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The influence of tumor microenvironment on the activity and gene expression profile of organospecific endothelial cells

Dissertation for the Doctor of medical sciences and health sciences in medical sciences

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Kinga Wilkus

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1. List of abbreviations

ACE	angiotensin converting enzyme
ADAMTS2	ADAM Metallopeptidase With Thrombospondin Type 1 Motif 2
AGTR2	angiotensin II receptor type 2
Ang	angiopoietin
αSMA	alpha smooth muscle actin
BBB	blood-brain barrier
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
cAMP	cyclic adenosine monophosphate
CCL21	chemokine (C-C motif) ligand 21
COL4A4	collagen type IV alpha 4 chain
COL6A3	collagen type VI alpha 3 chain
COL9A3	collagen type IX alpha 3 chain
CX3CL1	fractalkine; chemokine (C-X3-C motif) ligand 1
CXCR4	chemokine receptor type 4
EB	embryoid body
EC	endothelial cell
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ELN	elastin
EndoMT	endothelial-to-mesenchymal transition
eNOS	endothelial nitric oxide synthase
EP	ethyl pyruvate
EPC	endothelial progenitor cell
EphB4	ephrin type-B receptor 4 gene
ET-1	endothelin 1
FBLN2	fibulin 2
GAS6	growth arrest specific 6
GSPE	grape seed proanthocyanidin extract
HBH.MEC	human healthy breast tissue endothelial cell line
HBCa.MEC	human breast carcinoma endothelial cell line
HIF1	hypoxia-inducible factor 1

HUCB	human umbilical cord blood
HUVECs	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
ILs	interleukins
iPSC	induced pluripotent stem cell
ITPP	myo-inositol trispyrophosphate
ITGB6	integrin subunit beta 6
LFA-3	lymphocyte function-associated antigen 3
LAMA1	laminin subunit alpha 1
LLC	Lewis lung carcinoma
LYVE-1	lymphatic vessel endothelial hyaluronan receptor-1
MCAM	melanoma cell adhesion molecule
MHC	major histocompatibility complex
miRNAs	microRNAs
MMP-9	matrix metallopeptidase 9
MNC	peripheral blood mononuclear cell
NCAM	neural cell adhesion molecule
NECs	normal endothelial cells
NSCLC	non-small cells lung carcinoma
PDPN	podoplanin
PECAM -1	platelet endothelial cell adhesion molecule
PGE2	prostaglandin E2
PI3K	phosphoinositide 3-kinases
PODXL	podocalyxin-like protein 1
pO2	oxygen partial pressure
PTEN	phosphatase and tensin homolog deleted on chromosome ten
RO	4-(3-Butoxy-4-methoxybenzyl) imidazolidin-2-one)
RTKIs	receptor tyrosine kinase inhibitors
Sca-1	stem cells antigen-1
SPP1	osteopontin
TECs	tumor endothelial cells
TEER	transepithelial/transendothelial electrical resistance
TNF	tumor necrosis factor
UEA	ulex europaeus agglutining

VCAM-1	vascular cell adhesion molecule-1
VE-Cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VWF	von Willebrand factor
WPB	Weibel–Palade bodies
ZO-1	zonula occludens-1

2. Abstract

Introduction: Breast tumor is one of the most common cancer type among female population worldwide, still being the cause of thousands women's death. Thus, it is crucial to understand the mechanisms not only of tumor growth but also its progression and invasion. Angiogenesis, a process in which endothelial cells (ECs) are important players, is pathological in tumor effecting in blood vessels that do not function properly as in healthy tissue and this represents one of the hallmarks of cancer. The main goal of the project is to describe the influence of organo- and biological state- specificity of ECs, by their global and characteristic gene expression pattern. We demonstrate that ECs activity displays tissue-specific responses and that the cell model of tumor-derived ECs compared to healthy endothelium reflects the tumor microenvironment. Methodology: In this project, the study focused on the cells which are part of the tumor microenvironment and shape it during the disease i.e. the endothelial cells as they are responsible for the angiogenesis. The unique model of organospecific endothelial cells was used for the experiments. The cell lines were established from the endothelium of healthy tissue and primary tumor originating the same patient with breast cancer. These cells were immortalized in defined conditions which maintained their specific endothelial phenotype in terms of features and function according to the tissue origin. The cells were cultured *in vitro* in normoxia (21% pO2) and hypoxia (1% pO2). Functional assay, pseudo-tube formation, was used to evaluate the impact and significance of organospecificity on the activity of endothelial cells. Moreover, we used the cell viability assay to compare the proliferation rate of the cells in standard culture conditions according to their healthy or breast tumoral origin. Using flow cytometry and Western blotting, we characterised the ECs phenotype and the expression of selected molecules on the protein level. To determine how the microenvironment influenced important molecules as vascular endothelial growth factor A (VEGF-A) secretion by ECs, the level of VEGF-A was measured in medium by ELISA. Next generation sequencing (NGS) by the sequencing of the whole transcriptome, helped us to identify the key genes that are modulated by the tumor microenvironment. We identified a profile for a set of genes that indeed reflects endothelial tumor cells as distinct from normal tissues endothelial cells. Results: We review the current knowledge of ECs related to their organospecificity, plasticity and listed some selective angiogenesis models in pathologies. We presented characteristics and functions of ECs showing how endothelial progenitor cells or

endothelial precursor cells and mature ECs may be used in *in vitro* studies: in 3D models and co-culture with other cells to create a blood–brain barrier (BBB). Moreover, we sum up the role of endothelial cells in angiogenesis and pathologies. In the original paper, the global gene characteristics of the cell model was performed using whole transcriptome NGS. The most deregulated genes and biological processed between tumoral *vs* normal breast tissue-derived ECs, were identified. Pathological ECs were characterized by the increase of *Ephrin-B2* and *SNCAIP*, indicative of dedifferentiation and also lowered expression of CD31, EC marker. Therefore, other ECs specific proteins (ACE+, VWF+) and their differentiation markers (CD31+, CD 133+, CD105+, CD34), were assayed. We showed that their expression was downregulated in tumor-derived ECs. Moreover, pathological ECs had decreased levels of several other adhesion molecules (ICAM-1+, VCAM-1+), and barrier formation proteins as ZO-1+. By functional assays, such as pseudo-tube formation assay and permeability test, we confirmed the differences between both cell lines, what was also indicated by VEGF-A increased level released in response to low pO2. NGS data identified several genes involved in extracellular matrix (ECM) remodelling: collagens, laminin, fibronectin and integrin (ITGB6), as being deregulated in tumor-derived ECs. This further confirms pathological angiogenesis characteristics of HBCa.MEC evidenced in functional assays. Another process identified as altered in pathological ECs was endothelial to mesenchymal transition (EndoMT) correlated with the changes of ECM organization. Deregulated genes, included: *SPP1, ITGB6, COL4A4, ADAMTS2, LAMA1, GAS6, AGTR2, PECAM1, ELN, FBLN2, COL6A3, COL9A3*. ECM remodelling gene expression profile suggested that cancer ECs acquire migratory properties, what was later confirmed by functional assay- wound healing test (data not shown, under the preparation in the next publication).

Conclusion: The presently characterised unique model of breast tissue-derived ECs, representing the healthy and the tumoral tissue, demonstrates the necessity of proper cellular models to perform biologically relevant *in vitro* research. The endothelial cells differed significantly, both phenotypically and functionally, when originating from the tumor site as compared to the normal corresponding tissue. Not only the influence of the tumor microenvironment and the adaptive capacity of ECs were determined, but also their interactions with the stroma cells. This highlights the endothelial cells-to-microenvironment crosstalk which is crucial for tumor development: aside from disturbing angiogenesis, the tumor microenvironment rules deep phenotypic changes in pathological ECs by inducing EndoMT.

Streszczenie

Wstęp: Rak piersi jest jednym z najczęściej występujących nowotworów złośliwych wśród żeńskiej populacji na całym świecie, będąc przyczyną śmierci tysięcy kobiet. Dlatego tak istotne jest zrozumienie procesu wzrostu guza, jego progresji i formowania przerzutów. Angiogeneza- proces, w którym komórki śródbłonka (ECs- ang. *endothelial cells*) odgrywają kluczową rolę- nie przebiega prawidłowo w przypadku patologicznej tkanki w porównaniu do zdrowej tkanki, co stanowi jedną z głównych cech raka (ang. *hallmark of cancer*). Nadrzędnym celem projektu jest scharakteryzowanie organospecyficzności komórek śródbłonka wynikającej z mikrośrodowiska guza, poprzez określenie globalnego wzoru ekspresji genów oraz ocenę tkankowo-specyficznej odpowiedzi oraz aktywności ECs pochodzących z guza w porównaniu ze zdrowym śródbłonkiem.

Metodologia: W ramach realizacji projektu, badania koncentrowały się na komórkach wchodzących w skład i kształtujących mikrośrodowisko guza w trakcie choroby, tj. komórkach śródbłonka, ponieważ są one odpowiedzialne za angiogenezę. Do eksperymentów wykorzystano unikalny model organospecyficznych komórek śródbłonka, wyizolowanych ze zdrowej tkanki i guza pierwotnego, pochodzących od tej samej pacjentki z rakiem piersi. Komórki te unieśmiertelniono w określonych warunkach, zachowując ich charakterystyczny fenotyp śródbłonkowy, zarówno pod względem cech, funkcji i pochodzenia tkankowego. Komórki hodowano *in vitro* w warunkach normoksji (21% pO2) oraz hipoksji (1% pO2). Do oceny wpływu organospecyficzności na aktywność komórek śródbłonka wykorzystano test funkcjonalny tworzenia pseudonaczyń. Ponadto, użyliśmy testu żywotności komórek, aby porównać stopień proliferacji w standardowych warunkach hodowli między zdrowymi i patologicznymi komórkami śródbłonka. Za pomocą cytometrii przepływowej i metody Western blot scharakteryzowaliśmy fenotyp ECs i ekspresję wybranych cząsteczek na poziomie białka. Aby określić, w jaki sposób mikrośrodowisko wpłynęło na ważne cząsteczki, takie jak wydzielanie czynnika wzrostu śródbłonka naczyniowego A (VEGF-A- ang. *vascular endothelial growth factor A*) przez komórki śródbłonka, zmierzono poziom wydzielanego VEGF-A w pożywce hodowlanej za pomocą testu ELISA. Sekwencjonowanie nowej generacji (NGS- ang. *next generation sequencing*) całego transkryptomu, umożliwiło zidentyfikowanie kluczowych genów, które są modulowane przez mikrośrodowisko guza. Określono profil ekspresji genów, który charakteryzuje komórki patologiczne

w porównaniu ze zdrowymi komórkami śródbłonka.

Wyniki: Dokonano przeglądu aktualnej wiedzy na temat komórek śródbłonka, ich organospecyficzności i plastyczności. Przedstawiono w jaki sposób śródbłonkowe komórki progenitorowe/komórki prekursorowe śródbłonka oraz dojrzałe komórki mogą być wykorzystane w badaniach *in vitro*: w modelach 3D i łączonej hodowli z innymi komórkami w celu wytworzenia bariery krew-mózg (BBB-ang. *blood brain barier*). Podsumowano rolę komórek śródbłonka w angiogenezie i chorobach. W oryginalnej pracy przeprowadzono, przy użyciu sekwencjonowania transkryptomu, globalną charakterystykę genów nowego modelu komórkowego i zidentyfikowano najbardziej rozregulowane geny oraz procesy biologiczne. Patologiczne ECs charakteryzowały się wzrostem *Ephrin-B2* i *SNCAIP*, co wskazuje na ich mniejszą dojrzałość, a także obniżoną ekspresją CD31, markera ECs. Zbadano inne białka charakterystyczne dla śródbłonka naczyń (ACE+, VWF+) i markery ich różnicowania (CD31+, CD 133+, CD105+, CD34). Pokazaliśmy, że ich ekspresja była obniżona w śródbłonku pochodzącym z guza. Ponadto patologiczne ECs miały obniżony poziom białek adhezyjnych (ICAM-1+, VCAM-1+, CD62-L+) oraz białek tworzących barierę, takich jak ZO-1+. Za pomocą testów funkcjonalnych, testu tworzenia pseudonaczyń i testu przepuszczalności, potwierdziliśmy różnice między obiema liniami komórkowymi, na co wskazywał również zwiększony poziom VEGF-A uwalniany w odpowiedzi na niskie pO2. Dane z NGS wskazały geny zaangażowane w przebudowę macierzy pozakomórkowej (ECM-ang. *extracellular matrix protein*): kolageny, lamininę, fibronektynę i integrynę (ITGB6), które uległy deregulacji w ECs pochodzących z guza piersi. To dodatkowo potwierdza patologiczną angiogenezę HBCa.MEC wykazaną w testach funkcjonalnych. Innym procesem zmienionym w patologicznym śródbłonku było przejście endotelialno-mezenchymalne (EndoMT) związane z reorganizacją macierzy pozakomórkowej spowodowaną rozregulowaniem genów: *SPP1, ITGB6, COL4A4, ADAMST2, LAMA1, GAS6, AGTR2, PECAM1, ELN, FBLN2, COL6A3, COL9A3*. Profil ekspresji genów przebudowujących ECM sugerował, że nowotworowe ECs nabywają właściwości migracyjnych, co zostało potwierdzone testem funkcjonalnym – testem gojenia się ran (dane nieprzedstawione, znajdują się w przygotowanej do opublikowania kolejnej pracy naukowej).

Wnioski: Scharakteryzowany unikalny model ECs pochodzących z piersi- zdrowej i patologicznej tkanki, wskazuje na konieczność wykorzystywania odpowiednich modeli komórkowych do prowadzenia biologicznie istotnych badań *in vitro*. Komórki

śródbłonka z guza w porównaniu ze zdrową odpowiadającą tkanką różniły się fenotypowo i funkcjonalnie. Dzięki temu modelowi, określono nie tylko wpływ mikrośrodowiska guza i zdolności adaptacyjnych ECs, ale także ich interakcje z komórkami zrębu. Wskazano na komunikację między komórkami śródbłonka a mikrośrodowiskiem, które ma kluczowe znaczenie dla rozwoju guza: oprócz zakłócania angiogenezy, mikrośrodowisko guza zmienia fenotyp w patologicznych EC poprzez indukcję EndoMT.

3. Introduction

Breast tumor is one of the common cancer among the female population worldwide accounted for approximately 24.5% and 15.5% of cancer cases and deaths, respectively [1]. Thus, it is crucial to understand the basis of breast tumors biology, concerning not only tumor growth but also progression and metastasis that are crucial for the disease pathology. Years ago, anti-angiogenic therapy was supposed to improve the outcomes treatment of metastatic breast cancer patients. Anti-angiogenic therapy dedicated to inhibit the main angiogenesis mediator, vascular endothelial growth factor (VEGF), has been applied in clinical practice. Most known, monoclonal antibody - bevacizumab - directed against VEGF activity took the key place in anti-angiogenic breast cancer therapy. The clinical immunotherapy studies with bevacizumab showed that it must be combined with chemotherapy (ex. paclitaxel, capecitabine) in patients with metastatic breast cancer. Such adjuvant strategies led to a very limited overall survival improvement [2], [3]. Currently, Nehad M. Ayoub and colleagues reviewed clinically available anti-angiogenic molecules, also these targeting non-VEGF angiogenic pathways and normalization of tumor vasculature [4]. The strategy dedicated normalization of tumor vasculature arose because it was proven that anti-angiogenic strategies led to the destruction of the vasculature with the profound deleterious effects, namely the selection of cancer stem cells due to worsening of hypoxia [5], [6]. Nevertheless, they presented that breast cancer poorly responds to the inhibition of angiogenesis in terms of improved survival of patients [4]. This is one of the reson why it is pivotal to understand the mechanism of breast cancer angiogenesis taking under consideration heterogeneity, organospecificity and phenotype of ECs derived from this exact tumor. In this work we proposed and characterised a model of immortalized ECs obtained from the same patient but from distinct sites: pathological and healthy (Fig.1). The purpose is to use them to improve methods for breast cancer studies, instead of commonly used HUVECs (cells coming from the umbilical cord) assuming that biological phenomena are not differing among organs [7], [8]. We reviewed various endothelial cell models used in *in vitro* studies, underlying endothelial cells properties and underlying how important is to design proper cellular models for the study methods [8].

During the formation of vessels, angiogenesis is made by endothelial cells (ECs), similarly to pathological angiogenesis, a process in which ECs are also important players.

