and continuing through old age [1]. This process is both vital and dynamic and is required for the exchange of nutrients and metabolites. Angiogenesis has an important role to play in wound healing and recovery and fighting off the infections [2]. This

process is very dynamic in human uterus throughout the reproductive age. Such regular cycle of blood vessel formation and shedding is found nowhere else [3]. Angiogenesis in the female reproductive cycle occur in ovaries and endometrium, and they are interconnected process [4] However, human endometrium is the only dynamic organ that undergoes the most extensive proliferation, remodeling and vascularization in adult mammals compared with other organs, both under physiological and pathological conditions [5]. Angiogenesis in endometrium varies spatially and temporally including repair during menstrual cycle: rapid growth in the proliferative phase, extensive development in the secretory phase [6]. Unlike stable vascular system, endometrial vasculature undergoes cyclic growth and regeneration making it ideal model for angiogenic research [7]. Additionally, the endometrium's role in angiogenesis is supported by the interaction of stem cells present in the basalis layer of endometrium and endothelial cells.

Pathological angiogenesis is the major cause of many disorders occurs majorly in cancer contrasting the normal, regulated process of angiogenesis. In any pathological conditions, the physiological mechanism of angiogenesis is disturbed which leads to the blood vessel formation due to lack of regulatory feedback mechanism [8]. Though, most of the endometrial dysfunctions arise as a result of endocrine disturbance, it has a major pathological role associated with angiogenesis. Endometriosis is a multifactorial disease where endometrium grows outside of uterus [9], whereas endometrial hyperplasia (EIN) is a precancerous stage of endometrioid adenocarcinoma (type I), [10] with a common angiogenic progression profile. Targeting angiogenesis presents a promising approach to mitigate endometrial disorder, particularly endometrial carcinoma.

1.2 CD146/ Melanoma Cell Adhesion Molecule

Pericytes promote angiogenesis by wrapping around blood capillaries thereby interacting with endothelial cells. [11]. Pericytes exert multivariant role on proliferation, migration and stabilization of endothelial cells thereby contributing to different aspects of angiogenesis [12]. Recently, stem cells were found to possess many characteristics of pericytes and are capable of stabilizing endothelial cells [13]. Melanoma cell adhesion molecule known by several other names like MCAM/ CD146/ MUC18, membrane glycoprotein which belongs to mucin family; functions primarily as cell adhesion protein which is highly expressed by vascular endothelial cells and pericytes.

CD146 is 113 kDa glycoprotein present in the plasma membrane of human melanoma cells and discovered in 1987. In 1996, the detection of CD146 on endothelial cells in blood and different vascular systems was enabled by sendo-1 antibody. This detection establishes the vascular feature of CD146 [14]. CD146 is well established marker for endothelial cells to study endothelial cell circulation and also to sort endothelial cells from peripheral blood [15]. sCD146 activates endothelial cells to boost their survival by increasing various cellular activities like cell migration, proliferation, capillary formation and regenerative ability both in-vivo and in-vitro by promoting neovascularisation. [16-18]. Besides, stem cells were found to possess many characteristics of pericytes that are involved in angiogenesis [19-21]. The involvement of CD146 in endometrial stem cells and angiogenesis is signified here. While the connection between CD146, stem cells, and pericytes in relation to their angiogenic potential is being explored in various contexts, no Indian scientist has yet delved into the study of stem cell-pericyte interactions and their molecular mechanisms for treating vascular diseases. Thus, we intend to explore the role of endometrial stem cells supplemented or not with optimized concentrations of soluble CD146 and their isoforms for vascular regenerative applications.

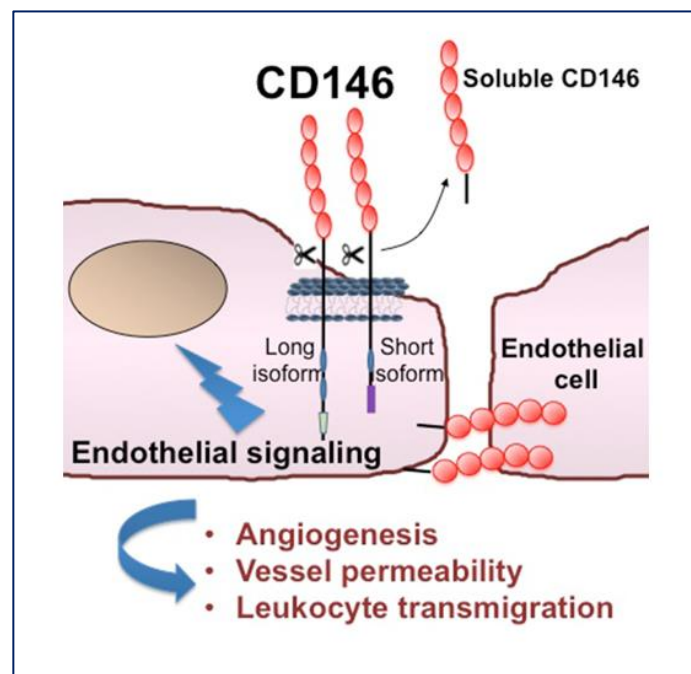


Fig. 1 Schematic representation of CD146 isoforms and their roles in endothelial signaling. The long and short isoforms of CD146 are depicted on the endothelial cell surface, with soluble CD146 generated through proteolytic cleavage. CD146 signaling is involved in key endothelial functions, including angiogenesis, vessel permeability, and leukocyte transmigration [14].

In addition to its physiological roles, CD146 has been implicated as a contributing factor in various pathological conditions, encompassing malignancies, autoimmune diseases, renal disorders, and endometrial-related pathologies. CD146 has been predominantly recognized as a biomarker for melanoma cells, with more than 80% of these cells exhibiting CD146 expression. [22]. Beyond melanoma, the expression of CD146 has also been documented in prostate carcinoma [23], breast carcinoma [24], pulmonary carcinoma [25], hepatocellular carcinoma [26], glioblastoma [27], endometrial carcinoma [28], among others. The biological roles of CD146 in both physiological and pathological contexts are currently under investigation.

1.3 Endometrium derived stem cells (eSCs)

Endometrium is the innermost lining of the human uterus which undergoes 400 cycles of regeneration throughout the reproductive phase of women. Endometrial stem cell is one such reason behind the dynamicity of endometrium, which is known for its high vasculature, dynamic angiogenic potential and high regenerative capacity [6,29,30]. Endometrial stem cells are reported to be safe, reproducible, and found superior to other post-natal stem cells due to their inherent angiogenic ability and their allogenic efficacy in treating diseases [31].

The endometrium is characterised by its glandular and luminal epithelial cells and a highly vascularised stroma, has attracted considerable attention from researcher investigating stem cells and progenitor cells within the tissue [12]. Endometrial stem cells have demonstrated superiority over the bone marrow derived stem cells and recognised for safe, reproducible and effective allogenic product that can be delivered through practical administrative system. eSCs have been noted for their immunomodulatory properties in comparison to other type of stem cells. These cells exhibit rapid proliferation in-vitro, possess anti-inflammatory properties, enhance wound healing and mitigate fibrosis.

CD146 have characteristic features of proliferation and multipotent differentiation ability, thus highly expressed in fresh stromal cells. In India, there has been no significant research stating stem cell and pericyte interactions for treating vascular diseases. [32,33]. There has been no significant research at national level where the interaction of stem cell pericyte and molecular mechanism in treating vascular diseases.

Thus, we intend to explore the role of CD146 and eSCs for vascular regenerative applications. Human endometrium sample obtained easily via hysterectomy is a medical waste, which could be used to isolate stem cells and convert this trash source to a treasure for its regenerative applications. It is a dynamic source which has a different physiologic angiogenic orientation unlike any other post natal stem cells. Thus, we propose that CD146 might activate endometrial stem cell proliferation and angiogenesis and might promote vascular regeneration of endometrial stem cells.

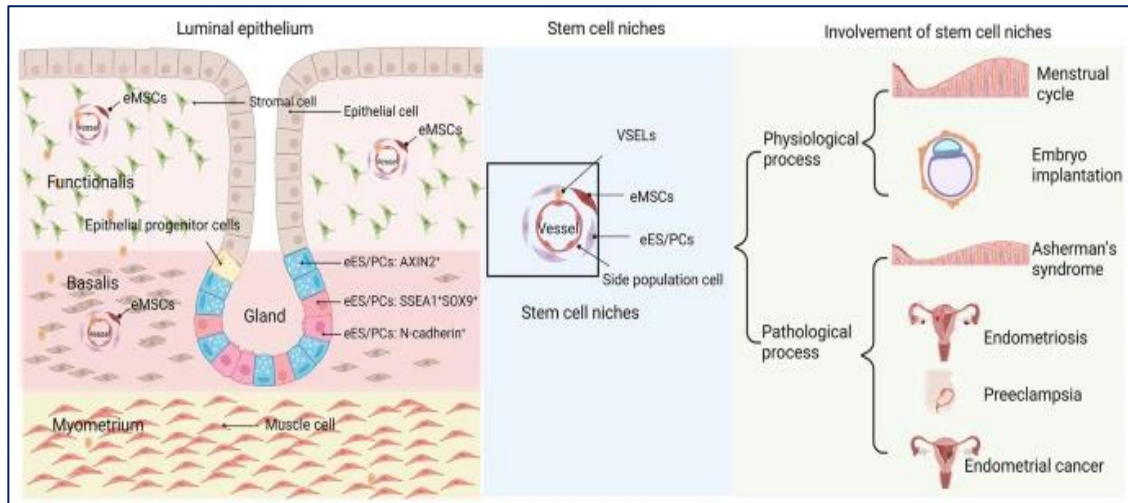


Fig. 2 The illustration shows the structural organization of the endometrium and the localization of endometrial stem cells. These stem cells reside specifically in the basalis layer, functioning as niche-specific cells. eSCs have a crucial role in various physiological processes, including the menstrual cycle, embryo implantation, and pregnancy support. However, they are also implicated in the development of endometrial pathologies [31].

1.4 Endometrial carcinoma

Dysfunctioning of endometrium results in several disorders like endometriosis, endometrial hyperplasia etc due to disrupted cellular and molecular mechanisms. Though, most of the endometrial dysfunctions arise as a result of endocrine disturbance, it has a major pathological role associated with angiogenesis. Endometriosis is a multifactorial disease where endometrium grows outside of uterus [34], whereas endometrial hyperplasia (EIN) is a precancerous stage of endometrioid adenocarcinoma (type I), [10] with a common angiogenic progression profile.

Endometrial cancer, which is often abbreviated as EC, is defined as the malignancy that originates in the innermost layer of the uterus, a tissue acknowledged as endometrium. This specific type of cancer has emerged as the most frequently diagnosed gynecological

malignancy, particularly prevalent in developed nations, and it ranks as the sixth most commonly occurring cancer among women on a global scale [36]. There exist two primary histopathological classifications of endometrial carcinoma, namely Endometrioid Adenocarcinoma, designated as Type I, and Serous Carcinoma, classified as Type II; it is noteworthy that Type I EC is the predominant form, comprising approximately 80% of all diagnosed cases, exhibiting a favorable prognosis, and is markedly influenced by estrogen levels [37]. In contrast, Serous Carcinoma is significantly less prevalent but aggressive type, accounting for less than 10% of all instances of endometrial cancer, characterized by its independence from estrogen, its aggressive nature, and a prognosis that is considered to be considerably poor [38]. Endometrial carcinoma represents the predominant gynecological malignancy within developed nations, predominantly impacting postmenopausal females [39]. This neoplasm arises in the endometrium, which constitutes the innermost lining of the uterus, and is distinguished by aberrant cellular proliferation that possesses the capacity to infiltrate adjacent tissues and possibly disseminate to various regions of the body. The frequency of endometrial carcinoma has exhibited a consistent upward trajectory over recent decades, a phenomenon largely ascribed to escalating rates of obesity, enhanced life expectancy, and alterations in reproductive behaviours [40].

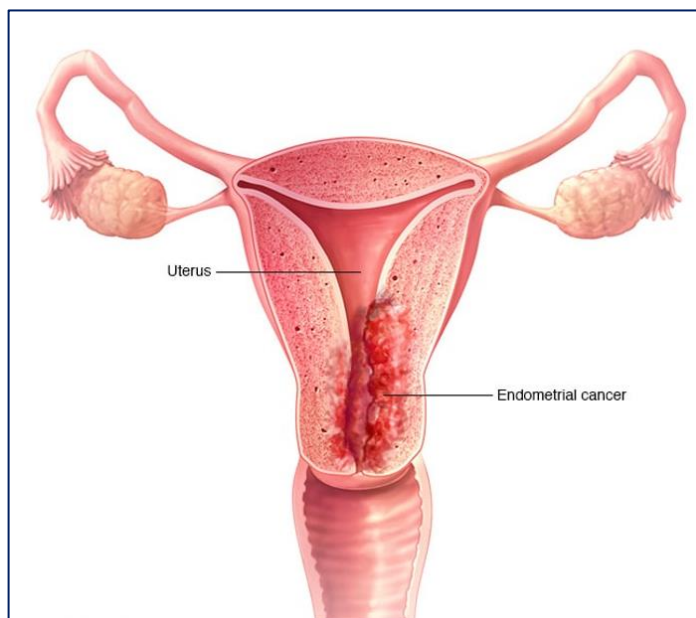


Fig.3 Illustration of the female reproductive system highlighting endometrial cancer. The cancer develops in the lining of the uterus (endometrium), shown in red, demonstrating its location and potential spread within the uterine cavity [43].

The risk factors associated with the onset of this malignancy encompass obesity, diabetes mellitus, hypertension, nulliparity, delayed onset of menopause, and exposure to

unopposed estrogen [41]. Endometrial carcinomas are broadly categorized into two principal types: 1. Type I (endometrioid): This variant is more prevalent, estrogen-dependent, and generally correlates with a more favorable prognosis. 2 Type II (non-endometrioid): This variant is less frequent, more aggressive, and typically does not correlate with estrogen exposure [42]. The early detection of this malignancy is imperative for enhancing clinical outcomes, as the condition is frequently identified at an incipient stage due to atypical vaginal bleeding, which serves as a prevalent presenting symptom. The diagnostic protocols generally encompass endometrial biopsy, transvaginal ultrasonography, and, in specific instances, hysteroscopy. The therapeutic modalities for endometrial carcinoma are contingent upon the stage and grade of the malignancy, in addition to individualized patient considerations [43]. The standard therapeutic approach typically involves surgical intervention (hysterectomy along with bilateral salpingo-oophorectomy), which may subsequently be complemented by adjuvant therapies such as radiotherapy, chemotherapy, or hormonal therapy in instances of advanced disease. Investigating the molecular mechanisms that underpin the development and progression of endometrial carcinoma represents a vibrant field of research, with promising implications for the advent of targeted therapies and personalized treatment modalities.

CD146 is a cell adhesion molecule, was originally identified as marker in Melanomas including endometrial carcinomas. The research indicates that, CD146 expression is significantly elevated in cancerous tissue compared to benign conditions like endometrial hyperplasia and endometrial polyp with a statistical difference $p < 0.05$. the expression levels of CD146 is associated with the clinical characteristics of endometrioid adenocarcinoma specially with the histological grade and depth of invasion. This suggests that CD146 may involve in tumor's aggressiveness and progression [28]. Given association with critical clinical parameters, CD146 proposed as potential biomarker for endometrial adenocarcinoma. This means, it could potentially aid in the diagnosis and assessment of the disease providing novel insights in treatment.

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Introduction:

In the previous chapter, we dealt with the basic ideology and reasoning behind this study. We got to know that angiogenesis is a dynamic process and is required during wound healing, fighting off infection, tissue growth, repair, and homeostasis. To carry out angiogenesis, many processes at molecular orchestrate together, for example, activation of pericytes and endothelial cell proliferation. Overall, angiogenesis is a process in which formation of new cells and new tissues take place. This is possible only when stem cells are directly involved or their secretions help in carrying out cells signalling processes and enhance the activation and formation of endothelial cells and blood vessels, respectively. We are in quest of analysing those cells and determining their true nature and role in angiogenesis. This will help in discovering its role in various vascular related diseases. Wound healing is another process in which MSCs play an important when there is a tissue injury. Therefore, in this study, we have not only tried to characterize the endometrial tissue derived MSCs but also analysed its angiogenic potential. These processes are led by certain intrinsic protein that mesenchymal stem cells (MSCs) inherit, therefore, in this study we have tried to unravel the role of CD146 in carrying out the process of angiogenesis in eSCs. In this chapter, we will /learn how far this research has already been conducted and what is already known or what has recently been known.

2.1 Angiogenesis and vascular diseases

Vascular diseases are mainly associated with the angiogenesis which involves formation of new blood vessels from existing ones [1]. As aforementioned, angiogenesis is crucial for the normal physiological processes, however, both inadequate and excessive angiogenesis can contribute to various disorders [2]. This necessitating the need of novel therapeutic strategies to modulate this process. Vascular diseases have become life threatening and cause of high morbidity in India. Despite several methods of treatment, there are minimal or at times no benefits to the patients.

Stem cell based therapeutic approach in vascular regeneration has gained interest in recent years. Treating vascular diseases through vascular regeneration approach by therapeutic angiogenesis to restore perfusion is underway [3]. Although several strategies have been used in both pre-clinical and clinical settings to treat vascular diseases including the angiogenic growth factor therapy with VEGF, FGF, or HIF-1 α ,

the vascular regeneration has not shown much effect [4-6]; due to short half life of recombinant growth factors in the body. Bone marrow mononuclear cells [7, 8], G-CSF mobilized peripheral blood mononuclear cells [9, 10] and endothelial progenitor cells [11, 12] are currently used for treating vascular diseases. However, extraction of bone marrow and adipose tissue involves a tedious, invasive procedure coupled with certain limitations [13]. Mobilization of G-CSF might lead to thrombosis [14]. Therefore, the addition of other cells capable of secreting growth factors and stabilizing endothelial cells has the advantage of delivering growth factors in a controlled and sustainable manner.

CD146 has been shown to display angiogenic properties and promote neovascularization in pre-clinical models [15, 16]. Stem cell therapies with an approach of neovascularization have so far included bone marrow [17, 18], dental pulp [19], adipose [20] and circulating stem cell [21]. Human endometrium sample obtained easily via hysterectomy is a medical waste, which could be used to isolate stem cells and convert this trash source to a treasure for its regenerative applications. It is a dynamic source which has a different physiologic angiogenic orientation unlike any other post natal stem cells. Evidences showing the regeneration of functional endometrium in women who underwent endometrium ablation have been reported [22]. Thus, a better therapeutic option that is safe, reliable, reproducible, cost-effective and non-invasive is required to reduce this morbidity. Thus, with the existing disadvantages in vascular regeneration coupled with the dynamic properties of endometrial stem cells, we postulate that endometrial stem cells could be an ideal source for vascular regeneration.

2.2 Existing research on endometrial stem cells (eSCs) and vascular disease

The link between endometrial stem cells and vascular diseases has gain significant attention in regenerative medicine [23]. Human endometrial stem is gaining more interest because of its different physiological angiogenic orientation than any other postnatal stem cells. They are known for its high vasculature, dynamic angiogenic potential and high regenerative capacity throughout reproductive cycle of women [22]. It undergoes more than 400 cycles of regeneration throughout the menstrual phase of women. Thus, endometrial stem cell have been found to be superior than bone marrow mesenchymal stem cells and are found to be safe, reproducible and an effective allogenic product with a practical delivery system [24].

The other salient feature is that it could be easily obtained via hysterectomies which are considered as a waste product. Endometrial stem cells derived from endometrium tissue are found to be superior due to their tissue remodeling capacity, inherent angiogenic ability and their allogeneic efficacy in treating diseases. As endometrium consists of glandular and luminal epithelial cells also have significant vascularized stroma, many researchers have focused on exploring the mesenchymal /progenitor cells of endometrium [25]. Endometrial regenerative cells (ERC) have demonstrated a greater efficacy compared to bone marrow stem cells and have been acknowledged as consistent and effective allogenic therapy with a feasible delivery approach [26]. On clinical aspects, regenerative endometrial cells of menstrual blood can be used for treating multiple sclerosis and potential therapeutic treatment of several cardiac diseases [27]. Santamaria X [28] used the animal model to study the potential of eSCs to treat the type 1 diabetes. The experimental group of animal treated with eSCs showed stabilization of glucose. The eSCs have the advantage to stabilizing endothelial cells as well as having the capacity to secrete growth factor, delivering them in a controlled manner. A study has revealed that human endometrium derived stem cells could be the good source of adult stem cells for vascular regeneration as they induce spontaneous angiogenesis enriching the number of endothelial progenitor cells when cultured with bioglass extracts [29]. Chan RW brings out the first evidence of clonogenicity of endometrial stem/ progenitor cells. He confirmed that, human endometrial cells were more clonogenic than epithelial cells and has ability to form large and small colonies depend on colony morphology and size [30, 31].

The recent studies indicate that endometrial stem cells may serve as a potentially valuable therapeutic modality for treatment [23, 32]. In experimental model of Peripheral artery disease (PAD), the transplantation of endometrial cells demonstrates the significant efficacy to promote angiogenesis and augmented perfusion in the affected fore further extremities [33,34]. Similarly, in ischemic heart diseases, endometrial stem cells exhibit superior angiogenic potential compared to the bone marrow mesenchymal stem cells. Endometrial stem cells promote cardiac repair and function by enhancing myocardial metabolism and angiogenesis as they possess greater proliferation and migration ability. Mechanistic assessment from the experimental model reveals, upregulation of angiogenic regulatory factors such as angiopoietin-1, FGF-9, fms like tyrosine kinase. This indicates that endometrial stem cells have ability for cardiac repair primarily through angiogenic effects rather than direct differentiation

into cardiomyocytes [35]. Further investigation explored the differentiation efficacy of eSCs into endothelial like cells, the building blocks of blood vessels. This ability to differentiation offers significant potential to treat cardiac related vascular disorders [36-38].

Pericytes, first identified over a century ago by Charles Rouget and also known as Rouget cells, are perivascular cells that surround blood capillaries. They are sometimes called mural cells or vascular smooth muscle cells (vSMCs). Pericytes are essential for stabilizing endothelial cells, as they regulate vascular growth, maturation, and remodeling, playing a key role in angiogenesis.

The newly isolated endometrial cells characterised by elevated expression of CD146, a recognised pericyte marker [39]. These cells exhibits capabilities for both multipotent differentiation and proliferation. This underscores the significance of CD146 in the context of endometrial stem cell functionality and the process of angiogenesis [40]. CD146, PDGF-R β stromal cells in both the functionalis and basalis layers of human endometrium through perivascular staining signifies that MSCs are pericytes. [41]. The perivascular staining by CD146 and PDGF-R β supports the possibility that MSC are pericytes. A study of mouse endometrium provides further evidence that MSC are perivascular, identifying stromal label-retaining cells, putative MSC, to a perivascular location [42]. CD146⁺ PDGF-R β ⁺ cells consist of 1.5% of endometrial stromal cells and identified the perivascular region in-vivo in both functionalis and basalis layer of endometrium [43]. A study was done where Human endometrial perivascular cells positive for CD146 and PDGFR β ⁺ induces and promotes repair of uterine injury in rat. Endometrial perivascular cells (CD146⁺PDGFR β ⁺) transplanted to the rat uterus overexpress CYR61 (Cysteine rich angiogenic inducer 1 plays vital role in vascular development) on structural and functional regeneration in rat models [44]. This highlights the function of CD146 in angiogenesis derived from endometrial stem cells. Thus, endometrial perivascular cells having dynamic properties mentioned above could be the ideal source for vascular regeneration.

The existing body of research underscores the intricate interplay between endometrial stem cells and CD146. The regenerative potential, angiogenic capabilities, immunomodulatory effects, and potential for endothelial differentiation position eSCs as multifaceted tools in the arsenal against vascular disorders. As ongoing research

continues to unveil the mechanisms and therapeutic applications of eSCs, the bridge between endometrial biology and vascular health holds the promise of innovative treatments that offer holistic solutions to the complexities of vascular diseases.

2.3 CD146

Recently, pericytes have been shown to play an efficient role in pathological and physiological angiogenesis as they migrate through basal lamina, a necessary step for stem cell therapy [45]. Pericytes are crucial for the stabilization of endothelial cells, as they control vascular growth, maturation, and remodeling, playing an essential role in angiogenesis. CD146, a marker important for pericyte-endothelial interactions, has been identified to have angiogenic properties and facilitate the formation of new blood vessels (neovascularization). [46]. In a recent study, scientists have shown that, CD146 constitute a new growth factor that stimulates angiogenesis in-vitro as well as in-vivo. Thereby, displays chemotactic activity on endothelial cells enhancing their angiogenic property as well as upregulates pro-angiogenic genes. Indeed, the in-vivo local injection of soluble form of CD146 boosts the blood flow in hind limb ischemia model of rat. A study conducted by Kebir A et al [47] established that, among the two isoforms of recombinant soluble CD146 viz. short and long isoforms, short isoforms of CD146 plays a key role in initial steps of angiogenesis.

Different studies have established a link between adult mesenchymal stem cells (MSCs) and pericytes for vascular regeneration. Pericytes are reported to behave as stem cells in the tissues, and give rise to these progenitor cells [48]. Pericytes are thought to migrate through the basal lamina and this migration is required for pericytes to activate their stem cell capacity [49]. They observed that the vasculogenic tubes were stabilized only in the presence of pericytes but not hMSCs [50]. Studies on adipose derived pericytes led to the finding that they are stem cell-like cells and possess better property than adipose stem cells [51]. Mendel TA et al. [52] had proven that pericytes isolated from adipose stem cells may represent an innovative cellular therapy for diabetic retinopathy and other retinal vascular diseases using multiple pre-clinical models of retinal vasculopathy. The fresh endometrial stromal cells also possess the markers of CD146 co-expressing both markers of pericytes (CD146⁺ PDGF-R β ⁺ cells) were discovered to have enriched colony-forming cells, expressing key MSC phenotypic markers and were multipotent, differentiating into four mesenchymal lineages in vitro [39]. Besides, their

phenotypic and lineage specific characterization have been demonstrated [40]. Although the significance of CD146 in endometrium is highlighted, its role in endometrial stem cells and angiogenesis pathway focusing towards vascular regeneration is uncertain.

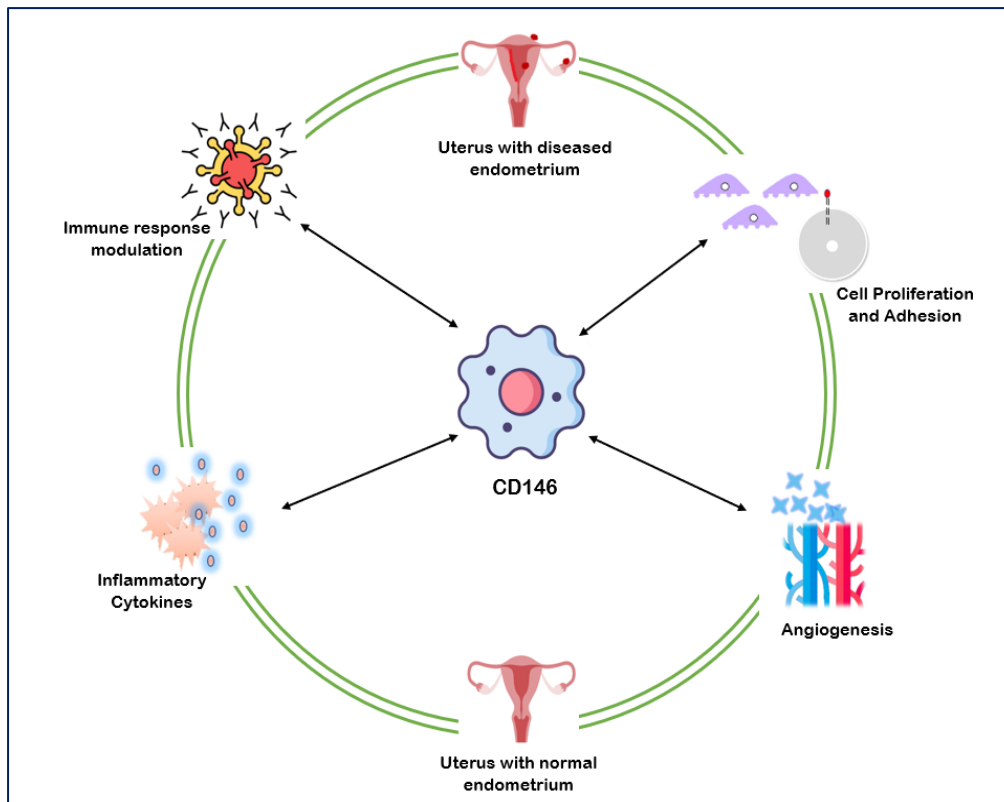


Fig. 1 The pivotal role of CD146 in regulating key processes

Unlike physiology, recent findings have been shown that CD146 is not only cell adhesion molecule but also serve as surface receptor for various growth factors and extracellular matrix. This dual role of allows CD146 to participate in cell signalling pathways that influences tumor behaviour [53]. Overexpression of CD146 is frequently observed in majority of malignancies and is implicated in nearly all stages of cancer development and progression [54]. CD146 induces activation of VEGFR-2 phosphorylation and AKT/p38 MAPK signalling pathway which encourages endothelial cell migration and micro-vessel formation in tumor and stabilizing tumor angiogenesis. It also regulates pro-angiogenic genes such as interleukin-8, ICAM-1 and MMP-9 in response to stimulation by tumor secreting factor. In addition to this, CD146 activates epithelial mesenchymal transition by interacting with extra cellular matrix leading remodelling of tumor environment and enhanced metastasis [55]. This signifies the pivotal role of CD146 in pathology. The absence or blocking of CD146 inhibits the proliferation, migration and tube formation of HUVEC cells as well as the activation of angiogenic pathways VEGFR2, p38 and ERK ½. This interaction between CD146 with

different pathways in different diseases has gain interest in its potential of diagnosis, prognosis as well as in targeted therapy. Recent studies reveal that CD146 targeted therapy represents a promising approach in cancer treatment particularly in solid tumors such as glioblastoma [56], breast cancer [57] etc. CD146 targeted therapy involves using an anti-CD146 monoclonal antibody to reverse immunosuppressive effect in tumor. Joshkon A observed, when this therapy combined with anti-VEGF α antibody there was potential elimination of tumor and also prevents immune escape [58]. CD146 Inhibition would effectively prevent cell state transition during the onset and progression of disease. Thus developing therapies that target CD146 carries considerable significance.