It is one of cancer hallmarks that enables the other tumoral features to occur. In addition, endothelial cells display the endothelial selectivity in various tissues called organospecificity [9]. This is the reason for further selectivity of adhesion/recognitions which makes ECs to be filters for molecules and cells during their homing and extravasation. Depending on the organ and the local microenvironment, endothelial cells select specific populations of circulating cells which may enter the surrounding tissues [10]. It was firstly described by Folkman and colleagues, that tumor growth and the metastasis is dependent on vascular development [11], [12]. In very early stages of tumorigenesis, pathological cells are provided with oxygen and nutrients by the diffusion from neighbour tissues. With time, pathological cells grow in uncontrolled manner what leads to hypoxia development in the core of tumor mass. Hypoxia, pO2 (oxygen partial pressure) lower than its physiological value in the corresponding tissue, modulates the activity of distinct cell populations and shapes the tumour microenvironment [13]. Angiogenesis, mediated by endothelial cells, is induced in response to hypoxia and does not function properly as in healthy tissue. Thus, the vasculature formed in pathologic environment further promotes hypoxia instead of compensating it, due to newly developing vessels that are unable to insure a blood flow and leaky. This circle of pathological processes is driven by the cancer cells acquisition of proangiogenic phenotype leading to uncontrolled production of proangiogenic factors [14].

Microenvironment, resulting from the action of hypoxia combined to the organ and the cell origin- healthy and pathological site- influences the ECs phenotype and activity what we presented in our original paper from 2021 [7].

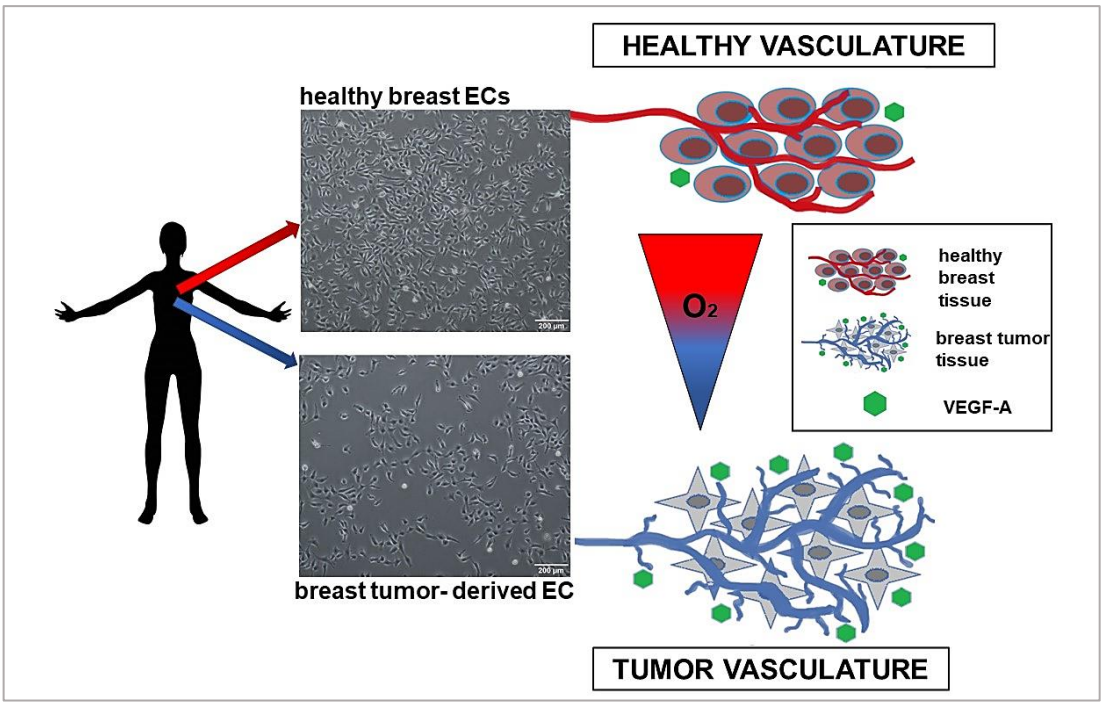

Fig.1 The graphical project overview. The immortalized endothelial cell lines of the breast tissue: healthy and pathological tissue derived, originated from the same patient. The picture shows the cell density after 48 h culture in normoxia (pO2=21%). Cells were seeded at t = 0 h in the same number. Magnification 4×.

4. Aim of the study

Firstly, the purpose of the study was to sum up the present knowledge about the endothelial models that are commonly used in not only cancer studies but also in other diseases that involve endothelium pathologies. We described the crucial ECs features correlated with their heterogeneity, functions and their organospecificity. Secondly, we indicated that the tumor microenvironment, with a strong focus to hypoxia, shapes ECs activity. Last but not least, we propose the new breast tumor endothelial cells model made of immortalized lines of endothelial cells isolated from the same patient and from the same site: Human healthy breast tissue (HBH.MEC) and Human breast carcinoma (HBCa.MEC). The study aimed to characterise the model, to identify new features that distinguish healthy ECs (healthy tissue-derived ECs) from tumor-derived ECs in the response to changes in the microenvironment and describe the global gene expression pattern characteristic for endothelial cells derived from breast tumour microenvironment.

5. Published papers

5.1 Endothelial Cells as Tools to Model Tissue Microenvironment in Hypoxia-Dependent Pathologies

International Journal of *Molecular Sciences*

Review

Endothelial Cells as Tools to Model Tissue Microenvironment in Hypoxia-Dependent Pathologies

Aleksandra Majewska, Kinga Wilkus, Klaudia Brodaczewska and Claudine Kieda

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Review

Endothelial Cells as Tools to Model Tissue Microenvironment in Hypoxia-Dependent Pathologies

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Abstract: Endothelial cells (ECs) lining the blood vessels are important players in many biological phenomena but are crucial in hypoxia-dependent diseases where their deregulation contributes to pathology. On the other hand, processes mediated by ECs, such as angiogenesis, vessel permeability, interactions with cells and factors circulating in the blood, maintain homeostasis of the organism. Understanding the diversity and heterogeneity of ECs in different tissues and during various biological processes is crucial in biomedical research to properly develop our knowledge on many diseases, including cancer. Here, we review the most important aspects related to ECs' heterogeneity and list the available in vitro tools to study different angiogenesis-related pathologies. We focus on the relationship between functions of ECs and their organo-specificity but also point to how the microenvironment, mainly hypoxia, shapes their activity. We believe that taking into account the specific features of ECs that are relevant to the object of the study (organ or disease state), especially in a simplified in vitro setting, is important to truly depict the biology of endothelium and its consequences. This is possible in many instances with the use of proper in vitro tools as alternative methods to animal testing.

Keywords: alternative methods; angiogenesis; endothelial cells; endothelial progenitors; hypoxia; induced pluripotent stem cells; in vitro 3-dimensional models; microenvironment; organo-specificity

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1. Introduction

A monolayer of endothelial cells (ECs composing the endothelium) lines the entire vascular system—blood and lymphatic vessels—forming an interface between circulating fluids and vessel wall. Endothelium plays a critical role in maintaining the homeostasis of the body through: regulation of blood pressure, blood coagulation and fibrinolysis, achieving the active transport of molecules and the participation in immunological processes (adhesion and/or transmigration of inflammatory cells and specific homing of immune cells) which is documented in many examples [1].

Due to the widespread occurrence of the vascular system in the body—endothelial cells are pervasive and occur in all parts of the organism—the weight of endothelium in an adult human is about 720 g [2]. However, endothelium is not a set of identical cells, but it is an extremely phenotypically diverse system (Figure 1). Another feature is structural, ECs differ according to their origin i.e., whether they are from microvessels (FSkMEC), macrovessels (FUmEC) (Patent number: 9228173) or lymphatics vessels (SVHEC SV40 immortalized murine endothelium cells line from peripheral lymph-node high endothelium) [3,4]. Moreover, activity of ECs varies in pathological states as compared to physiological ones (healthy breast-derived ECs and breast tumor-derived ECs). Differences are visible at the level of gene expression, surface antigens, cell morphology

and properties linked to the biological state of the organ they are in. In this summary, we present various endothelial cell models used in in vitro studies, taking into account distinct states of cell differentiation, organo-specificity, origin and functions, what will be valuable for the selection of proper, relevant to in vivo conditions, research model. The selection of ECs as models for in vitro studies requires detailed characteristics.

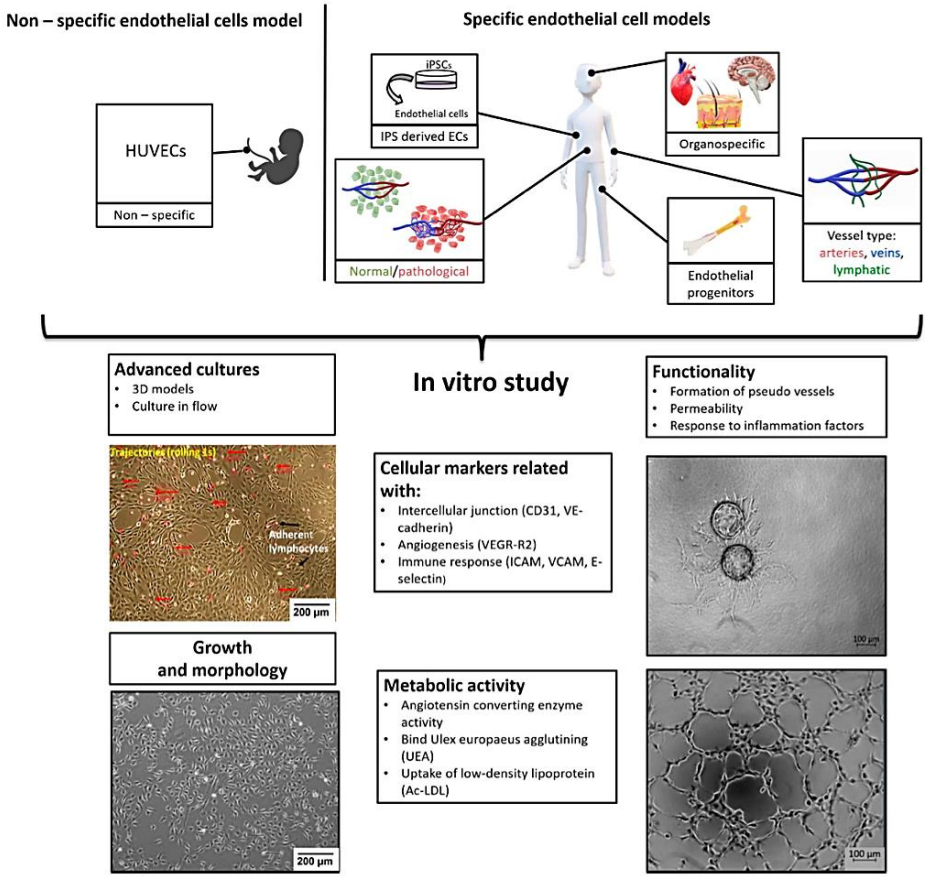

Figure 1. Organospecificity and plasticity of endothelial cells, selective angiogenesis model for signaling in pathologies and repair.

This review will consequently present a synthesis of the advancements of the knowledge about endothelial cells properties and show their necessity to design proper methods for the study of diseases. Indeed, a huge progress in deciphering the molecular mechanisms of pathologies progression is being achieved, pointing to the role of hypoxia. The lack of access to oxygen makes it crucial in the relationship of diseased cells to the vasculature and its state as deficient vs normal. Consequently, the angiogenesis-related strategies are very actively studied. They necessitate the design of relevant models that require a strong knowledge of the endothelium characteristics and biological properties.

2. Characteristic and Functions of EC

2.1. The Phenotype of ECs

The development of endothelial cells (ECs) from the mesoderm begins in the early stages of embryo gastrulation. During the process of vasculogenesis, endothelial progenitor cells (angioblasts) form a de novo primitive vascular plexus, which later differentiates into arterial, venous, lymphatic and capillary EC [5,6]. This process is also observed in adults during the recruitment of bone marrow progenitors in response to ischemic injury [7].

Endothelial cells are characterized on many levels (Figure 1): cell growth and morphology, cellular markers, metabolism and functionality. In standard 2D (two-dimensional) cultures, endothelial cells have cobblestone shape but in more advanced models with dynamic flow, due to shear stress, the cells elongate and more closely mimicking the shape, and thus the physiology, of the vessel in vivo [1]. Due to the high heterogeneity of endothelial cells in terms of surface and cytoplasmic markers, we can distinguish: markers universal for ECs such as CD31 (PECAM-1 Platelet endothelial cell adhesion molecule) [8] or VE-cadherin (CD144) [9], CD133 for endothelial progenitors [10] or function specific vessel markers such as Claudin-5 for tight junction in cerebral and lung ECs [11]. There are also markers specific to vessel types: LYVE-1 (Lymphatic vessel endothelial hyaluronan (HA) receptor-1) or in some cases IL-7 receptor for lymphatic endothelial cell [12], Ephrin-B2 for arterial endothelial cells, whereas EphB4 (Ephrin type-B receptor 4) marks venous endothelial cells [13]. Ephrin and Eph receptor tyrosine kinase families play an important role in angiogenesis and vasculogenesis both in development as in pathological processes [14]. ErphinA1 expression is strongly upregulated in hypoxic conditions in cancer cells and promotes angiogenesis through a coordinated cross-talk with PI3K/AKT dependent endothelial nitric oxide synthase (eNOS) activation [15]. The most commonly used endothelial cell markers are summarized in the Table 1.

Table 1. Cellular markers for the phenotyping of endothelial cells.

Marker	Characteristic	Reference
CD 31 (PECAM-1)	Platelet endothelial cell adhesion molecule that localizes the endothelial cell intercellular junction, is involved in the migration of leukocytes, and plays a role in angiogenesis	[8,16]
VEGFR2 (CD309, FLk-1, KDF)	Vascular endothelial growth factor receptor 2 transmembrane receptor tyrosine kinase that triggers angiogenesis; networks initiated by VEGF-A/VEGFR2 leads to endothelial cell proliferation, migration, survival and new vessel formation involved in angiogenesis	[17]
VEGFR3	Vascular endothelial growth factor receptor 3 transmembrane receptor tyrosine kinase; characteristic marker of lymphatic endothelial cells; VEGFR3 and its ligands (VEGF-C and VEGF-D) are involved in lymphangiogenesis and by forming complexes with VEGFR2 plays a role in angiogenesis	[18]
CD144 (VE-cadherin)	Endothelial specific adhesion molecule responsible for junction between cells, inhibition of VE-cadherin increases monolayer permeability and enhances neutrophil transendothelial migration	[9]
VWF	Von Willebrand factor (VWF) is a glycoprotein released from Weibel–Palade bodies (WPB) of endothelial cells and is associated with blood clotting by stabilizing factor VIII	[19]
EphB4	Receptor tyrosine kinase, marker of adult venous ECs	[13]
Ephrin-B2	Transmembrane ligand for EphB4, marker of arterial endothelial cells	[13]
CD 54 (ICAM-1)	Intercellular adhesion molecule-1 is involved in adhesion of immune cells during inflammation	[16]
CD106 (VCAM-1)	Vascular cell adhesion molecule-1 is involved in adhesion of immune cells during inflammation	[16]

Table 1. *Cont.*

Marker	Characteristic	Reference
CD146 (MCAM)	Melanoma adhesion molecule facilitates cell-cell interaction and is involved in inflammation and angiogenesis	[20]
CD105 (Endoglin)	Receptor for transforming grow factor β (TGF-β) affects angiogenesis by regulating ECs proliferation; induces the anti-apoptotic pathway of ECs in hypoxia	[21]
CD62e (E-selectin)	Endothelial leukocyte adhesion molecule-1, glycoprotein from the family of selectin (E-selectin, L-selectin, and P-selectin), it is expressed in endothelial cells after stimulation by TNF-α (tumor necrosis factor alpha), Il-1 (interleukin 1) or bacterial lipopolysaccharides, main player in early and specific adhesion of immune cells	[16]
Podoplanin	Membrane glycoprotein of podocytes, specific marker for lymphatic endothelial cells, plays a role in the regulation of lymphatic vascular formation and movement	[22]
LYVE-1	Membrane glycoprotein capable of binding to hyaluronic acid, marker of lymphatic endothelial cells	[12]
CD44	Cell surface adhesion receptor, is a marker of late endothelial progenitor (EPC) cells plays a role in ECs' regeneration	[23]
CD34	Glycoprotein first identified on hematopoietic stem and progenitor cells but it is also present in most micro-vessels in the umbilical artery but not in the endothelium of large vessels	[24]
CD133 (Prominin-1)	Tissue-specific stem cell marker, characteristic for EPCs	[10]
CD202b (Tie-2)	Hematopoietic stem cells marker also present in EPCs, receptor for Ang-1 and Ang-2	[25]

The assessment of typical endothelial biochemical pathways is another parameter of the endothelial cell characterization. Endothelial cells exhibit angiotensin converting enzyme activity (ACE; CD143), involved in the metabolism of angiotensin and inactivation of bradykinin [26]. ECs express receptors for acetylated low-density lipoprotein (Ac-LDL), which can be easily detected after incubation with labeled ligand (DiL-Ac-LDL) [27]. Binding of Ulex europaeus agglutining (UEA) is also characteristic for endothelial cells [28].