2.4 CD146 and Endometrial carcinoma

As CD146 is highly expressed surface marker in metastatic lesions and other type of cancers, it can be used as potential tumor diagnosis, prognosis and treatment. [59,60]. Mills L et al [61] demonstrated the inhibition of growth and metastasis of MUC/MCAM in nude mice by neutralizing anti-MUC18 Ab (ABX-MA1) thereby suppressing homotypic and heterotypic interactions, angiogenesis, and invasion. In one study, the authors showed that an anti-CD146 antibody, mAb AA98, displayed antiangiogenic properties in chicken chorioallantoic membrane assays and inhibited tumor growth in different xenografted human tumor models in mice. In a model of human umbilical vein endothelial cells (HUVECs), it was also shown that silencing CD146 with specific siRNA inhibited the proliferation and migration of the cells [62, 63]. The study from Wang P et al [64] reported a novel angiomiR, miRNA-329, and its negative correlation with CD146 expression in angiogenesis. These in vitro and in vivo studies introduce an appealing new way of treating angiogenesis by targeting CD146.

Endometrial stem cells have the double-edged sword approach as they have the efficacy to treat vascular diseases as well as they are culprit in the pathophysiological diseases such as endometriosis, endometrial hyperplasia and carcinoma due to their dysregulatory mechanism [65, 66]. There are several factors like miRNA, VEGF, macrophages inhibitors are responsible in etiology of endometriosis [67]. Endometriosis, an estrogen dependant disease where estrogen may stimulate angiogenesis by increasing VEGF expression [68]. Endometrial hyperplasia is a condition characterized by the thickening of the endometrial lining due to an imbalance between estrogen and progesterone often display as abnormal uterine bleeding and can lead to endometrial carcinoma if untreated

[69]. endometrial hyperplasia, particularly atypical hyperplasia is a premalignant condition with a strong association to endometrial carcinoma 25-45% [70]. Endometrial carcinoma (EC) is prevalent malignancy of female reproductive system, particularly in developed countries. It primarily affects postmenopausal women, although the significant proportion is diagnosed before menopause. In western world, with an annual incidence estimated at 10-20 per 100,000 women is affected with endometrial carcinoma [71]. Considering Indian scenario, S. Agarwal proves, about 82% of the Indian patients were reported to have endometrial carcinoma [72].

CD146 was originally identified as marker in Melanomas. In addition to that, CD146 was found to be overexpressed in several other carcinomas like Epithelial Ovarian Cancer [73], Clear Cell Renal cell Carcinoma [74], Gastric Cancer [75], Triple negative Breast Cancer [76] etc. however, its expression in endometrial cancer was unclear. For this, Zhang et al studied 80 endometrioid Adenocarcinoma samples and 7 non Endometrioid Adenocarcinoma samples for the expression level of CD146 against AA4 antibody, showed positive correlation of CD146 expression with the myometrial invasion in endometrial cancer. CD146 was detected in majority of tumor cells and vascular endothelial cells at higher level as compared to corresponding control, which suggests that CD146 may be involved in the onset and development of endometrial cancer [77]. Zhou Y et al also demonstrated the overexpression of CD146 along with P53, and Ki-67 in Uterine Sarcoma, signifying correlation of lymph node metastasis and poor overall survival of leiomyosarcoma [78]. A retrospective study done by Kikalishvili N, revealed that as grade of malignancy increases, the proliferation/stem cell index also increases [79]. In addition to this, there is a rare condition called Epithelioid trophoblastic tumors (ETTs), a gestational trophoblastic Neoplasia, also has focally positive expression of CD146, proved immunohistologically by Li J et al. [80]. Though aforementioned studies throw a light on targeting CD146 in order to suppress tumor angiogenesis and eventually tumor regression, there are no any references regarding role of CD146 in causing pathoangiogenesis derived endometriosis, endometrial hyperplasia etc. as well as targeting CD146 at initial precancerous stages to prevent its further progression.

This study explores the function of CD146 in eSCs, and the significance of CD146 pathway within both healthy physiologic and pathological samples of endometrial tissues. Using CD146 mimics, which would improve the process of angiogenesis within

healthy endometrial tissues, this study proposes the use of inhibitors to ablate the dysfunctional process of angiogenesis in disease-prone endometrial tissues. The underlying hypothesis is that CD146 could serve as a critical regulator in two key aspects: first, as a boosting agent that enhances endometrial stem cells for vascular regeneration, and second, as a therapeutic target for overcoming or treating endometrial carcinoma. Understanding mechanistic contributions of CD146 through this research is aimed at illuminating potential therapeutic strategies for endometrial disorders.

Considering the research problem and relevant literature, This study has following aims and objective:

AIM:

1. To assess the angiogenic potential of CD146⁺ endometrial stem cells
2. Utilizing a CD146 inhibitor to suppress pathological angiogenesis.

OBJECTIVES:

1. To isolate and characterize endometrial stem cells
2. To sort CD146⁺ endometrial stem cells and assess for their angiogenic potential.
3. To check the prognostic significance of CD146 in endometrial carcinoma
4. To inhibit the CD146 using M2J-1 mAb in endometrial carcinoma.

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3.1 Introduction

The endometrium in humans, forming the uterus's inner layer, exhibits an impressive ability to regenerate, happening over 400 times during a woman's reproductive years. This extraordinary regenerative phenomenon is underpinned by the presence of endometrial stem cells (eSCs), which significantly augment the tissue's intricate vascular architecture, distinguished angiogenic capabilities, and robust regenerative potential [1]. eSCs are regarded as a reliable, safe and superior alternative to other adult stem cells, attributable to their inherent angiogenic characteristics and efficacy in addressing vascular pathologies [2]. CD146, a pivotal marker linked to pericytes and endothelial cells, has recently attracted scholarly interest due to its critical involvement in facilitating angiogenesis [3]. Endometrial cells exhibiting elevated CD146 expression possess the capacity to proliferate and differentiate into diverse cell lineages [4]. The objective of this investigation is to elucidate the role of CD146 within eSCs, highlighting its prospective to enhance the angiogenic as well as regenerative functions of these cells. The originality of this research resides in its analysis of CD146's influence on eSC functionality, thereby offering novel insights into their therapeutic potential within the realms of regenerative medicine and vascular diseases.

3.2 Materials and Methods

3.2.1 Sample Collection

Normal endometrial samples were procured from D. Y. Patil Hospital and Research Center in Kolhapur after receiving ethical approval. Participants were made aware of the study's objectives, and written consent was secured prior to the collection of samples. All specimens were delivered to the laboratory in sterile containers with a transport medium. Normal endometrial biopsies were obtained from reproductively active women (n=3) aged 20, 30, and 45 years, all of whom had no prior history of endometrial disorders. Each sample was processed within two hours post-collection.

3.2.2 Isolation and culture of endometrial stem cells

Endometrial tissue was meticulously scraped from the underlying myometrium following the hysterectomy procedure. The obtained tissue was rinsed with Dulbecco's phosphate-buffered saline (DPBS) (Gibco, Cat. No. 10010023) containing antibiotics (Gibco, Cat. No. 15240096). The rinsed tissue was subsequently incubated with 0.25%

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trypsin (Gibco, Cat. No. 25200056) at 37°C for 15 minutes in a humidified atmosphere with 5% CO₂. To stop the action of trypsin, Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Cat. No. 11965092) enriched with 10% fetal bovine serum (FBS) (Gibco, Cat. No. 26140079) was added. The resulting cell suspension was washed two times with DPBS through centrifugation at 450g for 10 minutes to eliminate any remaining trypsin and cellular debris. The cell pellet obtained was then re-suspended in complete DMEM, seeded, and cultured in a T-25 flask (Nunc™ EasYFlask™ Cell Culture Flasks, Cat No. 156367). Cells were incubated at 37°C in a humidified 5% CO₂ environment and passaged every 3-5 days. To reduce contamination by somatic cells, endometrial stem cells (eSCs) were cultivated in growth media supplemented with fibroblast growth factor (FGF) (Sigma-Aldrich, Cat. No. F3685) and epidermal growth factor (EGF) (Sigma-Aldrich, Cat. No. E9644), which supported their proliferation and maintenance.

3.2.3 Flow Cytometry Analysis of Endometrial Stem Cells

3.2.3.1 Sample Preparation:

Following passage five, cells were harvested using 0.25% trypsin and fixed in IC fixation buffer. The cells were then thoroughly washed and incubated with specific antibodies (Table 3.1) at the suggested concentrations in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA). The incubation was carried out for 45 minutes at room temperature.

Table 3.1: list of antibodies used for surface marker analysis

Sr. No.	Antibody	Isotype	Host	Dilution	Make and Cat. no.
1.	CD29 FITC conjugate	IgG1	Mouse	0.25ug/test	Invitrogen, 11-0299-42
2.	CD56 PE conjugate	IgG1	Mouse	0.125ug/test	Invitrogen, 12-0567-42
3.	CD117 FITC conjugate	IgG1	Mouse	20 µL/1x10 ⁶ cells	Invitrogen, MA1-19597

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4.	CD90 FITC conjugate	IgG1	Mouse	1ug/test	Invitrogen,11-0909-42
5.	CD 73 FITC conjugate	IgG1	Mouse	0.25ug/test	Invitrogen, 11-0739-42
6.	CD105 PE conjugate	IgG1	Mouse	1ug/test	Invitrogen, 12-1057-42
7.	CD13 PE conjugate	IgG1	Mouse	0.25ug/test	Invitrogen,12-0138-42
8.	CD44 PE conjugate	IgG2	Rat	0.125ug/test	Invitrogen,12-0441-82
9.	CD140b PE conjugate	IgG2	Mouse	20ul/test	BD Biosciences, 558821
10.	CD146 PE conjugate	IgG2	Mouse	20ul/test	BD Biosciences, 561013
11.	CD166 PE conjugate	IgG1	Mouse	0.06ug/test	Invitrogen,12-1668-42
12.	CD106 PE conjugate	IgG1	Mouse	0.125ug/ml	Invitrogen, 12-1069-42
13.	HLA-DR FITC conjugate	IgG1	Mouse	0.125ug/ml	Invitrogen,11-9956-42
14.	CD34 PE conjugate	IgG1	Mouse	1:50	Cell Signaling technology, #79253
15.	CD45 PE conjugate	IgG1	Mouse	0.2mg/ml	Biolegend, 202207
16.	CD14 PE conjugate	IgG1	Mouse	1:20	Cell Signaling technology, #59896
17.	CD19 PE conjugate	IgG1	Mouse	10 μ L/ 10^6 cells	R&D Systems, FAB4867P

3.4.3.2 Data Acquisition and Analysis

Following antibody incubation, cells were washed with PBS and resuspended in PBS for flow cytometry analysis. A minimum of 10,000 events were acquired for each sample. Data acquisition and analysis for cells stained with CD44, CD133, and CD146 were performed using BD FACS Diva software. Data for CD73, CD90, and CD105 were acquired using MACSQuantify™ software and analyzed with FlowJo™ v10 software.

3.2.4 Growth Factor analysis of eSCs

This experiment used the LEGENDplex™ Human Growth Factor Panel (Cat. No. 741282) to comprehensively evaluate growth factor secretion from ESCs. The cells were cultured in serum-free DMEM (Gibco, Cat. No. 11965092) for forty-eight hours before the cell culture supernatant was collected. The supernatants were appropriately diluted and processed concomitantly with the manufacturer's instructions. The 96-well plate (Nunc™ MicroWell™ 96-Well Microplates, Cat No. 260887) was prepared with the reconstituted beads, detection antibodies, and examined samples subsequent to an incubation period. Analysis was performed using the Attune NxT acoustic focusing cytometer, which is designed to simultaneously flow cytometrically detect 13 human growth factors in a single sample: Angiopoietin-2 (Ang-2), EGF, EPO, FGF-basic, G-CSF, GM-CSF, HGF M-CSF, PDGF-AA, PDGF-BB, SCF, TGF- α , and VEGF.

3.2.5 Angiogenesis PCR array

The angiogenesis PCR array for HUVECs and cultured eSCs was conducted using the RT² Profiler PCR Array Kit (Qiagen, Cat. No. 330231) and RT² First Strand Kit (Qiagen, Cat. No. 330404), following the manufacturer's protocol. RNA was isolated from HUVECs, and cultured eSCs (P3) using a RNeasy Mini Kit (Qiagen, Cat. No. 74104), and after quality control, the RNA was converted into cDNA using cDNA Reverse Transcription Kit (Applied Biosystems, Cat. No. 4368814). The cDNA was stored at -20°C until further use. For the PCR array, the SYBR Green Master Mix (Qiagen, Cat. No. 330502) was thawed, briefly centrifuged, and combined with PCR components as per the instructions. The mixture was added to a 96-well RT² Profiler PCR Array plate, sealed, and run on a QuantStudio 3 system for qPCR. Following the run, CT values were recorded, and relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Housekeeping genes, including GAPDH, ACTB, and B2M, were used for normalization.

3.2.6 Tri-Lineage Differentiation

Differentiation studies were conducted based on previously published protocols, with a brief description of each differentiation process outlined below.

3.2.6.1 Osteogenic Differentiation

Once cells reached 80-90% confluence, the complete culture medium was replaced with osteogenic induction medium, which was refreshed periodically. The osteogenic medium consisted of DMEM-LG (Gibco, Cat No. 11885084), 10% FBS (Gibco, Cat. No. 26140079), 1% antibiotic, dexamethasone (0.1 μ M) (Sigma Aldrich, Cat. No. D4902), β -glycerophosphate (10 mM) (Sigma Aldrich, Cat. No. G-9422), and ascorbic acid (2 mM) (Sigma Aldrich, Cat. No. A4544). Osteogenic differentiation was verified on day 21 using Von Kossa staining, and the stained cells were observed under a phase-contrast microscope.

3.2.6.2 Adipogenic Differentiation

Adipogenic differentiation was induced by culturing cells in adipogenic induction medium containing DMEM-LG (Gibco, Cat No. 11885084), 10% FBS (Gibco, Cat. No. 26140079), 1% antibiotic, dexamethasone (1 μ M) (Sigma Aldrich, Cat. No. D4902), isobutyl methyl xanthine (0.5 mM) (Sigma Aldrich, Cat. No. 15879), insulin (10 μ g) (HiMedia, Cat. No. TCL035), and indomethacin (200 μ M) (Sigma Aldrich, Cat. No. 17378). Differentiation was assessed on day 18 by staining the MSC monolayer with Oil Red O, a triglyceride-specific dye.

3.2.6.3 Chondrogenic Differentiation

Cells were seeded at a density of 5000 cells/cm² in a 12-well plate and incubated for 24 hours. Chondrogenic differentiation was induced using a medium composed of DMEM-LG (Gibco, Cat No. 11885084), ITS supplement (1X) (Gibco, Cat. No. 41400045), sodium pyruvate (1 mM) (Gibco, Cat. No. 11360070), dexamethasone (100 nM) (Sigma Aldrich, Cat. No. D4902), ascorbate-2-phosphate (50 mg/ml) (MedChemExpress, Cat

No. HY-103701), TGF- β 3 (10 ng/ml) (Sigma Aldrich, Cat. No. H8791), and L-proline (40 mg/ml) (Sigma Aldrich, Cat. No. P0380). The medium was replaced every two days. Control cells were cultured in standard growth medium without induction. Chondrogenic differentiation was evaluated after 28 days by staining for glycosaminoglycans (GAGs) using 2% Alcian Blue.

3.2.7 Mesenchymal marker analysis of eSCs by flowcytometry

The detailed procedure is explained in 3.2.3

3.2.8 Stemness-related genes analysis using RT-PCR

Total RNA was isolated with the Qiagen RNeasy Mini Kit (Qiagen, Cat. No. 74104) according to the manufacturer's instructions. Subsequently, it was used to synthesize cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, India, Cat. No. 4368814), based on the supplied protocol, starting with 2 μ g of RNA in a single cycle reaction at 48°C for 30 min followed by 5 min at 95°C, and kept frozen at -20°C. mRNA expression levels of Oct-4, SOX-2, c-Myc, NANOG, ALDH1A3, PPAR γ , SOX-9, Runx2, and KLF-4 were quantified using the QuantStudio™ 5 Real-Time PCR System, with GAPDH as the housekeeping gene. Specific primers (IDT) were used for amplification, and real-time monitoring was carried out with Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). The reaction was performed by using 2 μ l of template cDNA over 40 cycles of (95°C for 15 seconds, and 60°C for 1 minute). The expression levels of the target gene were normalized to human GAPDH by using the $\Delta\Delta C_t$ method. The C_t values of each gene were normalized to the mean C_t value, and RT-qPCR data were analyzed using the $2^{-\Delta\Delta C_t}$ method with relative gene expression normalized to the average C_t of the human β -actin gene.

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Table 2: list of primer sequence used in stemness- related gene analysis

Sr. no.	Primer	Forward sequence	Reverse sequence
1.	GAPDH	5'- GAGAAGGATGTGGTCCGA GTGTG-3'	5'- GGCAGATGGTCGTTTGGCTG AATA-3'
2.	Oct-4	5'- CGCCCCCAGCAGACTTCA CA-3'	5'- CTCCTCTTTTGCACCCCTCCC ATTT-3'
3.	Sox-2	5'- AGAGGAGCCCAAGCCAA AGAG-3'	5'- CGAATTTCCATCCACAGCCG TC-3'
4.	KLF-4	5'- CAGAGTGCATCGACCCCT CG-3'	5'- TTCCTCCTCAGAGTCGCTGC- 3'
5.	c-Myc	5'- TGAACCTCAGCTACAAAC AGGTG-3'	5'- AACTGCATGCAGGACTGCAG AG-3'
6.	Nanog	5'- CTCCGTGTCCAACCAGCA G-3'	5'- CACGTTGAACCACTTACAGA TGC-3'
7.	ALDH 1A3	5'TGGATCAACTGCTACAA CGC-3'	5'- CACTTCTGTGTATTTCGGCCA- 3'
8.	PPAR γ	5'-GCAGGAGCAGAGCAA AGAGG-3'	5'- CCAGGAATGCTTTTGGCATA C-3'
9.	Runx2	5'- GTCAGTGTGCTGAAGAGG CT-3'	5'- GGTTAATCTCCGCAGGTCAC- 3'
10.	SOX-9	5'- CCCCAACAGATCGCCTAC AG-3'	5'- GAGTTCTGGTCGGTGTAGTC- 3'

3.2.9 In-ovo angiogenic assay

Yolk Sac Membrane (YSM) Model (Experimental Days 4-6)

Fertilized eggs of Black australorp were received from "Government hatchery center, Kolhapur, Maharashtra". The shells of fertilized eggs were disinfected using 70% alcohol and incubated at 37.5°C in humidified egg incubator till day 5-8 according to the experiment performed. The experimental group were divided into Control, Vehicle control (PBS control) and eSCs. Using forceps, a gap was created in the egg by holding

the egg at 5 'o' clock position. Albumin was aspirated using a 2ml syringe. The gap was then sealed properly using tape and parafilm. The egg was then gently cracked from the top (blunt end) and the bubbles along with more albumin sucked out using a pipette. 25000 eSCs were then injected onto the chick vasculature and sealed by parafilm. The eggs were then sent to the incubator. In the same way, 1X PBS was injected onto other eggs as vehicle control. The experiment was performed in triplicate and the surviving embryos were further harvested for analysis.

3.2.9.1 Macroscopic analysis

After 48 hours of incubation with the treatment, the eggs were carefully opened to evaluate the angiogenic effect. Images of the vasculature in both control and treated eggs were captured using a digital camera and transferred to a computer for analysis with ImageJ software.

3.2.9.2 Histological Preparation

The YSM was carefully excised and fixed in 10% buffered formaldehyde for 10 hours, followed by dehydration in a graded alcohol series, clearing with xylene, and embedding in paraffin. Sections of 5µm thickness were cut parallel to the surface, stained with hematoxylin-eosin, and examined under a light photomicroscope.

3.2.10 Magnetic Cell sorting

The CD146 MicroBead Kit and MACS® Column (Make: Miltenyi Biotec, Cat. No. 130-093-596) were used to sort CD146⁺ cells from eSCs in accordance with the manufacturer's instructions. In a nutshell, The kit's CD146 microbeads and FcR blocking agent were used to interact with the cells. The treated cells were then exposed to a magnetic field and loaded onto a MACS LS column for separation.

3.2.11 Marker analysis of CD146⁺ cells

CD146⁺ cells were tested for CD73 (Invitrogen, 11-0739-42), CD90 (Invitrogen, Cat No. 11-0739-42), CD105 (Invitrogen, Cat. No. 12-1057-42), PDGFRb (R&D systems, Cat No. MAB1263), Wnt-5a (Novus Biologicals, Cat No. NBP2-24752F) and HLA-DR (Invitrogen, Cat No. 11-9956-42). The detailed procedure explained in 3.2.3

3.2.12 Growth factor analysis of CD146⁺ cells

The growth factor analysis of CD146⁺ cells was conducted to evaluate their secretion of growth factors. The procedure is described in 3.2.4

3.2.13. Ring formation assay

The ring formation assay was performed to evaluate the endothelial cell differentiation potential of CD146⁺ cells. 24 well plate (Nunc™ Cell-Culture 24 well plate, Cat No. 142475) was coated with 200ul of Matrigel (Make: Corning, Cat No. 354234) per well and incubated at 37°C for an hour to solidify. The CD146⁻ and CD146⁺ cells are seeded into the wells at a density of 1×10^3 cells/well and then incubated in the growth media without any exogenous growth factor or serum. Endothelial ring formation was monitored at various time points, and images were captured using a bright-field microscope. (Nikon-Eclipse TE 2000-S).

3.2.13.1 Characterization of differentiated endothelial cells

Matrigel was washed from the plates carefully and endothelial cells were harvested. RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Cat. No. 74104) following the manufacturer's protocol. The High-Capacity cDNA Reverse Transcription Kit (Make: Applied Biosystem, India, Cat. No. 4368814) was used to synthesize cDNA using 2 ug of RNA following the manufacturer's instruction. Master mix was made for KDR, Tie2, FLT1, vWF and β actin. Reactions were carried out in an Applied Biosystems™ ProFlex™ 96-well PCR equipment. The PCR machine was programmed to execute 30 cycles of 5 minutes at 95°C, 45 seconds at 55°C, and 1 minute at 72°C. In the meantime, a 2% agarose gel (UltraPure™ Agarose Cat No. 16500500) was prepared with EtBr (UltraPure™ Ethidium Bromide Cat No. 15585011), and once the gel solidified, the PCR product was loaded into the appropriate wells using a DNA ladder (100 bp DNA Ladder, Cat No. 15628019) in a different well. HUVEC cells were used as positive sample.

3.2.14 Wound Scratch assay

The migratory potential of CD146⁺ cells was assessed using a wound scratch assay. In this assay, both CD146⁻ and CD146⁺ cells were seeded at a density of 0.04×10^6 cells and cultured to about 95% confluence. A linear scratch was introduced across the cell

monolayer using a 100 µL pipette tip to make a wound. Wound closure progression was observed at specific time points: 0, 12, and 24 hours, by taking bright-field phase-contrast microscopy images. These images were used to analyze cell migration and efficiency of wound healing. The extent of wound healing was measured in terms of the remaining cell-free area at each time point in comparison with the initial wound size. The software ImageJ was used for calculating the percentage wound closure to obtain the quantitative measurement of cell migration and repair processes.

3.2.15 Chemokine analysis

A customized panel was designed for the selected chemokines using BioLegend reagents specific to human reactivity. The protocol was carried out following the manufacturer's instructions. The assay began with prewetting the plate by adding 100 µL of 1X Wash Buffer per well, followed by loading standards and samples in duplicate according to a predetermined layout. Mixed beads (25 µL per well) were then added, and the plate was sealed and incubated at room temperature for two hours. Subsequently, 25 µL of detection antibodies per well were added, the plate was resealed, and incubation continued for another hour. This was followed by two wash cycles using 200 µL of 1X Wash Buffer per well. Next, SA-PE (25 µL per well) was added without vacuuming, and the plate underwent a final incubation step. The samples were then analyzed using a flow cytometer.

3.2.16 Cytokine analysis

The cytokine analysis was performed for EGF, Endothelin 1, GM-CSF, IL-1 β , IL-6, and Angiopoietin 1 using ELISA kits sourced from Invitrogen. All procedures were conducted following the manufacturers' guidelines.

3.2.17 In-ovo angiogenic assay

Yolk Sac Membrane (YSM) Model (Experimental Days 4-6)

Chick Chorioallantoic Membrane (CAM) Model (Experimental Days 8-10)

The eggs were divided according to experimental groups viz. Control, CD146⁻ and CD146⁺ cells. For both of the models, in the case of direct injection, on day 4 of incubation, 2-3 ml of albumin was removed from the tapering end of the egg and the aperture was sealed with sterile adhesive tape. The blunt end was gently opened using

sterile forceps in an area covering the air sac with the help of sterile forceps, and according to the experimental groups, cells incorporation was done by sealing the opening with sterile adhesive tape, following the procedure mentioned above. Macroscopic analysis was also done as per 4.2.9.1 and quantified using ImageJ. The statistical analysis of quantified data was carried out using GraphPad Prism.

3.2.17.1 Immunohistochemistry

Yolk sac membrane was carefully removed and fixed tissue is fixed, dehydrated, cleared, infiltrated with paraffin wax, and finally embedded in a mold to form a solid block for sectioning. Following the sectioning, tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval using 0.01 M sodium citrate buffer (pH 6.0) (Invitrogen, Cat No. 005000) in a pressure cooker for 20 minutes at 100°C, followed by cooling it for 20-minute. To prevent non-specific binding, the sections were incubated with BSA for 60 minutes at room temperature. The sections were then incubated with CD31 (Abcam, cat No. ab28364), VEGF (Abcam, Cat No. ab2350), VAP (Santa Cruz, Cat No. sc-293278) and vWF (Invitrogen, Cat No. MA5-14029) at 1:200 dilution overnight at 4°C overnight. After incubation, the sections were thoroughly washed with distilled water containing 0.05% Tween 20, followed by a 60-minute incubation with the secondary antibody in the dark. The sections were washed again and counterstained with DAPI for 30 seconds before being mounted using a fluorescent mounting medium. For negative control, sections were processed without primary antibodies and stained only with the secondary antibody and DAPI.

3.3 Results:

3.3.1 Isolation and culture of Endometrial Stem Cells (eSCs)

The endometrial stem cells (eSCs) were obtained through the application of a trypsin digestion methodology, and the cells derived from human endometrial tissue were maintained in culture until passage 5 (Fig. 3.1). The eSCs attained confluency within five days at passage 0 (P0) and passage 1 (P1), and subsequently reached confluency within a span of three days from passage 2 (P2) onward.

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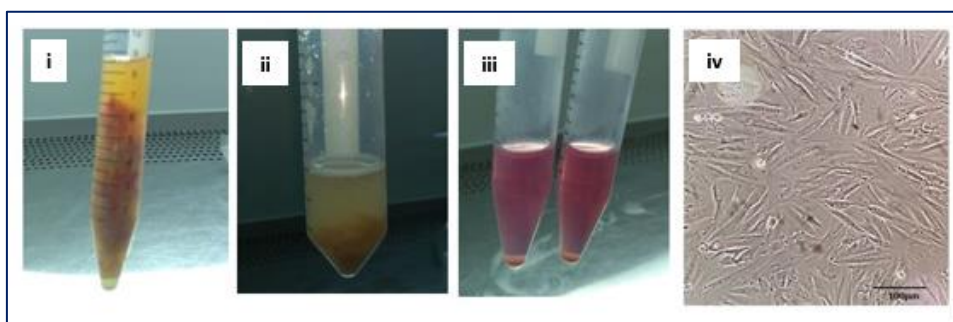


Fig. 3.1: Isolation of eSCs and culture. The biological specimen was procured from the medical facility (i), and subsequently, trypsin was employed to facilitate its digestion (ii). Centrifugation was then utilized to isolate the cellular pellet (iii), after which the separated cells were cultured in complete DMEM medium (iv). The resultant image was captured at a magnification of 10X.

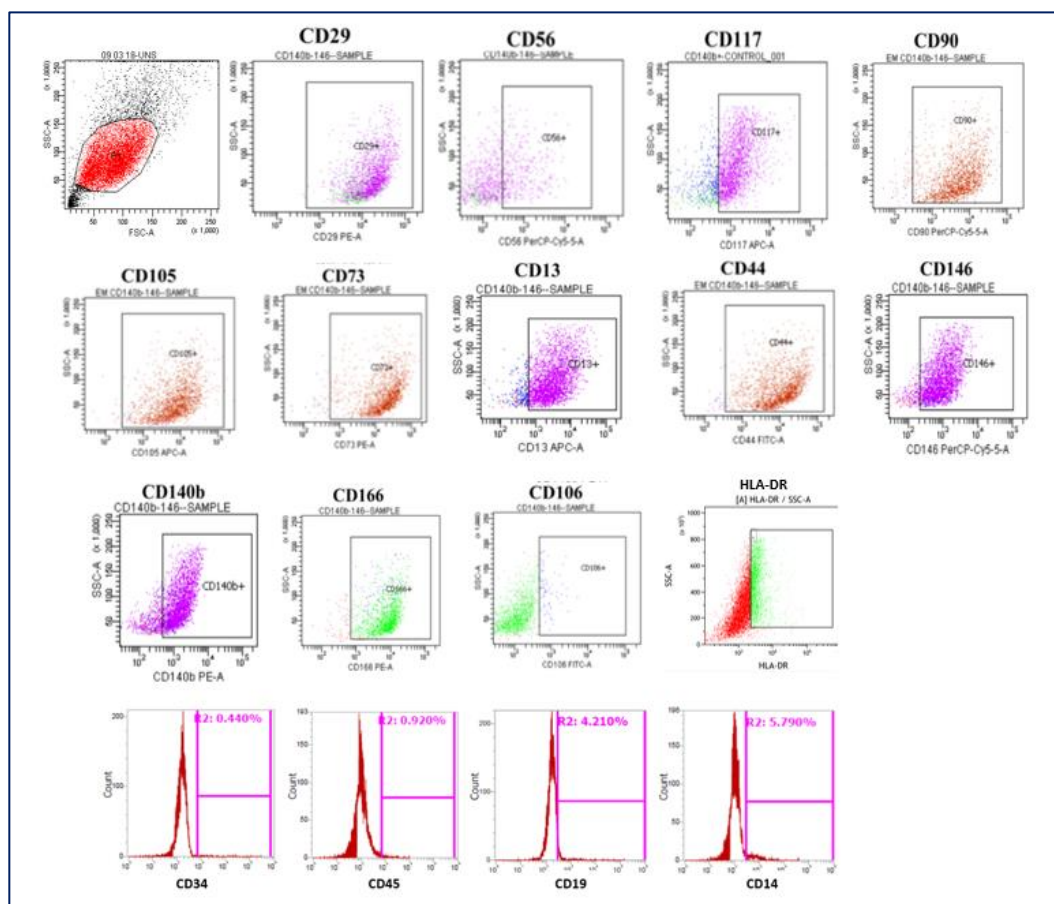


Fig. 3.2 Marker profile of eSCs. The endometrial cells exhibits significant expression of mesenchymal markers (CD90, CD105, CD73, CD140b, CD146) as well as cell adhesion proteins (CD29, CD44, CD166). Conversely, the expression levels of HLA-DR, CD34, CD45, CD14, and CD19 are negative.

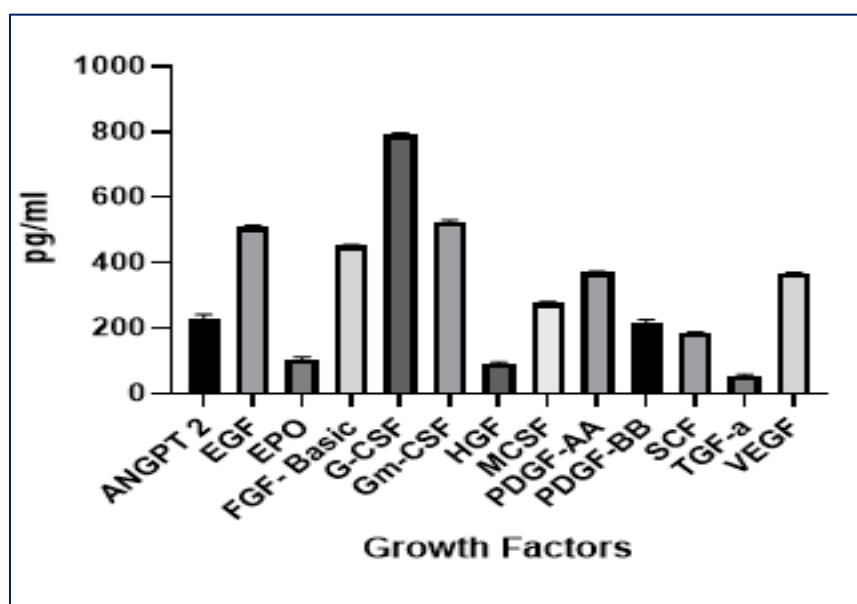


Fig. 3.3 Growth factor analysis of eSCs. In the analysis of growth factor secretion profiles, the concentrations of GM-CSF and G-CSF were observed to be elevated in comparison to other factors. This was followed by EGF, basic FGF, PDGF-AA, and VEGF, with a p-value of <0.0001.

3.3.2 Flowcytometry characterization of endometrial stem cells

The presence of endometrial stem cells (eSCs) within the perivascular niche of the endometrium, potentially exhibiting mesenchymal characteristics, prompted the characterization of endometrial cells via flow cytometry (Fig 3.2). The eSCs exhibited significant positivity for CD16 and CD140b, thereby corroborating their origin from the perivascular niche. The processes of cell differentiation, development, and fate specification are influenced by CD49. This marker plays a crucial role in cellular polarity, proliferation, adhesion, motility, survival, and apoptosis. The expression of CD90, CD73, and CD105 in eSCs signifies their mesenchymal nature. Furthermore, CD117 is essential for the processes of cell survival, proliferation, and differentiation. A marker known as CD13 is expressed on stem cells and throughout various stages of myeloid cell development, particularly during the initial phases of B and T cell maturation. The CD44 marker serves as a transmembrane adhesion molecule associated with various mesenchymal markers and cell adhesion molecules. The weak positivity of HLA-DR suggests that eSCs arise from the perivascular cells of the endometrium, as documented by Gargett. Moreover, the eSCs tested negative for hematopoietic markers

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CD34, CD45, CD14, and CD19, thereby affirming their identity as mesenchymal stem cells.

3.3.3 Growth factor analysis

The secretory profile of growth factors produced by endometrial stem cells (eSCs) is illustrated in Fig. 3.3. The secretion levels of Granulocyte-Colony Stimulating Factor (G-CSF) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) were observed to be the highest among all analyzed factors. These two factors are predominantly acknowledged for their significant immunomodulatory capabilities. In addition to these two, Epidermal Growth Factor (EGF), Basic Fibroblast Growth Factor (FGF basic), Platelet-Derived Growth Factor AA (PDGF-AA), and Vascular Endothelial Growth Factor (VEGF) exhibit substantial expression within eSCs. These factors are esteemed for their roles in enhancing cellular proliferation, facilitating cell migration, and/or promoting the development of tubular morphology. The data analysis was conducted utilizing GraphPad Prism software, yielding a p-value of <0.0001.

3.3.4 angiogenic gene array of endometrial stem cells

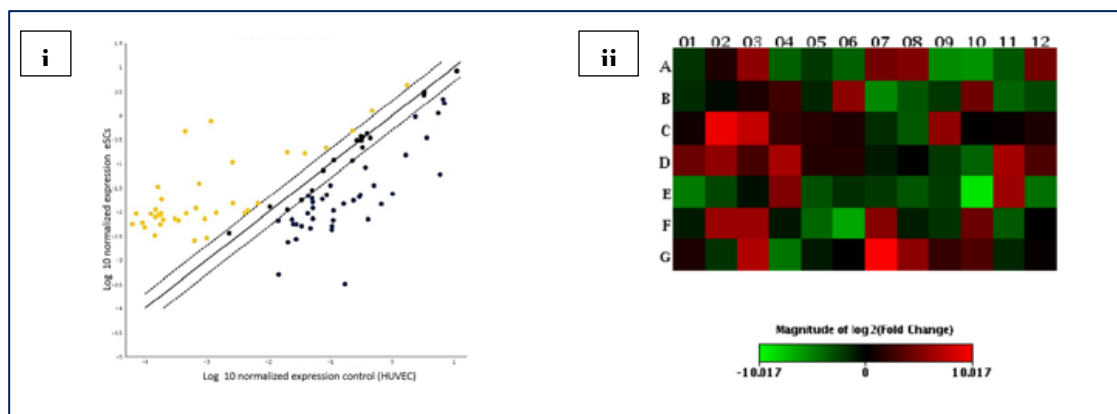


Fig.3.4 A (i) The scatter plot and heat map illustrate the expression patterns of angiogenic genes. The scatter plot compares the normalized expression of each gene across the array between two selected groups, allowing for a quick visualization of significant gene expression changes. The central line represents unchanged gene expression, while the dotted lines denote the predefined fold regulation threshold. Data points positioned beyond these dotted lines in the upper left and lower right quadrants meet the selected threshold for fold regulation. The heat map, shown in (ii), depicts the gene expression profile of angiogenesis-related genes in HUVEC and eSCs.

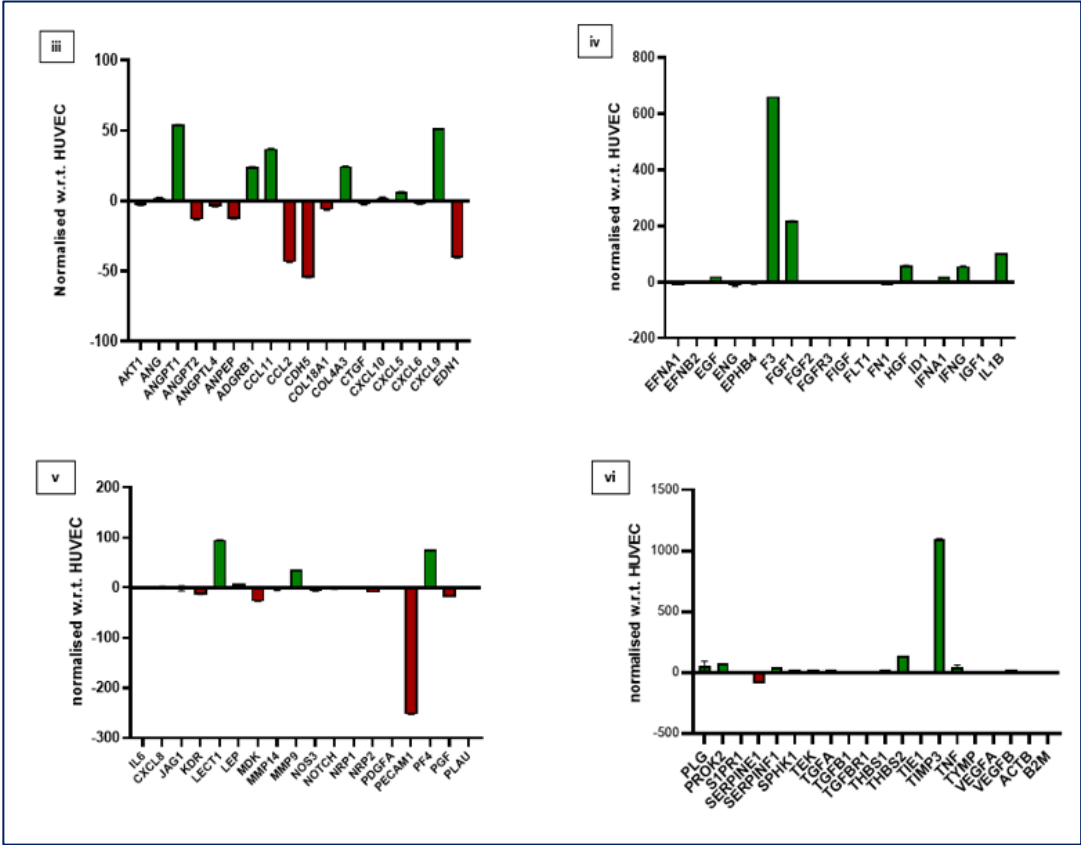


Fig 3.4 B Up and downregulation of angiogenic genes. represents Up-regulated and Downregulated genes of eSCs normalized with respect to HUVEC (iii, iv, v and vi). F3, FGF1 and TIMP3 genes were highly regulated along with ANGPT1, CXCL9, ILB1. However, PECAM1 is highly downregulated in eSCs.

To investigate the function of various genes associated with angiogenesis, a PCR array was employed. The scatter plot depicted in Fig 3.4 A (i) delineates the normalized expression levels of each gene present on the array across two distinct groups by juxtaposing them, thereby facilitating a rapid visualization of substantial alterations in gene expression. The central line serves as an indicator of unaltered gene expression. The dashed lines signify the predetermined threshold for fold regulation. Data points situated beyond the dashed lines in the upper left and lower right quadrants fulfill the established fold regulation criteria. Fig 3.4 A (ii) presents a heat map that illustrates the gene expression profile of angiogenesis-related genes in human umbilical vein endothelial cells (HUVECs) and endometrial cells. Fig 3.4 B (iii, iv, v, and vi) delineate the profiles of both upregulated and downregulated genes. Endometrial stem cells (eSCs) demonstrated a fold change of 1097 relative to HUVEC cells, indicating a significant overexpression of TIMP3. Following TIMP3, notable regulatory changes were observed

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for F3, FGF1, THBS2, IL1 β , and LECT1, with respective fold changes of 660.8, 218.3, 132, 103.3, and 95.7.

Conversely, SERPINE and CDH5 exhibited downregulation with fold changes of -93.1 and -54.2, respectively, while PECAM experienced a pronounced downregulation in eSCs with a fold change of -252.6.

3.3.5 Mesenchymal characterization of endometrial stem cells

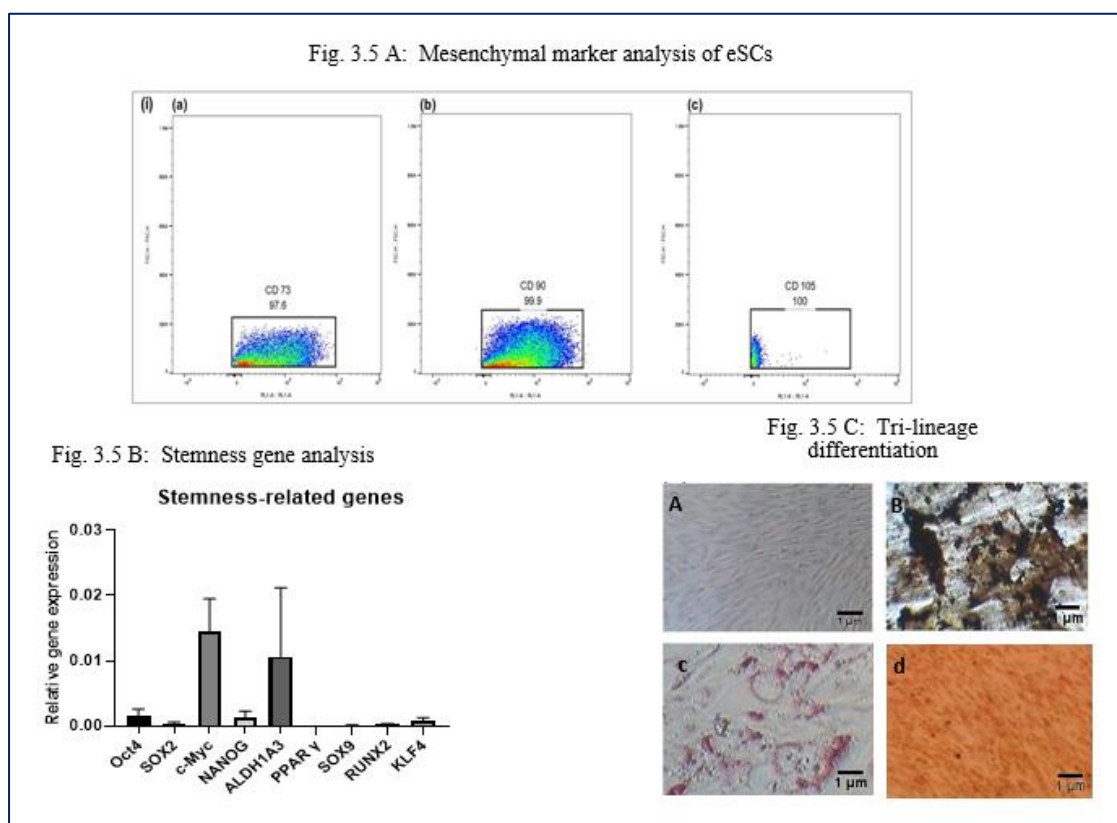


Fig. 3.5 Mesenchymal characterization of endometrial stem cells exemplifies the characterization and functional evaluation of endometrial stem cells (eSCs). Panel 3A illustrates the flow cytometric analysis of mesenchymal stem cell surface markers, indicating that eSCs exhibit a high level of expression for CD73 (a), CD90 (b), and CD105 (c). Panel 3B delineates the relative expression levels of genes associated with stemness (Oct4, Sox2, c-Myc, Klf4, ALDH1A3, PPAR γ , SOX9, RUNX2) within eSCs, with the data presented as mean $\pm$ standard deviation. Panel 3.5 C showcases the tri-lineage differentiation capabilities of eSCs, with visual representations depicting eSCs (a), adipogenic differentiation, which is stained with Oil Red O (c), osteogenic differentiation, which is stained with vankosa (b), and chondrogenic differentiation, which is stained with Alcian Blue (c) and Safranin O (d).

endometrial stem cells (eSCs) exhibit a propensity to express prominent mesenchymal stem cell (MSC) surface markers such as CD73, CD90, and CD105, in addition to their capability for differentiation [5]. The characteristics of eSCs were thoroughly examined

and validated through flow cytometry, revealing expression levels of 97.6% for CD73, 99.9% for CD90, and 100% for CD105, as illustrated in Fig 3.5A.

Subsequently, the expression of stemness-related genes in these cells, including Oct-4, SOX 2, c-Myc, NANOG, ALDH1A3, PPAR γ , SOX 9, RUNX2, and KLF4, was systematically evaluated. As depicted in Fig. 3.5 B, the gene expression analysis underscored the stemness characteristics of the cells. The pronounced expression of ALDH1A3 and c-Myc indicated a significant potential for cellular proliferation and expansion. Elevated levels of c-Myc expression are correlated with the capacity of eSCs to undergo cellular proliferation, facilitate angiogenesis, and enhance cellular metabolism within the endometrial microenvironment. Furthermore, ALDH1A3 plays a critical role in the maintenance, proliferation, and differentiation of stem cells, as well as in the synthesis of retinoic acid within cells. Previous investigations have established that endometrial cells exhibit increased levels of ALDH1A3 expression. The results obtained are statistically significant, with a p-value of 0.0012. Additionally, eSCs demonstrate notable differentiation potential. To validate the differentiation efficacy of endometrial stem cells, tri-lineage differentiation was meticulously conducted. Endometrial stem cells, as shown in Fig. 3.5 C (a), were successfully differentiated into Osteocytes (Fig. 3.5 C (b)), Chondrocytes (Fig. 3.5 C (c)), and Adipocytes (Fig. 3.5 C (d)). The differentiation process was corroborated through specific staining techniques, employing von Kossa staining for osteoblasts and Oil Red O staining for adipocytes.

3.3.6 Endometrial stem cells possess angiogenic potential

Figure 6A illustrates the Yolk Sac Model (YSM) assay conducted in ovo, which serves as an indicator of the angiogenic capacity of endometrial stem cells (eSCs). The fig. delineates the neoangiogenic influence exerted by eSCs on the YSM models. In Fig. 3.6A (a), the control samples are depicted where the eggs were left undisturbed. Fig 3.6A (b) illustrates the vehicle control group in which phosphate-buffered saline (PBS) was administered. Fig 3.6A (c) displays the models that received injections of eSCs. Fig. 3.6A (d, e, f) present the histological staining of the YSM models in respective order. The pro-angiogenic effect of eSCs is evidenced by the observed pattern and density of blood vessel formation (angiogenesis) in the YSM models. The quantitative analysis of nodes, junctions, and segments was performed utilizing the AngioAnalyzer plugin of

ImageJ, as shown in Fig. 6 B. These results were subjected to statistical analysis using GraphPad, yielding significant findings with a p-value of <0.0001 . The histological staining corroborates that the models receiving eSC injections display a distinct branching morphology.

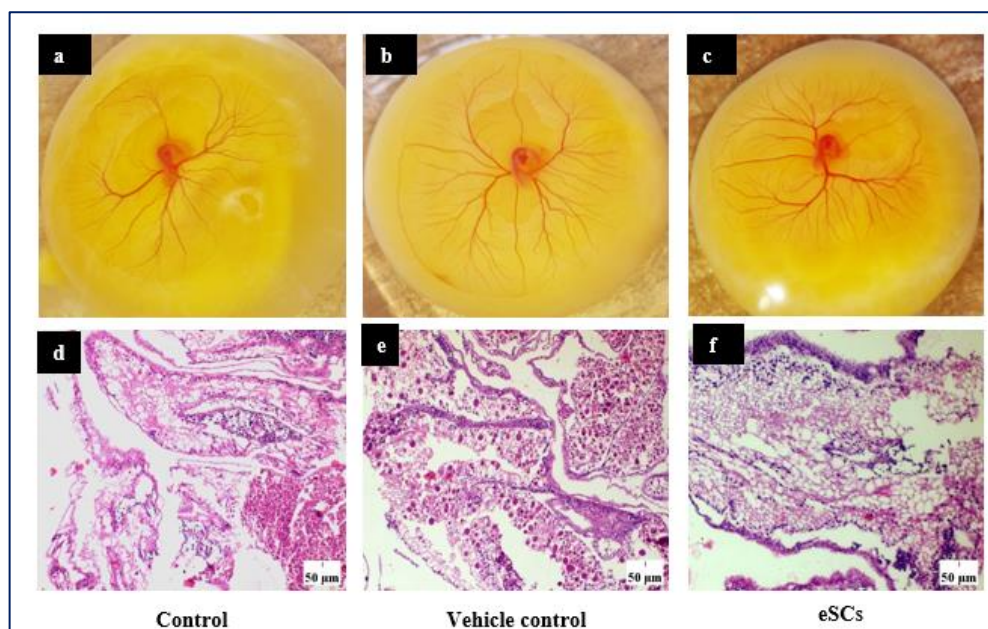


Fig 3.6 A In-Ovo angiogenic assay: Chick embryo model presents a three-dimensional representation of the in-ovo angiogenic assay utilizing a chick embryo model, thereby demonstrating the angiogenic capabilities of endometrial stem cells (eSCs) through visual documentation of control (a), vehicle control (b), and eSC-treated cohorts (c), accompanied by histopathological evaluation of vascular formation within these cohorts (d-f), with images captured at a magnification of 20X

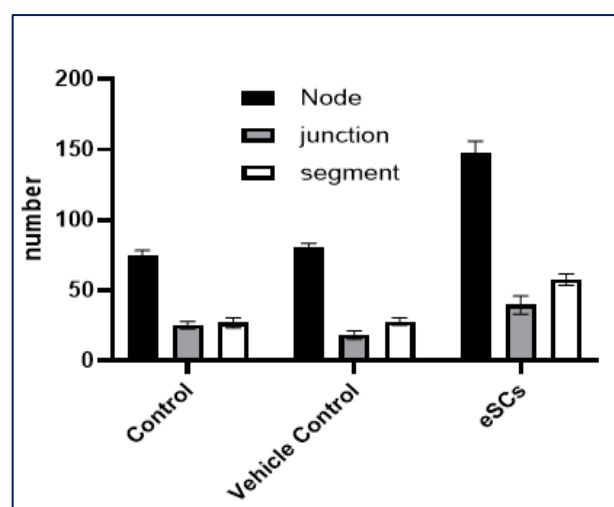


Fig. 3.6 B provides a quantitative analysis of the number of nodes, junctions, and segments within the control, vehicle control, and eSC-treated cohorts, indicating a marked enhancement in angiogenesis within the eSC-treated cohort, with the data expressed as mean $\pm$ standard deviation.

3.3.7 Sorting CD146⁺ cells and their marker characterization

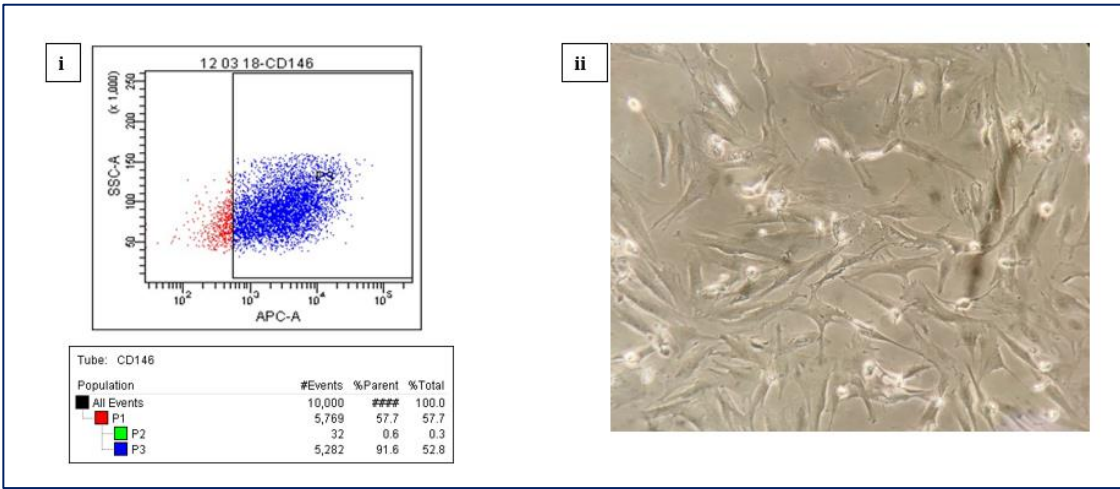


Fig 3.7 A: Characterization and functional assays of endometrial stem cells (eSCs) and CD146⁺ sorted cells.