2.2. *Regulation of ECs' Heterogeneity*

As Aird W.C. describes, endothelial cell heterogeneity is associated with epigenetic modifications (DNA methylation, histone methylation and histone acetylation), caused by extracellular signals, which negatively or positively affect gene expression. Epigenetic changes can persist after removal of signals and are transmitted during mitosis. It is different in the case of the micro-environmental impact, which also influences endothelial cells' heterogeneity—it is represented by receptor-mediated posttranslational modification of transcription factors and other proteins, but the removal of the external factor causes a loss of such effects and a phenotype change linked to each subsequent mitosis [29]. These changes make the primary endothelial cells ineffective in in vitro studies because their phenotype becomes less specific with each passage due to the lack of a unique microenvironment. Chi et al. demonstrated that ECs isolated from different sites of the human vasculature after multiple passages had different transcriptional profiles between not only macrovascular and microvascular ECs but also between arterial and venous ECs [30]. These findings were supported by Lacorre et al. who showed that in vitro culture of differentiated ECs resulted in downregulation of genes, which were upregulated in natural tissue microenvironment [31]. These data indicate that epigenetics and microenvironment play role in mediating regulation of tissue-specific EC genes expression. Moreover, Burridge and Friedman, presented in their DNA microarray study, that origin influenced on differences in endothelial transcriptome of coronary and iliac arteries [32]. Taken altogether,

heterogeneity of ECs may be caused by effects of different extracellular environments and epigenetic modifications induced by extracellular signals, and these have to be taken into account when culturing ECs in vitro, devoid of these signals. Not only epigenetic modification but also biochemical modification, such as glycosylation of the cells, affects modulation and regulation of biological processes [33,34].

2.3. microRNA Mediated ECs' Modulation

Small non-coding RNA molecules (microRNA, miRNA) that regulate gene expression are also involved in maintaining homeostasis and ECs' functionality. Fish et al. found that miR-126, which is known as an endothelial-specific miRNA, regulated the response of ECs to VEGF (vascular endothelial growth factor) by repressing negative regulators of the VEGF pathway, including the Sprouty-related protein (SPRED1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2/p85-β) what promoted angiogenesis and indicated that miR-126 regulates vascular integrity [35]. miR-31 and miR-17-3p induced by TNFα (tumor necrosis factor α) are associated with increased expression of adhesion molecules in ECs (selectin-E and ICAM-1), therefore are involved in maintaining important immunological functions of ECs [36]. In in vitro, studies miR-125b was shown to inhibit the translation of VE-Cadherin mRNA and tube formation by ECs, in-vivo miR-125b induced nonfunctional blood vessel formation [37]. Regulation of angiogenesis at the miRNA level (both promoting and inhibiting) is important in many pathological states. Proagniogenic miRNAs include, for example, miR494 targeting PTEN (phosphatase and tensin homolog deleted on chromosome ten) in lung cancer [38] or miR -155 targeting VHL (Von Hippel–Lindau tumor suppressor) in breast cancer [39], in turn, miR622 has an anti-angiogenic effect through by suppressing the CXCR4-VEGFA axis in colorectal cancer [40]. The effect of the microRNA may differ depending on the type of tumor—miR-27-b has pro-angiogenic activity in lung [41] but anti-angiogenic in ovarian cancer [42]. MicroRNA-103 regulates the vascular permeability by inhibiting VE-cadherin as well as other molecules associated with endothelial integrity as p120-catenin (p120) and zonula occludens 1 (ZO-1) which results in the promotion of metastasis [43]. Patients with ischemic stroke had a low miR-221 level and a study by Peng H. et al. confirmed that miR-221 caused a decrease in the viability and migration of ECs by targeting the PTEN/PI3K/AKT pathway [44]. The above examples show that miRNAs, originating from ECs and surrounding cells, regulate ECs' activity and functionality by targeting various signaling pathways and modulate EC function on many levels. This regulation plays an important role in many pathological states, which shows the possibilities of using miRNA-based therapies acting on endothelial cells. The detailed role of miRNAs in endothelial cells has been reviewed elsewhere [45–47].

2.4. Functional Heterogeneity of ECs

Endothelial cells display many functions, most of which are performed by vascular beds or specific subsets of blood vessel types. Apart from hemostasis, leukocyte trafficking or regulation of vasomotor tone, ECs take part in angiogenesis, permeability and acquired immunity [48]. Angiogenesis is the process of forming vessels from pre-existing ones and plays an important role in physiological processes (e.g., in adults: wound healing, in the female reproductive tract under control of the estrous cycle). On the other hand, pathological angiogenesis is associated with the development of many diseases such as cancer, rheumatoid arthritis or retinopathies [49]. The tube formation assay or sprouting assay are used to test the functionality of ECs in in vitro conditions (Figure 2) [50,51].

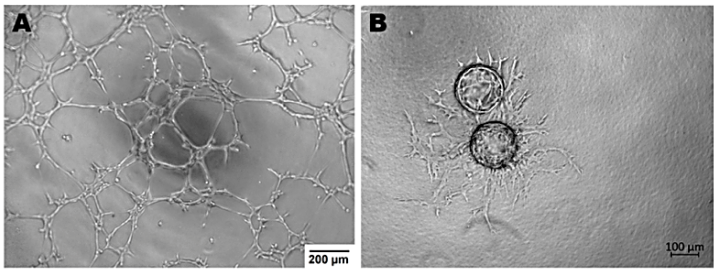

Figure 2. Endothelial cells ability to angiogenesis in vitro. (**A**)—Tube Formation Assay, magnification 4×; (**B**)—Sprouting Assay based on Cytodex beads, magnification 5×; both assays were performed with murine brain derived endothelial cells (MBr MEC FVB).

In addition to testing for angiogenesis, further functional tests based on EC properties include the endothelial permeability assay which is particularly important in studies related to the blood–brain barrier or metastasis. Vascular permeability is closely related to the TEER (Transepithelial/transendothelial electrical resistance) value, which is a strong indicator of the integrity of the endothelial cell layer and is evaluated prior to drug and chemical transport testing [52]. In permeability tests, Transwell filters covered with ECs are usually used and after adding the test substance to the top of the filter, its concentration in the bottom of the filter is measured. Permeability is also tested in advanced microflow models, which further ensures shear stress, also affects permeability and better reflects in vivo conditions [53,54].

The function of endothelial cells is also associated with immunity and response to inflammation, by regulating the migration of immune cells through the vascular wall to inflammatory sites. Adhesion molecules (such as ICAM-1, VCAM-1, Selectins, PECAM-1) play an important role in this process [55]. Therefore, in in vitro conditions the degree of activation of adhesion molecules after cytokine stimulation can be assessed, such as E-Selectin expression after TNFα or IL-1 stimulation [16]. Functional studies include a simple test for the adhesion of immune cells to a monolayer of endothelial cells [56,57] or a more advanced microfluid test, where the rolling and adhesion of leukocytes can be observed in real time [20,58]. Bielawska-Pohl et al. showed that interaction between immune cells and organospecific endothelial cells can be a potential target to block vascular injury [59].

3. ECs for in vitro Research

3.1. Immature Endothelial Cells—Endothelial Progenitor Cells/Endothelial Precursor Cells

Endothelial progenitor cells (EPCs) are defined as cells that are able to differentiate into mature endothelial cells and contribute to the formation of new blood vessels. Putative EPCs were first described in 1997. Asahara T. et al. isolated cells enriched for expression of CD34 and Flk-1 (receptor for vascular endothelial growth factors A and B; VEGFR2) among peripheral blood mononuclear cells (MNCs) in the circulation. These putative EPCs, upon transplantation, localized in the vessels and contributed to promoting vascular regeneration at sites of ischemia [60]. This initiated an increased interest in the topic of EPC and the development of methods to use EPC to repair ischemic organ functions through enhanced vasculogenesis [61,62]. At the same time, many controversies related to the classification, characteristics and nomenclature of these cells arose. The main problems are: the lack of specific markers, as well as high heterogeneity of EPCs, associated with various proliferative and differentiation abilities and EC plasticity conditioned by the environmental composition which results in the lack of unequivocal data [34]. Expression of markers changes during the trafficking and during the process in which EPCs differentiate

into endothelial cells—in bone marrow these cells express CD34, CD133 and VEGFR2, while in the circulation, they express CD31 and CD146, and once reaching maturity markers characteristic for ECs as VE-cadherin, von Willebrand factor (VWF) are detected [63]. EPCs types distinguished by their origin and isolation methods (MNCs culture or flow cytometry) were reviewed by Medina R.J. et al. [64]. The source of EPCs for in vitro tests is most often peripheral blood [60] and bone marrow [65], cord blood [66,67], but also fetal liver [68], aorta gonad [69] and adipose tissue [70]. As indicated by Yoder M.C. et al. various methods of EPC isolation provide cells with different phenotypes—capable or unable to integrate into existing vessels in human model [71].

Significant differences in both gene expression and functional abilities, were observed in immortalized endothelial cell progenitors isolated from mouse aorta-gonads: 10.5 or 11.5 days post conception (dpc)—MAgEC 10.5 and MAgEC 11.5, which represent a model of EPCs at different stages of maturation. Both lines highly expressed stem cell markers (Sca-1 and CD34), but also EPCs/mature endothelial cell markers (PECAM-1 (CD31)) and the angiotensin converting enzyme, VWF, while expression of EphB4 indicates their venous vessel commitment. Loss of CD133, increase of VE-cadherin, VEGFR2 and PODXL (podocalyxin-like protein 1) (phenotype associated with mature EC) protein expression were observed in more differentiated MAgEC11.5. There are also significant differences in the ability to form pseudo-vessels between these two cell types. MAgEC 11.5 generate pseudo-vascular structures and networks, while MAgEC 10.5 do not have such abilities, but acquire them after stimulation with CX3CL1 and CCL21, which confirms that MAgEC 10.5 is present in the phenotype of early progenitors. Both cell lines effectively cooperate with mature MLuMEC lung microvascular cells to produce pseudo vessels in vitro, which gives hope for their influence in neovascularization in diseases such as cancer [69,72].

Primary cells of EPC characteristics could be obtained by culture of MNCs isolated from human umbilical cord blood (HUCB)—up to day 3 of culture, cells display monocytic characteristics (CD14+, CD45+), but after 6 days in culture the expression pattern shifts to an endothelial character (cells positive for VEGFR2, VE-cadherin, took up Ac-LDL and bound the endothelial specific lectin UEA-1); however, CD133 and CD34 are not expressed. In the same study, the phenotype of EPCs obtained after prior selection of CD45+ cells was also assessed—these cells also expressed endothelial cells phenotype over time, but also retained progenitor markers CD133 [73].

Circulating (recruited from the bone marrow) endothelial cell progenitors appear to be an important marker and significance for potential therapy in many diseases, such as cancer [74,75] cardiovascular [76,77] or pulmonary diseases [78], endometriosis [79] and diabetes [80,81].

Two cell lines, human endothelial progenitor cells—cord blood: HEPC-CB.1 and HEPC-CB.2 were isolated from umbilical vein and immortalized in vitro as described the authors Paprocka et al. [67]. These cell lines are early progenitor cells and are characterized by high proliferation ability. Both HEPC-CB.1 and HEPC-CB.2 were investigated by flow cytometry in order to assess their immunophenotype. It has been shown, that they are positive for the following markers: general stem cells (CD133), hematopoietic stem cells (CD13), non-hematopoietic stem cells (CD271), mesenchymal stem cells (CD90) and endothelial cell markers; mature as CD202b, VEGFR2, CD146 and stem-like or activated as CD105 [67,82]. Both cell lines are positive for CXCR4 (fusin, CD184); the unique receptor for the CXCL12 (SDF1 stromal cell-derived factor 1) chemokine, and weakly express markers of stem cells and endothelial cells: CD44 and CD15s. Both, HEPC-CB.1 and HEPC-CB.2 were weakly positive for the markers of progenitors but also differentiated endothelial cells (UEA-1 and Dil-Ac-LDL). On the other hand, both cell lines did not present markers of hematopoietic origin (CD34, CD117, CD45) and were negative for some markers of differentiated endothelial cells (CD31 and VWF). Moreover, when cultured in various oxygen partial pressures, these lines showed different patterns of chemokine secretion. The levels of IL-8, VEGF and angiogenin were higher in hypoxia than in normoxia. Moreover, both cell lines secrete IL-6 and IL-10 (anti-angiogenic factor) [67]. Furthermore, they

were CD133+, CD34−, VEGFR2+ and CD31− what characterizes immature endothelial progenitor cells [83]. In addition to the above, endothelial nitric oxide synthase (eNOS) mRNA was present in both cell lines confirming the endothelial character of these cells [67, 84]. However, it should be mentioned that there were differences between both cell lines. Firstly, the higher expression of CD133 and lower of CD271 was presented by HEPC-CB.2 as compared to HEPC-CB.1 [82]. IL-10 was secreted at a lower level by HEPC-CB.2 than second cell line [67]. Taking into account migration ability, both cell lines were more prompt to migrate to the increasing VEGF-concentration medium. Although both cell lines present a clear angiogenic potential as indicated by the expression of receptors: VEGFR2, and CXCR4 the cells did not achieve angiogenesis in in vitro assay [67]. Taken altogether, both cell lines were positive for the early endothelial markers but did not or poorly express markers of differentiated endothelial cells and can provide a relevant in vitro model of EPCs due to their phenotype and angiogenic properties.

3.2. Mature Organospecific ECs

3.2.1. HUVECs

Human umbilical vein endothelial cells (HUVECs) have been the most frequently used in vitro models in oncology or cardiovascular EC research since 1973, when they were described for the first time by Jaffe E. et al. [85]. These primary cells are obtained by collagenase digestion of the interior of the human umbilical vein, could only be cultured up to a few months (5 months) because they undergo cellular senescence which finally leads to cell death [85,86]. Due to this fact, only short-term research could be performed on with HUVECs as models. However, Folkman J. et al. proposed a culture up to 8 months for longer experiments, but still different donors of the cell line were sources of errors [87]. This heterogeneity of donors results in complication when the data are compared [88]. It has been shown that responsiveness to IL-8 may vary between culture conditions of HUVECs [88]. Moreover, the vascular origin plays a crucial role in morphology of the cells. Human placental endothelial cells (HPEC) are more elongated and form networks in low density cultures compared to human umbilical vein endothelial cells. Taken altogether, it could be advised to use the immortalized endothelial cell line which will be a model of experiments giving repeatable and comparable results [88].

To avoid all limitations of primary cultures of ECs described above and to investigate large-vessel endothelium, another cell line was obtained—EA.hy926—by the fusion of HUVECs with A549 (human lung carcinoma cell line) [89]. This cell line expressed only one marker of differentiated vascular endothelium, factor VIII-related antigen. Although, Edgell C. J. et al. established EA.hy926 cells that maintained VIII-Ag for 100 cumulative population doublings, including more than 50 passages and three cloning steps, the absolute criteria for predicting the immortality of this cell line were not established [89]. Ahn. K. et al. demonstrated that EA.hy926 preserves the endothelin converting enzyme (ECE) activity of HUVEC and is useful for the study of ECE and its regulation of endothelin-1 (ET-1) production [90]. EA.hy926 has been used as a model in the research investigating ethyl pyruvate (EP) as an inhibitor of LDL oxidation, which is a central element in the development of atherosclerosis [91]. Furthermore, this cell line was used to evaluate the effects of grape seed proanthocyanidin extract (GSPE) under high glucose condition [92]. Taken altogether, EA.hy926 is a permanent human cell line that is widely used in the EC research, though not being well characterized in the criteria of endothelial markers (CD202b, CD146, CD105, CD143), markers of finally differentiated endothelial cells (CD31, VWF, CD45) and activity (pseudo-tube formation, wound healing).

3.2.2. Adult ECs from Various Sources

Primary ECs have been described according to their origin: artery or vein, macrovascular or microvascular vessel. Accordingly, we can distinguish macrovascular cardiac ECs as the following: human aortic endothelial cells (HAEC) [93], human coronary artery endothelial cells (HCAEC) [94], human pulmonary artery endothelial cells (HPAEC) [95]. The

microvascular ECs isolated from humans are: human dermal microvasculature endothelial cells (HDMEC) [96], human pulmonary microvasculature endothelial cells (HPMEC) [97], human brain microvasculature endothelial cells (HBMEC) [98,99].

To demonstrate the effective organo-specificity of endothelial cells, tissue-specific ECs were isolated, characterized phenotypically and functionally by Kieda C. et al. [4]. These include ECs isolated from peripheral lymph nodes from surgical biopsies: HPLNEC.B3 (cervical), HPLNEC.S1R1 (inguinal), HMLNEC.MEL (mesenteric); cells isolated from patient with ovarian carcinoma (HOMEC.J6B), appendicitis (HAPEC.S1), cells originated from normal skin (HSKMEC.1) microvasculature and normal intestine (HIMEC.1). Furthermore, immortalized HS888Lu cells from normal lung tissue of a patient with osteosarcoma metastatis to the lung (HLMEC) were described [4]. These cell lines, maintaining their organo-specific characteristics, were well characterized (Patent number: 9916169A, 9631178). All cell lines were positive for ACE, VE-cadherin and E-selectin what confirms that they maintain their endothelial phenotype. P-selectin positive cells, EC activation marker, were found in the following cell lines: HSKMEC.1, HAPEC.S1 and HIMEC.1 [4]. Furthermore, other markers, related to the EC maturation level or organ of origin, were tested: CD34 (positive cell lines: HPLNEC.B3, HPLNEC.S1R1, HOMEC.J6B), CD54 (positive cell lines: HSKMEC.1, HMLNEC.MEL, HAPEC.S1, HIMEC.1), CD49e (positive cell lines: HSKMEC.1i, HAPEC.S1, HIMEC.1) and CD44 for which all cell lines were negative, except HIMEC.1 [4]. These phenotypes were maintained after immortalization what makes described cell lines a valuable tool to study organo-specific ECs in long-term in vitro experiments (Patent Number 9916169A).

Another cell line that omits limitations of primary cultures and donor-dependent variable results used in microvascular endothelium studies, is HMEC-1, obtained by transfection of human dermal microvascular ECs (HDMECs) with SV40T sequences [100–102]. Yuelin Xu et al. demonstrated that HMEC-1 constitutively expressed platelet-endothelial cell adhesion molecule (CD31), ICAM-1 (CD54), LFA-3, and MHC class I but lacked of CD36 (thrombospondin receptor), NCAM, and MHC class II. These phenotypic characteristics were identical to those of HUVECs but differed from HDMECs, which expressed both CD36 and NCAM HMEC-1 EN4, PAL-E, H3/5-47, and CD36 [102]. They also express the cell adhesion molecules ICAM-1 and CD44 [100]. Moreover, early studies indicated morphologic similarities with HDMECs and that HMEC-l formed tubes on Matrigel^{TM}, took up Ac-LDL, and expressed VWF. These characteristics remained stable, with the exceptions of VWF and CD36. HMEC-1 no longer expresses either of them, although mRNA for VWF is still present [102]. Ades. E. et al. have established HMEC-1 and present that the cell line might be passaged more than 95 times and does not undergo senescence, showing a life span 10-times longer than the primary culture [100,101]. Muñoz-Vega, M. et al. showed that HMEC-1 present similar morphology, size and granularity of the cells as HUVECs. HMEC-1 expressed ICAM-1 and VCAM-1 after TNF-α stimulation [101]. Moreover, Kryczka J. et al., showed that HMEC-1 adopt the mixed amoeboid-mesenchymal migration type during EndoMT (Endothelial-to-Mesenchymal Transition) (downregulation of the endothelial marker VE-cadherin) [103].

The next promising cell lines to study microvascular endothelium are dermal vascular endothelial cells that were immortalized by stably expressing human telomerase catalytic subunit hTERT (hTERT-HDMEC) [104]. All hTERT-EC lines resembled young primary ECs in their morphology and growth response, with little or no staining with SA β-galactosidase activity, defining hTERT-EC line as immortalized [104]. Moreover, data indicate that hTERT(+) ECs retain EC characteristics such as: expression of Von Willebrand factor, high PECAM-1 reactivity, LDL uptake. In the addition, TNF-α-stimulated cell surface expression of ICAM-1, VCAM-1 and E-selectin [104]. Data also show pro-angiogenic responses on 3D (three-dimensional) collagen culture by formation of tubes in vitro [104].

Besides the human cells, similar endothelial cells' immortalization and lines establishment in conditions that keep the phenotype stability were performed from murine, canine and bovine origins, which not only allowed the confirmation of the high degree

of organo-specificity, but also demonstration of the species-specificity of infectious diseases [105]. Moreover, the immortalization of cell lines was performed from cat and eleven lines were obtained from distinct tissue origin (Patent number: 9228173). The feline skin endothelial cells (FSkMEC) derived from the microvasculature and the feline umbilical cord endothelial cells (FUmEC) derived from the macrovasculature were used to demonstrate the cellular process of specific EC skin bacterial invasion by Bartonella Hansellae in cats and the species specific transmission to human [105]. The similar cell line establishment strategies in bovine were performed to study, in proper models, the organ and specific molecular mechanisms of viral infections [106].

Considering the above described characteristics of cells, the use of HUVECs as a standard model is risky because it does not reflect the in vivo state and ECs' heterogeneity. Furthermore, the isolation of organospecific cells is a new way to obtain cell models for in vitro (iPSCs- induced pluripotent stem cells) that are more relevant to in vivo conditions. It opens to future use in personalized medicine as a tool for targeted therapy.

3.3. iPSCs Derived Endothelial Cells

The possibility of reprogramming somatic cells into pluripotent stem cells by introducing the genes coding for four factors, Oct3/4, Sox2, c-Myc and Klf4, described by Yamanaka S. and Takahashi K. [107,108] did facilitate advances in regenerative medicine, but also contributed to the development of new strategies in in vitro research. iPSCs (induced pluripotent stem cells) have the potential to differentiate into other cells including endothelial cells, which allows for generating patient-specific ECs and their uses in in vitro tests as well as taking advantage of their therapeutic potential. Many methods of iPSC differentiation into endothelial cells have been developed, including: co-culture with stromal cells, embryoid body (EB), two-dimensional culture on a matrix-coated surface with the addition of appropriate molecules and growth factors as well as three-dimensional culture [109,110]. Choi K.D. et al. co-cultured various human iPSC cell lines with OP9 feeders for 8 days, which resulted in the generation of hematopoietic precursors and endothelial cells (CD31+, CD43−). These ECs also expressed CD105, CD144, VE-cadherin, VEGFR2, and were also able to form tubes on Matrigel^{TM} [111]. Similar results were obtained by Taura et al., but in both cases a low efficiency (<6%) of differentiation was noted and the resulting cell culture was strongly heterogeneous [112]. Better efficiency was obtained using the embryoid body (EB) differentiation relying on the spontaneous differentiation of aggregated iPSC. Adams W.J. et al. characterized the timescale of EC differentiation from EBs—after 10 days they obtained 18 ± 4% CD144 and CD31 positive cells which are typical mature EC makers. In addition to assessing endothelial-specific markers (presence of CD144, CD31, VEGFR2 and others), the authors also evaluated the functionality of such differentiated ECs. Cells were able to form pseudo-vessels on Matrigel^{TM}; in response to inflammation factors the expression of adhesion molecules (E-selectin, ICAM-1, VCAM-1) was upregulated, which is critical for endothelial cells-to-leukocyte interactions and they also released several proinflammatory cytokines and chemokines. Cells were able to interact with T lymphocytes and neutrophils. iPSC-EC barrier capabilities were reduced after the addition of histamine and VEGF, and increased after PGE2 stimulation [113]. Rufaihah A.J. et al. showed that ECs obtained using the EB differentiation method could be directed for differentiation into: (a) arterial iPSC-ECs, using higher concentrations of VEGF-A and 8-bromoadenosine3′:5′-cyclic monophosphate; (b) venous phenotype with low concentrations of VEGF-A; and (c) lymphatic, using VEGF-C and Ang1 (angiopoietin-1) [114]. Arterial and venous endothelial cells were also obtained under biochemically defined conditions, in monoculture or seeded in a scaffold by two-stage differentiation—first into mesoderm precursors, and then into endothelial precursor cells (EPCs), which express residual venous and arterial markers [115]. Despite the apparent possibility to modulate the differentiation of one differentiated cell into another, Hu S. et al. showed that the differentiation of ECs depends on the origin of iPSC—from one patient they obtained three different iPSC lines (from fibroblasts (FB-iPSC), endothelial cells (EC-iPSC) and cardiac progenitor cells (CPCiPSC) and differentiated them

into ECs using activin bone marrow protein 4 (BMP4), basic fibroblast growth factor (bFGF) and VEGF. Among the three lineages, EC-iPSC had the highest ability to differentiate into ECs. This was further confirmed functionally showing the importance of the cellular origin which dictates the lineage differentiation propensity of iPSCs [116]. This demonstrates the limit of iPSC potential, the importance of tissue specificity as well as the limit of the ability of differentiation. Called the somatic memory of iPSCs, this takes its importance and must be taken into account to delineate the application to clinical translation.

Although the effect of miRNA on endothelial cells has already been discussed, there is little evidence on the role of these small, non-coding RNAs in iPSC-EC. MicroRNA-21 mediates endothelial differentiation from iPSCs in the presence of VEGF—overexpression of miR-21 in iPSCs induced EC marker up-regulation accordingly, inhibition of miR-21 produced the opposite effects. The direct target of miR-21 is the PTEN/AKT pathway and *PTEN* knockdown is required for endothelial differentiation via miR-21 [117]. miR199a also affects EC differentiation—the use of a mimic of this miRNA increased expression of CD144 and CD31 as well as the ability to form tubes on Matrigel^{TM} [118]. Wang L. et al. identified several miRNAs: miR-125a-5p, miR-149, miR-296-5p, miR-100, miR-27b, miR-181a and miR-137, which were up-regulated in hiPSC-ECs during endothelial differentiation [119]. Numerous studies have compared the functional potential of iPS-derived ECs with the standard HUVECs used; however, the results are not conclusive. Among others, it has been shown that iPSC-ECs displayed a five-fold reduction in capillary network formation compared to HUVECs when co-cultured with human lung fibroblasts NHLFs in a 3D fibrin matrix, at the same time it has been proven that it is associated with a weaker expression and activity of MMP-9 (matrix metallopeptidase 9) [120]. Other studies on hiPSC-EC functionality in the vasculogenesis by in vivo testing, showed that CD34 + hiPSC and CD31 + hiPSC formed very dense sprouting networks with numbers of junctions and total vessel lengths significantly higher than HDMEC and HUVEC [121]. Moreover, iPSC-derived endothelial cells displayed in vitro inflammatory responses comparable to primary cells—an increase of ICAM-1 and E-selectin levels after stimulation with TNFα was observed, although the leukocyte adhesion ability was lower than towards HUVECs [121]. In the in vivo model of zebrafish, iPSCs displayed a greater ability to integrate with the vascular system than HUVEC [122].

Obtaining organospecific endothelial cells from iPSC seems to be a very promising solution, especially for tissues from which it is difficult to isolate primary ECs. To date, several protocols obtaining brain-types of microvessels from iPSC have been developed. These cells display many attributes that permit to reconstitute in vitro a blood–brain barrier model: high expression of adherent and tight junction proteins, VE-cadherin, ZO-1, Occludin and Claudin-5, as well as transporters (LAT-1) and efflux pumps (P-glycoprotein). Such a model also presents appropriate permeability indicators: TEER values after stimulation with retinoic acid or by co-culture with astrocytes do reach maximal levels above 1000. These are comparable to levels reported for rat brains in vivo (1000–1500 Ω cm^2) [123–125]. Patient-specific brain endothelial cells obtained from iPSCs are a promising model for studying the underlying mechanisms and potential therapy of neurological disorders. Lim R.G. et al. showed that in Huntington's disease, endothelial cells have intrinsic impairments in angiogenic potential and drug efflux capacity, they form abnormal blood–brain barrier, and have WNT signaling defects [126].

This method of differentiating iPSC also allowed for the production of other organospecific types of ECs, among others cardiovasculature-specific endothelial cells (expressing markers MEOX2, GATA4, GATA6 and ISL1) [127], as well as corneal endothelial cells [128] or coronal-like ECs [129]. However, the lineage somatic memory and the primary cells instability in culture should be taken into account in the iPSC dedifferentiation assays. This was particularly demonstrated in the EC line production and gets more significant considering the endothelial cells organospecificity [4]. As described earlier, EC specific phenotype and function in distinct organs are key to proper biological phenomena and homeostasis. This demonstrates the importance of careful consideration of the biological

environment of a cell in the organ it is located in, together with its plasticity as a function of its origin.

3.4. The Limitations of Primary Isolated Cells and Cell Lines

In many cases, immortalized ECs offer a very valuable model for preliminary in vitro studies to omit disadvantages of primary cell lines. These include the presence of contaminating cells and limited numbers of cells, as well as the progressive loss of cell viability [130]. On the other hand, many cell lines with extended life span showed disadvantages: tumorigenicity or chromosomal instability, loss of primary endothelial features [131]. Checked for non tumorigenicity, transformed cells with a longer life span may still present different transcriptomes and phenotypes from the tissue of origin when compared to primary ECs which save important markers and functions more relevant to human physiology [132]. Although primary ECs present such advantages, they also undergo fast aging processes [133]. Thus, they show morphological and functional changes and have limited potential for self-renewal and differentiation and are known to lose their specific characters over 4 to 5 passages. Nevertheless, the choice of a suitable model should be chosen according to the project assumptions.

4. Endothelial Cells in Advanced In Vitro Models

The widespread presence of endothelial cells in tissues and organs and the importance of the vascular system in the pathophysiology of many diseases resulted in the need to reconstitute in vitro models based on ECs, taking into account their characteristic, organo-specific and microenvironment-dependent plasticity in: co-cultures, invasion/migration assays, angiogenesis, 3D (three dimensional) culture, organoids, organ-on-chips. These methods allow better imitation of in vivo conditions [72]. They intend to consider the presence and the role of the various relevant types of cells and their crosstalk, together with the extracellular matrix components, oxygen partial pressure conditions and variations or shear stress-fluid flow. They provide a good alternative to study the mechanisms of diseases and the design of new therapies.

Advanced methods are used to study, among others, the mechanisms of tumor angiogenesis. Buchanan C.F. et al. showed that co-culture of breast cancer cells and endothelial cells (telomerase-immortalized human microvascular endothelial cell line—TIME) in 3D microfluidic collagen hydrogel increases the permeability of ECs-formed structures. Changes in gene expression associated with angiogenesis (VEGF, MMP, ANG1, ANG2) depend on the values of microvascular wall shear stress, resulting, among others, in changes in ECs' permeability [54]. In the 3D models of melanoma cells, Klimkiewicz K. et al. showed the recruitment of both mature and progenitor endothelial cells to tumor spheroids (in both mouse and human models) [72]. This recruitment was an active process, observed only for live cancer cells. ECs' recruitment by melanoma spheroids were higher in hypoxia than in normoxia indicating the hypoxia-dependent signaling specific for early endothelial progenitors (MAgEC 10.5). Moreover, in normoxia the recruitment process of ECs began with a delay, and the previously non-organized ECs evolved in the long term into a complex network suggesting the appearance of hypoxia inside the spheroid, which also shows the sensitivity of endothelial cells to the aerobic conditions of the micro environment [72]. The 3D model with endothelial cells (HUVEC) was also developed for hepatocellular carcinoma and used to study the effectiveness of anti-angiogenic drugs (sorafenib, sunitinib, axitinib) [134]. Although the authors have proven that the 3D model well reflects the hypoxic core of the tumor, true gradient penetration of drugs and observed that all three drugs prevented the formation of vessels, it is necessary to mention the limitations: vessels remain stable in this model only for a short time (3 days) due to, among others, lack of pericytes, fibroblasts and vascular smooth muscle cells. This makes the researchers unable to investigate the long-term effect of anti-angiogenic drugs. This could be a key effect as Pàez-Ribes M. et al. indicate that the effect of anti-angiogenic drugs is temporary, later on

the tumor becomes resistant to such treatment, what leads to revascularization, induction of more aggressive phenotype and increases metastasis [135].

Another use of endothelial cells in 3D models and co-culture with other cells is to create an in vitro blood–brain barrier (BBB). Wilhelm I. et al. established a rat in vitro BBB model using Transwell filters with primary rat brain endothelial cells (RBECs), pericytes and astrocytes. After stimulation with hydroxycortisone cAMP (cyclic adenosine monophosphate) and RO (4-(3-Butoxy-4-methoxybenzyl) imidazolidin-2-one)) they obtained high TEER values of 264 ± 67 Ohm × cm^2 and low permeability, which makes this model useful for drug transport testing [136]. Stone N.Z. et al. point to the need to enrich the three-cell model with neurons which make BBB integrity more sensitive to oxygen-glucose deficiency, as evidenced by a decrease in TEER, increased permeability and markers of cell damage [137]. Another method of recreating the BBB in in vitro conditions using ECs in co-culture are self-assembling spheroids, of which the core is comprised mainly of astrocytes, while brain endothelial cells and pericytes encase the surface, acting as a barrier that regulates transport of molecules. The spheroid surface exhibits high expression of tight junction proteins ZO-1, Claudin5, Occludin, VEGF-dependent permeability, efflux pump activity and receptor-mediated transcytosis of angiopep-2 [138]. The more advanced 3D microfluid BBB model was designed by Campisi M. et al. based on the ability of hiPSC derived ECs to differentiate and self-assemble into brain microvascular cells in cooperation with pericytes and astrocytes which, by facilitating the organization of the endothelium, stabilize the mature vascular system, with tight connections and low permeability [139].