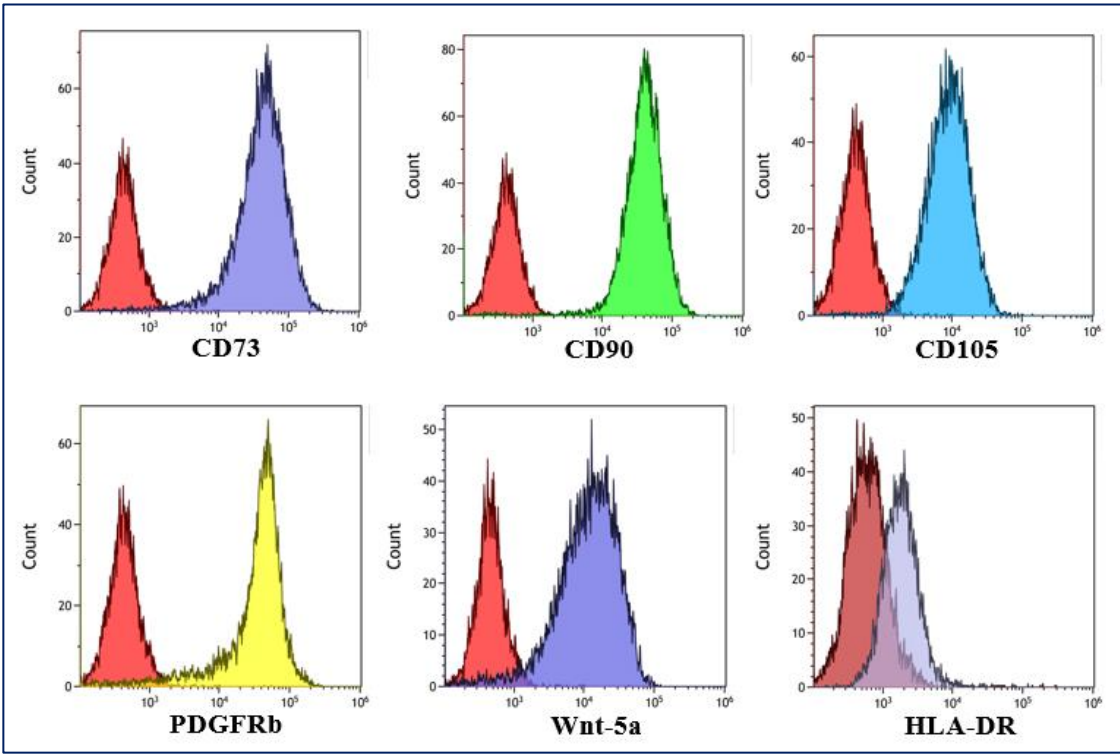


Fig. 3.7 B represents, flowcytometry marker analysis which shows eSCs are positive for CD73, CD90, CD105, PDGFRb, Wnt5-a and slightly positive for HLA-DR.

3.3.8 CD146⁺ population of stem cells are highly potent in inducing angiogenesis

After delineating the characteristics of the isolated cells and identifying the growth factors secreted by these cells as well as the angiogenic genes expressed therein, we proceeded to culture the isolated endometrial stem cells (eSCs) and conduct further analyses.

The cells extracted from the endometrial tissue were subjected to analysis and subsequently compared with the cultured cells derived from the identical source (endometrium). CD146 serves as a significant marker for mesenchymal stem cells and plays a crucial role in angiogenesis and cellular proliferation.

Fig. 3.7A (i) illustrates the expression levels of CD146 in the cultured eSCs. The cultured eSCs exhibited an elevated expression of CD146 at a remarkable 91.6%. This increased expression in the cultured cells may be attributed to the functional involvement of CD146 in the process of cellular proliferation. A study investigating a related topic reported a similar finding of heightened CD146 expression in cultured cells.

Flow cytometry marker analysis (Fig. 3.7 B) demonstrates that the sorted CD146 positive cells express the mesenchymal markers CD90, CD73, and CD105, indicating the presence of mesenchymal characteristics. The cells exhibit positive expression for PDGFR β , a marker correlated with vascular growth and neovascularization. Expression of Wnt-5a exhibits cell migration potential. The sorted cells are found to be slight positive for HLA-DR.

3.3.9 Angiogenic characterization of sorted CD146⁺ from eSCs

3.3.9.1 Growth Factor analysis

Fig 3.8 compares the secretion levels of various cytokines and growth factors between CD146⁻ and CD146⁺ cell populations, measured in pg/ml. The most striking differences are observed in VEGF (Vascular Endothelial Growth Factor) and M-CSF (Macrophage Colony-Stimulating Factor), where CD146⁺ cells produce significantly higher levels compared to CD146⁻ cells. This suggests that CD146⁺ cells may play a crucial role in angiogenesis and immune modulation. Additionally, CD146⁺ cells secrete higher levels of PDGF-AA and TGF- α , which are involved in cell proliferation, differentiation, and tissue repair. In contrast, for other cytokines such as ANG-2, EGF, EPO, BFGF, G-CSF, GM-CSF, HGF, PDGF-BB, and SCF, the differences between the two cell populations are relatively minor, with CD146⁺ cells generally showing slightly elevated secretion.

These findings indicate that CD146 expression is associated with a pro-angiogenic and pro-survival phenotype, suggesting a functional role for these cells in vascular development and tissue homeostasis.

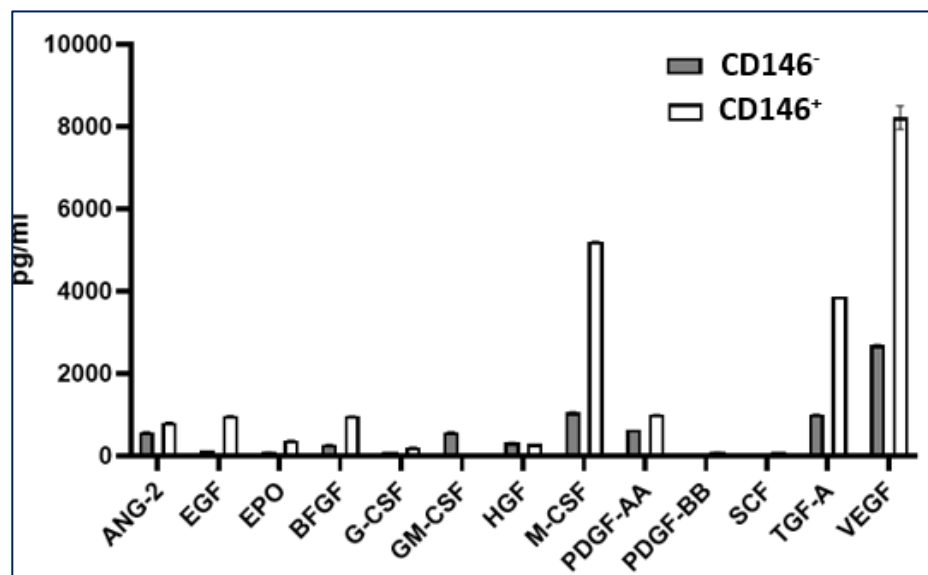


Fig. 3.8. Growth factor analysis of CD146⁺ cells. Growth factor profiling demonstrates higher levels of VEGF, TGF- α , and other factors in CD146⁺ cells compared to CD146⁻.

3.3.9.2 Endothelial Ring formation assay

The capacity of CD146⁺ cells to differentiate into endothelial lineage was thoroughly examined in the present investigation. The Matrigel tube formation assay was employed for this purpose (Fig. 3.9 A). At intervals of 0 h, 6 h, 10 h, and 15 h, the spontaneous arrangement of the endothelial ring comprising CD146⁻ and CD146⁺ cells was meticulously observed. Within a span of 15 hours of incubation, a ring-like configuration rapidly emerged in both CD146⁺ cells and CD146⁻. Nevertheless, CD146⁻ exhibit the formation of sustained endothelial tubes 4 hours subsequent to the emergence of those in CD146⁺ cells. Figure 4C illustrates the minute images captured at various time points. Consequently, the sorted CD146⁺ cells demonstrate superior angiogenic potential in comparison to CD146⁻. This observation is consistent with previous research wherein the authors underscored the enhanced angiogenic capacity of CD146⁺ cells over differentiated counterparts. Additional studies have also indicated that CD146⁺ cells display heightened responsiveness to growth factors, such as platelet-derived growth factor (PDGF) [6].

Characterization of endothelial rings:

The assessment of pro- and angiogenic markers, conducted for the differentiated endothelial cells after 15 hours via an RT-PCR methodology, facilitated the validation of the endothelial identity of the cells. For this analysis, a comprehensive markers panel encompassing KDR, Tie2, FLT1, and vWF was utilized (Fig. 3.9 B). Human umbilical vein endothelial cells (HUVEC) were utilized as the standard reference. The findings unequivocally demonstrate the commitment of CD146⁺ cells to the endothelial lineage, with the expression of HUVEC markers remaining consistent.

3.3.9.3 Wound scratch assay

Wound scratch assays were conducted on both CD146⁻ and sorted CD146⁺ cells derived from eSCs to evaluate their migratory capacity. No statistically significant differences were observed in the initial scratch width at the 0-hour mark across both experimental groups. The outcomes pertaining to cellular migration are encapsulated in Fig. 10 A. An additional graphical representation, Fig 3.10 B, generated via ImageJ, illustrating the data points is also included. At the 12-hour and 24-hour time intervals post-scratch, the group of sorted CD146⁺ cells exhibited a significant reduction in migration compared to the group of CD146⁻. Quantitative analysis was performed utilizing GraphPad Prism, yielding a p-value of 0.0020.

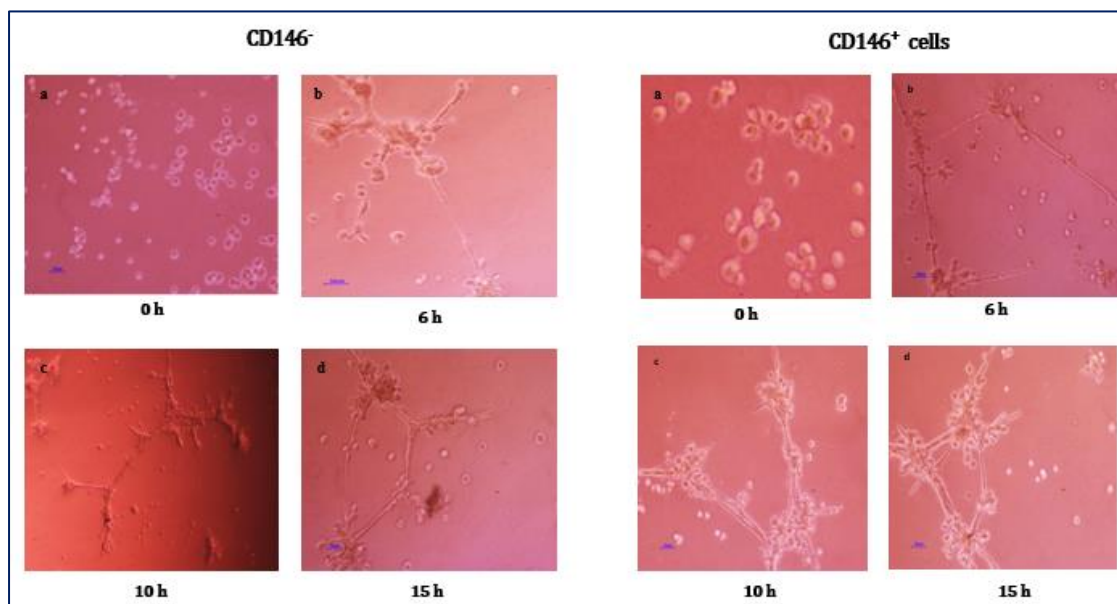


Fig. 3.9 A: Endothelial ring formation assay. demonstrates enhanced differentiation in CD146⁺ cells over time (0, 6, 10, and 15 hours) compared to CD146⁻.

CHAPTER III: Characterization and Angiogenic Potential of CD146⁺ Endometrial Stem Cells

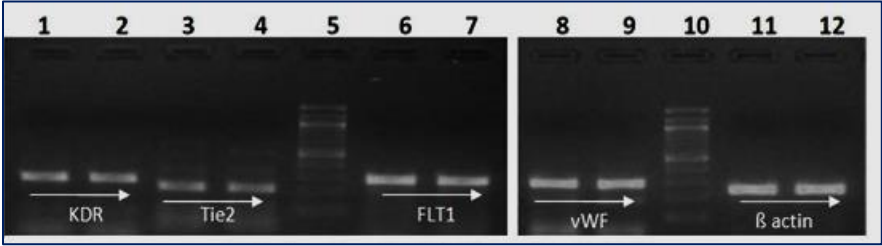


Fig. 3.9 B Semi-quantitative PCR confirms endothelial differentiation, with markers such as KDR, Tie2, FLT1, and VWF detected in differentiated cells (lanes 2, 4, 7, 9, and 12), similar to HUVEC controls (lanes 1, 3, 6, 8, and 11)

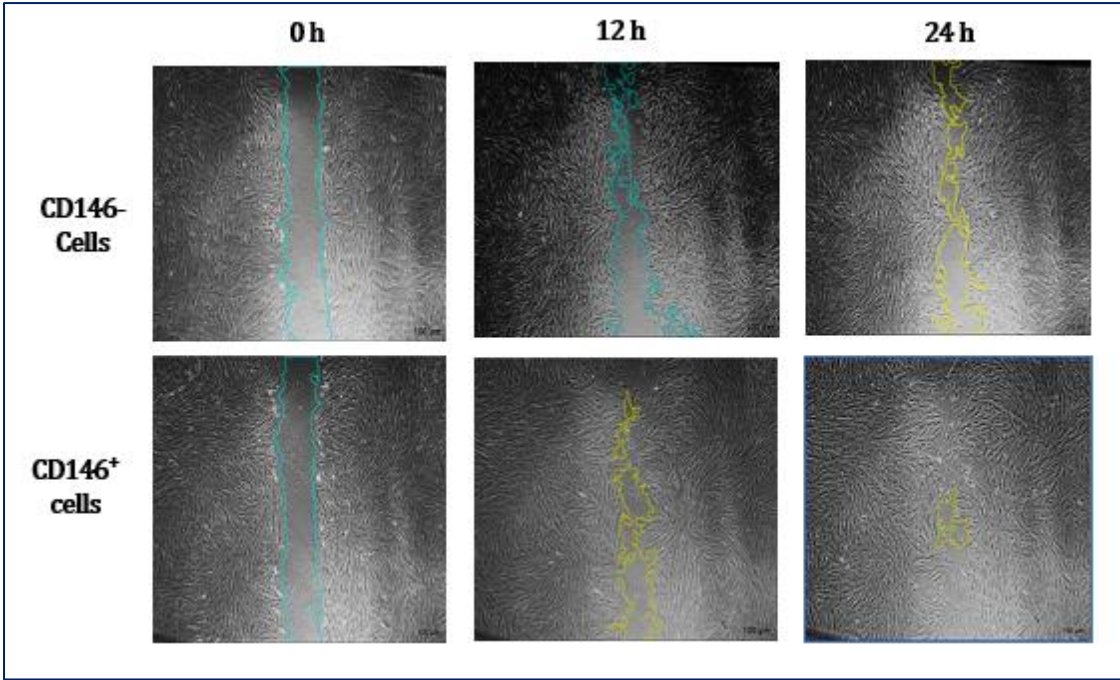


Fig. 3.10 A The wound scratch assay, with images captured at 4X magnification, shows faster migration and wound closure in CD146⁺ cells at 0, 12, and 24 hours.

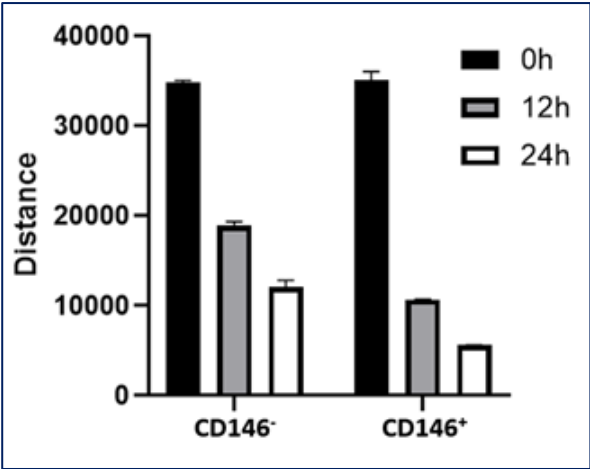


Fig. 3.10 B Quantitative analysis of wound scratch assay. highlights significantly greater migration distances in CD146⁺ cells, indicating their superior regenerative potential.

3.3.10 Chemokine Analysis

Chemokines are essential for recruiting leukocytes to inflammation sites and regulating angiogenesis. Fig 3.11 A presents the cytokine profile of CD146⁺ cells and CD146⁻. Notably, CXCL5 and CXCL8, both known for their pro-angiogenic properties, show significantly higher levels in CD146⁺ cells ($P \leq 0.0001$ for both), highlighting their role in promoting endothelial cell migration and proliferation. Additionally, CCL3 and CCL20, which are critical for angiogenesis and immune cell recruitment, also exhibit highly significant differences in their levels ($P \leq 0.0001$). These findings emphasize the complex molecular regulation of angiogenesis mediated by chemokines. In contrast, CXCL11, an angiostatic factor, and CXCL1 are found to be downregulated in CD146⁺ cells.

3.3.11 Cytokine Analysis

Fig. 3.11 B illustrates the cytokine profile of CD146⁺ cells compared to CD146⁻. The analysis shows that CD146⁺ cells express higher levels of GM-CSF, IL-1 β , IL-6, PDGF AA/BB, EGF, Endothelin-1, and Angiopoietin-1 than CD146⁻. Angiopoietin-1 (Ang1) plays a critical role in endothelial survival and vascular stabilization by inhibiting leukocyte–endothelium interactions and reducing endothelial permeability. Epidermal growth factor (EGF) regulates the mesenchymal stem cells (MSCs) proliferation by acting as a receptor ligand that promotes MSC proliferation and enhances their paracrine function. EGF also stimulates MSCs to produce VEGF and HGF, key factors in tissue regeneration and wound healing. Endothelin-1 (ET-1), a potent vasoconstrictor, can enhance the regenerative capacity of bone marrow-derived MSCs. Additionally, IL-6, a pleiotropic cytokine, is essential for hematopoiesis, immune response, cell survival, and proliferation. All results were highly significant, with a p-value of <0.0001 .

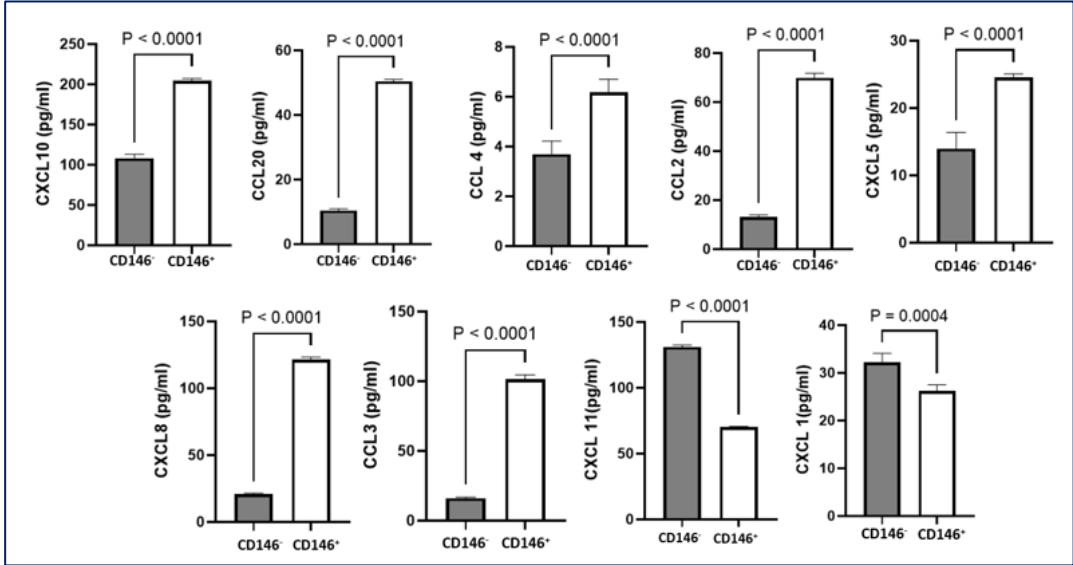


Fig. 3.11 A: Chemokine analysis of CD146⁺ cells, reveals that CXCL5, CXCL8, CCL3, and CCL20 play key roles in angiogenesis and immune cell recruitment, emphasizing their involvement in inflammation and blood vessel formation. Additionally,

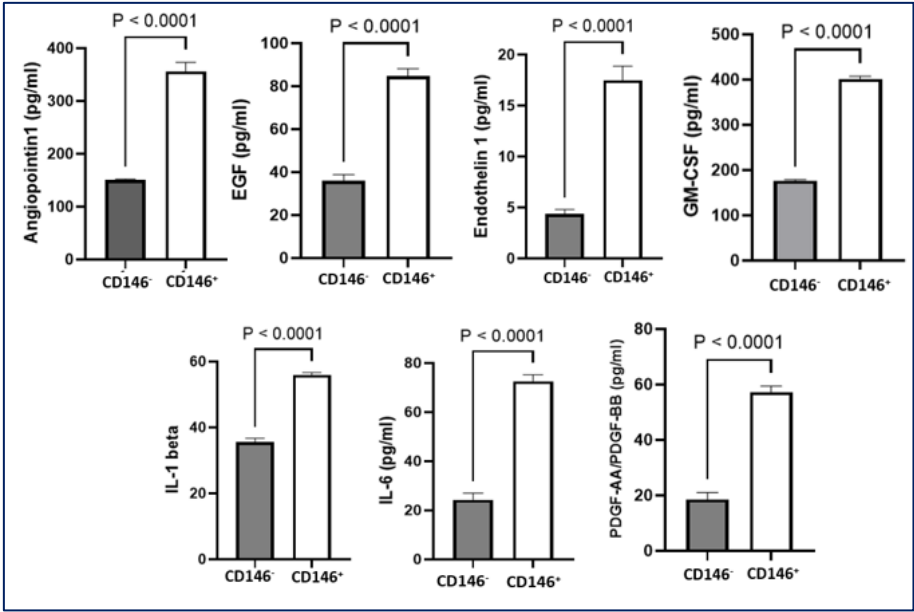


Fig. 3.11 B: cytokine profiling of CD146⁺ cells, shows that CD146⁺ cells express higher levels of cytokines such as GM-CSF, IL-1 β , IL-6, PDGF AA/BB, EGF, Endothelin-1, and GM-CSF, which are essential for endothelial cell survival and vascular stabilization.

3.3.12 CD146 possess angiogenic potency: In-ovo assay

To further investigate CD146's role in promoting angiogenesis, a yolk sac membrane (YSM) assay was conducted to evaluate the angiogenic potential of eSCs and to understand the specific contribution of CD146. Three groups were analyzed: control, CD146⁻, and CD146⁺ cells. In YSM assay, the patterns of vessel formation, density,

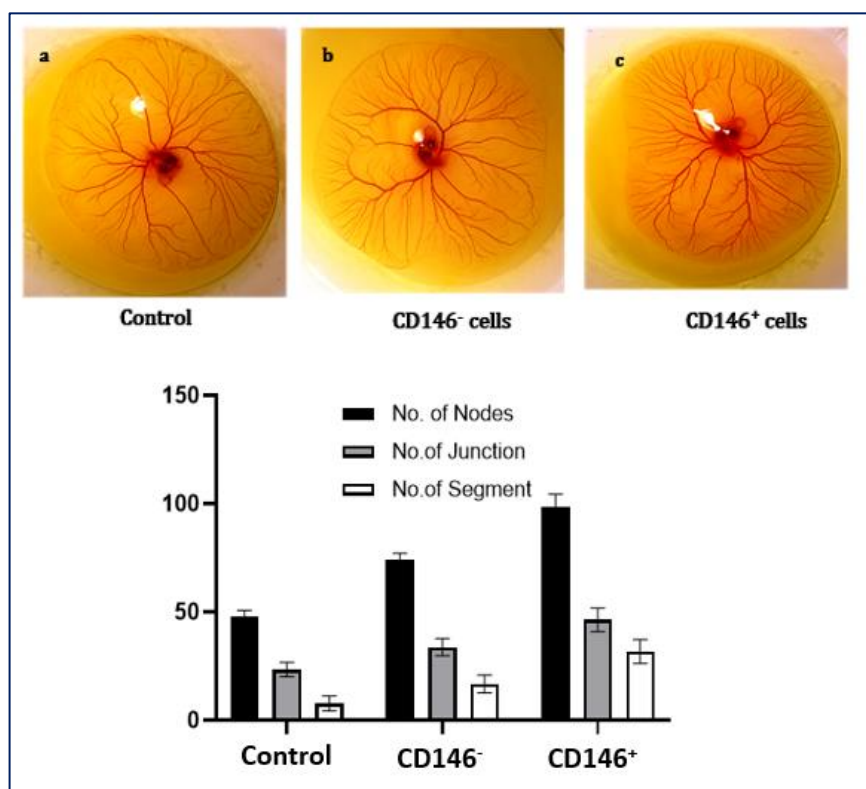
CHAPTER III: Characterization and Angiogenic Potential of CD146⁺ Endometrial Stem Cells

radial symmetry, and spatial arrangement are depicted in Figure 12 A (i), with a graphical representation shown in Fig 3.12 A (ii). Following treatment, the number of junctions, nodes, and segments was quantified using ImageJ software.

In the control group, the number of nodes was higher compared to the treatment groups. However, CD146⁺ sorted cells exhibited a greater number of nodes, junctions, and segments than the other groups, reinforcing the finding that CD146⁺ cells are more effective in angiogenesis induction than other stem cell populations.

The CAM assay results are presented in Fig 3.12 B (i), illustrating the patterns of vessel formation, density, radial symmetry, and spatial arrangement. Figure 12 B (ii) provides a graphical representation of the CAM assay quantification, showing the number of junctions, nodes, and segments analyzed using ImageJ. The statistical analysis yielded a p-value of 0.0023 for the YSM assay and 0.0016 for the CAM assay, indicating significant differences.

Fig. 3.12 A: in-ovo study. Fig.12 A (i) The study conducted in Yolk Sac Model (YSM) found that CD146⁺ sorted cells, which are involved in angiogenesis, have a higher number vasculature compared to



other groups viz control and CD146⁻. Fig 12 (ii); The study found that CD146⁺ sorted cells, which are involved in angiogenesis, have a higher number vasculature compared to other groups viz control and CD146⁻.

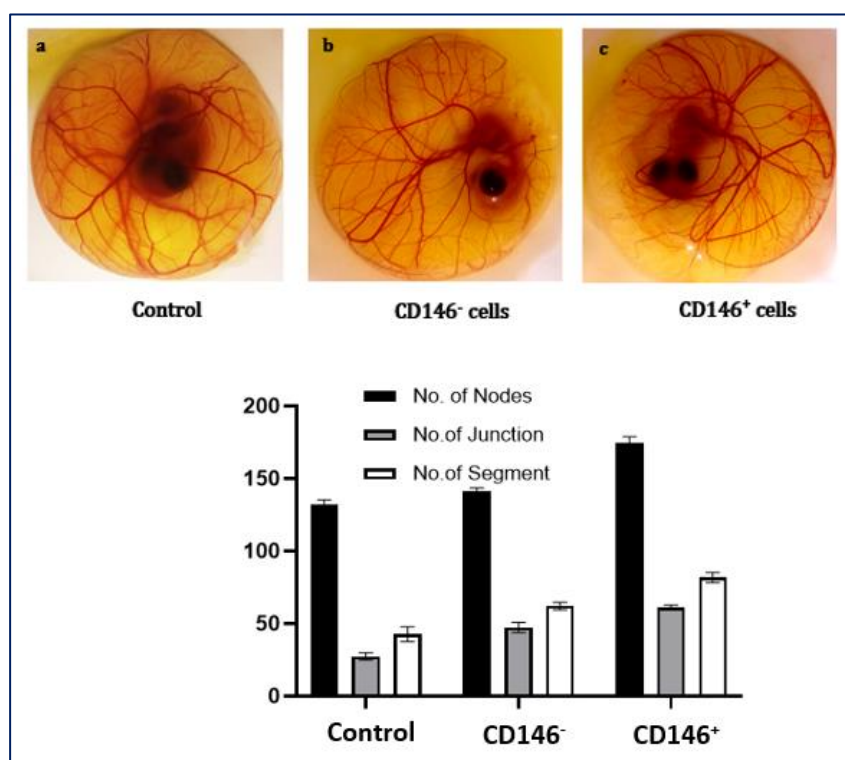


Fig.3.12 B: in-ovo study. Fig.12 B (i) The study conducted in Chorionallentoic membrane model (CAM) found that CD146⁺ sorted cells, which are involved in angiogenesis, have a higher number vasculature compared to other groups viz control and CD146⁻. Fig 12 B (ii); The study found that CD146⁺ sorted cells, which are involved in angiogenesis, have a higher number vasculature compared to other groups viz control and CD146⁻.