5. Endothelial Cells in Angiogenesis and the Role of ECs in Pathologies

The key players of angiogenesis are endothelial cells (ECs) which form blood vessels with other cell types, called mural cells, that include the vascular smooth muscle cells (vSMCs) and pericytes [140]. Endothelial cells are responsible for the regulation of transport across the vessel [141]. The pathological angiogenesis, one of the hallmarks of cancer plays a crucial role during solid tumor growth and metastatic spread [142]. Angiogenesis is stimulated by chemical signals from tumor cells in a phase of rapid growth when nutrients and oxygen must be provided. The imbalance between inhibitors (among others: angiostatin, endostatin, interferon) and activators of this process is caused by several factors including: hypoxia, low pH, hypoglycemia, immune/inflammatory stimuli [143]. These signals come from the angiogenic activators, including: growth factors (VEGF-A, bFGF), interleukin-8, angiogenin, transforming growth factor (TGF)-α, TGF-β, tumor necrosis factor (TNF)-α, platelet-derived endothelial growth factor, granulocyte colony-stimulating factor, placental growth factor, hepatocyte and epidermal growth factor [143,144]. Among the variety of these angiogenic modulators, VEGF-A is the essential player in the formation of blood vessels.

In low pO_2, hypoxia present in the growing tumor mass has the direct effect to increase expression of hypoxia-inducible factor 1 (HIF1α) induces VEGF-A synthesis triggering ECs' activation by binding to VEGF receptors on the ECs' surface [145]. Tumor cells release VEGF-A which stimulates ECs to break down the basement membrane in blood vessel by the release of proteolytic enzymes [146]. Plasmin, which is the active form of plasminogen, activated by urokinase-plasminogen activator (uPA), is one such key proteolytic enzyme [147]. The activation of endothelial cells from preexisting blood vessels to form new vessels in hierarchical manner is mediated by motile endothelial cells located at the ends of newly formed vessels, known as tip cells [148,149]. The other endothelial cells that follow the migrating tip cell, differentiate under the influence of the tip cell into stalk cells that proliferate and create a lumen [148]. When, endothelial cells adhere to one another and lumenogenesis takes place, the basement-membrane formation and pericyte attachment occurs.

Newly formed blood vessels should supply nutrients and oxygen for tissue; however, in the tumor, their permeability is disrupted due to pathologic structure and differences between ECs originated from healthy and pathological site [150]. Firstly, the cells are

structurally organized in different ways. Although, ECs are structurally heterogenous and functionally dependent on their origin called organospecificity, in general healthy ECs form layers sealed by tight junctions on the luminal surface of the vessel [4,150]. On the contrary, cancer-derived ECs generate chaotic formation of the inner cellular lining of blood vessels [151]. Moreover, the pathologic endothelium is immature and thin-walled [152]. Secondly, the permeability and perfusion vary between healthy and pathologic vessels [153]. The way the vessels are structured defines their function. In healthy-derived endothelium, the ordered layers allow easy flow and free passage of blood. Oppositely, pathological EC structure is associated with increased permeability and decreased perfusion in the blood vessel [154]. Thirdly, tumor endothelial cells have even higher glycolytic metabolism comparing to the healthy ones [33,155]. Last but not least, the differences between tumor suppressors and oncogenes might be the reason of the dissimilarity described above. It has been shown that in pathologic ECs, the main controller of angiogenesis—PTEN, tumor suppressor gene (endothelial phosphatase and tensin homolog) is one of the most deregulated genes [156,157]. Moreover, in pathologic angiogenesis, VEGF synthesis is increased and permanently activated by hypoxia at the tumor cells level. This maintains the angiogenic anarchic growth and results in pro-tumorigenic signals [156,158,159]. Such disrupted phenotype of pathological vessel can be maintained in vitro. Indeed, ECs isolated from a tumor site differ from healthy ones on molecular and functional level (Figure 3, data not published). Additionally, in vitro hypoxia (culture of cells in low, non-physiological pO_2, usually 1%) is an appropriate tool to mimic tumor micro-environmental conditions [160–162]. Figure 4 displays the observed growth differences evidenced upon hypoxia treatment of the cells, while cells differences appear in their ability to form vessels in vitro estimated by functional assay for pseudo-tube formation (data not published, Figure 3). Understanding the mechanisms related to pathological angiogenesis in the tumor microenvironment revealed ECs as a new, potential therapeutic target for the treatment of many solid tumors. In 2004 the US Food and Drug Administration approved the first VEGFA inhibitor, bevacizumab for the first-line treatment of metastatic colorectal cancer [163]. The new developments in this area contributed to the identification and approval of many anti-angiogenic drugs, which offered better outcomes (often in combination with standard chemotherapy or immune checkpoint blockers) [164,165]. The aim of anti-angiogenic therapies is mainly to inhibit VEGF-VEGFR signaling at different levels: (a) blocking the activity of VEGFR by tyrosine kinase inhibitors (TKIs) (b) neauralizing circulating VEGF molecules with a monoclonal antibody (c) blocking the activity of VEGFR by monoclonal antibody [166–168]. However, the effectiveness of anti-angiogenic therapies in the light of recent reports and clinical observation is being more and more often denied [166,169]. Effects of anti-angiogenic treatment in vivo is only temporary which is mediated by hypoxia-resistance mechanisms such as: inducing new vascular mimicry properties by tumor cells, increased production of proangiogenic factors, or autophagy [170–172]. Studies show that such therapy can in fact, promote the metastatic potential of tumor by increasing collagen deposition [173], modification of its properties and show harmful clinical side effects (ex. Bevacizumab—a monoclonal antibody that neutralizes the vascular endothelial growth factor A (VEGF-A) [174,175]. RTKIs, which inhibit the vascular endothelial growth factor receptors (VEGFRs) tend to be insufficient [168]. Due to this fact, it is extremely important to develop therapies that will be more effective and durable. One of the approaches is to alleviate hypoxia by bringing back proper permeability and perfusion of the vessels. Such normalization of the vessels might be an invaluable tool to improve the outcome of anti-cancer therapies by reconstituting the vessels proper function, thus increasing chemotherapeutics penetration [176,177]. Another potential tool in cancer therapy is offered by microRNAs-mediated (angiomiRs) regulation that modulate angiogenesis, regulate cancer immunity (miR-424) and can be combined with T cell-based therapy (miR-17-92,-155,-181a) or suppression of VEGF (miR-16-like family) and interference with TGF-β signaling [47,162,178–180]. MicroRNA-34a has been reported as an anti-metastatic and suppresses angiogenesis in bladder cancer by directly targeting

CD44 [181]. An interesting concept is also the attempt to use precursor endothelial cells to deliver therapeutic miRNAs to the tumor site [182].

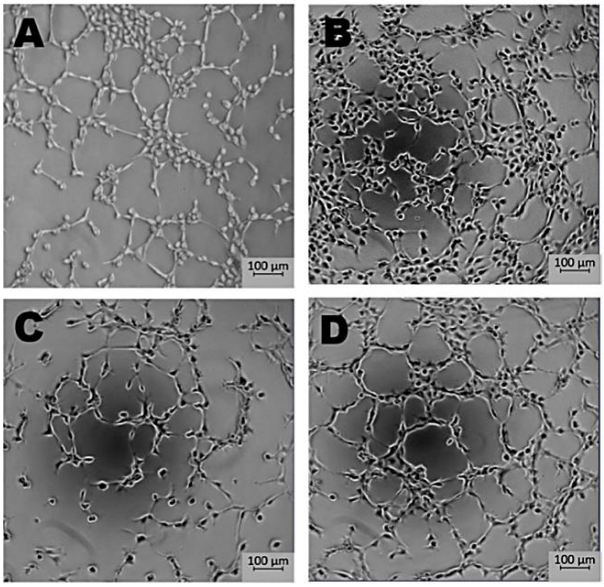

Figure 3. The effect of microenvironment and organospecificity on the angiogenic potential of Endothelial cells (ECs). Morphology of tubes formed in vitro by healthy and cancer-derived EC cells after 5 h in normoxia or hypoxia. Low pO_2 reduces the pro-angiogenic response in ECs derived from tumor site when compared to normoxia. In the case of healthy tissue-derived ECs, hypoxia does not significantly influence tube formation. (**A**)—healthy ECs, normoxia; (**B**)—healthy ECs, hypoxia; (**C**)—cancer-derived ECs, normoxia; (**D**)—cancer-derived ECs, hypoxia; magnification 5×.

Based on the need for an efficient vessel normalization, strategies are developed to increase oxygen partial pressure in the tumor site. It has been shown that allosteric effector of hemoglobin (Hb), *myo*-inositol trispyrophosphate (ITPP) inhibits hypoxia-induced pathological angiogenesis by activation of PTEN in the ECs [156,183]. ITPP primarily acts as an allosteric effector enhancing the capacity of hemoglobin to release bound oxygen, which influences vessel normalization by reducing the HIF-1α activation thus reducing the VEGF-A synthesis [183,184]. The regulation by PTEN, was shown. As PTEN is the main control of the PI3K/mTOR/p53 axis, ITPP based strategies can be a key method for angiogenesis regulation and vessel normalization. Additionally, PTEN activation which leads to the inhibition of PI3K (phosphoinositide 3-kinases) and decreasing tumor AKT (protein kinase B)phosphorylation results in tumor growth reduction [156]. Moreover, ITPP was described as an effective tool in the treatment of hypoxia-dependent cardiovascular diseases (heart failure) by similar molecular mechanisms [185,186].

Another blood vessel normalization strategy, resulting from tumor hypoxia alleviation, is based on cell-gene therapy with the use of endothelial progenitors in murine model (MAgEC11.5) [69]. These progenitors are able to target pathological angiogenesis and by cell mediated gene therapy allow the expression, locally, of soluble VEGF-receptor2 in a hypoxia-driven manner. It acts as a VEGF trap and its action results in vessel normalization and functionalization, microenvironment composition modification and ultimately, in

tumor growth reduction [69]. This is a direct demonstration and application of angiogenesis normalization cell-gene therapy.

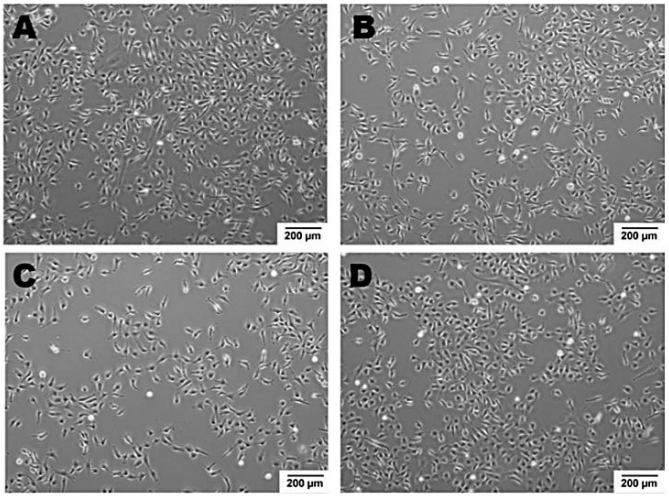

Figure 4. ECs' growth modulated by hypoxic micro-environmental conditions. Cell density after 48 h in normoxia or hypoxia: Cells were seeded at 0 h in the same number. Growth of healthy ECs is a little slower upon hypoxia vs. normoxia, oppositely to cancer derived ECs, whose growth is higher in hypoxic conditions as compared to normoxia. (**A**)—healthy ECs, normoxia; (**B**)—healthy ECs, hypoxia; (**C**)—cancer-derived ECs, normoxia; (**D**)—cancer-derived ECs, hypoxia; magnification 4×.

In addition, Alk1 (activin receptor-like kinase 1) has been described as another therapeutic target which is responsible for promoting vascular remodeling and maturation [187]. It has been proven that the Alk1 ligand -BMP9- promotes vascular normalization in Lewis Lung Carcinoma (LLC) tumors what results in deep microenvironment changes [187].

Moreover, the combination of immunotherapy with anti-angiogenic treatment in cancer has been described. Recently, most clinical trials have been devoted to PD1 (Programmed cell death protein 1)/PD-L1 (Programmed death-ligand 1) immune checkpoint neutralization. PD-L1 is largely used as the anti-tumor immunity target and VEGF-A is the anti-angiogenic target which are applied in trials concerning non-small cells lung carcinoma (NSCLC), renal cell carcinoma ovarian cancer patients and many other cancers as melanoma [165] where the data, although promising, remain partial.

This points to the deep interest that should be devoted to conducting experiments and alternative assays in conditions as close as possible to those encountered in vivo, namely hypoxia, which appears fundamental for angiogenesis-based studies.

6. Conclusions

To sum up, the choice of proper in vitro cellular models to study pathologies by alternative methods is crucial to perform biologically relevant research involving endothelial cells. Several in vitro tools are accessible to properly mimic ECs' heterogeneity, organo-specificity and plasticity in response to environmental stimuli. Growth and unification of methods of isolation and culture of ECs may help elucidate the mechanisms of endothelium development and function in health and disease, which in turn, can bring effective adaptations for the development of new treatment strategies for angiogenesis-dependent diseases. The large and fast increase in the endothelial cell lines devoted to defined studies, points to

the great advantage of using comparable sets of similarly treated cells. The main routes opened by the knowledge of the endothelial cells organo-specificity is the development of cell-carried tissue-specific therapies for repair strategies. As alternative methods are being developed, competition favors the models that present properties as close as possible to the in vivo context in terms of micro-environmental parameters as well as dynamics effects. The importance of hypoxia in diseases is getting primordial, and pathologies classified as hypoxia-dependent are increasing. This cannot be dissociated from angiogenesis as the most direct effect of hypoxia and for its consequences when abnormal. Means for the reconstitution of proper vessels are essential to learn about the potential and effects of a successful repair of the pathologic vasculature. In this line, advantages provided by the early endothelial progenitor cells lie in their ability to target the hypoxic sites. They naturally counteract and compensate the damaged cell wall and may reconstitute homeostasis. In addition to such cellular processes, this review points to the molecular modifications of the pathologic angiogenesis and shows how this may help to, consequently, succeed in the control of the tumor microenvironment through hypoxia compensation.

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Abbreviations

2D	two-dimensional
3D	three-dimensional
Ac-LDL	acetylated low-density lipoprotein
ACE	angiotensin converting enzyme
ALK1	activin receptor-like kinase 1
Ang	angiopoetin
BBB	blood-brain barrier
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
cAMP	cyclic adenosine monophosphate
CCL21	chemokine (C-C motif) ligand 21
CX3CL1	fractalkine; chemokine (C-X3-C motif) ligand 1
CXCR4	chemokine receptor type 4
EB	embryoid body
EC	endothelial cell
EndoMT	Endothelial-to-Mesenchymal Transition
eNOS	endothelial nitric oxide synthase
EP	ethyl pyruvate
EPC	endothelial progenitor cell
EphB4	ephrin type-B receptor 4 gene
ET-1	endothelin 1

GSPE	grape seed proanthocyanidin extract
HIF1	hypoxia-inducible factor 1
HUCB	human umbilical cord blood
ICAM-1	intercellular adhesion molecule-1
ILs	interleukins
iPSC	induced pluripotent stem cell
ITPP	myo-inositol trispyrophosphate
LFA-3	lymphocyte function-associated antigen 3
LLC	Lewis Lung Carcinoma
LYVE-1	lymphatic vessel endothelial hyaluronan receptor-1
MCAM	melanoma cell adhesion molecule
MHC	major histocompatibility complex
miRNAs	microRNAs
MMP-9	matrix metallopeptidase 9
MNC	peripheral blood mononuclear cell
NCAM	neural cell adhesion molecule
NSCLC	non-small cells lung carcinoma
PECAM -1	platelet endothelial cell adhesion molecule
PGE2	prostaglandin E2
PI3K	phosphoinositide 3-kinases
PODXL	podocalyxin-like protein 1
PTEN	phosphatase and tensin homolog deleted on chromosome ten
RO	4-(3-Butoxy-4-methoxybenzyl) imidazolidin-2-one)
RTKIs	receptor tyrosine kinase inhibitors
Sca-1	stem cells antigen-1
TEER	transepithelial/transendothelial electrical resistance
TNF	tumor necrosis factor
UEA	ulex europaeus agglutining
VCAM-1	vascular cell adhesion molecule-1
VE-Cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VWF	von Willebrand factor
WPB	weibel–Palade bodies
ZO-1	zonula occludens-1

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5.2 Distinctive Properties of Endothelial Cells from Tumor and Normal Tissue in Human Breast Cancer

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Distinctive Properties of Endothelial Cells from Tumor and Normal Tissue in Human Breast Cancer

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Abstract: Tumor microenvironments shape aggressiveness and are largely maintained by the conditions of angiogenesis formation. Thus, endothelial cells' (ECs) biological reactions are crucial to understand and control the design of efficient therapies. In this work, we used models of ECs to represent a breast cancer tumor site as well as the same, healthy tissue. Cells characterization was performed at the transcriptome and protein expression levels, and the cells functional biological responses (angiogenesis and permeability) were assessed. We showed that the expression of proteins specific to ECs (ACE+, VWF+), their differentiation (CD31+, CD 133+, CD105+, CD34-), their adhesion properties (ICAM-1+, VCAM-1+, CD62-L+), and their barrier formation (ZO-1+) were all downregulated in tumor-derived ECs. NGS-based differential transcriptome analysis confirmed CD31-lowered expression and pointed to the increase of Ephrin-B2 and SNCAIP, indicative of dedifferentiation. Functional assays confirmed these differences; angiogenesis was impaired while permeability increased in tumor-derived ECs, as further validated by the distinctly enhanced VEGF production in response to hypoxia, reflecting the tumor conditions. This work showed that endothelial cells differed highly significantly, both phenotypically and functionally, in the tumor site as compared to the normal corresponding tissue, thus influencing the tumor microenvironment.