Immunohistochemistry of the YSM is shown in Figure 13, demonstrating the expression of endothelial and angiogenic markers CD31, VEGF, VAP, and vWF in the control, eSC-treated, and CD146⁺ cell-treated groups. In the control group (Fig. 3.13 B, F, J, N), baseline expression of these markers is minimal, as evidenced by sparse staining, indicating low inherent endothelial and angiogenic activity. The eSC-treated group (Fig. 3.13 C, G, K, O) shows moderate expression of CD31, VEGF, VAP, and vWF, with arrows highlighting areas of positive staining, reflecting some endothelial cell presence and angiogenic potential.

In contrast, the CD146⁺ cell-treated group (Fig. 3.13 D, H, L, P) demonstrates significantly higher expression of all four markers, with extensive and intense staining highlighted by arrows. This robust expression indicates a high level of endothelial differentiation and angiogenic activity. The stark contrast between the CD146⁺ group and the other groups underscores the superior ability of CD146⁺ cells to promote

endothelial differentiation and vascular formation, highlighting their potential for applications in vascular regeneration and tissue engineering.

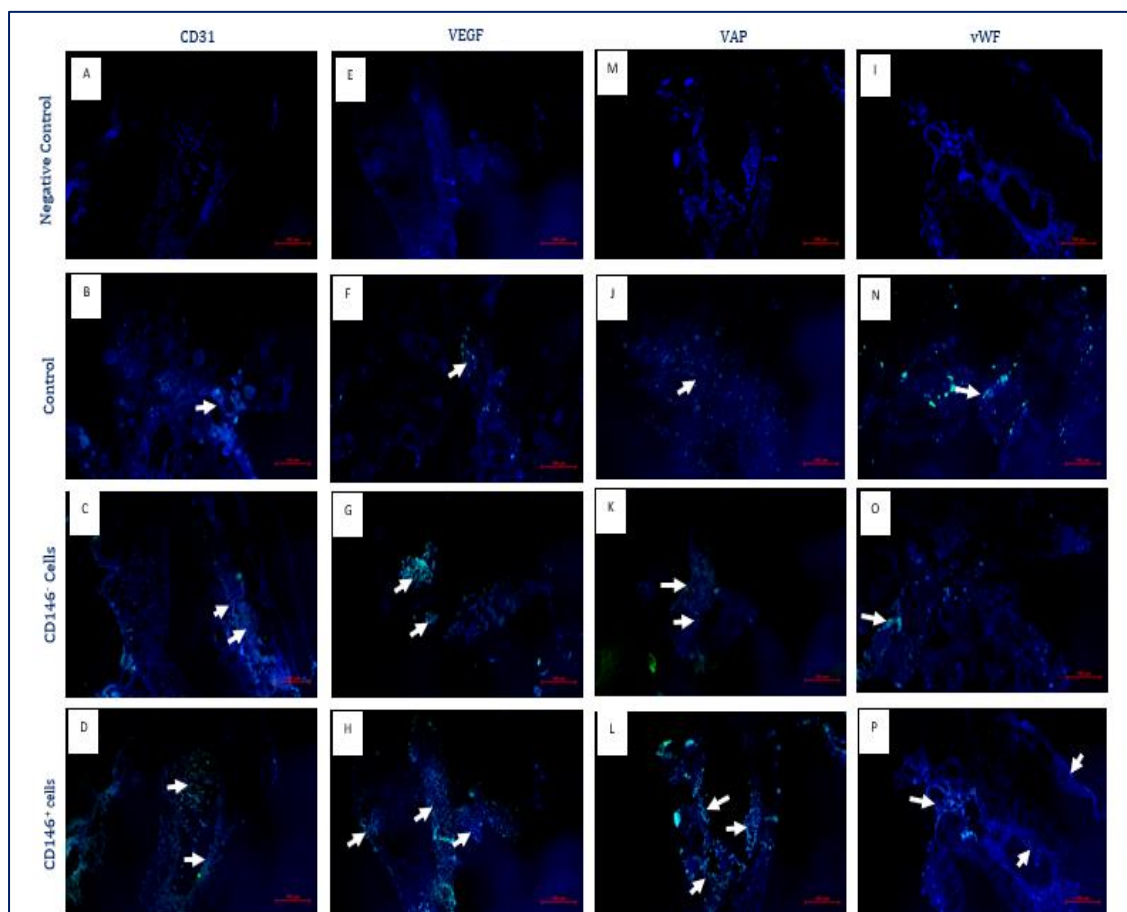


Fig 3.13: Immunofluorescence staining of tissue sections for endothelial and angiogenic markers at 20X magnification. The panels display staining for CD31 (A-D), VEGF (E-H), VAP (I-L), and vWF (M-P). The negative control group (A, E, M, I) shows minimal background fluorescence. The control group (B, F, J, N) represents untreated tissue, displaying baseline expression of CD31 (B), VEGF (F), VAP (J), and vWF (N). In the CD146⁻ treated group (C, G, K, O), an enhanced expression of CD31 (C), VEGF (G), VAP (K), and vWF (O) is observed compared to the control. The CD146⁺ cell-treated group (D, H, L, P) exhibits significantly increased expression of CD31 (D), VEGF (H), VAP (L), and vWF (P) compared to both the control and CD146⁻-treated sections. The scale bar represents 100 μ m. Blue staining indicates DAPI-labeled nuclei, while green indicates specific antibody staining for the respective markers. Panel (Q) shows the bright-field images of the cells.

3.4 Discussion:

This study offers novel insights of angiogenic potential and characterization of endometrial stem cells (eSCs), with a specific emphasis on CD146⁺ cells and their role in tissue regeneration. eSCs were isolated by trypsin digestion and characterized via flow cytometry, demonstrating the expression of cell adhesion markers CD146 and CD140b,

indicative of their niche-specific properties. These findings align with prior research that identifies CD146 as a marker of perivascular stem cells with significant angiogenic potential in various tissues, including the endometrium [7,8].

The endometrium reaches full development at puberty, undergoes dynamic changes throughout the menstrual cycle, as well as during pregnancy, menopause, and aging. This inherent capacity for tissue remodeling and regeneration positions eSCs as a unique stem cell population with a high angiogenic potential compared to other adult stem cells [9]. Our results demonstrate the significant expression of angiogenesis-related genes and the secretion of angiogenic growth factors such as GM-CSF [10], G-CSF [11], VEGF [12], EGF [13], FGF-basic [14], and PDGF-AA [15], all of which contribute to angiogenesis and vascular stability. During angiogenesis, CD146 expression increases in endothelial cells, functioning as a co-receptor for VEGF and facilitating signaling pathways that drive endothelial proliferation and migration. CD146⁺ pericytes play a crucial role in enhancing angiogenesis by supporting endothelial survival, migration, and tube formation [16]. They exhibit a strong response to PDGF-BB stimulation, contributing to more efficient and sustained endothelial sprouting and vascular stabilization. This highlights CD146 as a key marker in pericyte-mediated angiogenesis.

Endometrial stem cells display a distinct balance between pro- and anti-angiogenic markers. Notably, the expression of TIMP3, a tissue inhibitor of metalloproteinase, is significantly higher in eSCs compared to HUVEC cells. TIMP3 plays a crucial role in angiogenesis and regulating extracellular matrix remodeling by inhibiting matrix metalloproteinases (MMPs) [17]. This suggests a regulatory mechanism within eSCs to regulate angiogenesis and preserve tissue homeostasis in the endometrium.

The F3 gene, also known as tissue factor, promotes angiogenesis by binding to integrins and activating signaling pathways [18] such as FAK, PI3K/AKT, and MAPK. FGF1, another critical factor, promotes stemness, inhibits senescence, and enhances cell proliferation. THBS1, although less prominent, enhances endothelial cell proliferation in capillary sprouts [19], while IL-1 β drives angiogenesis and facilitates wound healing. The downregulation of PECAM, an endothelial hematopoietic marker, confirms the purity of the eSC population, which only exhibits angiogenic activity upon differentiation into endothelial cells. Additionally, downregulation of SERPINE1, which

codes for PAI-1 involved in clot formation as well as in tumor angiogenesis, further underscores the homeostatic role of eSCs. eSCs, which can be obtained non-invasively through endometrial biopsy, exhibit mesenchymal stem cell characteristics, expressing markers such as CD73, CD90, and CD105 [20]. The expression of ALDH1A3 in endometrial tissues is associated with progenitor cell populations, while c-Myc, a regulator of cell proliferation and self-renewal, enhances stem cell division during the menstrual cycle. Consistent with other studies, eSCs display a broad differentiation capacity, including osteogenic, adipogenic, chondrogenic, and pancreatic β -cell lineages [21,22].

In an in-ovo yolk sac membrane (YSM) model [23], eSCs demonstrated a pro-angiogenic effect, evidenced by increased vessel formation. This highlights the inherent angiogenic potential of eSCs. Pericytes, known for their role in vascular stabilization and remodeling, contribute significantly to pathological and physiological angiogenesis [24]. CD146, a key pericyte marker, has been shown to promote neovascularization and support endothelial proliferation and differentiation [25]. In this study, CD146⁺ cells isolated from the endometrium showed over 90% surface marker expression by passage 5. These cells expressed VEGF and TGF- α , both of which are critical for angiogenesis and tissue regeneration [26]. The Matrigel tube formation assay demonstrated that CD146⁺ cells formed stable vascular structures, highlighting their superior angiogenic potential compared to CD146⁻. Although CD146⁺ cells exhibited reduced migration in a wound scratch assay, their primary role appears to be in promoting angiogenesis rather than cell migration. Chemokine analysis revealed significant differences in CXCL5, CXCL8, CCL3, and CCL20 levels in CD146⁺ cells, suggesting their involvement in angiogenesis and immune modulation [27, 28]. The analysis suggests that CD146⁺ cells show higher expression of GM-CSF, IL-1 β , IL-6, PDGF AA/BB, EGF, Endothelin-1, and Angiopoietin-1 compared to CD146⁻ [29]. Angiopoietin-1 (Ang1) is a significant survival factor for endothelial cells and vascular stabilization through the inhibition of leukocyte-endothelium interactions and decreased endothelial permeability. EGF is a receptor ligand that induces MSC proliferation and increases paracrine activity, which, in turn, stimulates VEGF and HGF production. Both are significant factors in tissue repair and wound healing. ET-1, an extremely potent vasoconstrictor, has been found to be involved in increasing the regenerative capacity of MSCs derived from bone marrow.

Another multifunctional cytokine is IL-6, which is involved in hematopoiesis, immune regulation, cell survival, and proliferation. In the in-ovo assay, CD146⁺ cells demonstrated enhanced angiogenic potential compared to other stem cell populations, with increased nodes, junctions, and segments in vessel formation. Immunohistochemical analysis confirmed these findings, showing elevated expression of vascular markers such as CD31, VEGF, VAP, and vWF in CD146⁺ eSCs compared to control groups.

Despite these promising findings, the study has limitations, including a small sample size and the absence of in vivo validation of eSC angiogenic potential. Additionally, the focus on angiogenesis limits the exploration of other therapeutic applications such as immunomodulation and tissue engineering. Future research should address these limitations and further elucidate the mechanisms underlying the angiogenic properties of eSCs.

3.5 Conclusion:

This study highlights the strong angiogenic potential of CD146⁺ endometrial stem cells (eSCs), demonstrating their ability to promote vascular formation, endothelial differentiation, and immune modulation. CD146⁺ cells exhibited superior vessel formation in the in-ovo model and formed stable vascular structures in Matrigel, confirming their role in endothelial support and tissue regeneration. Their chemokine profile further supports their function in vascular remodeling and immune interactions. These findings establish sorted CD146⁺ from eSCs as a promising candidate for angiogenesis-driven regenerative therapies.

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4.1 Introduction:

Endometrial carcinoma (EC) originates from the innermost layer of the uterus, referred to as the endometrium, and it represents the most prevalently identified gynecological neoplasm, underscoring its importance in women's health [1]. The risk factors linked with EC includes obesity, diabetes mellitus, hypertension, and a history of unopposed estrogen exposure [2]. The main signs of endometrial carcinoma are postmenopausal hemorrhage and pelvic pain. Timely identification of EC is necessary for efficacious management and therapeutic intervention. The diagnostic process generally involves pelvic examinations, imaging investigations, and endometrial biopsy to confirm the presence of malignant cells by histology or immunohistochemistry [3]. The staging of endometrium carcinoma follows the guideline established by the international federation of gynecology and obstetrics (FIGO) with the most recent update in 2023 [4]. This revision includes molecular classification and histological factors to enhance prognostic accuracy.

CD146 a melanoma cell adhesion molecule and a membrane glycoprotein play a significant role in the progression ad pathology of EC [5]. Its expression is associated with various mechanism that contribute to tumor growth, angiogenesis and poor prognosis in patients. The over-expression of CD146 in EC indicates its potential as diagnostic marker when compared to normal tissue [6]. EC is one of the most significant health burdens on women, and proper prediction of tumor characteristics is a requirement for effective treatment planning. CD146 expression is related to tumor aggressiveness [7, 8]. The rapid steps taken in the realm of computational biology have brought data scientists and oncologists alike in a closer manner to an efficient and cost-effective computer-aided surveillance of cancer systems. Machine Learning (ML) a branch of artificial intelligence holds vast applications ranging from drug discovery, cancer detection and survival predction to optimize treatment starergies. ML techniques have improved significantly in oncology to make better predictions regarding susceptibility, reccurnce and survival [9]. This retrospective study investigates the relationships between CD146 expression, tumor characteristics, and patient survival outcomes. Special attention is given to tumor dimensions, thickness, and FIGO grades to assess their predictive value for overall survival. By employing various statistical methods and visualizations, this analysis aims to provide understandings into tumor biology and implications for patient prognosis.

4.2 Methods

4.2.1 Study patients and sample collection

The study samples were obtained from Kolhapur Cancer Centre, Kolhapur, Maharashtra. Clinical records, and tissue embedded paraffin blocks of endometrial carcinoma cases were retrieved from archives. The study included, endometrial cancer patients who underwent diagnostic and therapeutic resections for the age group of 38-76. The tumor was diagnosed by histopathological criteria and categorized based on FIGO staging. A comprehensive clinical history was taken which includes age of the patient, type of disease, FIGO grade, TNM staging, treatment history, survival status etc.

4.2.2 Immunohistochemistry

The paraffin block retrieved were processed for sectioning and staining. Briefly, Sections were fixed on positively coated slide and proceed for deparaffinization and rehydration using xylene and alcohol grades. Antigen unmasking was done by Tri sodium citrate buffer with pH of 6.0

while heat treatment was done at the temperatures of 95°C and 98°C between 10 and 20 minutes. Blocking was done followed by antigen retrieval with serum for about an hour. An overnight incubation of the sections with a monoclonal CD146 antibody (Cusa Biotech, CSB-RA013563A0HU) was carried out in a fridge at four degrees containing a recommended amount of moisture and temperature. Non-specific binding was also blocked. Three thorough PBS washes preceded a 30-minute room temperature incubation with a biotinylated secondary antibody, after which the biotin-streptavidin-peroxidase system was employed to amplify the signal. The HRP conjugated system was observed using DAB substrate. The optimal staining which is most visually appealing is reached when the reaction is suspended with distilled water. Slides were then counterstained using hematoxylin and sealed with mounting media. Images were taken at 10X and 20X. Further, the expression of CD146 in endometrial carcinoma was quantified using ImageJ. The quantification was done separately for endometrium and myometrium regions.

4.2.3 Data Collection and preparation

Clinical data of 29 endometrial carcinoma patients were obtained from Kolhapur Cancer Centre, Kolhapur. The data set obtained, contains essential tumor-related variables. After meticulous cleaning and preprocessing, tumor dimensions (Measured in cm²),

Tumor thickness, FIGO grading, Overall survival status and CD146 expression were considered in order to predict the survival outcome.

CD146 expression of both endometrium and myometrium was calculated using following formula:

CD146 Expression quantification Formula:

$$\text{CD146 Expression quantification} = \frac{\text{Endometrium Percent Area} + \text{Myometrium Percent Area}}{2}$$

4.2.4 Statistical analysis and machine learning

Statistical analysis was done using SPSS software. The significant Correlation between the considered groups and the association between CD146 expression and different clinicopathological parameters were evaluated using the correlation analysis. T test and ANOVA was done to evaluate the variation between CD146 expression in FIGO grade 1 and 2. Survival analysis was done to examine the relation between CD146 and survival status of the patient. The statistical analysis was done by using IBM SPSS software and some analysis was also done on stat models in python.

Machine learning model is developed to predicted the survival analysis using CD146 expression and tumor characteristics. The received clinical data was cleaned by removing unnecessary information and made ready according to the machine learning program. The data set was then was split into training and test model in 80-20 ratio. Machine learning models such as logistic regression, random forest, Decision tree and Support vector machine (SVM) were developed in order to predict the survival of the endometrial carcinoma patient.

4.3 Results:

CD146 mediated angiogenesis plays a crucial role in tumor progression. The purpose of this study is to investigate how tumor features and survival outcomes relate to the prognostic value of CD146 expression. In this retrospective study, CD146 expression of 29 tumor samples were examined and correlated with number of pathological and clinical factors. The data was obtained from cancer hospital for EC patients treated between 2017-2024.

4.3.1 Patient clinical characteristic

The clinical data obtained from the hospital reveals, the mean age of the patients affected by endometrial carcinoma is 60.28 years and the average tumor size is 16.81cm². The

most common histological subtype was moderately differentiated endometrial adenocarcinoma accounting for 13.79% of cases and other subtypes with varying differentiation levels accounts of 3.45% and 6.89%. In FIGO grading system, grade 1 is most prominent contributing for 64.29%, followed by grade 2 contributes 32.14% and lastly grade 3 for 3.57%.

In the study, Immunohistochemical expression of CD146 was quantified using ImageJ as percentage of total area. The mean % are in the endometrium was observed to be relatively low compared to the myometrium with values ranging significantly among the patients this difference in expression level could potentially contribute in understanding the tumor behavior.

4.3.2 T-Test and ANOVA of expression of CD146 correlated with FIGO grades

T test was performed to compare CD146 expression between FIGO grades 1 and 2. t-test was performed, yielding a t-statistic of 1.01 and a p-value of 0.32, indicating no significant difference.

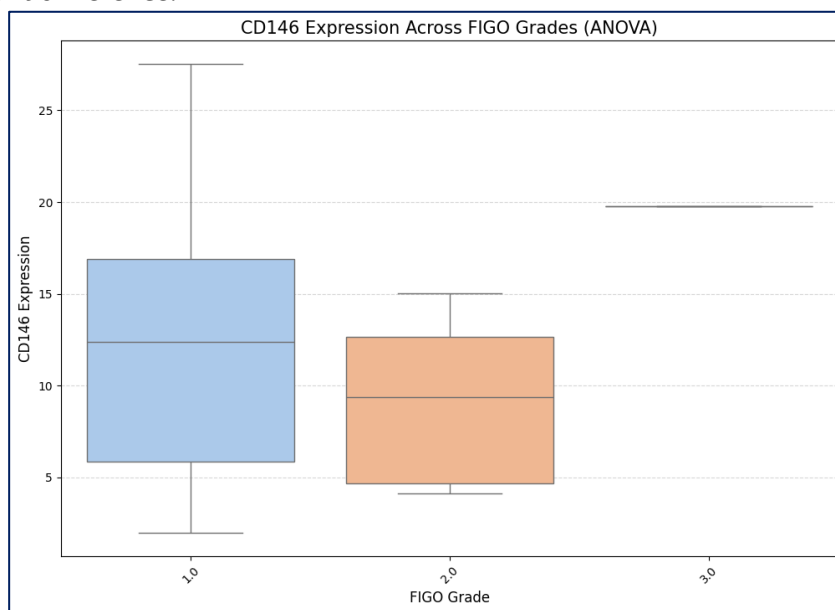


Fig 4.1. CD146 Expression Across FIGO Grades: Boxplot showing the variation in CD146 expression levels across different FIGO grades, highlighting differences in median values, interquartile ranges, and potential outliers, analyzed using ANOVA

The box plot represents the CD146 expression levels across FIGO grades 1, 2, and 3. The distribution of CD146 expression appears to decline as the grade of the FIGO increases from grade 1 to grade 2. The median expression level was significantly higher

CHAPTER IV: Prognostic significance of CD146 in Endometrial Carcinoma

for FIGO Grade 1 compared to FIGO Grade 2. The range of expression levels was also significantly different between these two grades. FIGO Grade 1 shows a higher IQR, meaning an increased variation in the gene expression level among these patients as compared to those placed in Grade 2. The t-test and ANOVA results indicated that CD146 expression was not significantly different across FIGO grades, implying that FIGO classification alone may not be sufficient to differentiate CD146-related tumor progression

Table 4.1. T-Test results for CD146 expression across FIGO grades.

Comparison	t-statistic	p-value
FIGO Grade 1 vs 2	1.01	0.32

An ANOVA test confirmed the absence of significant differences in CD146 expression across different FIGO grades, with an F-statistic of 1.29 and a p-value of 0.29.

Table 4.2: ANOVA results for CD146 expression.

Metric	F-statistic	p-value
ANOVA (Across FIGO Grades)	1.29	0.29

4.3.3 Correlation Analysis

Correlation analysis was performed to examine the relationship between CD146 and survival status, CD146 expression and tumor dimensions, CD146 expression and tumor thickness.

Table 3: Correlation matrix of key variables.

Variable	CD146 Expression	Survival Status	Tumor Dimension (cm ²)	Thickness (cm)
CD146 Expression	1.000000	0.322189	-0.263036	-0.253236
Survival Status	0.322189	1.000000	-0.185643	-0.184248
Tumor Dimension (cm ²)	-0.263036	-0.185643	1.000000	0.695527
Thickness (cm)	-0.253236	-0.184248	0.695527	1.000000

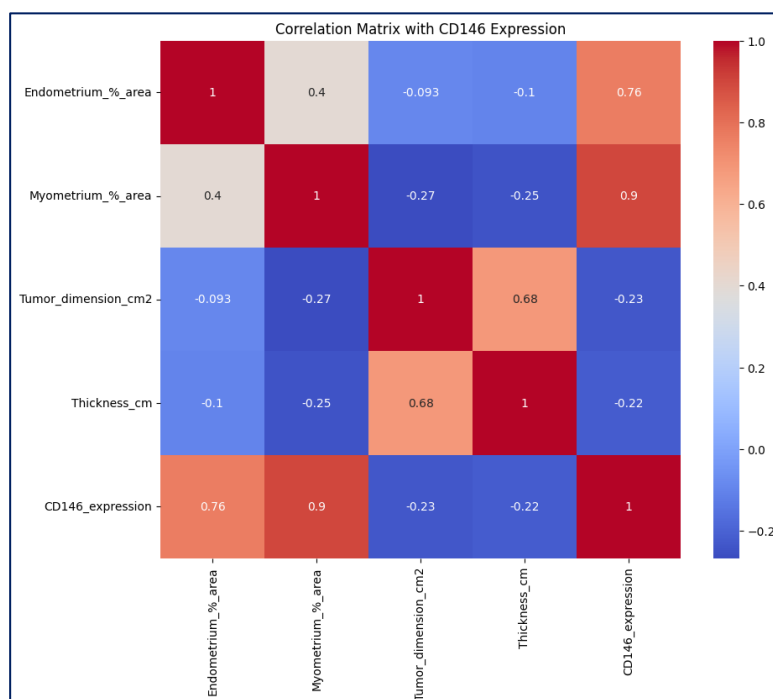


Fig. 4.2 Correlation matrix showing the relationships between CD146 expression and various clinical parameters, including Endometrium % area, Myometrium % area, Tumor dimension, and Thickness.

Considering correlation matrix, higher expression levels are correlated with larger areas of myometrium and endometrium. The strong positive association between CD146 expression and both myometrium % area (0.9) and endometrium % area (0.76) is seen in the correlation matrix. On the other hand, there are weak to moderate negative associations between CD146 expression and thickness (-0.22) and tumor dimension (-0.23), which suggests that slightly smaller tumor thicknesses and dimensions may be linked to higher CD146 expression. Moreover, the matrix shows a moderately positive association of 0.68 between tumor thickness and tumor dimension, which indicates that tumors tend to be thicker as they get bigger. The correlation analysis showed a weak positive correlation ($r = 0.322$) between CD146 expression and survival status, suggesting a potential but modest influence of CD146 on survival outcomes.

4.3.4 Survival analysis

A binary variable was created to represent survival status, categorizing patients as deceased (1) or alive (0). Figure 3 shows the distribution of CD146 expressions across these survival groups.

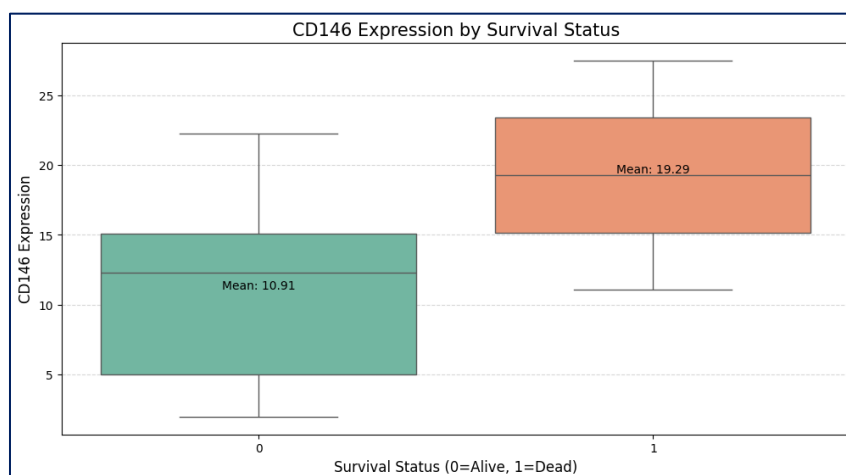


Fig 4.3: Boxplot of CD146 expression categorized by survival status (0 = Alive, 1 = Dead). Box plot showing CD146 expression levels by survival status, with survival status categorized as 0 for alive and 1 for deceased. The mean expression levels are 10.91 for those alive and 19.29 for those deceased.

The boxplot illustrates the distribution of CD146 expression across two survival groups (alive and deceased), revealing a significant difference in expression levels between them. The mean CD146 expression is notably higher in the deceased group (19.29) compared to the alive group (10.91), suggesting a potential correlation between elevated CD146 levels and poorer survival outcomes. Also, considering the well-documented involvement of CD146 in the processes of angiogenesis, tumor advancement, and metastatic spread, its heightened expression may be implicated in the manifestation of more aggressive tumor characteristics and reduced survival probabilities. These observations imply that CD146 has the potential to function as a prognostic biomarker.

4.3.5 Machine Learning Models

4.3.5.1 Classifier Performance

The machine learning models exhibited remarkable efficacy in forecasting survival outcomes. Logistic Regression, Random Forest, and Support Vector Machine (SVM) models attained an accuracy rate of 100%, underscoring their robustness in distinguishing between survival status. The Decision Tree classifier, although exhibiting a marginally lower accuracy of 89%, still demonstrated considerable predictive capability, highlighting the potential applicability of these models in clinical prognostic assessments. The metrics of precision, recall, and F1-score substantiated these

conclusions, revealing nearly flawless performance across all models with the exception of the Decision Tree, which displayed minimal classification inaccuracies.

Table 4.4: Model performance comparison across classifiers.

Model	Accuracy (%)	Precision	Recall	F1-Score
Logistic Regression	100	1.00	1.00	1.00
Random Forest	100	1.00	1.00	1.00
Decision Tree	89	1.00	0.89	0.94
SVM	100	1.00	1.00	1.00

4.3.5.2 Feature importance analysis

The feature importance from the random forest identifies CD146 expression as the most critical variable for survival prediction. This aligns with the hypothesis that CD146 provide valuable information beyond standard clinicopathological information. By incorporating all these features together, the model could become more accurate in predicting outcomes from endometrial carcinoma patients.

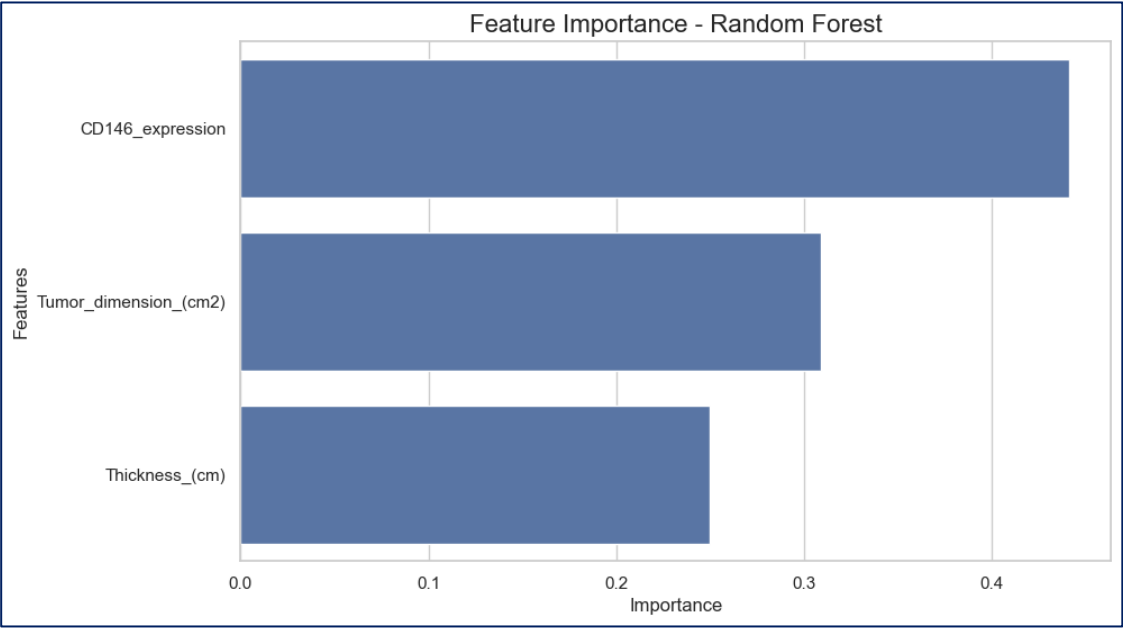


Fig 4.4: Feature importance plot from a Random Forest model, showing that CD146 expression has the highest importance, followed by tumor dimension (cm²) and thickness (cm) in predicting the target variable.

The decision tree analysis explains the significance of three tumor-related parameters, namely CD146 expression, tumor size, and tumor thickness, concerning the prediction of patient survival. The highest score, approximately 0.45, shows that CD146 serves as

the most essential factor in predicting patient survival outcomes. This observation suggests that differences in CD146 expression may employ a significant influence on survival potential, thereby supporting its status as a critical prognostic biomarker. Moreover, tumor size, assigned a score of 0.03, looks to be the most relevant predictor following CD146 expression. An increased tumor size is correlated with advanced disease stages and adverse prognostic outcomes. In contrast, tumor thickness, which demonstrates the lowest performance score of approximately 0.25, implies that the depth of tumor invasion does not adequately serve as a predictor of survival when contrasted with CD146 expression and tumor size.

4.4 Discussion

The finding of this investigation highlights the significance of CD146-mediated angiogenesis plays in the progression of endometrial cancer (EC). Although CD146, a cell adhesion molecule known for its roles in angiogenesis and tumor propagation, has been thoroughly studied in several cancers [10-13], nothing is known about its prognostic relevance in EC. This study aims to fill this knowledge gap by examining how CD146 expression correlates with characteristics of EC tumors and their behavior well as survival rates. Providing new insights into its potential as a predictive marker, for EC prognosis.

The analysis of clinical and pathological features in this cohort provides valuable insights into EC progression. EC mainly affects postmenopausal women [14], which explains the mean patient age of 60.28 years in line with epidemiological patterns. Tumor size was usually large (16.81 cm²), in line with advanced stages of disease in late-diagnosed EC. Furthermore, moderately differentiated adenocarcinomas became the most common histologic subtype, which is consistent with previous studies reporting its high frequency in EC [16].

The endometrium and myometrial display distinct expression patterns of CD146, indicating its potential for regulation by specific microenvironments. The higher expression of CD146 in myometrium was consistent with its documented role in promoting an invasive phenotype in cancer cells [17]. These findings were supported by evidence linking CD146 to epithelial-mesenchymal transition (EMT), an important driver of metastasis [18]. Immunohistochemical analysis confirmed that CD146 expression was significantly higher in myometrium, suggesting its role in tumor

invasion and stromal interactions. This warrants further investigation of microenvironmental factors that regulate CD146 expression in myometrial tissues.

The expression of CD146 did not differ significantly between FIGO grades, with p values of 0.32 for the t-test and 0.29 for ANOVA. Increasing FIGO grade resulted in decreased expression of CD146, especially among individuals with FIGO grade 1 tumors, which had a higher interquartile range (IQR). According to this pattern, early-stage tumors likely exhibit higher CD146-mediated angiogenic activity because of an expansion required during initial tumor growth [19], but these findings underscore the limitations of FIGO classification in accounting for molecular complexity and emphasize need for integration of biomarkers such as CD146 with better prognostic accuracy [20].

An interesting correlation was found between CD146 expression and tumor characteristics in correlative studies. The correlation between CD146 expression and tumor size (-0.26) and thickness (-2.25), suggested that early tumors were essential for angiogenesis through CD146, as an important factor in vascular expansion during the initial growth. Early-stage breast and ovarian cancers exhibit similar patterns, with the expression of CD146 peaking early during growth and declining late in development [21, 22].

Survival analysis further highlights the prognostic relevance of CD146. Patients with higher CD146 expression (mean 19.29) had significantly poorer survival outcomes compared to those with lower expression levels (mean 10.91). These findings support prior evidence linking elevated CD146 levels to enhanced angiogenesis, immune evasion, and therapy resistance (Huang et al., 2021). Furthermore, the strong correlations of CD146 with myometrial (0.90) and endometrial (0.76) areas underscore its role in modifying the tumor microenvironment to promote invasion and metastasis. High precision machine learning (ML) models, particularly those in logistic regression, random forest, and support vector machine (SVM) types, have been used to predict survival outcomes with high precision, while decision tree classifiers have achieved accuracy of up to 89% [23,24]. A feature importance analysis revealed that CD146 was the most crucial variable for survival prediction, with a prognostic value exceed commonly accepted clinicopathological parameters. The proof is provided by these findings, which suggest that ML can capture intricate biological patterns to improve personalized medicine approaches [25].

Despite its benefits, this study contains some drawbacks. Larger multicenter cohorts require validation due to the limited sample size (29 patients). These studies cannot be generalized, and the retrospective design does not account for causation, so prospective studies are necessary to verify these results. In the future, it is important to investigate the molecular mechanisms that underlie CD146's role in EC progression, including its interaction with angiogenic factors such as VEGF and PDGF. Also, preclinical studies on CD146-targeted therapies with monoclonal antibodies [26] and small molecules inhibitors could result in new therapeutic prospects.

4.5 Conclusion

In conclusion, this study highlights the importance of CD146 in endometrial cancer prognosis. High CD146 expression is associated with poorer survival outcomes, positioning it as a promising biomarker for the aggressive disease phenotype. Incorporating CD146 into machine learning-based prognostic models shows the potential to advance personalized medicine by improving risk stratification and guiding treatment planning. Although further research is needed to confirm these findings and explore therapeutic implications, this study highlights the importance of CD146 in advancing our understanding of endometrial cancer pathogenesis and improving patient outcomes.

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5.1 Introduction:

Endometrial carcinoma (EC) arises from the endometrium, the innermost lining of the uterus, and is the most frequently diagnosed gynecological malignancy worldwide. Its incidence is particularly high in postmenopausal women, making it a significant health burden [1]. Several risk factors, including obesity, diabetes mellitus, hypertension, unopposed estrogen exposure, and genetic predispositions, are implicated in the pathogenesis of EC [2]. The disease is often identified through clinical symptoms such as abnormal uterine bleeding and pelvic discomfort, underscoring the need for timely diagnosis and intervention. Diagnostic approaches typically involve pelvic examinations, imaging studies, and histopathological evaluation of endometrial biopsy specimens. Endometrial carcinoma (EC) arises from the endometrium, the innermost lining of the uterus, and is the most frequently diagnosed gynecological malignancy worldwide [3]. Its incidence is particularly high in postmenopausal women, making it a significant health burden [4]. Tumor grading and staging are crucial in guiding the management and prognosis of EC. The International Federation of Gynecology and Obstetrics (FIGO) staging system, recently updated to include molecular classification, provides a comprehensive framework for assessing disease progression [5]. Despite advancements in staging and treatment, predicting disease behavior and outcomes remains challenging due to the heterogeneous nature of EC. CD146, also known as melanoma cell adhesion molecule (MCAM), is a membrane glycoprotein that has garnered attention for its role in tumor progression, angiogenesis, and metastasis in various cancers [6]. In EC, CD146 overexpression has been associated with tumor aggressiveness and poor prognosis, highlighting its potential as a diagnostic and prognostic biomarker. Studies suggest that CD146 promotes tumor growth by facilitating angiogenesis and enhancing cell migration and invasion [7]. However, the precise role of CD146 in EC pathogenesis and its clinical utility remain underexplored, necessitating further research. The integration of molecular markers such as CD146 with clinical and pathological features offers a promising avenue for improving risk stratification and treatment strategies in EC. Furthermore, the application of machine learning and advanced statistical methods has the potential to enhance our understanding of complex tumor biology and predict patient outcomes with greater accuracy. This study aims to investigate the expression of CD146 in EC tissues, its correlation with

tumor characteristics, and its prognostic value in patient survival outcomes, contributing to the growing body of evidence on molecular markers in gynecological cancers.

Endometrial carcinoma is one of the most widespread gynecological carcinoma and frequently distinguished by aggressive migration and proliferation and invasive characteristics [9]. Angiogenesis play a critical role in the development of endometrial tumor by influencing tumor progression, metastasis and overall development [10]. High vascular density of endometrial tumor directly correlates to the advancement of the disease [11]. Despite the advancement in the treatment, the prognosis is recurrent, emphasizing the need of novel treatment modalities [12]. CD146 is cell adhesion molecule primarily expressed on endothelial cells in physiological scenario and serves as potential biomarker [13] for various cancers including endometrial carcinoma also a promising therapeutic target [14, 15]. Overexpression of CD146 has been associated with tumor progression, survival and metastatic potential [16, 17]. CD146 targeted therapy is emerging as a promising approach in the treatment of various diseases particularly in systemic sclerosis and various cancers. This targeted therapy is promising mode of treatment as patient can receive multiple dosages without any adverse immunological response.

Targeting CD146 could be one of the promising way to treat the endometrial carcinoma. Physiologically, CD146 is primarily expressed on endothelial cells and has fundamental role is vascular stabilization in angiogenesis [18]. However, its over expression has documented in various types of cancers [19-21]. In endometrial carcinoma, CD146 is recognized as key biomarker for tumor cell survival, proliferation and migration [22-23]. However, targeting CD146 is a challenge as it also express on other physiological cells as well. Recently, a group of scientists It has been discovered that CD146 positive tumors secrete soluble CD146, which is released from membrane-anchored CD146 and causes cancer cells to express tissue factors, generate cancer stem cells, and undergo EMT. M2J-1 mAb, a new antisoluble CD146 antibody, prevented these effects. Therefore, M2J-1 mAb may represent a novel therapeutic approach to stop procoagulant activity and tumor spread in a variety of invasive CD146 positive malignancies [24,25].

Although it is reported so, wide studies on CD146 in endometrial stem cells of endometrial disorders including endometrial cancer is uncertain. This, on the other side of the coin, the role of CD146 in promoting endometrial dysfunctions and their targeted

therapeutic approach would be identified. Targeting CD146 with M2J-1 could be the interesting therapeutic approach to treat endometrial carcinoma by reducing the proliferation, invasion and angiogenesis.

5.2 Materials and Methods

5.2.1 Sample collection

Endometrial carcinoma samples were obtained from Kolhapur Cancer Centre, Kolhapur, following ethical approval. Participants were informed about the purpose of the study, and written informed consent was obtained before sample collection. All specimens were transported to the laboratory in sterile containers containing transport medium. Diseased endometrial biopsies were collected from abdominal hysterectomy specimens of women aged 35 to 70, undergoing surgery for endometrial adenocarcinoma. All samples were processed within two hours of collection.

5.2.2 Isolation and culture of endometrial carcinoma cells

Endometrial carcinoma tissue was carefully scraped from the underlying myometrium following hysterectomy. The collected tissue was rinsed with Dulbecco's phosphate-buffered saline (DPBS) containing antibiotics. The rinsed tissue was then incubated with 0.25% trypsin at 37°C for 15 minutes in a humidified 5% CO₂ incubator. To neutralize the trypsin, Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was added. The digested cell suspension was washed twice with DPBS by centrifugation at 450g for 10 minutes to remove residual trypsin and cellular debris. The resulting cell pellet was re-suspended in complete DMEM, seeded, and cultured in a T-25 flask. Cells were incubated at 37°C in a humidified 5% CO₂ incubator and passaged every 3-5 days.

5.2.3 CD146 characterization of endometrial carcinoma cells using flowcytometry

Cultured endometrial carcinoma cells were analyzed for percentage of CD146⁺ cells using flowcytometry. Detailed procedure is described in 3.2.3.

5.2.4 Endometrial carcinoma cell sorting and marker analysis

Endometrial carcinoma cells were sorted for CD146⁺ cells using magnetic cell sorter. The detailed procedure is explained in 4.2.10. Sorted cells were then assessed for cancer stem cell marker CD133 (Invitrogen, Cat No. 12-1338-42) and CD44 (Invitrogen, Cat.

No. 11-0441-82) along with CD146 (BD Biosciences, Cat. No. 561013). Detailed methodology of marker analysis is described in 3.2.3.

5.2.5 The impact of CD146⁺ EC cells on proliferation, migration and invasion was studied by inhibiting CD146 with humanised monoclonal antibody M2J-1.

5.2.5.1 Proliferation assay of Sorted Cell population

Sorted CD146 endometrial carcinoma stem cells were cultured and analyzed for their proliferation capacity. Approximately 5000 cells from each population were seeded into 96-well plates (Nunc™ MicroWell™ 96-Well Microplates, Cat No. 260887) and incubated for 24 to 48 hours until reaching 70% confluency. WST-1 (CELLPRO-RO Roche, Cat No. 5015944001) was then added to each well and incubated for 1 hour. Optical density (OD) was measured at 450 nm using a spectrophotometer. The experiment was repeated three times with the sorted cell populations to ensure result consistency, and the combined mean of each outcome was determined using the Mann-Whitney test.

Inhibition of sorted CD146⁺ endometrial carcinoma cell proliferation

To assess the impact of CD146 inhibition on the proliferation capacity of endometrial carcinoma, CD146⁺ sorted cells were utilized. Approximately 5000 sorted CD146⁺ cells were plated, with four wells designated for each group: control and treated (four wells for the control and four for the treated group). The control wells were treated with IgG, while the treated group received a monoclonal anti-CD146 antibody (M2J-1). After 24 hours, 10 µL of WST-1 was added to each well, and optical density (OD) was measured at 450 nm using a spectrophotometer. The experiment was repeated three times with the sorted cell population to ensure reproducibility, and the combined mean of each result was calculated using the Mann-Whitney test.

5.2.5.2 Migration assay of Sorted CD146 Cell population

Sorted CD146⁺ endometrial carcinoma stem cells were cultured and analyzed for their migration capacity. Approximately 10,000 sorted CD146⁺ cells were seeded into 24-well plates and incubated for 24 to 48 hours until reaching 70% confluency. A scratch was created at the center of each well using a 10 µL tip, and the cells were allowed to grow further. The scratch width was measured at 0 hours and 8 hours using ImageJ

software, and the mean was calculated. To ensure result consistency, the experiment was repeated five times with the sorted cell population, and the combined mean of each outcome was determined using the Mann-Whitney test in GraphPad Prism Software.

Inhibition of sorted CD146⁺ endometrial carcinoma cell migration:

To evaluate the effect of CD146 inhibition on the migration capacity of endometrial carcinoma, CD146⁺ sorted cells were utilized. A total of 10,000 sorted CD146⁺ cells were seeded into 24-well plates for both the control and treatment groups. The control group was treated with IgG, while the treated group received a humanized monoclonal anti-CD146 antibody (M2J-1). Fresh media was added to the wells, and a scratch was created at the center of each well perpendicularly using a 10 µL tip. Images were captured for each well using a confocal microscope at 0 and 8 hours post-scratch. The scratch width was measured using ImageJ software, and the mean was calculated. To ensure reproducibility, the experiment was repeated five times, and the combined mean of all results was determined using the Mann-Whitney test.

5.2.5.3 Invasion assay of Sorted CD146 Cell population

Sorted CD146⁺ endometrial carcinoma stem cells were cultured and analyzed for their invasion capacity. Inserts with a diameter of 0.8 µm (Nunc, Cat No. TMO140629) were activated by incubating them with 1 mL of PBS^{-/-} (Gibco, Cat. No. 10010023) at 37°C for 1 hour. A 100 µL layer of Matrigel (Corning, Cat. No. 354234) was then applied to the inserts and allowed to solidify in the incubator for 1 hour. Approximately 20,000 sorted CD146⁺ cells were seeded into 24-well plates (Nunc® Cell Culture Inserts in 24-well Nunclon Delta surface plate, Cat No. TMO140620). After 48 hours, the inserts were transferred to 4% PFA (Himedia, Cat No. TCL119) for 20 minutes, followed by staining with crystal violet (Sigma Aldrich, Cat No. C6158) for 30 minutes. The inserts were then washed with PBS^{-/-} and distilled water. Microscopic images were captured and analyzed using ImageJ software. To ensure result consistency, the experiment was repeated five times with the sorted cell population, and the combined mean of all outcomes was determined using the Mann-Whitney test.

Inhibition of CD146 sorted cell invasion:

To evaluate the effect of CD146 inhibition on the migration capacity of endometrial carcinoma, CD146⁺ sorted cells were used. Inserts with a diameter of 0.8 µm were activated by incubating them with 1 mL of PBS-/- at 37°C for 1 hour. A 100 µL layer of prepared Matrigel was then applied to the inserts and allowed to solidify in the incubator for 1 hour. Approximately 20,000 CD146⁺ cells from both the control and treated groups were seeded into 24-well plates (Nunc® Cell Culture Inserts in 24-well Nunclon Delta surface plate, Cat No. TMO140620) using incomplete media, while complete media was added to the wells. Four wells were designated for the control group treated with IgG, and four wells for the treatment group, which received a humanized monoclonal anti-CD146 antibody (M2J-1). Cells were incubated for 48 hours, after which the inserts were fixed in 4% PFA (Himedia, Cat No. TCL119) for 20 minutes, followed by staining with crystal violet (Sigma Aldrich, Cat No. C6158) for 30 minutes. The inserts were then washed with PBS-/- (Gibco, Cat. No. 10010023) and distilled water. Microscopic images were captured and analyzed using ImageJ software. The experiment was repeated five times with the sorted cell population to ensure reproducibility, and the combined mean of each result was determined using the Mann-Whitney test.

5.2.6 In-ovo angiogenic assay

Chick Chorioallantoic Membrane (CAM) Model (Experimental Days 8-10)

The in-ovo angiogenic assay was performed using Chick Chorioallantoic Membrane (CAM) model (days 8-10) to evaluate the angiogenic potential of sorted CD146⁺ EC cells as well as the inhibition assessment of M2J-1. Fertilized Black Australorp eggs were disinfected and incubated at 37.5°C with high humidity. On day 8 of incubation, 2-3 ml of albumin was extracted from the narrow end of the egg, and the aperture was sealed with adhesive tape. A small opening was made at the blunt end near the air sac using sterile forceps, and a sterile silicon ring was placed onto the chick vasculature. 20,000 CD146⁺ EC cells were injected directly into the silicon ring place onto the vasculature. Similarly, in another group, cells were injected followed by the anti-CD146 antibody M2J-1 to check the inhibition potential. The openings were sealed with adhesive tape and parafilm, and the eggs were returned to the incubator for 48 hours.

Following incubation, the eggs were carefully opened to assess angiogenesis. Macroscopic images of the vasculature were captured for analysis.

5.3 Results

5.3.1 Isolation, culture and characterization of endometrial carcinoma cells

The endometrial carcinoma (EC) sample was procured from the cancer hospital with consideration of ethical parameter and brought to the lab in sterile condition. Fig 5.1 shows the cancerous uterus (a) and tumor within (b). The endometrial carcinoma cells were isolated using trypsin digestion method and the cells isolated from human endometrium tumor were cultured until P5. eSCs reached confluency within 5 days P0 and P1 and within 3 days subsequently from P2 onwards. EC cells were appeared in fibroblastic nature under culture condition (Fig 5.2).

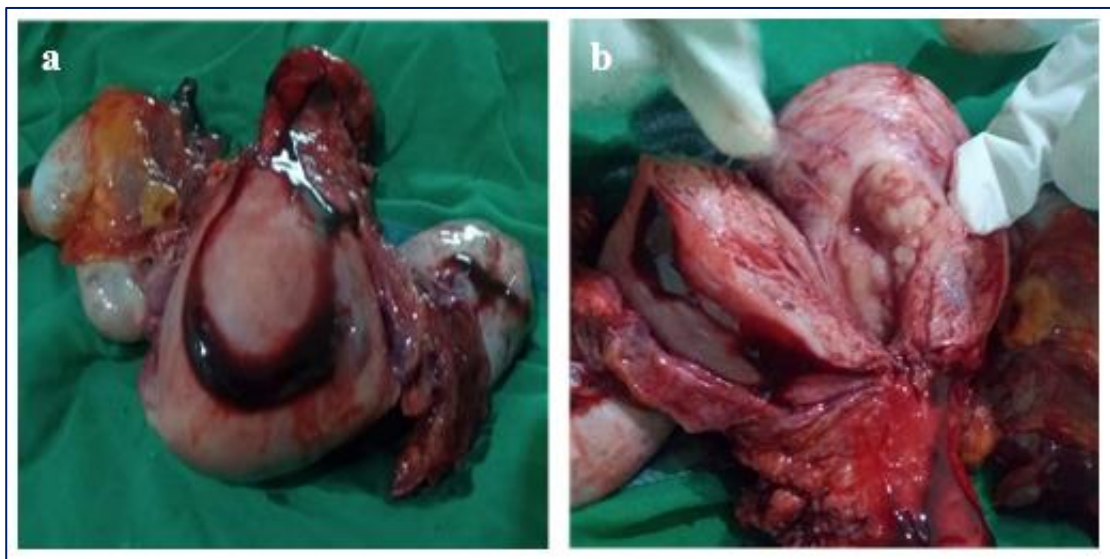


Fig. 5.1 Endometrial Carcinoma (EC) specimen. (a) uterus with endometrial carcinoma; (b) EC tumor associate with endometrial wall

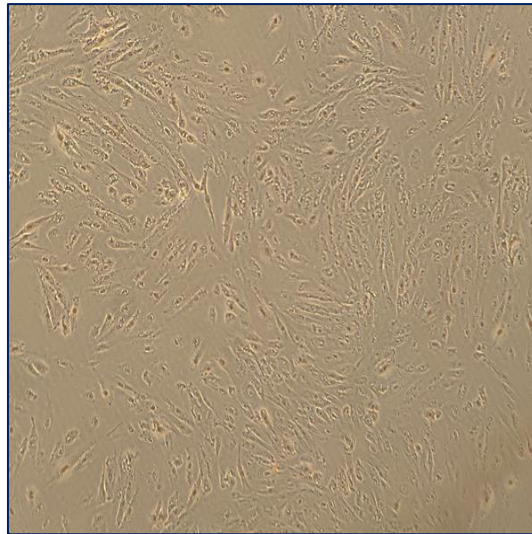


Fig 5.2. Morphology of EC cells under culture. EC cells were appeared with fibroblastic morphology. Cell isolation was done using trypsin digestion and cells were passaged at 80% confluency

The cells isolated from endometrium tumor tissue were assessed to understand the percentage of CD146⁺ cell population. The cultured endometrial cancer cells showed good expression of CD146 which is above 50%. This expression in cultured cells is probably due to the involvement of CD146 in tumor survival. A study examining a similar aspect found increased CD146 expression in cultured cells. From the cultured cells, CD146⁺ cells were sorted and analyse further for their proliferation, migration, invasion with the inhibition of Anti-CD146 and compare with unsorted cells.

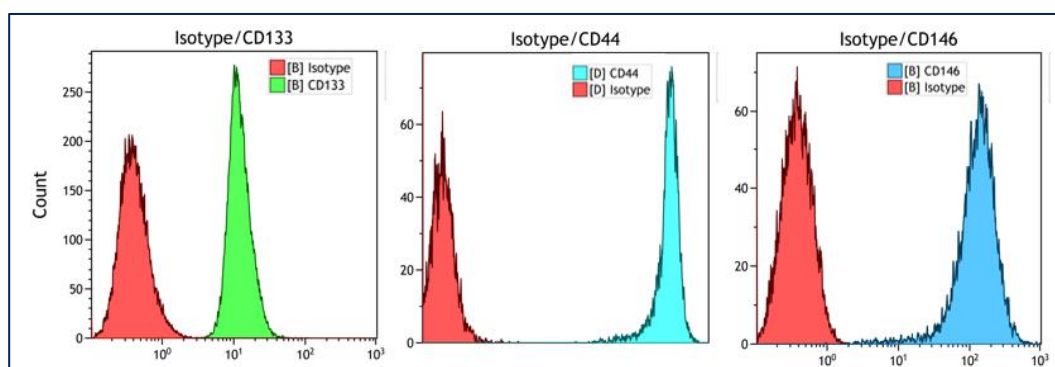


Fig. 5.3. Flowcytometry marker analysis of sorted CD146⁺ EC cells. The cells were positive for cancer stem cell markers CD133 and CD44. While ensuring that sorted cells are pure CD146⁺ population

The sorted endometrial carcinoma cells were characterised for cancer stem cell (Fig. 5.3) marker and CD133, CD44 and CD146. Sorted CD146 was found highly expressing

cancer stem cell marker CD133, CD44. In the CD146 sorted cell population derived from endometrial carcinoma, cell surface characterization was conducted to assess the expression of cancer stem cell marker. The finding from the analysis clearly demonstrate that the cell which exhibits expression of CD146 co-currently displayed significant elevated levels of the cancer cell marker CD133 and CD44, there by indicating a profound and strong association between the expression of CD146 and manifest stem cell like property within the endometrial carcinoma cells.

5.3.2 The impact of CD146⁺ on proliferation, migration and invasion was studied by inhibiting CD146 with humanised monoclonal antibody M2J-1.

5.3.2.1 Proliferation assay

The cell proliferation assay (Fig. 5.4) reveals significant decrease in the proliferation of sorted CD146 endometrial carcinoma cells when treated with M2J-1 compared to control group. The absorbance at 450nm indicates cell viability, with treated cells showing a low absorbance value, highlighting a reduction in their proliferative potential upon CD146 inhibition ($p=0.0145$).

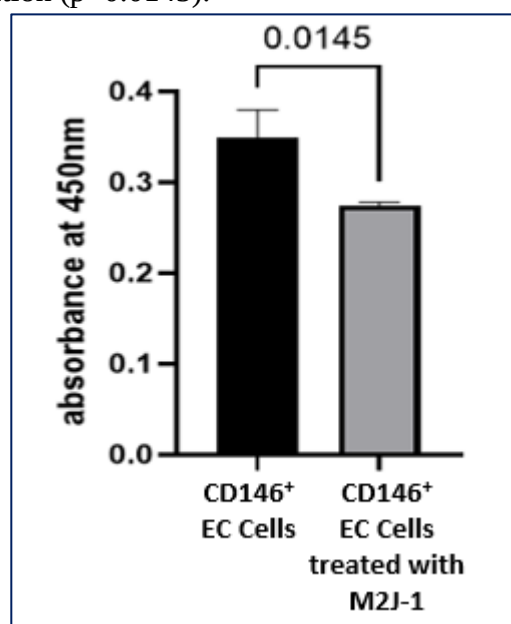


Fig. 5.4 Proliferation assay comparing CD146⁺ endothelial cells (EC) with CD146⁺ cells treated with M2J-1. A significant reduction in absorbance at 450 nm ($p = 0.0145$) is observed in treated cells, indicating significant decreased cell proliferation.

5.3.2.2 Migration assay

The migration assay results illustrated through wound healing images (Fig. 5.5 (i)) and quantitative analysis (Fig. 5.5 (ii)) is done by ImageJ. The scratched area in endometrial carcinoma cells shows decreased in the migration distance after 8hours. However, the scratch area distance in the group treated with M2J-1 remains wider. The quantification analysis was done using Image j by calculating the scratch area after desired time points. The P value obtained is 0.0005 suggests significance of the experiment. This suggests, CD146 is involved in the development of endometrial carcinoma and could be the novel treatment option.