Keywords: microenvironment; angiogenesis; endothelial cells; breast cancer; organospecificity; vascular dysfunction

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1. Introduction

As breast cancer is one of the major causes of death among the female population worldwide, numerous studies are carried on breast tumor angiogenesis [1]. In the great majority of them, human umbilical vein endothelial cells (HUVECs) were used as the universal model of endothelial cells (ECs). However, such a widespread use may lead to skewed observations due to the heterogeneity of donors and changes in phenotype caused by long-term culture [2]. Furthermore, they do not represent, by any means, the organs in which cancers originate. The actual, recognized organospecificity of endothelial cells has not been considered. Moreover, the pathologic conditions of the tumor microenvironment are neglected, what is another source of misinterpretation of results. Therefore, setting up a relevant experimental models for angiogenesis is crucial to better understanding the molecular mechanisms triggered during tumor growth and metastasis and to provide diagnostic, prognostic and treatment opportunities.

Endothelial cells form monolayer in all blood vessel walls and fulfill multiple functions, helping to maintain vascular homeostasis, regulate blood flow and blood clotting, control vessel wall permeability and regulate proper trafficking and recirculation of leukocytes [3,4]. Besides its physiological function, the endothelium plays an important role

in processes that occur during the progression of diseases particularly those affecting the vascular system. Angiogenesis is a fundamental mechanism in cancer development. In the hypoxic microenvironments of tumors, endothelial cells perform angiogenesis, leading to the abnormal structure and function of the blood vessels, which further maintains hypoxia instead of compensating for it [5]. The biological differences between tumor endothelial cells (TECs) and normal endothelial cells (NECs) in tissues or organs add to the heterogeneity that must be considered for endothelial cell properties between organs and vessel classes in view of data interpretations [6].

Endothelial cells exhibit numerous characteristic markers that define the cell type. Endothelial cells present von Willebrand factor (VWF) [7] and platelet endothelial cell adhesion molecule 1 (PECAM-1, CD31) [8]. Moreover, ECs, on their surface, present endoglin (CD105), which regulates their proliferation [9]. Considering their differentiation steps, known endothelial progenitor cells (EPCs) are CD133+, CD34+, CD44+ and CD202b+. This set of markers is a combination that evolves according to maturation and specialization, leading to organospecificity. Considering the vessel type, lymphatic ECs are characterized by the presence of VEGFR3 [10], podoplanin and lymphatic vessel endothelial receptor 1 (LYVE-1). Venous ECs present Ephrin-B4 receptor tyrosine kinase, whereas arterial endothelial cells exhibit Ephrin-B2 [11]. After stimulation by tumor necrosis factor (TNF), bacterial lipopolysaccharide (LPS) and interleukin-1 (IL-1), ECs express cell adhesion molecules such as intercellular cell adhesion molecule 1 (ICAM-1, CD54) [12], vascular endothelial cell adhesion molecule 1 (VCAM-1, CD106) [13] and E-selectin (E-Sel, CD62-E) [14] on the cell surface. n their surfaces, ECs also express CD309 (VEGFR-2, KDR-1), which controls cell proliferation and migration and may modulate endothelial permeability [15,16]. Endothelial cells exhibit angiotensin converting enzyme activity (ACE; CD143), involved in the metabolism of angiotensin [17] and the inactivation of bradykinin. In functional assays, endothelial cells are able to form pseudo-vessels in vitro on Matrigel^{TM} as a mimicry of the extracellular matrix [18,19].

Herein, we showed that newly established cell lines, organo-specifically representing normal healthy breast tissue (HBH.MEC (healthy ECs)) and tumor site-derived endothelial cells (HBCa.MEC), though isolated from the same breast tumor patient, differed from one another. Our study presented new insights into the phenotype and established differences between healthy and pathological breast endothelia for valid mimicry of breast tumor conditions. Our data validated established endothelial cell lines that maintained their EC character during long-term culture as more relevant tools than those generally known and used [20,21].

2. Results

2.1. Tumor Microenvironment Influences EC Morphology, Proliferation and Expression of BCL6/p53

Both cell lines maintained their cobblestone morphology in monolayer cultures along numerous passages (Figure 1A). Healthy tissue-derived ECs showed a tendency to display a higher proliferation rate in standard culture conditions than breast tumor-derived ECs (Figure 1B,C). The level of BCL6 protein, a negative regulator of apoptosis, was lower in pathological ECs than in healthy ECs, whereas p53 protein levels displayed an opposite tendency (Figure 1D).

2.2. HBH.MEC and HBCa.MEC Lines Display ECs Phenotype

Cells were stained for endothelial cell-specific markers (CD31, VWF, ACE, CD133, CD34, CD105) and related to pathological states of tissue (PDPN, AP, αSMA, EGFR). The unstained cells were used to test for autofluorescence and unconjugated primary antibodies (VWF, ACE). Controls were set using secondary labeled antibodies for nonspecific labeling levels.

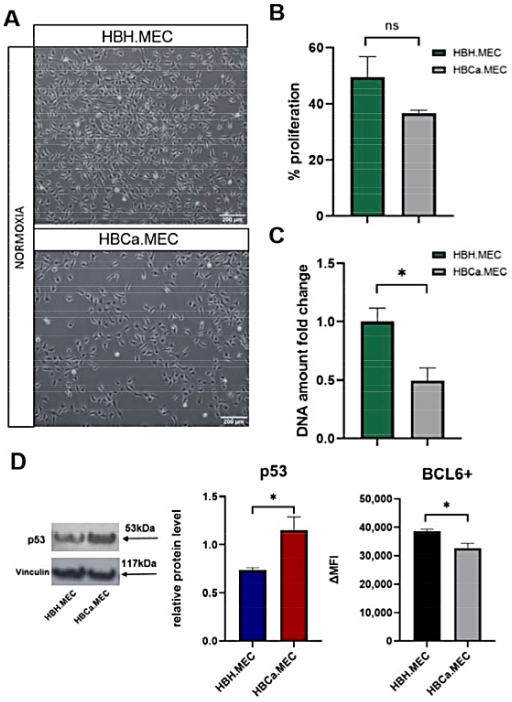

Figure 1. The influence of new endothelial cell lines phenotype on morphology, viability and apoptosis induced proteins. (**A**) Cell density after 48 h in normoxia. Cells were seeded at t = 0 h in the same number. Magnification 4×. (**B**) The influence of ECs pathological state on cell proliferation. Cells were seeded at 0 h in the same number and cultured in normoxia for 48 h. (**C**) DNA proliferation confirmed that tumor-derived ECs multiplied slower in normoxia when compared to healthy ECs. * $p < 0.05$ in Student *t* test vs. HBH.MEC normoxia (**D**) The protein level of BCL6 and p53. Cells were stained for BCL6 and undergo flow cytometry analysis. Data were recorded for 10,000 events using CellQuest software (v.2.3.0.84) and presented as delta MFI. The level of p53 (53 kDa) was evaluated on WB, relatively to loading control, Vinculin (117 kDa). Representative bands are shown. Bar chart presents data from ImageJ analysis (v.1.52p). Data are reported as the means ± SEM (n = 3). Ns—not significant; * $p < 0.05$ in Student *t* test vs. HBH.MEC.

The mean fluorescence of cells for each marker is presented on graphs and representative histograms are shown (Figure 2). In general, healthy ECs and tumor-derived ECs were positive for all tested markers except for CD34 (VWF+, ACE+, CD31+, CD133+, CD105+), but pathological ECs were characterized by lower levels of expression (Figure 2). Moreover, both cell lines expressed the adhesion molecules ICAM-1, VCAM-1 and CD62L+. ICAM displayed a similar level of expression in both cell lines (Supplementary Materials, Figure S1). In addition, both cell lines expressed PDPN, EGFR and AP with higher levels of expression of these markers in healthy ECs than in pathological ECs. PDPN expression, however, showed a certain degree of variability. The entire tumorous EC population showed higher levels of αSMA and a tendency to express EGFR more effectively than healthy ECs (Figure 2B).

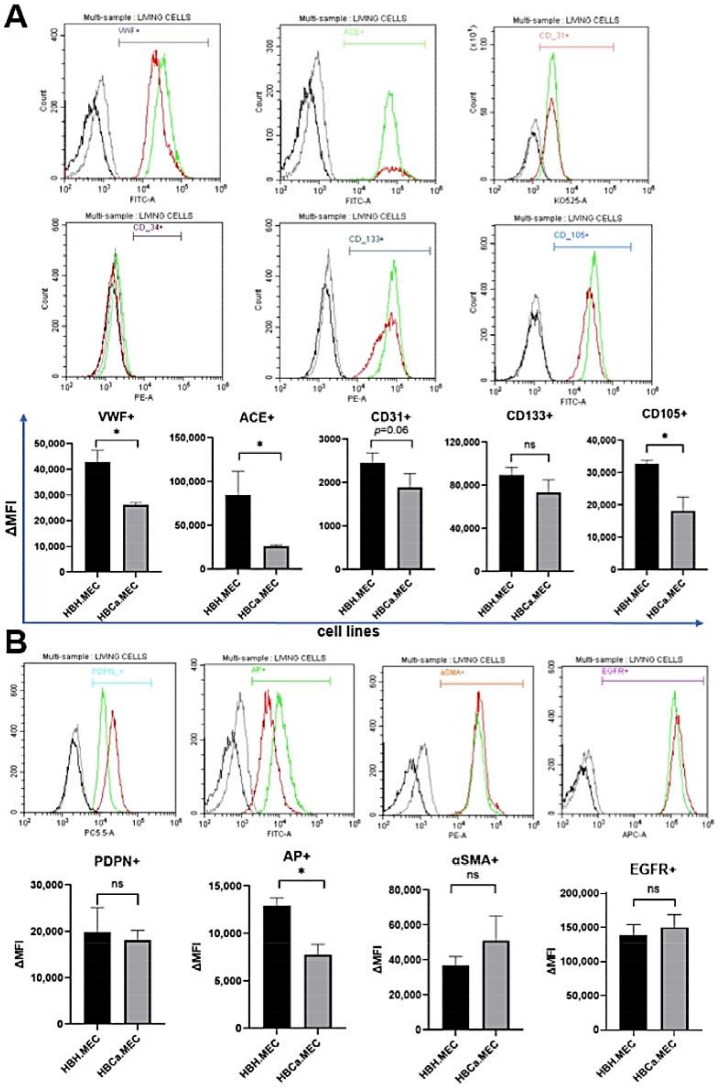

Figure 2. Characterization of ECs by flow cytometry markers. The cells were stained for the following markers: VWF, ACE, CD31, CD34, CD133, CD105, PDPN, AP, αSMA, EGFR. Data were recorded for 10,000 events using CellQuest software (v.2.3.0.84) and presented as histogram overlays. (**A**) The most characteristic markers for ECs and EPCs. (**B**) Markers associated with cancerous phenotype. Histogram overlays display representative repetitions; gray—healthy ECs unstained, black—tumor ECs unstained, green—healthy ECs stained, red—tumor ECs stained. Y axis = the number of events; X axis = fluorescence intensity; the bar charts present delta MFI. Ns—not significant; * $p < 0.05$ in Student *t* test vs. HBH.MEC. Data are reported as the means ± SEM (n = 3).

2.3. Hypoxia Induces the Production of VEGF-A by HBH.MEC and HBCa.MEC

To determine how the microenvironment influenced VEGF-A secretion by mature ECs, cells were cultured under normoxia or hypoxia for 48 h. Then, the level of VEGF-A was measured in medium. The production of VEGF-A was stimulated upon hypoxia in the case of both cell lines, with significantly higher rates and differential expressions in pathological ECs than in healthy ECs (Figure 3). Moreover, tumor-derived ECs secreted a lower levels of VEGF-A in normoxia (Figure 3).

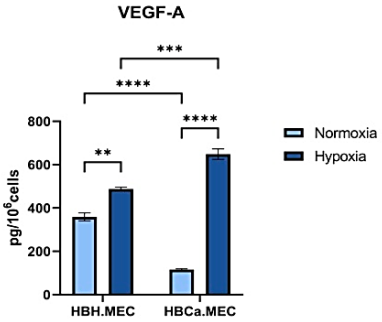

Figure 3. Secretion of VEGF-A produced by HBH.MEC and HBCa.MEC cultured upon normoxic and hypoxic conditions. Supernatants from both cell lines were collected after culture for 48 h in normoxia (19% O_2) or in hypoxia (1% O_2). Secretion of VEGF-A was evaluated by using the ELISA. Results are expressed as pg/million cells ± SEM, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ in two-way ANOVA (n = 3).

2.4. Proangiogenic Response In Vitro Depends on the Pathological State of ECs

To observe the functional activity of the studied cells, tube formation assay was performed. Healthy and tumor-derived ECs were assessed for their angiogenic potential on Matrigel™-coated plates. Both cell lines were able to perform pseudotube formation, as previously shown [22]. In the case of HBH.MEC, angiogenesis was achieved after 5 h, whereas HBCa.MEC created networks but did not achieve fully-formed tubes (Figure 4A). Healthy ECs presented a higher number of both nodes and junctions than tumor-derived ECs (Figure 4B). We also checked the levels of CD309 (FLK1), which induces angiogenesis as well as permeabilization of cell monolayers, in both cell lines (Supplementary Materials, Figure S2). Although both cell lines were CD309+, the levels of vascular endothelial growth factor receptor 2 were higher in healthy ECs than in HBCa.MEC cultured in normoxia (Figure 4B).

2.5. The Transcriptome Analysis of Breast Tumor-Derived ECs Compared with Healthy ECs Revealed a Distinct Expression Profile Implicated in Vascular Development and Angiogenesis

Validation of the organo- and biological significance of the differences between normal and pathological ECs in the same organ was performed. Transcriptome comparison of healthy and tumor-derived ECs demonstrated significantly differentially expressed genes. as shown in the graph, the red and blue dots represent significantly up-regulated and down-regulated genes, respectively (Figure 5A). NGS data indicated for 350 up-regulated genes and 396 down-regulated genes. We chose 279 genes with logFC > or < ±1.5 and q value < 0.05. Among these, we found the ten with the most-changed mRNA levels between both cell lines (Figure 5B). Then, analysis was performed in Cytoscape (v.3.8.0) in order to assess the protein–protein interaction network of ECs' differentially expressed molecules. The interaction network showed nodes representing PECAM-1

downregulation and Ephrin-B2 upregulation; these are EC markers and are significant for the differentiation state (Figure 5C). SNCAIP suggested a strong relation to tumoral plasticity. To assess the transcriptomic characteristics based on gene ontology, we selected the 279 most-expressed genes per cell type, based on UniProt analysis. We detected enrichment of multiple vasculature-related biological processes, as well as processes related to muscle differentiation. The 10 most activated processes per cell type are shown in Figure 5D.

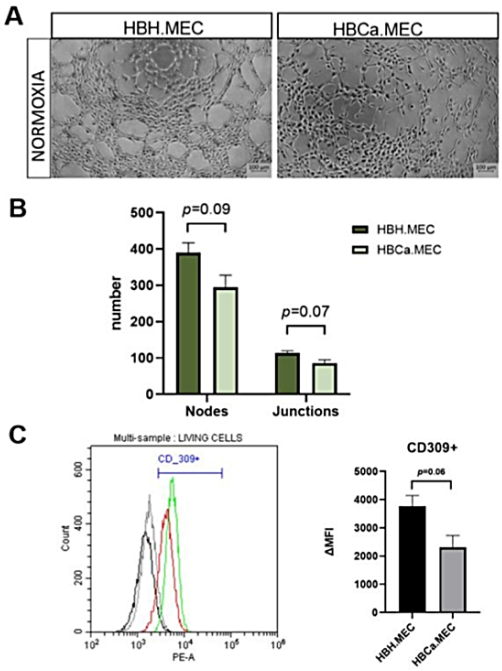

Figure 4. The effect of pathological state of ECs on the angiogenic potential. (**A**) Morphology of tubes formed in vitro by HBH.MEC/HBCa.MEC after 5 h in normoxia, magnification 5× (representative pictures). (**B**) The number of nodes, junctions was estimated by Image J software (v.1.52p). $p < 0.05$ in Student t test vs. HBH.MEC. Data are reported as the means ± SEM (n = 3). (**C**) The level of CD309 in both cell lines. The cells were stained for CD309 and data were recorded for 10,000 events using CellQuest software (ver.2.3.0.84) and presented as histogram overlay (shows representative repetitions; gray—healthy ECs unstained, black—tumor ECs unstained, green—healthy ECs stained, red—tumor ECs stained. Y axis = the number of events; X axis = fluorescence intensity). The bar chart presents delta MFI. $p = 0.06$ in Student t test vs. HBH.MEC. Data are reported as the means ± SEM (n = 3).

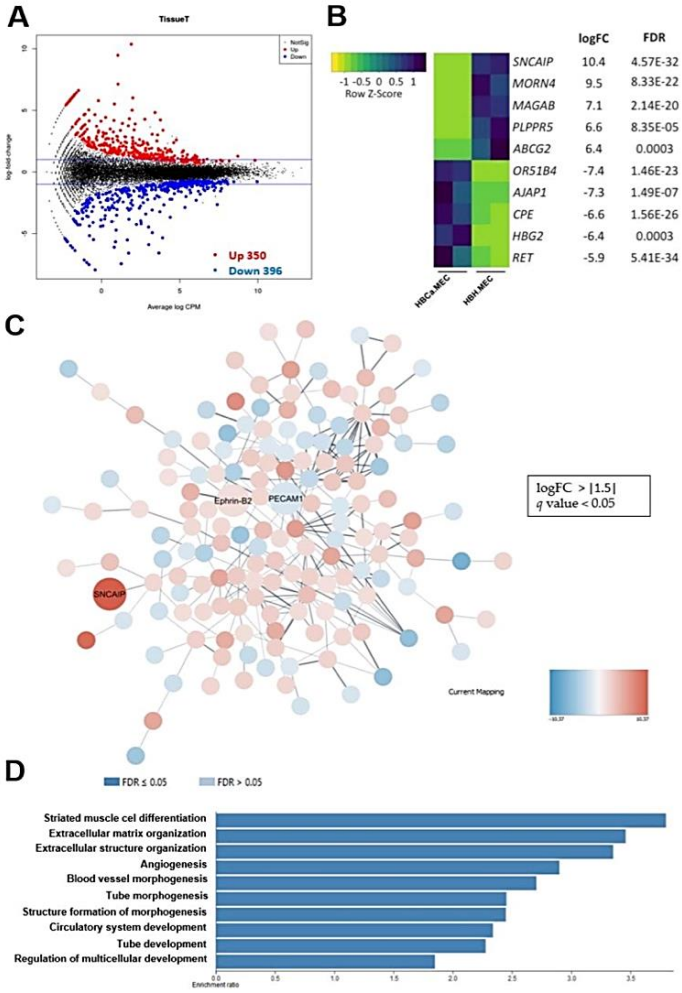

Figure 5. Differences in the transcriptome profiles of tumor-derived ECs and healthy ECs. (**A**) Volcano plot of significantly differentially expressed genes of pathological ECs vs. healthy ECs. (**B**) top 10 of the most differentially expressed genes of pathological ECs vs. healthy ECs; FDR-false discovery rate. (**C**) Functional enrichment network performed in Cytoscape software (v.3.8.0). (**D**) Enrichment analysis based on Gene Ontology biological processes shows the top 10 activated processes in both cell types (web-based gene set analysis toolkit enrichment method: ORA; organism: homo sapiens, enrichment categories: geneontology biological_process).

3. Discussion

Due to the heterogeneity of ECs, it is extremely important to use ECs that are as similar as possible to in vivo conditions in order to better mimic the cancer microenvironment. We proposed new cell lines as a model for angiogenesis studies in breast cancer biology,

as breast cancer is one of the common cancer types among women worldwide [1]. The proposed ECs are human, mature, organospecific, immortalized cell lines and were isolated from the same patient, which decreases variability between donors. In this paper, we focused on the characterization and comparative analysis of these cell lines in terms of cell growth, morphology, cellular markers, gene expression patterns and their functionality in vitro.

After immortalization, as patented, and many passages, the ECs presented the endothelial phenotype and clearly displayed biologically and phenotypically significant differences. Both cell lines were positive for ACE, the criterion that kept the previously-immortalized human endothelial cell lines [20]. Additionally, these ECs expressed another endothelial marker, CD105, similarly to those studied by Grange et al. [23]. Importantly, we found that both ECs lines presented main markers of differentiated endothelial cells: VWF and CD31 [24]. PECAM-1 was also identified in NGS as being downregulated in breast TECs vs. breast NECs, which underlines the fact that the tumor microenvironment alters the EC phenotype. The characteristic surface marker expression pattern was highly representative of endothelial cells. At the same time, the cell lines were negative for CD34 and positive for CD133, with the latter being a marker of not only endothelial progenitor cells, but also hematopoietic lineage, as shown by Ohga et al. [25]. The expression of CD133 was previously detected in high (but not low) metastatic tumor blood vessels [25]. Nevertheless, the present cells' surface phenotype confirmed their endothelial origin, excluding a hematopoietic lineage evolution as well as their response to hypoxia. As Paprocka et al. observed, exposure to hypoxia induced VEGF-A secretion in both cell lines [26]. Although tumor ECs produced less VEGF than healthy EC at a basal level, the level of induction in response to hypoxia was much stronger. This reflected the pathologic angiogenic response and explained the ineffectiveness of the tumor vessels as well as their permeability and inability to alleviate hypoxia.

ECs derived from healthy breast tissue displayed higher proliferation rates- measured by metabolic activity- than tumor–derived ECs. They also expressed higher BCL6 protein levels. Additionally, p53 appeared to be less active in these cells, contrary to breast-tumor derived ECs. This could indicate that the tumor microenvironment, from which the ECs were isolated, had a prolonged influence on their growth and the expression of proapoptotic proteins. The cells' origin also affected also their proangiogenic potential. Indeed, the obtained endothelial cell lines could form capillary-like structures in Matrigel^{TM} coated plates, which is a feature of mature ECs and not of undifferentiated breast tumor progenitor cells [23]. Both of our cell lines were able to form vessels in vitro, but the efficiency of this process depended on their origin. HBCa.MEC achieved a complete network in the studied timeframe, showing lower angiogenic potential than healthy ECs. Moreover, both cell lines were CD309+, with higher expressions of CD309 in healthy ECs. Newly formed networks, initiated by VEGF-A/VEGFR2, resulted in a higher rates of healthy EC proliferation, survival and new vessel formation than tumor-derived ECs [27]. This phenomenon was partly observed in present study, as the permeability assay indicated that tumor-derived EC monolayers leaked more than healthy EC monolayers. This distinct permeability may have occurred due to the lower ZO-1 protein level in pathologic cells. In our study, we detected a lower expression of alkaline phosphatase in cancer ECs. This protein is largely characteristic of blood–brain barrier derived ECs but has been shown to increase upon re-induction of barrier properties [28,29]. This may further suggest a defective angiogenesis regulation in the tumor endothelial cell line.

Our cell lines also displayed differences in leukocyte rolling-related protein levels such as ICAM-1, PECAM-1, VCAM-1, which were expressed on the cell surface as on the nonlymphoid tissue–derived microvascular ECs, HIMEC.1 and HSKMEC.1 [20,27]. CD54 was less expressed in breast tumor-derived ECs than in healthy ones, corroborating the phenotype of tumor ECs vs. the normal features of healthy ECs. VCAM-1 and ICAM-1, which mediate leukocyte—endothelial cell adhesion, were expressed in our model [30]. Furthermore, the presence of L-selectin—the receptor responsible for the initial steps of

leukocyte rolling [31]—suggested that the obtained cell lines could be used as a model for endothelium—immune cell interaction [32].

Apart from EC-specific markers, we also evaluated proteins related to of the ECs' origin and tumor-related proteins. Both of our cell lines were PDPN+, a controversial marker for both arterial and lymphatic ECs, as Furukoji et al. and Hatakeyama et al. showed [33,34]. The expression of EphrinB2 served as further evidence that both ECs originated from the arteries [11], as shown by NGS. Additionally, this gene has also been associated with poor prognosis in HER2-positive breast cancer [35] and has been up-regulated in breast TECs. Moreover, NGS analysis indicated upregulation of SNCAIP in tumor-derived ECs, which is almost exclusively expressed in triple-negative breast tumors (protein atlas), suggesting the tumor-related phenotype of the obtained cell lines. Another tumor-related marker that is up-regulated in pathological ECs vs. healthy ECs is ABCG2, a multidrug-resistance receptor (MDR) in breast cancer cells [36] that is also expressed by various ECs [37,38].

Moreover, pathologic ECs tend to present increased level of mesenchymal markers (α-SMA) than healthy ones. SMA is used to identify vascular smooth muscle cells and pericytes [39] but is also present on arterioles/venules, rather than capillaries [40]. However, some endothelial cells, especially in in vitro culture, have also been shown to express this marker [41]. Importantly, SMA was shown to increase in vessels upon inflammation and during fibrosis, which confirms the pathological features of cancer-derived ECs [42].

Global gene analysis identified several genes that showed differential expression in healthy and tumor-derived ECs. The 10 most changed expressed genes in cancer-derived EC were previously shown to play roles in cancer cells. Upregulated genes in pathological ECs vs. healthy ECs include ABCG2, also known as breast cancer resistance protein (BCRP) and lipid phosphate phosphatase-related protein type 5 (PLPR5), found in lung cancer and in breast tumors [36,43]. Melanoma-associated antigen 11 (MAGAB) is expressed in several types of tumors, such as melanoma, head and neck squamous cell carcinoma, lung carcinoma and breast carcinoma [44]. Overexpression of MORN4 is found in breast cancer tissue [45]. SNCAIP mutation is mostly found in cutaneous melanoma but also occurs in breast invasive ductal carcinoma [46]. Additionally, O51B4, which plays a role in olfactory receptors and in some cancers, was decreased in TECs [47]. The downregulated genes specific to cancer ECs were characterized before dropping in tumor cells. The genes with lower expression in HBCa.MEC are related to AJAP1(a tumor suppressor), HBG2 (down-regulated in ovarian cancer) [48] and CBPE(a modulator of actin filaments' organization) [49]. Apparently, tumor-derived ECs display gene expression patterns characteristic of both endothelium and cancer cells.

4. Materials and Methods

4.1. Endothelial Cell Lines Culture

Endothelial cells were established according to the method previously described [20,21] (CNRS patent 99-16169). The samples were obtained from a female patient (INSERM UMR 1186, Integrative Tumor Immunology and Genetic Oncology, Gustave Roussy, EPHE, Villejuif, France), diagnosed with breast cancer (stage IIA: T2-N1-M0; HR-/HER2-). Resection specimens of primary tumor and healthy tissue, were received freshly after surgery, with informed written patient consent. All procedures were performed in accordance with generally accepted guidelines for the use of human material. The samples of healthy tissue and primary breast tumor were named HBH.MEC and HBCa.MEC, respectively. HBH.MEC and HBCa.MEC were seeded at density 5×10^4 cells/10cm2. Both ECs lines were cultured on Primaria Tissue Culture Flask (Corning, NY, USA, #353808) in the presence of Opti-MEM I Reduced Serum Medium (Gibco, Paisley, UK #31985070) supplemented with 2% (*vol/vol*) fetal bovine serum (Gibco, Paisley, UK #A3840402). All cells were maintained at 37 °C and 5% CO_2 in humidified atmosphere. Lastly, prior to experiments, cells were detached with enzyme cell detachment medium: Accutase supplied in Dulbecco's PBS containing

0.5 mM EDTA and phenol red (Invitrogen, Carlsbad, CA, USA #E136579). The cell lines were mycoplasma-free (PromoKine, Hamburg, Germany #PK-CA91-1096).

4.2. Cell Viability Assay

HBH.MEC and HBCa.MEC, were seeded on 96-well plates, 1500 cells/well. Cells were cultured in 200 µL of normoxic medium for 48 h. Then, to assess cell viability, Alamar Blue Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA #DAL1100) was added to the wells and incubated for 4 h at 37 °C. After this time, the absorbance of alamarBlue was read at 570 nm against the blank established by cell-free wells filled with medium.

4.3. Flow Cytometry Analysis

After the cells were collected and washed twice with PBS, they were incubated with the recommended dilution of antibodies. The samples were stored at 4 °C for 30 min and washed with PBS. Then, cells were analyzed by flow cytometry using CYTOFLEX software v.2.3.0.84 (Becton Dickinson, Franklin Lakes, NJ, USA). The lower threshold was used to exclude debris and live cells with gating (10,000 cells), according to forward scatter (FSC) × side scatter (SSC), followed by sections containing antibodies. The following antihuman antibodies were used: APC-conjugated EGFR Antibody (BioLegend, San Diego, CA, USA #352905), PerCP-conjugated Podoplanin Antibody (eBioscience, San Diego, CA, USA #46-9381-42), PE-Cy7-conjugated CD34 Antibody (BD Biosciences, Franklin Lakes, NJ, USA #560710), PE-conjugated CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany#130-098-046), FITZ-conjugated CD105 (BioLegend, San Diego, CA, USA #323204), PE-conjugated Anti-CD309 Antibody (Beckman Coulter Life Sciences, Marseille Cedex, France #a64615), Alexa Fluor 700-conjugated BCL-6 Antibody (BD Biosciences, Franklin Lakes, NJ, USA # 566993), PE-conjugated Anti-CD62L Antibody (Beckman Coulter Life Sciences, Marseille Cedex, France #IM2214U), FITC-conjugated Anti-CD54 Antibody (Beckman Coulter Life Sciences, Marseille Cedex, France #IM0726U), KO525-A-conjugated Anti-CD31 Antibody (BD Horizon, Franklin Lakes, NJ, USA #563454), Alexa Fluor 488-conjugated Alkaline Phosphatase (BD Biosciences, Franklin Lakes, NJ, USA #561495), VWF Antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA #sc-53466), ACE Antibody (R&D Systems Minneapolis, MA, USA #AF929). For αSMA, VWF and ACE, cells were fixed and permeabilizated according to manufacturer protocol (Beckman Coulter Life Sciences Marseille Cedex, France #B31168). For the nonconjugated antibodies VWF and ACE, secondary FITZ-conjugated antibodies were used: Alexa Fluor 488-conjugated anti-rabbit (Jackson ImmunoResearch, Ely, UK #115-545-003) and Alexa Fluor 488-conjugated anti-goat (Jackson ImmunoResearch, Ely, UK #115-545-003), respectively.

4.4. Western Blotting

ECs cultured after 48 h in normoxia were washed with PBS and lysed in radio-immune precipitation assay (RIPA) buffer (Thermo Fisher Scientific, Rockford, IL, USA #89900) containing Protease Inhibitors Cocktail (Sigma-Aldrich, Darmstadt, Germany #P8340) and then incubated overnight at -80 °C. The same amounts of protein in the samples were assessed by BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA#23225) and then heated at 95 °C for 10 min. Next, separation was performed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA#1620094). After blocking nonspecific binding sites for 2 h using 5% nonfat dried milk in Tris-buffered saline/Tween at room temperature, membranes were incubated overnight at 4 °C with specific Abs: anti-p53 (1C12, Cell Signaling, Warsaw, Poland #2524), anti-ICAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA #sc-8439), anti-ZO-1 (Cell Signalling, Warsaw, Poland #5406), anti-VCAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA #sc-18864) and anti-Vinculin (V284, Santa Cruz Biotechnology, Santa Cruz, CA, USA #sc-59803). Then, incubation took place for 2h at RT with horse anti-mouse, goat anti-rabbit IgG or goat anti-rat secondary antibodies conjugated with horseradish peroxidase (HRP) (1:10,000; Vector Laboratories, Janki, Poland

#PI-2000, #PI-1000, #PI-9400). Next, signals were detected by chemiluminescence substrate (Santa Cruz Biotechnology, Santa Cruz, CA, USA #sc-2048) on X-ray films (Carestream, Rochester, NY, USA #7711468). The density of bands was quantified by the ImageJ software (v.1.52p). Band intensities were normalized to the intensities of their corresponding loading controls (Vinculin, 117 kDa).