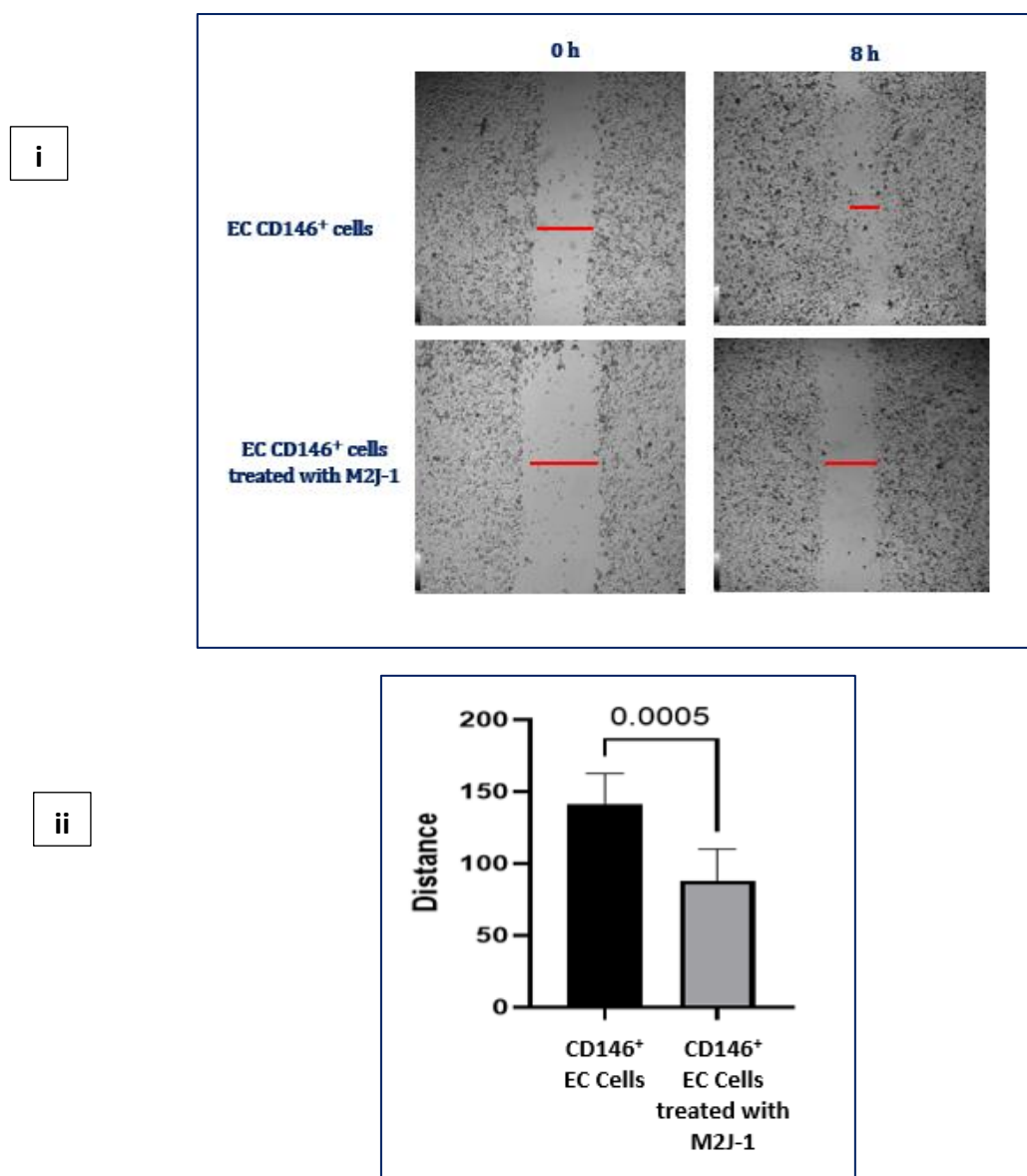


Fig 5.5. Migration assay. Migration assay of CD146⁺ EC cells with and without M2J-1 treatment. (i) Representative images of wound closure at 0 hr and 8 hr. The red lines indicate the migration front. (ii)

CHAPTER V: Role and inhibition of CD146⁺ cells sorted from endometrial carcinoma

Quantification of migration distance using ImageJ shows a significant reduction in migration distance for CD146⁺ EC cells treated with M2J-1 compared to untreated cells ($p = 0.0005$).

5.3.2.3 Invasion assay

In the invasion assay, untreated CD146⁺ sorted cells display robust invasive capacity, filling the field with cells that have penetrated the matrix. In contrast, M2J-1-treated cells show a substantial reduction in invasion, as seen in the representative images Fig. 5.6 (i) and quantified in images Fig. 5.6 (ii). The amount of invaded cells is significantly lower in the treated group ($p = 0.0003$), indicating that CD146 inhibition impairs the invasive potential of endometrial carcinoma cells

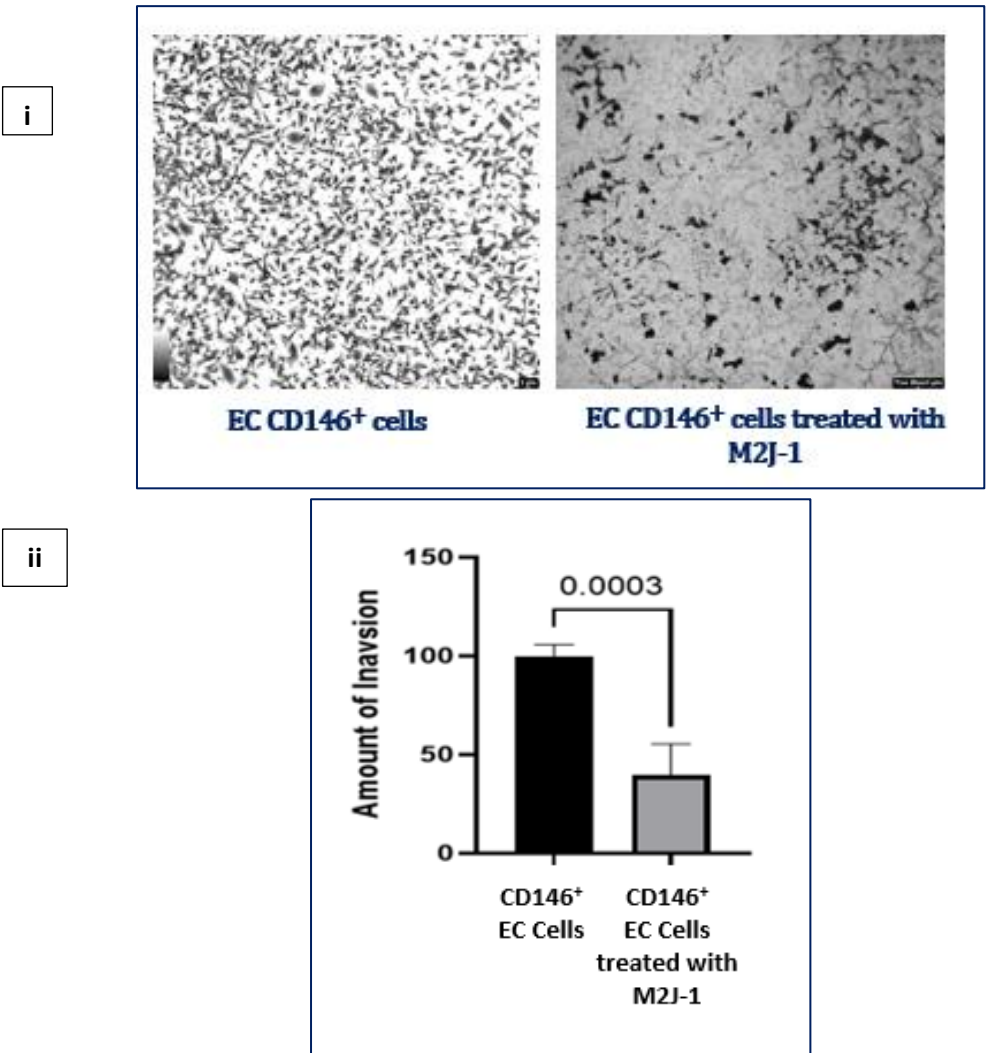


Fig. 5.6 Invasion assay of EC CD146⁺ cells with and without M2J-1 treatment. (i) Representative images of EC CD146⁺ cells showing a high level of invasion in the EC CD146⁺ group and a significantly reduced invasion in the presence of M2J-1. (ii) Quantification of invasion assay results. The bar graph represents the mean number of invaded cells, showing a significant reduction in invasion upon M2J-1 treatment compared to untreated CD146⁺ EC cells ($p = 0.0003$).

5.3.3 Growth factor analysis of CD146⁺ EC cells

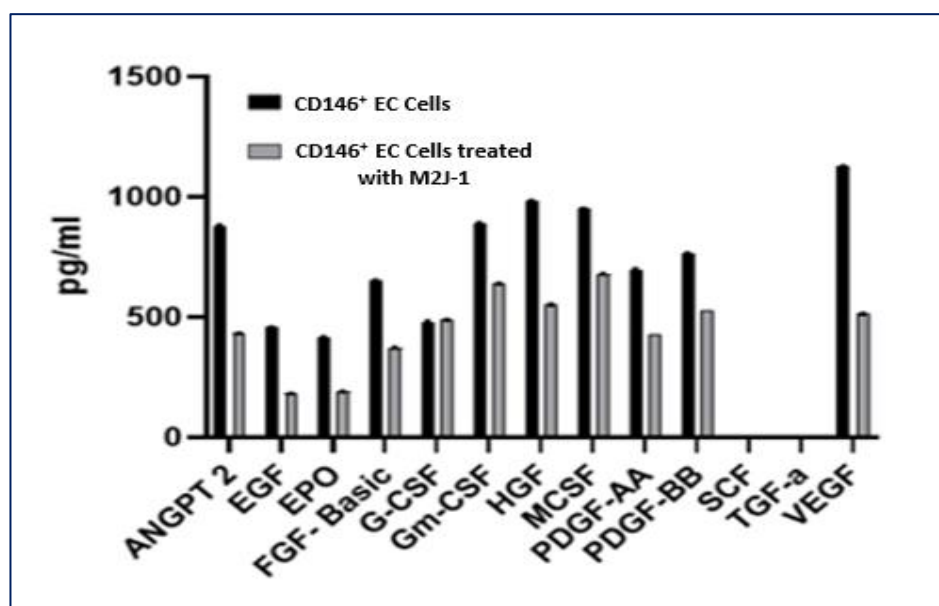


Fig. 5.7 Growth factor secretion profile of CD146⁺ EC cells with and without M2J-1 treatment. A significant reduction in the levels of key angiogenic factors, including VEGF, ANGPT2, PDGF, and others, is observed upon M2J-1 treatment, indicating its inhibitory effect on pro-angiogenic signaling. Data are presented as mean $\pm$ SEM.

The examination of growth factor dynamics was undertaken to assess the secretion patterns of isolated CD146⁺ EC and those subjected to M2J-1 treatment. The secretory profile of CD146⁺EC indicates a pronounced secretion of growth factors. Among these factors, VEGF, HGF, and ANGPT2 exhibited the highest levels of secretion which are recognized for tumor cell proliferation, angiogenesis and invasion. Conversely, upon treatment of CD146⁺ cells with M2J-1, a significant reduction in the secretion of growth factors was observed in fig. 5.7.

5.3.4 In-ovo angiogenic assay

Further, the role of sorted CD146⁺ EC cells and cells treated with M2J-1 was analyzed for angiogenesis. CAM assay was conducted in three groups as control, Sorted CD146⁺ EC cells and CD146⁺ cells treated with M2J-1 (Fig.5.8). The stereomicroscopic observation of CAM, post 48 hrs of the focal application of CD146⁺ EC cells illustrated high vasculature. While, in the group CD146⁺EC cells treated with M2J-1 the vasculature has been reduced suggesting that M2J-1 inhibits CD146 resulting in lowering angiogenesis.

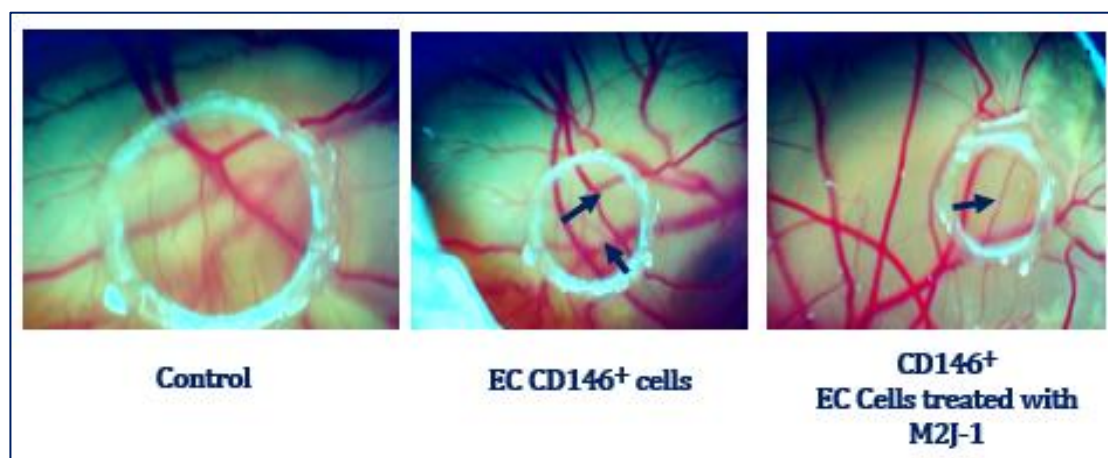


Fig. 5.8 In-ovo angiogenic assay using CAM model to evaluate the angiogenic nature of CD146⁺ EC cells and also after the treatment of M2J-1. In the group CD146⁺ EC high vasculature was observed compared to normal indicates the angiogenic potential of CD146⁺ cells. While in the treatment group, the vasculature on CAM reduced significantly after the treatment of M2J-1.

5.4 Discussion:

Angiogenesis is crucial in the pathogenesis of endometrial carcinoma (EC) that influence tumor progression, metastasis and overall survival. The advancement of disease stage depends on the vascular density. CD146 is known to be involved in the process of angiogenesis where it influences endothelial cell proliferation, migration and stabilization in physiology as well as in pathology.

CD146 being the marker for endothelial cells suggest that CD146 contributes in the vascularization tumor in endometrial carcinoma [10]. This study investigates the role of CD146 in endometrial carcinoma progression particularly through angiogenesis. The study shows, CD146 is not only the crucial biomarker for tumor growth but also a possible therapeutic target by classifying and analyzing CD146⁺ cells isolated from endometrial carcinoma tissue. The inhibition of CD146 using the monoclonal antibody M2J-1 significantly reduced cell proliferation, migration, invasion, and angiogenic potential, indicating its critical involvement in tumor pathophysiology. Flowcytometry analysis showed high expression of CD146 (~80%) in whole population of endometrial carcinoma cells confirms that CD146 could be the tumor survival marker. Since CD146 is essential for enhancing cellular adhesion, proliferation and metastatic activity, this upregulation suggests its participation in tumor growth [25]. Furthermore, expression of CD133 and CD44 in CD146⁺ sorted cells indicates that CD146 is associated with cancer-stem cell like properties. This highlights the importance of CD146⁺ cells in tumor

aggressiveness and resistance to conventional therapies [26]. Endometrial carcinoma cells' viability was dramatically decreased by inhibiting CD146 with M2J-1, according to the proliferation assay utilizing WST-1, indicating that CD146 signaling plays a role in tumor progression. A decrease in cellular metabolism and proliferation is indicated by the treated cells' decreased absorbance at 450 nm ($p=0.0145$). The wound healing migration assay also shown that CD146⁺ cells have increased migratory potential, which was markedly diminished after M2J-1 therapy. The expanded wound area seen in cells that were treated highlights how important CD146 is for cellular mobility. This implies that CD146 promotes the adhesion and cytoskeletal remodeling processes that propel the migration of cancer cells. These results were further supported by the invasion assay, which showed that untreated CD146⁺ cells exhibited strong invasive characteristics.

However, as seen by the notable decrease in the number of invading cells ($p=0.0003$), the M2J-1 therapy significantly reduced their capacity to pierce the extracellular matrix. According to these findings, CD146 is a crucial regulator of invasion [27], most likely through modifying integrin-mediated interactions and matrix breakdown enzymes. The release of growth factors that promote the invasion, angiogenesis, and proliferation of cancer cells is a crucial component of tumor development [28]. According to the study's findings, CD146⁺ cells secrete higher amounts of VEGF, HGF, and ANGPT2, three known mediators of tumor growth. In particular, VEGF is essential for maintaining tumor vasculature and encouraging angiogenesis [29]. The decrease in growth factor release following M2J-1 treatment suggests that CD146 may control these factors' expression, highlighting its function in tumor maintenance. By successfully interfering with this signaling network, targeting CD146 reduces the tumor microenvironment's capacity to support aggressive cancer cell activity [30]. Because it promotes the disposal of waste and the provision of nutrients, angiogenesis is a crucial mechanism in the development of tumors. There was strong evidence of CD146's pro-angiogenic function from the in-ovo CAM assay.

Increased vascular production was seen with application of CD146⁺ cells, indicating that CD146 amplifies angiogenic signaling pathways. Nevertheless, a significant decrease in vasculature was noted after M2J-1 therapy, supporting the idea that CD146 is essential for neovascularization [31]. The therapeutic potential of targeting CD146 is shown by the suppression of angiogenesis using M2J-1. The treatment may successfully deprive

the tumor of its vital vascular supply by interfering with CD146-mediated angiogenic pathways, which would hinder its growth and propensity for metastasis. According to the study's results, CD146 shows promise as a treatment target for endometrial cancer. significant reduction in proliferation, migration, invasion, and angiogenesis following M2J-1 treatment suggests that targeting CD146 could provide an effective strategy for controlling tumor progression. The inhibition of CD146 not only disrupts cancer stem-like properties but also interferes with tumor-associated angiogenesis, making it a dual-functional target [32]. Future studies should explore the molecular mechanisms underlying CD146-mediated tumor progression in greater detail. Investigating the downstream signaling pathways influenced by CD146 could provide deeper insights into its role in cancer biology. Additionally, in vivo studies are warranted to validate the efficacy of CD146-targeted therapies in preclinical models.

5.5 Conclusion

In conclusion, this study highlights the critical role of CD146 in endometrial carcinoma progression, emphasizing its involvement in tumor proliferation, migration, invasion, and angiogenesis. The inhibition of CD146 using M2J-1 significantly attenuates these tumor-promoting properties, demonstrating its potential as a novel therapeutic target. The findings from this research contribute valuable knowledge to the field of cancer biology and lay the foundation for further investigations into CD146-targeted therapies for endometrial carcinoma.

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General Discussion:

This study provides groundbreaking insights into the angiogenic potential and unique characterization of endometrial stem cells (eSCs), with a specific focus on CD146⁺ cells and their critical role in tissue regeneration. The isolation and comprehensive characterization of endometrial stem cells using trypsin digestion revealed the expression of key adhesion markers like CD146 and CD140b. The distinct identity of eSCs is highlighted by the presence of these markers that indicate niche-specific properties [1,2]. CD146 has been extensively documented as a marker for perivascular stem cells with significant angiogenic potential in various tissues, including the endometrium [2,3]. The findings confirm the existing literature's emphasis on CD146's role in endothelial proliferation, migration, and stabilization, making it a co-receptor for VEGF during angiogenic processes [4-6]. The endometrium's natural ability to undergo cyclical remodelling, regeneration, and vascular adaptation further validates the distinct traits of eSCs as stem cell populations [7]. eSCs have a greater capacity for angiogenesis compared to other post-natal stem cells because they secrete critical growth factors like GM-CSF, VEGF, EGF, and FGF-basic [8,9]. These findings emphasize the potential of CD146⁺ eSCs in contributing to tissue repair and regeneration, marking a significant advancement in the understanding of their therapeutic applications.

CD146⁺ eSCs have a remarkable regenerative potential that can be used in therapeutic applications for tissue repair and vascular regeneration. CD146, a marker for perivascular stem cells, holds an essential role in angiogenesis and tissue remodelling, especially in circumstances that necessitate substantial vascular support [10]. CD146⁺ eSCs have a multifaceted regenerative capacity, which includes the ability to support vascular stability, endothelial proliferation, and immune modulation [11,12].

The study found that CD146 was expressed by more than 90% of eSCs by passage 5, indicating a highly enriched population of perivascular stem cells. The secretion of angiogenic growth factors, including VEGF, EGF, and PDGF-AA, by these cells is crucial for endothelial cell survival, proliferation, and migration. The expression of Angiopoietin-1 (Ang1), a vital factor in vascular stabilization [13], was found to be significant in CD146⁺ cells, resulting in inhibition of endothelial permeability and leukocyte-endothelial interactions. These findings highlight the synergistic role of CD146⁺ cells in maintaining vascular integrity while fostering new vessel growth. CD146⁺ eSCs have a remarkable regenerative potential that can be used in therapeutic

applications for tissue repair and vascular regeneration [14]. CD146, a marker for perivascular stem cells, holds an essential role in angiogenesis and tissue remodelling, especially in circumstances that necessitate substantial vascular support [10]. CD146⁺ eSCs have a multifaceted regenerative capacity, which includes the ability to support vascular stability, endothelial proliferation, and immune modulation.

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The pro-angiogenic potential of CD146⁺ eSCs was further confirmed through functional assays. In Matrigel-based tube formation assays, CD146⁺ cells formed stable and extensive vascular networks compared to unsorted eSCs. This underscores their ability to support neovascularization, a crucial process in tissue regeneration and repair [17]. In an in-ovo yolk sac membrane model, CD146⁺ cells demonstrated superior angiogenic potential, with significantly increased vascular nodes, junctions, and segments. Immunohistochemical analysis further validated these results, showing enhanced expression of vascular markers such as CD31, VEGF, VAP, and vWF in CD146⁺ eSCs. These findings collectively suggest that CD146⁺ eSCs are highly effective in promoting and stabilizing new blood vessel formation, which is essential for regenerative processes.

In addition to their angiogenic properties, CD146⁺ cells contribute to immune modulation and extracellular matrix remodelling. Chemokine analysis revealed elevated levels of CXCL5, CXCL8, CCL3, and CCL20, indicating their role in orchestrating immune responses and supporting tissue homeostasis [18- 21]. High expression of TIMP3, a tissue inhibitor of metalloproteinases, further highlights their ability to regulate extracellular matrix dynamics, thereby controlling angiogenesis and ensuring tissue stability [22].

Despite their strong regenerative potential, CD146⁺ eSCs exhibited reduced migratory capacity in wound scratch assays, suggesting that their primary role lies in angiogenesis rather than direct migration to injury sites. Nonetheless, their robust paracrine activity and ability to form vascular structures position them as a potent tool for regenerative medicine. Future studies should explore their applications in clinical models, focusing on optimizing their therapeutic efficacy in tissue engineering and vascular regeneration.

CD146, a cell adhesion molecule, plays a pivotal role in angiogenesis and tumor progression, making it a compelling candidate for prognostic evaluation in endometrial carcinoma (EC) [23, 24]. This study highlights the intricate relationship between CD146 expression and EC progression, providing novel insights into its potential as a prognostic biomarker [25]. Although extensively studied in various cancers, the role of CD146 in EC remains underexplored. This investigation bridges that gap by analysing CD146 expression patterns in EC tissues and correlating them with clinical and pathological features, tumor behaviour, and survival outcomes.

CD146 expression exhibited distinct patterns in endometrial and myometrial tissues, with significantly higher expression observed in myometrium. This aligns with previous evidence suggesting that CD146 facilitates an invasive tumor phenotype by promoting epithelial-mesenchymal transition (EMT), a process central to metastasis [26]. The myometrial expression of CD146 suggests its involvement in tumor invasion and stromal interactions, underscoring its potential role in mediating tumor progression. Immunohistochemical analysis further validated these findings, revealing enhanced CD146 expression in stromal regions, indicative of its contribution to tumor microenvironment remodeling.

Interestingly, CD146 expression did not differ significantly across FIGO grades, with a p-value of 0.32 in t-tests and 0.29 in ANOVA. However, a trend was observed where higher CD146 expression was associated with early-stage tumors (lower FIGO grades), supporting the hypothesis that angiogenesis is a critical process during the initial stages of tumor growth [27]. Early-stage tumors likely depend on CD146-mediated vascular expansion to sustain rapid proliferation and survival [28]. Conversely, advanced-stage tumors may exhibit alternative angiogenic mechanisms or reduced reliance on CD146, reflecting the complexity of tumor progression pathways.

The correlation analysis revealed intriguing relationships between CD146 expression and tumor characteristics. A moderate negative correlation was observed between CD146 expression and tumor size (-0.26) and thickness (-2.25), suggesting that CD146-driven angiogenesis plays a more prominent role in early tumors with smaller dimensions. This implies that as tumors grow larger and progress to advanced stages, their angiogenic dependence on CD146 may diminish, possibly due to the activation of alternative signaling pathways.

Beyond its angiogenic role, CD146 is implicated in modulating cell migration, adhesion, and immune response, which are vital for tumor progression and metastasis [29]. The ability of CD146 to promote endothelial cell proliferation and stabilize vascular structures further reinforces its role in creating a supportive tumor microenvironment. Moreover, the involvement of CD146 in pathways such as PI3K/AKT and MAPK highlights its broader implications in tumor cell survival and proliferation [30].

While these findings provide a solid foundation for understanding CD146's role in EC, the study acknowledges certain limitations, including the need for larger sample sizes and longitudinal analyses to better define its prognostic value. Future research should focus on integrating CD146 expression data with machine learning approaches to enhance the precision of EC prognostic models. Such efforts can pave the way for personalized therapeutic strategies targeting CD146 and its associated pathways, offering improved outcomes for patients with endometrial carcinoma.

The intersection of regenerative medicine and oncology represents a transformative paradigm in both therapeutic and research landscapes. Regenerative medicine focuses on repairing and restoring damaged tissues using stem cells, bioengineered constructs, and biomolecules, while oncology aims to understand and combat cancer. Despite their seemingly divergent goals, these fields share commonalities in cellular processes such as proliferation, differentiation, angiogenesis, and immune modulation, paving the way for innovative cross-disciplinary approaches.

One of the critical links between regenerative medicine and oncology is the angiogenic process. Angiogenesis, essential for tissue repair and regeneration, is also a hallmark of tumor progression, as cancer cells co-opt this process to establish vascular networks that support growth and metastasis. CD146, a molecule central to angiogenesis, exemplifies this dual role. In regenerative medicine, CD146⁺ stem cells demonstrate significant

potential in promoting vascular stability, endothelial sprouting, and tissue remodeling. Conversely, in oncology, CD146 is implicated in the epithelial-mesenchymal transition (EMT) and tumor invasion [26]. This duality highlights the importance of understanding context-specific roles of angiogenic factors to harness their therapeutic potential while mitigating oncogenic risks.

Immune modulation is another critical area where these fields converge. In regenerative medicine, mesenchymal stem cells (MSCs) and other progenitor cells regulate immune responses to create a conducive environment for tissue repair [31]. Similarly, cancer exploits immune modulation to escape immune surveillance and promote tumor growth [32]. Identifying molecular pathways that delineate regenerative and oncogenic immune responses is essential for therapeutic advancements. For example, cytokines such as IL-6 and growth factors like VEGF play a pivotal role in both wound healing and tumor progression, further underscoring the need for precise targeting in clinical interventions.

Additionally, the study of cellular microenvironments presents a valuable avenue for collaboration. In regenerative medicine, the stem cell niche is engineered to optimize cellular function and support tissue regeneration [33]. In contrast, the tumor microenvironment is a target for disrupting cancer progression [34]. Understanding how the microenvironment influences cell behavior in these contexts offers opportunities for shared insights. For instance, research on extracellular matrix (ECM) remodeling in tissue engineering can inform strategies to inhibit ECM-mediated cancer progression.

Emerging technologies such as 3D bioprinting, organ-on-chip models, and machine learning further bridge these fields. While regenerative medicine uses these tools to create functional tissues, oncology benefits from their application in modeling tumor biology and screening therapeutics. Collaborative research that integrates regenerative principles with cancer biology has the potential to address complex clinical challenges, such as reconstructing tissues post-tumor resection and delivering localized, targeted cancer therapies.

By fostering a dialogue between regenerative medicine and oncology, researchers can develop safer, more effective therapies. This convergence not only enhances our understanding of cellular dynamics but also paves the way for innovations that benefit patients across diverse clinical settings.