4.5. Next Generation Sequencing

Total RNA was isolated from the cell cultured in normoxia for 48 h, according to manufacturer's protocol, with RNeasy Plus Mini Kit (Qiagen, Hilden, Germany#74136). Then, total concentration purity of the isolated material was evaluated using the fluorometer Qubit and Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Singapore,#10210), according to manufacturers' instructions. Samples were also investigated in order to assess the quality and integrity of RNA with Qubit RNA IQ Assay Kit (Thermo Fisher Scientific, Singapore, #33221). Next, NGS libraries were prepared with the NEBNext Library Prep Kit (BioLabs, Ipswich, MA, USA #E7770S). Finally libraries underwent quality assessment using the Bioanalyzer 2100 and High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA # 5067-4626), according to the manufacturers' protocols. NGS assay were performed as an outsourced service. Differentially expressed genes (DEGs) were determined as those with $p < 0.05$ and fold change > 1.5. Functional enrichment analysis was performed using Cytoscape software (v.3.8.0) to identify gene ontology (GO) biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways represented by DEGs with statistical significance.

4.6. Secretion of VEGF-A

Both cell line's media were changed to normoxic or hypoxic conditions (pO_2 = 19% and pO_2 = 1%, respectively) after overnight incubation in standard culture and previous incubation in the appropriate condition for 24 h. Next, cells were moved to culture in normoxia or hypoxia for 48 h. Then, media were collected and stored at −80 °C. ELISA was performed according to manufacturer protocol (R&D Systems, Minneapolis, MA, USA #DVE00) in three independent biological repetitions and three technical repetitions for each.

4.7. In Vitro Angiogenesis Assay

Angiogenesis was performed on Matrigel^{TM}-coated 24-well plates (BD Biosciences, Franklin Lakes, NJ, USA). Cells were seeded at 5×10^4 cells/cm^2 and observed for 5 h in standard culture conditions using a Zeiss AxioObserver.7. The rearrangement of the cells and the formation of pseudo-vessels were followed for 5 h with a time step of 30 min at 5× magnification using Z1/7—software Zen v.2.6 (Zeiss, Oberkochen, Germany).

4.8. Permeability Test

Briefly, 0.4 nm filters (VWR, Warsaw, Poland #734-2746P) were coated with collagen IV (Sigma-Aldrich, Darmstadt, Germany #C752) and fibronectin (Sigma-Aldrich, Darmstadt, Germany #F1141) and left to solidify in sterile conditions. Then, HBH.MEC or HBCa.MEC, (1500 cells/well) were seeded on each filter placed into each well on a 96-well plate. Cells were cultured in 200 µL of normoxic medium for 48 h. Then fluorescein dye was added (Sigma-Aldrich, Darmstadt, Germany #46960-100G-F) and cells were incubated in standard culture conditions. After 30 min the measurement of fluorescence was measured at 488–520 nm.

4.9. Cell Proliferation Assay

In total, 1500 cells/well (HBH.MEC) and 2000 cells/well (HBCa.MEC) were seeded on 96-well plates in normoxia/hypoxia for 48 h. Then, CyQUANT Cell Proliferation Assay (Invitrogen, Rockford, IL, USA #C7026) was performed according to manufacturer protocol.

Fluorescence of the samples was measured using a fluorescence microplate reader set up with 480 nm excitation.

4.10. Statistical Analysis

Statistical significance was calculated by Student's *t*-test and two-way ANOVA, and p-value was calculated in GraphPad PRISM v.9.0. The data are represented by histograms and bar charts, each of which consisted of results from at least three independent experiments. For all experiments with error bars, the standard error of the mean (SEM) was calculated to indicate the variation within each experiment. p value of <0.05 was considered statistically significant and denoted with: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

5. Conclusions

Despite some limitations, our model offered a relevant tool for angiogenesis studies. Both cell lines, healthy ECs as well as pathological ECs, were obtained and cultured in normoxia— which is not a natural microenvironment. Some of the features, especially those of cancer ECs, might have been modified during prolonged culture, as indicated by exposure to hypoxia, which revealed further differences between cell lines. Nevertheless, breast TECs still possessed features of dysfunction in proangiogenic response and permeability. In this study, we demonstrated the properties of two cell lines deemed suitable for in vitro models of endothelial organospecific cells reflecting the phenotypes of healthy and tumor tissues. This entire characterization indicated that HBH.MEC and HBCa.MEC provide a valuable in vitro model of breast tumor angiogenesis, permeability and leukocyte rolling studies which mimic cell behavior and (dys)function in pathological vessels of the most lethal breast cancer subtype. Furthermore, it confirmed the validity of the endothelial organospecificity for the design of biologically relevant tumor models.

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Data Availability Statement: All data generated or analyzed during this study are included either in this article or in the Supplementary Figures. The data that support the findings of this study are available from the corresponding author kwilkus@wim.mil.pl upon reasonable request. NGS data are available at GEO database (7 July 2021) https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179509.

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6. Conclusions

We pointed the importance of using organospecific endothelial cells in breast cancer studies. Moreover, it was presented that the tumor microenvironment, with emphasis of the cell origin, shapes ECs activity. The breast derived- endothelial cells differ phenotypically and functionally depending on their origin: healthy and tumor tissue, indicating modulatory role of tumor microenvironment, which aside from disturbing angiogenesis, influences the changes in pathological endothelium inducing endothelial to mesenchymal transition genotype.

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8. Ethics Committee Opinion

This study uses strains of cells established previously as endothelial cell lines from the fresh specimens of breast tissues, that were gifted by prof. Salem Chouaib (INSERM UMR 1186, Gustave Roussy Institute, Villejuif, France) under a collaborative research project program. The Ethics committee of the Gustave Roussy Institute did not require the study to be reviewed or approved by an ethics committee because samples were medical waste (year 2011). The study only required the patient consent as all patient data were anonymised and deidentified. Therefore we did not add the information on ethics approval but necessary data are included in the manuscript.

Cell lines named HBH.MEC and HBCa.MEC represent endothelial cells of healthy tissue and primary breast tumor, respectively, and are deposited in the National Collection of Cells and Microorganisms (Pasteur Institute).

9. Statements of all co-authors of publications

Warszawa 04/01/2023.....
(miejscowość, data)

..Claudine KIEDA.....
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Endothelial Cells as Tools to Model Tissue Microenvironment in Hypoxia-Dependent Pathologies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie i przedstawienie pracy w formie publikacji stanowi:
koncepcję oraz poprawki edytorskie końcowej wersji publikacji przed jej przesłaniem do czasopisma.
Mój udział procentowy w przygotowaniu publikacji określam jako 15 %.
Wkład .....Kingi Wilkus..... w powstawanie publikacji określam jako 35 %,
(imię i nazwisko kandydata do stopnia)
obejmował on: ogólne opracowanie założeń pracy, przygotowanie oryginalnego szkicu pracy przeglądowej na podstawie dostępnej literatury, edycję końcową przed publikacją w czasopiśmie.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek/mgr .....Kingi Wilkus.....
(imię i nazwisko kandydata do stopnia)

.....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

…...Warszawa, 9.01.23.....
(miejscowość, data)

.....Klaudia Brodaczewska.....
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Endothelial Cells as Tools to Model Tissue Microenvironment in Hypoxia-Dependent Pathologies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie i przedstawienie pracy w formie publikacji stanowi:
ogólne opracowanie założeń pracy oraz poprawki edytorskie końcowej wersji publikacji przed jej przesłaniem do czasopisma.
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Wkład Kingi Wilkus w powstawanie publikacji określam jako 35 %,
(imię i nazwisko kandydata do stopnia)
obejmował on: ogólne opracowanie założeń pracy, przygotowanie oryginalnego szkicu pracy przeglądowej na podstawie dostępnej literatury, edycję końcową przed publikacją w czasopiśmie.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek/mgr Kingi Wilkus.
(imię i nazwisko kandydata do stopnia)

Adiunkt
Laboratorium Onkologii Molekularnej
i Terapii Innowacyjnych
Wojskowego Instytutu Medycznego
.....
dr n. biol. Klaudia BRODACZEWSKA
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa 3.01.20
(miejscowość, data)

Aleksandra Majewska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Endothelial Cells as Tools to Model Tissue Microenvironment in Hypoxia-Dependent Pathologies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie i przedstawienie pracy w formie publikacji stanowi:
Opracowanie koncepcji pracy, przygotowanie pierwszej wersji manuskryptu, edycję końcową przez publikacją w czasopiśmie.
Mój udział procentowy w przygotowaniu publikacji określam jako 35 %.
Wkład Kingi Wilkus w powstawanie publikacji określam jako 35 %,
(imię i nazwisko kandydata do stopnia)
obejmował on: koncepcję, przygotowanie oryginalnego szkicu pracy przeglądowej na podstawie dostępnej literatury, edycję końcową przez publikacją w czasopiśmie.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Kingi Wilkus.
(imię i nazwisko kandydata do stopnia)

A. Majewska
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa 04/01/2023.....
(miejscowość, data)

.....Claudine KIEDA.....
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Distinctive Properties of Endothelial Cells from Tumor and Normal Tissue in Human Breast Cancer", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Koncepcję i nadzór na postępami badań, zaplanowanie metodologii oraz poprawki edycyjne do końcowej wersji publikacji.

Mój udział procentowy w przygotowaniu publikacji określam jako 21 %.

Wkład .....Kingi Wilkus..... w powstawanie publikacji określam jako 51 %,
(imię i nazwisko kandydata do stopnia)

obejmował on: opracowanie ogólnych założeń pracy, optymalizację, planowanie i przeprowadzenie wszystkich etapów badań, analizę wraz z interpretacją otrzymanych danych, przygotowanie oryginalnego szkicu pracy, edycję końcową pracy.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek/mgr..... Kingi Wilkus....
(imię i nazwisko kandydata do stopnia)

Claudine Kieda

.....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

…….Warszawa, 9.01.23…..
(miejscowość, data)

…….Klaudia Brodaczewska……
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Distinctive Properties of Endothelial Cells from Tumor and Normal Tissue in Human Breast Cancer", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

Koncepcję, zaplanowanie metodologii, interpretację otrzymanych wyników oraz poprawki edycyjne do końcowej wersji publikacji.

Mój udział procentowy w przygotowaniu publikacji określam jako 20 %.

Wkład ……Kingi Wilkus……. w powstawanie publikacji określam jako 51 %,
(imię i nazwisko kandydata do stopnia)

obejmował on: opracowanie założeń pracy, optymalizację i wykonanie badań, analizę i interpretację otrzymanych wyników, przygotowanie oryginalnego szkicu pracy, edycję końcową przed publikacją w czasopiśmie.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr……..Kingi Wilkus……..
(imię i nazwisko kandydata do stopnia)

Adiunkt
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*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Poznań, 04.01.2023 r.
(miejscowość, data)

Arkadiusz Kajdasz
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Distinctive Properties of Endothelial Cells from Tumor and Normal Tissue in Human Breast Cancer", oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: wstępne opracowanie analizy na podstawie otrzymanych wyników NGS.
Mój udział procentowy w przygotowaniu publikacji określam jako 7 %.
Wkład..... Kingi Wilkus..... w powstawanie publikacji określam jako 51 %,
(imię i nazwisko kandydata do stopnia)
obejmował on: koncepcję, przeprowadzenie wszystkich etapów eksperymentów, analizę otrzymanych wyników i ich interpretację, przygotowanie oryginalnego szkicu pracy.
(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek/mgr..... Kingi Wilkus.....
(imię i nazwisko kandydata do stopnia)

Arkadiusz Kajdasz

.....
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

10. Abstracts of papers in preparation/ submission

10.1 Microenvironment commits breast tumor ECs to dedifferentiation by micro-RNA-200-b-3p regulation and extracellular matrix remodelling

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Keywords: breast cancer, ECM-receptor interaction, EndMT, endothelial cells, heterogeneity, miR-200b-3p

Abstract

Introduction: Hypoxia shapes the tumor microenvironment and modulates distinct cell populations activity. Low pO2 activates pathological angiogenesis in cancer- process in which endothelial cells (ECs) are the most important players. The main goal of the study was to evidence the hypoxic tumor microenvironment influence on the global gene expression pattern characteristic for ECs and evidence the distinct and specific responses displayed by the tumor-derived ECs compared to the healthy endothelium during the endothelial to mesenchymal transition (EndMT) and its regulation by miR-200-b-3p. Methodology: Immortalized lines of ECs from the same patient with diagnosed breast cancer- healthy breast tissue (HBH.MEC) and primary tumor (HBCa.MEC) were used. The experiments were performed in normoxia and hypoxia for 48 hours. By wound healing test, we investigated the migration abilities of ECs. Using the established model, global gene expression analysis with NGS was carried out to detect new pathways altered in pathological ECs and to find the most changed miRNAs. The validation of NGS data from RNA and miRNA was estimated by qPCRs.

Results: Hypoxia influences ECs migration properties in wound healing assay. In hypoxia, healthy ECs migrate slower than in normoxia, as opposed to HBCa.MEC where no decreased migration ability was induced by hypoxia, due to EndMT features. NGS

data identified this process to be altered in cancer ECs through extracellular matrix (ECM) organization. The deregulated genes, confirmed by qPCR, included: *SPP1*, *ITGB6*, *COL4A4*, *ADAMST2*, *LAMA1*, *GAS6*, *AGTR2*, *PECAM1*, *ELN*, *FBLN2*, *COL6A3*, *COL9A3*. NGS also identified collagens, laminin, fibronectin and integrin, as being deregulated in tumor-derived ECs. Moreover, the analysis of ten most intensively modified miRNAs when breast tumor-derived ECs were compared to healthy ECs, put a light on miR-200b-3p which is strongly up-regulated in HBCa.MECs as compared to HBH.MECs.

Discussion and Conclusions: The pathological ECs differed significantly, both phenotypically and functionally, from the normal corresponding tissue, thus influencing their microenvironment cross- talk. The gene expression profile confirms the EndMT phenotype of tumor-derived ECs and migratory properties acquisition. Moreover, it indicates the role of miR-200b-3p, regulating EndMT in pathological ECs, silencing several angiogenic growth factors and their receptors by directly targeting their mRNA transcripts.

10.2 PTEN differentially regulates the Mdm2-p53 pathway in breast tumor *vs* normal endothelial cells evidenced by hypoxic microenvironment

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Key words: ECs, Mdm2-p53, Nutlin-3, pathological angiogenesis, PTEN

Abstract

Introduction and aim: Hypoxia, lower than physiological oxygen tension, shapes the tumor microenvironment and modulates distinct cell populations activity. Low pO2 activates pathological angiogenesis, the process in which endothelial cells (ECs) are crucial and is highly regulated by PTEN protein – a tumor suppressor. What is more, PTEN was shown to modulate the activity of p53 which also contributes to the angiogenic activity of ECs. To find out which of these tumor suppressors is the best target to repair the pathologic angiogenesis, the present study compares tumoral-to-normal tissue-

derived ECs. The main aim of the research was to evaluate how, in the real microenvironmental conditions of oxygen availability, PTEN and p53/Mdm2 pathways are modified in ECs originating from breast cancer site (HBCa.MEC) as compared to healthy breast tissue (HBH.MEC).

Results and conclusion: The pro-angiogenic response of ECs from healthy and pathological sites appears to be different when cells were cultured in hypoxia vs normoxia. do not HBCa.MEC showed increased ability to form vessels *in vitro* as numbers of nodes and junctions were significantly increased upon hypoxia. At the same time hypoxia reduced the total level of the proteins but pMdm2/Mdm2 and p-PTEN/PTEN ratios were elevated, indicative for a higher level Mdm2 active form (p-Mdm2) and non-active form of PTEN (p-PTEN), what may have mediated increased angiogenesis, as these proteins were not changed in healthy ECs. Additionally, when Mdm2-p53 interaction was inhibited (by Nutlin-3), hypoxic cancer ECs angiogenic activity was reduced reaching the normoxic ECs response. To establish the role of PTEN in Mdm2/p53 interaction, PTEN was silenced in both cell lines. Hypoxia-induced Mdm2 increase is reduced upon PTEN silencing in the tumor-derived ECs as opposed to normal tissue derived ECs. At the same time, PTEN silencing, had similar effect in both cell lines, on the p-p53/p53 ratio, reducing it. Altogether, we conclude that the PTEN-mediated control of pathological angiogenesis occurs by modulation of Mdm2/p53 interaction in the context of breast tumor microenvironment and can be a specific target to consider restoring the normal ECs status.