This study establishes a crucial foundation for understanding the role of CD146 in endometrial physiology and pathology; however, it highlights several areas requiring further exploration. The limited sample size restricts the broader applicability of the findings, underscoring the need for larger, multicenter studies to validate these results across diverse populations. While the *in vitro* and *in ovo* assays provided valuable preliminary insights, the absence of comprehensive *in vivo* studies limits the understanding of the therapeutic and oncogenic potential of CD146⁺ endometrial stem cells (eSCs). Mechanistic studies are essential to elucidate the precise signaling pathways involved in CD146-mediated angiogenesis and its implications in tumor progression, offering potential targets for therapeutic intervention. Additionally, the exploration of strategies to modulate CD146 activity could open new avenues for treatments in both regenerative medicine and oncology. Integrating advanced machine learning (ML) algorithms with larger datasets, incorporating additional biomarkers and clinical parameters, could significantly enhance the predictive accuracy of prognostic models, thereby advancing precision medicine. Addressing these limitations through interdisciplinary research and technological innovation will be critical to fully harnessing the therapeutic potential of CD146 while mitigating associated risks.

The findings presented in this thesis hold significant translational potential, bridging the gap between foundational research and clinical applications in both regenerative medicine and oncology. One of the key outcomes of this work is the identification of CD146⁺ endometrial stromal cells (eSCs) as a viable, non-invasive cell source for regenerative therapies. Their robust angiogenic potential opens avenues for addressing unmet needs in various clinical domains [35]. For example, CD146⁺ eSCs could be utilized to treat endometrial disorders, including Asherman's syndrome and thin endometrium, where impaired vascularization and stromal function are key pathological features [36]. Beyond gynecological applications, these cells hold promise for promoting wound healing in chronic ulcers or ischemic injuries by facilitating angiogenesis and tissue remodelling [37]. Furthermore, their application in bioengineered constructs, such as vascularized tissue scaffolds, could enhance the functional integration and survival of transplanted tissues.

In oncology, this thesis underscores the role of CD146 as a dual biomarker and therapeutic target, particularly in endometrial cancer (EC). The identification of CD146 as a prognostic marker suggests its potential for refining risk stratification and

therapeutic decision-making. Patients with high CD146 expression may represent a subset with more aggressive disease phenotypes, warranting closer monitoring and more intensive treatment regimens [38]. Incorporating CD146 expression into machine learning (ML)-driven prognostic models could provide a personalized approach to oncology, improving patient stratification and treatment outcomes. By combining clinical parameters with molecular data, ML models can predict disease progression and therapeutic response more accurately, facilitating tailored interventions [39].

From a therapeutic standpoint, blockage of CD146 presents various novel approaches. For instance, antibody-based treatments in the form of monoclonal antibodies could be applied to effectively inhibit CD146-mediated angiogenesis and thus depritalize tumors from their vascular supply [40]. Moreover, small-molecule inhibitors that disrupt CD146 signaling mechanisms would simultaneously knock down its role in EMT and metastasis, both critical processes for cancer progression [4]. The strategies may enhance presently used therapeutic regimens or act as stand-alone therapies, particularly in drug-resistant tumor cases. Additionally, the angiogenic role of CD146 in endothelial cells suggests that inhibition of this molecule would hypothetically reduce the pro-tumorigenic effects of VEGF and other angiogenic factors; hence it represents a new approach for therapeutic intervention [41].

The potential applications of CD146 extend beyond direct therapeutic targeting [42]. In the realm of diagnostics, CD146 expression could serve as a biomarker for early detection or disease monitoring, enabling clinicians to track disease progression and treatment efficacy [43,44]. This is particularly relevant for EC, where early-stage detection significantly improves survival rates [45]. Additionally, leveraging the angiogenic role of CD146 in the tumor microenvironment could guide the development of combination therapies that simultaneously target tumor cells and their supportive niches.

In a combination, these results show the functional and clinical importance of CD146 in regenerative medicine and cancer therapy. The capacity to exploit therapeutic angiogenesis with CD146⁺ eSCs, as well as the possibility to use CD146 as a marker in cancer, is indicative of the versatility of this molecule as a clinical instrument. Further research should first focus on applying preclinical research to confirm the efficacy and feasibility of these approaches in clinical settings.

This thesis makes a step further in the development of the field of oncology by examining CD146⁺ eSCs. The results indicate that these cells may possess angiogenic activity and contribute to effective tissue repair, and have potential prognostic significance in EC. As an interdisciplinary approach, this work lays the groundwork for future investigations that seek to develop smarter therapies based on exploiting the beneficial features of CD146⁺ cells while minimizing their destructive effects. With more focused research and creativity in science, the outstanding promise of therapeutic CD146⁺ eSCs can be delivered to patients in many different fields of medicine.

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Summary and Conclusion

This PhD dissertation provides a comprehensive exploration of the role of CD146⁺ cells in both normal and malignant endometrial environments, shedding light on their potential for regenerative medicine and cancer therapy. The work investigates the functional properties, characterization, and prognostic significance of CD146⁺ endometrial stem cells (eSCs) while also examining the contribution of CD146 to the progression of endometrial carcinoma (EC). This research is notable for its integration of experimental biology with advanced machine learning approaches, offering fresh insights into how CD146 can inform prognosis and therapeutic strategies for EC patients. Through these investigations, the study establishes CD146 as an important biomarker and therapeutic target in both tissue regeneration and oncology, offering promising avenues for clinical translation.

The first part of the dissertation focuses on the characterization of CD146⁺ eSCs, a stem cell population within the endometrium with significant regenerative potential. Endometrial stem cells play a crucial role in maintaining the integrity and function of the endometrium across the menstrual cycle, and their ability to regenerate tissue after injury is key to their therapeutic potential. CD146, a well-established endothelial marker, is implicated in regulating endothelial cell adhesion, migration, and vascular remodeling, but its specific role in eSCs was not well understood before this study.

The research demonstrates that CD146⁺ eSCs have a robust angiogenic potential, making them promising candidates for tissue regeneration, particularly in situations where vascularization is inadequate. Through in vitro Matrigel assays and in vivo chick chorioallantoic membrane (CAM) assays, it was shown that these cells can form stable and organized vascular networks. This ability to induce vascular formation is essential for tissue repair and regeneration, as the formation of blood vessels is a key process in healing and recovery from ischemic injuries. The study also highlighted the chemokine profile associated with CD146⁺ eSCs, which supports their role in vascular remodeling and immune modulation. The secretion of specific chemokines indicates that these cells may influence the immune environment, making them useful in developing regenerative therapies targeting both the vasculature and the immune system.

The findings suggest that CD146⁺ eSCs could have broad therapeutic implications in regenerative medicine. In particular, their ability to promote angiogenesis could be harnessed for treating conditions characterized by poor vascularization, such as ischemic diseases, wound healing, and organ regeneration. Additionally, their potential for immune modulation suggests that they might be useful in treating autoimmune diseases and inflammatory conditions, offering new possibilities for cell-based therapies. Thus, CD146⁺ eSCs emerge as valuable candidates for the development of angiogenesis-driven regenerative treatments.

The second section of this thesis explores the role of CD146 in endometrial carcinoma, one of the most common gynecological cancers. Understanding the molecular mechanisms driving endometrial carcinoma is crucial for improving the prognosis and treatment of this disease. The research establishes that CD146 expression is significantly correlated with poorer survival outcomes in endometrial carcinoma patients, positioning it as a potential biomarker for identifying aggressive disease phenotypes. High CD146 expression was found to be associated with more advanced stages of cancer and a higher likelihood of recurrence, suggesting that it could be an indicator of poor prognosis.

To further explore the clinical utility of CD146 as a prognostic marker, the study integrates CD146 expression data into a machine learning-based model, alongside other clinicopathological parameters. This novel approach demonstrated that incorporating CD146 into predictive models improves risk stratification and could inform treatment planning. By using advanced machine learning algorithms to analyze molecular and clinical data, this study represents an innovative step toward personalized cancer care. These findings highlight the potential for CD146 to contribute to more accurate and individualized prognostic models, guiding therapeutic decisions and improving patient outcomes.

The integration of CD146 into machine learning-based prognostic models adds a valuable tool for clinicians in managing endometrial carcinoma. By identifying patients at higher risk for aggressive disease, these models could allow for more precise and effective treatment strategies. Although further validation is required, the application of machine learning to integrate molecular markers like CD146 into clinical practice represents a promising avenue for improving personalized cancer treatments.

The final part of this dissertation delves into the functional role of CD146⁺ cells in endometrial carcinoma. The study investigates how these cells contribute to the pathophysiology of cancer, particularly in relation to tumor proliferation, migration, invasion, and angiogenesis. These characteristics are central to cancer metastasis and are often associated with a more aggressive disease phenotype. The findings from this research suggest that CD146⁺ cells are actively involved in driving these processes, making them key players in the progression of endometrial carcinoma.

One of the most significant findings of this study is the therapeutic potential of targeting CD146 to inhibit tumor progression. Using the CD146-specific inhibitor M2J-1, the research demonstrated that CD146 inhibition significantly reduced tumor cell proliferation, migration, invasion, and angiogenesis in vitro. These results strongly suggest that CD146 could serve as an effective therapeutic target in endometrial carcinoma, particularly for patients with metastatic or aggressive disease. By inhibiting CD146, it may be possible to limit tumor growth and spread, thereby improving patient outcomes.

The study also provides valuable insights into the tumor microenvironment, showing that CD146⁺ cells contribute to the creation of a pro-tumorigenic niche that supports cancer progression. This highlights the dual role of CD146 as both a biomarker for prognosis and a functional contributor to tumor biology. Inhibition of CD146 could, therefore, not only improve prognosis by reducing tumor burden but also disrupt the supportive tumor microenvironment, offering a novel strategy for cancer therapy.

In conclusion, this dissertation makes significant contributions to our understanding of CD146's role in both the normal regenerative processes of the endometrium and in the progression of endometrial carcinoma. The work underscores the importance of CD146⁺ eSCs as promising candidates for regenerative therapies, particularly those aimed at promoting angiogenesis and immune modulation. Additionally, the research establishes CD146 as a critical prognostic marker for endometrial carcinoma, with potential applications in machine learning-based predictive models for personalized cancer treatment. The findings also highlight the therapeutic potential of targeting CD146 to inhibit tumor progression and metastasis, opening up new avenues for treatment.

The integration of CD146 into both regenerative and oncology contexts presents exciting possibilities for the development of targeted therapies that could improve patient care. While the therapeutic strategies discussed in this dissertation are still in the early stages of exploration, the evidence supporting CD146 as a therapeutic target is compelling. Future research should focus on further validating the role of CD146 in cancer biology and regenerative medicine, as well as refining therapeutic interventions that target CD146 for improved clinical outcomes.

Ultimately, the work presented in this thesis emphasizes the importance of CD146 as both a marker of regenerative potential and a key driver of tumor progression, offering a valuable target for therapeutic intervention in both cancer treatment and tissue regeneration. The findings set the stage for future research that could significantly impact clinical practices in gynaecological oncology and regenerative medicine, advancing the potential for CD146-targeted therapies to improve patient outcomes and quality of life.

Recommendations:

- 1. Exploration of CD146 as a Therapeutic Target:** Future research should focus on the development of targeted therapies aimed at CD146, a molecule intricately involved in angiogenesis and tumor progression. CD146 has emerged as a critical player in the angiogenic processes that support tumor growth, particularly in endometrial carcinoma. Monoclonal antibodies such as M2J-1 or small molecule inhibitors offer promising avenues for disrupting CD146-mediated pathways. To advance this area, preclinical studies should evaluate the specificity and efficacy of these targeted therapies in laboratory models. These studies should examine the ability of CD146 inhibitors to suppress angiogenesis and tumor growth in both isolated cellular environments and animal models of endometrial carcinoma. Following successful preclinical validation, clinical trials will be necessary to assess safety, tolerability, and therapeutic efficacy in humans. Additionally, it is important to explore the potential applications of CD146-targeted therapies beyond endometrial carcinoma, such as in other cancers or pathological conditions where CD146 is implicated, such as melanoma and vascular disorders.
- 2. Advanced Characterization of Endometrial Stem Cells (eSCs):** eSCs hold significant regenerative potential, and their interplay with CD146 warrants deeper investigation. Future studies should focus on unraveling the molecular mechanisms underlying CD146 expression in eSCs, particularly its role in promoting angiogenesis and vascular regeneration. This includes exploring specific isoforms of CD146 and their distinct contributions to physiological processes, such as wound healing, and pathological states, including tumor angiogenesis. Advanced molecular profiling techniques such as single-cell RNA sequencing, proteomics, and CRISPR-Cas9 gene editing could be employed to dissect the role of CD146 isoforms in eSCs. Furthermore, studies should aim to elucidate how CD146 interacts with signaling pathways, including VEGF, Notch, and Wnt, which are critical for vascular and stem cell biology. Understanding these mechanisms could pave the way for the development of innovative therapeutic approaches that exploit the regenerative and angiogenic properties of eSCs while minimizing their pathological contributions.
- 3. Translation to Clinical Applications:** Despite the immense therapeutic promise of eSCs, their clinical application remains in its infancy. Future research should prioritize translational studies to harness the potential of eSCs in vascular

regenerative medicine. Particular focus should be placed on addressing their therapeutic utility in treating peripheral arterial disease, ischemic heart diseases, and chronic wounds, including diabetic ulcers.

Genetic modification of eSCs to enhance their regenerative capacity and ensure safety profiles could be a crucial step. For example, gene-editing technologies can be used to modify eSCs to overexpress anti-inflammatory cytokines or angiogenic factors, which may amplify their therapeutic efficacy. Moreover, rigorous preclinical studies should evaluate the scalability, safety, and efficacy of these gene-modified cells compared to other stem cell sources, such as mesenchymal stem cells or induced pluripotent stem cells.

The next logical step involves conducting well-designed clinical trials to evaluate the therapeutic potential of eSCs. These trials should assess critical parameters such as the optimal dosage, route of administration, and potential side effects. Establishing standardized protocols for isolation, culture, and delivery of eSCs will be essential for ensuring reproducibility and regulatory compliance.

4. **CD146 Biomarker Development:** CD146 has significant potential as a biomarker for the early diagnosis, prognosis, and monitoring of endometrial carcinoma progression. To fully realize this potential, efforts should focus on developing sensitive and specific diagnostic tools that leverage CD146 expression levels.

Advanced imaging modalities, such as positron emission tomography (PET) and near-infrared fluorescence imaging, could be integrated with CD146-targeted probes to non-invasively monitor CD146 expression in tumors. Similarly, liquid biopsy approaches, including circulating tumor cell analysis and exosomal CD146 detection, could provide minimally invasive methods for tracking tumor progression and therapeutic response.

Bioassay development is another critical area. High-throughput assays using CD146 antibodies or aptamers could facilitate early cancer detection and personalized treatment planning. Additionally, longitudinal studies examining CD146 expression across different stages of endometrial carcinoma could help establish it as a robust prognostic marker. The validation of CD146 as a clinical biomarker requires large-scale studies involving diverse patient cohorts to ensure its reliability and applicability across populations.

5. **Targeting Pathological Angiogenesis in Early Stages:** CD146-targeted interventions hold promise not only in advanced cancer stages but also in preventing

the progression of precancerous conditions such as endometrial hyperplasia. By disrupting pathological angiogenesis in its early stages, these interventions could reduce the likelihood of malignant transformation and improve patient outcomes.

Future research should explore the role of CD146 in endometrial hyperplasia and other precancerous states. Studies could investigate how CD146 contributes to the angiogenic switch, a critical event in the transition from benign to malignant tissue. Preclinical models of endometrial hyperplasia should be developed to test the efficacy of CD146-targeted therapies in halting or reversing disease progression.

Additionally, combinatorial approaches involving CD146 inhibitors and other therapeutic agents, such as hormonal treatments or anti-inflammatory drugs, could be explored to enhance efficacy. Public health initiatives aimed at early detection and intervention in high-risk populations could further amplify the impact of these therapeutic strategies.

6. **Immunomodulatory Applications:** The immunomodulatory properties of eSCs represent an exciting avenue for therapeutic exploration. These cells secrete a plethora of cytokines and growth factors that modulate inflammation and promote angiogenesis, making them valuable candidates for treating autoimmune and inflammatory diseases.

Future research should focus on characterizing the secretome of eSCs under different physiological and pathological conditions. Proteomic and metabolomic analyses could identify key bioactive molecules responsible for their immunomodulatory effects. These findings could inform the development of cell-free therapies, such as exosome-based treatments, which leverage the therapeutic potential of eSC-derived secretomes without the challenges associated with cell-based therapies.

Preclinical studies should evaluate the efficacy of eSC-derived therapies in models of autoimmune diseases, such as rheumatoid arthritis, and inflammatory conditions, such as inflammatory bowel disease. Additionally, clinical trials should be designed to assess the safety and effectiveness of these therapies in human patients, with a focus on long-term outcomes and potential side effects.

7. **Addressing Resistance Mechanisms for enhancing Therapy Outcomes through CD146-Targeted Interventions:** One of the major challenges in cancer treatment is the development of resistance to conventional therapies, including chemotherapy and immune checkpoint inhibitors (ICIs). Resistance arises from the adaptive changes within the tumor and its surrounding microenvironment, making it difficult to achieve

long-term therapeutic success. Targeting CD146, a molecule that plays a pivotal role in tumor progression and angiogenesis, offers a promising approach to overcome these resistance mechanisms and improve the efficacy of existing therapies.

8. Assess the applicability of findings in endometrial carcinoma to other diseases (ovarian or cervical cancers, or non-reproductive cancers) where angiogenesis plays a critical role.

CD146 is a key player in angiogenesis, tumor invasion, and metastasis, making it relevant beyond endometrial carcinoma. Expanding research to other diseases can maximize the therapeutic potential of CD146-targeted interventions and eSC-based therapies:

Ovarian and Cervical Cancers: Both ovarian and cervical cancers share similar angiogenic and metastatic pathways with endometrial carcinoma. Evaluating CD146 expression and its role in these cancers could validate it as a universal target for gynecological malignancies. Preclinical models and patient-derived xenografts could be used to test the efficacy of CD146 inhibitors in these cancers.

Non-Reproductive Cancers: Many solid tumors, such as breast, lung, and colorectal cancers, rely on pathological angiogenesis for growth and metastasis. Investigating the role of CD146 in these cancers can open new avenues for treatment. For example, CD146-targeted therapies could be combined with anti-angiogenic agents like bevacizumab to enhance therapeutic outcomes.

Comparative Studies: Conducting comparative studies across different diseases will help identify shared and unique roles of CD146 and eSCs, enabling tailored therapeutic strategies. This approach may also highlight specific disease contexts where these therapies are most effective.

Fulfilling these recommendations will significantly advance our understanding of CD146 and eSCs, paving the way for novel therapeutic strategies to address angiogenesis-associated diseases and cancers. By exploring CD146 as a therapeutic target, advancing the characterization of eSCs, and translating these findings into clinical applications.



D. Y. PATIL MEDICAL COLLEGE, KOLHAPUR

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Outward No. DMCK/1591/2018
INSTITUTIONAL ETHICS COMMITTEE, D. Y. PATIL MEDICAL COLLEGE, KOLHAPUR.

Date: **14 MAY 2018**

This is to certify that the research project titled,

"Role of CD146 in Endometrial Stem Cells and Angiogenesis under Normal and Diseased Conditions."

Submitted by : Miss. Priyanka P. Hilage

Under the supervision of appointed Guide (if any): **Dr. Indumathi Somasundaram**

Has been studied by the Institutional Ethics Committee (IEC) at its meeting held on **14/05/2018** and granted approval for the study with due effect with the following caveats:

1. If you desire any change in the protocol or standard recording document at any time, please submit the same to the IEC for information and approval before the change is implemented.
2. All serious and/or unexpected adverse events due to the drug/procedures tested in the study must be informed to the IEC within 24 hours and steps for appropriate treatment must be immediately instituted.
3. In case of injury/disability/death of any participant attributable to the drug/procedure under study, all compensation is to be made by the sponsor of the study.
4. The Chief investigator/Researcher must inform the IEC immediately if the study is terminated earlier than planned with the reasons for the same.
5. The final results of the study must be communicated to the IEC within 3 months of the completion of data collection.
6. The researcher must take all precautions to safeguard the rights, safety, dignity and wellbeing of the participants in the study.
7. The researcher must be up to date about all information regarding the risk/benefit ratio of any drug/procedure being used and any new information must be conveyed to the IEC immediately. The IEC reserves the right to change a decision on the project in the light of any new knowledge.
8. Before publishing the results of the study, the researcher must take permission from the Dean of the Institution.
9. Annual progress report should be submitted for all sponsored projects to the committee.
10. Unethical conduct of research in non-sponsored projects will result in withdrawal of the ethics approval and negation of all data collected till that date.



Dr. (Mrs) Shimpa Sharma
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Characterization and angiogenic potential of CD146⁺ endometrial stem cells

Priyanka Hilage¹, Apurva Birajdar¹, Tejesh Marsale⁴, Dhanashree Patil⁵, Ashwini Mane Patil⁶, Gaurang Telang⁷, Indumathi Somasundaram¹, Rakesh Kumar Sharma^{2*} and Meghnad G. Joshi^{1,3*}

Abstract

Background The human endometrium, lining the inner uterus, regenerates over 400 times uniquely during a woman's reproductive life. Endometrial stem cells (eSCs) enrich the tissue, resulting in a dense vascular network, significant angiogenic potential, and effective regeneration power. Being of natural angiogenic properties and proven effective in the treatment of vascular disorders, eSCs can be considered safe, reliable, and superior to other post-natal stem cells. Cluster of Differentiation 146 (CD146) has emerged as a pivotal marker associated with pericytes and endothelial cells for promoting angiogenesis. Endometrial cells with high CD146 expression could proliferate and differentiate into multiple lineages. This study will explore the role of CD146 in eSCs, focusing on the potential to boost the angiogenic and regenerative functions of the cells. The novelty of this study lies in the investigation of CD146 on eSC function, which may open new possibilities for eSC-based therapy in regenerative medicine and vascular disorders.

Methods The study involved obtaining endometrial biopsies from active reproducing women to isolate and cultivate eSCs. eSCs were assessed for growth factor secretion pattern, characterized for their mesenchymal properties. Finally, eSCs were tested for their angiogenic potential by angiogenic gene expression profile and in-ovo chick embryo model. As aimed, to check the role of CD146 in eSC angiogenesis, CD146⁺ cells were magnetically sorted and cultured. The sorted cells underwent various analyses, including flowcytometry to identify mesenchymal markers and human growth factor panel to analyze growth factor secretion profiles. The study evaluated the angiogenic potential of CD146⁺ cells using functional assays, including ring formation, endothelial differentiation, and wound scratch assays, to evaluate cell migration and healing capabilities. Molecular insights were obtained through chemokine and cytokine investigations. In-ovo Chick model assay was conducted to check the angiogenic potential and evaluated through macroscopic as well as through immunohistochemistry.

Result Endometrial stem cells (eSCs) were successfully isolated using a combination of mechanical and enzymatic digestion, followed by culturing in complete DMEM media. The secretion profile of eSCs revealed significant production of various angiogenic growth factors, including Granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), Vascular endothelial growth factor (VEGF), Fibroblast

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Melanoma Cell Adhesion Molecule (CD 146) in Endometrial Physiology and Disorder

Priyanka Hilage, Mrunal N. Damle, Rakesh Kumar Sharma, and Meghnad G. Joshi

Abstract

The human endometrium, the innermost lining of the uterus, is the anatomic prerequisite for pregnancy. It is the only dynamic tissue that undergoes more than 400 cycles of regeneration throughout the reproductive life of women. Key to this function are endometrial stem cells as well as cell adhesion molecules. Melanoma cell adhesion molecule (MCAM/CD146/MUC18) is a membrane glycoprotein of the mucin family and a key cell adhesion protein, highly expressed by endometrial cells. CD146 is a significant molecule pivotal in endometrial physiology, assisting tissue regeneration and angiogenesis. Endometrium also acts as a culprit in causing several endometrial dysfunctions, such as endometriosis, endometrial hyperplasia, and endometrial carcinoma,

due to interrupted molecular and functional mechanisms. Though most of the endometrial dysfunctions arise as a result of endocrine disturbance, it has a major pathological role associated with angiogenesis. It has already been proven that CD146 is a potential marker for the diagnosis of angiogenic dysfunctions and malignancy, including endometrial cancer. However, its mechanistic role in causing the pathology is a mystery. This chapter explores the role of CD146 in normal and pathological endometrial conditions and the therapeutic implications of CD146.

Keywords

CD146 · Endometrial disorders · Endometrium physiology

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Abbreviations

AA4	Anti-CD146 monoclonal antibody clone
AA98	Anti-CD146 monoclonal antibody clone
AKT-mTOR	Protein kinase B (Akt)/mammalian target of rapamycin (mTOR)
ALCAM	Activated leukocyte cell adhesion molecule



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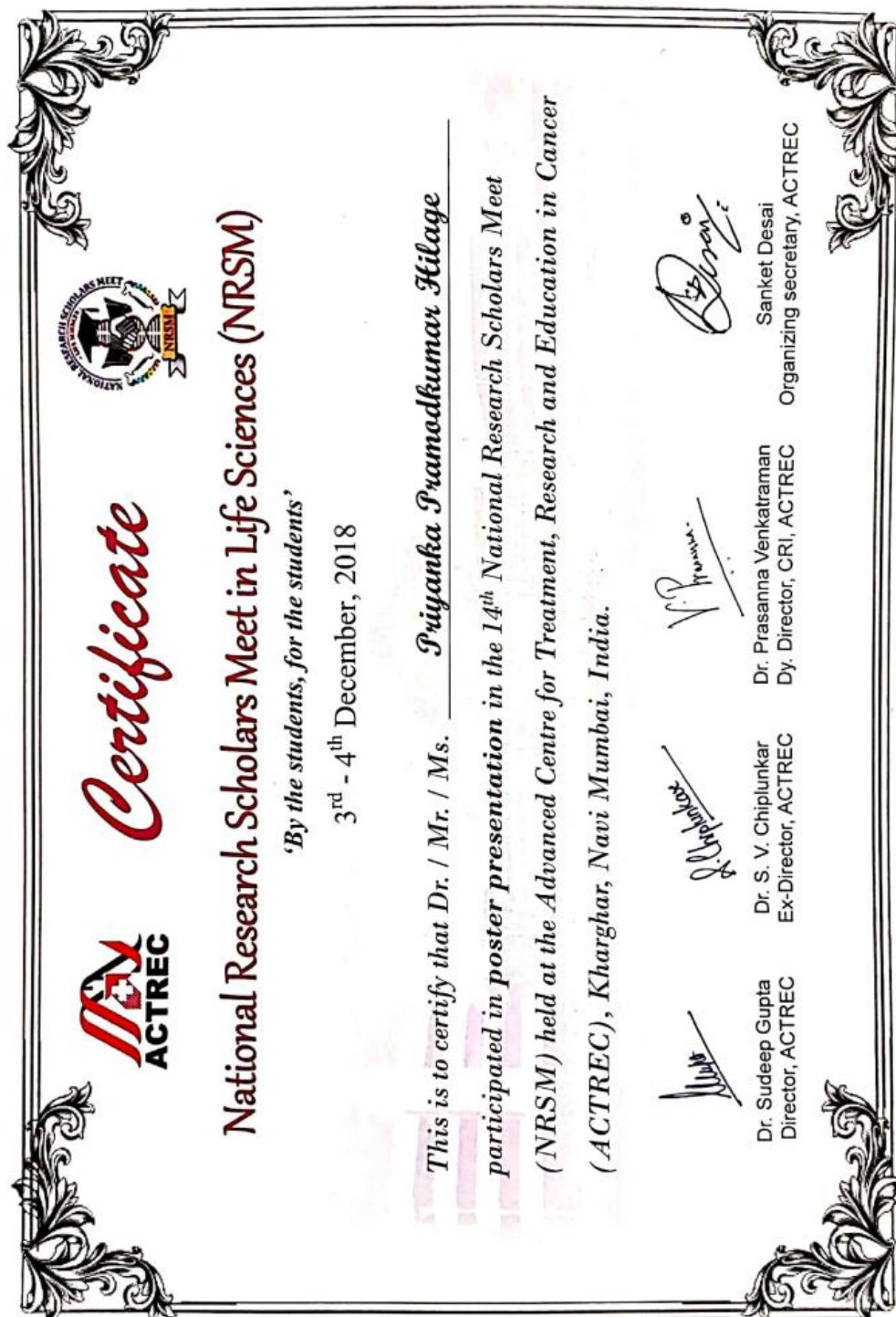
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