Dissertation

The role of succinate-SUCNR1 axis in human placental endothelial cells

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

Graz, April 2022 Reham Atallah

Disclosures

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Abbreviations*

ADA	American Diabetes Association
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
ECIS	Electric Cell-substrate Impedance Sensing
EPO	Erythropoietin
ERK	Extracellular signal-regulated kinase
FCCP	Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FCS	Fetal calf serum
FGF2	Fibroblast growth factor 2
FpECA	Fetoplacental arterial endothelial cells
FpECAd	Fetoplacental arterial endothelial cells from diabetic pregnancies
FpECs	Fetoplacental endothelial cells (denoting both arterial and venous cells)
FpECV	Fetoplacental venous endothelial cells
FpECVd	Fetoplacental venous endothelial cells from diabetic pregnancies
GDM	Gestational diabetes mellitus
GLUT	Glucose transporter
GPCR	G-protein coupled receptor
GSH	Reduced glutathione
HAPO	Hyperglycemia and Adverse Pregnancy Outcome
HBSS	Hanks Balanced Salt Solution
HEK	Human embryonic kidney

HIF1	Hypoxia inducible factor-1
HK	Hexokinase
HRE	HIF response elements
HUVECs	Human umbilical vein endothelial cells
IADPSG	International Association of Diabetes and Pregnancy Study Group
INS/IGF	Insulin/Insulin-like growth factor
ISH	In situ hybridization
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDMs	Monocytes derived macrophages
mtDNA	Mitochondrial DNA
NAD	Nicotinamide adenine dinucleotide
NF-KB	Nuclear factor-κB
NO	Nitric oxide
OGTT	Oral glucose tolerance test
PBS	Phosphate buffer saline
PE	Preeclampsia
PHD	Prolyl hydroxylase
PlGF	Placental growth factor
ROS	Reactive oxygen species
RT	Room temperature
SDH	Succinate dehyrdogenase
SiRNA	Small interfering

T2DM	Type 2 diabetes mellitus
TCA	Tricarboxylic acid cycle
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor
VWF	von Willebrand factor
WGA	Wheat germ agglutinin

*Abbreviations are arranged in alphabetical order

Zusammenfassung

Plazentainsuffizienz, welche zu einer Minderversorgung des Fötus führt, ist eine erhebliche Schwangerschaftskomplikation wie sie unter anderem bei Patientinnen mit Gestationsdiabetes (GDM) angetroffen wird. Insuffiziente Plazenten zeichnen sich durch einen hyper-vaskularisierten Aufbau aus, welcher bereits in der Vergangenheit auf das Zusammenspiel von Hypoxie und Angiogenese untersucht wurde. Im Zuge dieser Nachforschungen wurde aber, bis heute, ein mögliches Zusammenspiel zwischen Plazenta und fötalen Metaboliten nicht aufgeklärt. Diese Doktorarbeit stellt eine Abhandlung über Succinat und dessen Rezeptor, SUCNR1, als mögliche Regulatoren für plazentale Angiogenese unter dem pathophysiologischen Umfeld von GDM dar.

Initial ließen sich in plazentalen Gewebshomogenaten von Patientinnen mit GDM höhere Spiegel von Succinat und vaskulären endothelialen Wachstumsfaktoren (VEGF) nachweisen als in Plazenten gesunder Vergleichsmütter. Diese positive Korrelation von Succinat und VEGF bereits auf Organebene deutete auf die potenzielle Rolle des Succinat-SUCNR1 Zwischenspiels als Angiogenese-Regulator hin. Es war uns nicht nur möglich die Präsenz des SUCNR1 Proteins in arteriellen humanen plazentalen Endothelzellen (FpECAs) nachzuweisen, sondern auch dass dessen Expression unter GDM Bedingungen zunimmt. In in-vitro Experimenten mit Endothelzellen aus humanen Nabelschnurvenen (HUVECs) führte die Gabe von Succinat zur Ausprägung mehrerer Eigenschaften, welche als pro-angiogenetisch angesehen werden können, wie vermehrte Zellteilung, Zellmigration, vermehrte Sprossung aus Zell-Sphäroiden und eine Reduktion der Zellbarriere. Die Verabreichung von Succinat hatte auch eine erhöhte Produktion von VEGF zur Folge. All diese Befunde ließen sich in FpECAs bestätigen. Ebenso konnte durch einen Knockdown von SUCNR1 eine Regression der pro-angiogenetischen Wirkung von Succinat beobachtet werden. Die weiterführende Signaltransduktion wurde in der endothelialen Zelllinie EA.hy926 untersucht und es gelang uns eine Verbindung zu Extracellular Regulated Kinase (ERK1/2) herzustellen.

Zusammenfassend zeigt diese Doktorarbeit wie wichtig das Zusammenspiel von Succinat und SUCNR1 für FpECS ist. Die regulatorischen Eigenschaften im Zuge des Angiogenese-Prozesses konnten nachgewiesen werden und stellen somit einen möglichen Mechanismus dar, welche die Hypervaskularisierung der GDM Plazenten erklärt.

Abstract

Placental insufficiency is a hallmark of pregnancy complications such as gestational diabetes mellitus (GDM). Placentas from GDM pregnancies are commonly reported to be hypervascularized. This phenomenon has been addressed in contexts of hypoxia and angiogenic imbalance. However, a possible contribution of placental/fetal metabolites to this anomaly has been overlooked to-date. Within this thesis, I investigated an axis comprised of succinate and its cognate receptor, SUCNR1, in human placenta as possible regulators of placental angiogenesis, which might be aggravated in GDM.

I measured significantly higher succinate levels in placental tissue lysates from women with GDM relative to matched controls in parallel to an increase in vascular endothelial growth factor (VEGF). A positive correlation of SUCNR1 and VEGF protein levels in tissue lysates indicated a potential link between succinate-SUCNR1 axis and placental angiogenesis. At the cellular level, our data showed expression of SUCNR1 in human placental endothelial cells. SUCNR1 protein expression was upregulated in diabetic fetoplacental arterial endothelial cells (FpECAds) in comparison to normal cells (FpECAs). In our in vitro experiments, succinate prompted hallmarks of angiogenesis in human umbilical vein endothelial cells (HUVECs) such as cell proliferation, migration and spheroid sprouting in addition to barrier disruption. These results were further validated in FpECAs, where succinate induced endothelial tube formation. VEGF gene expression was increased in response to succinate in both HUVECs and FpECAs. Yet, knockdown of SUCNR1 in HUVECs led to suppression of VEGF gene expression and abrogated cell migratory ability, wound healing and barrier response upon treatment with succinate. To study the signaling machinery downstream of SUCNR1, we used endothelial cell line EA.hy926 and we demonstrated that extracellular signal-regulated kinase (ERK1/2) pathway was regulated upon treatment with succinate.

In conclusion, this thesis highlighted succinate-SUCNR1 as an important signaling axis in human FpECs. The role of this axis in regulating angiogenesis was demonstrated. Our findings proposed a mechanism to the increased angiogenesis in GDM placentas.

1. Introduction

1.1. Human placenta

Pregnancy is a challenging condition that requires the mother's body to undergo a series of physiological changes. Adaptations of the cardiovascular, renal, hematologic, respiratory and metabolic systems all synchronize to support the needs of the fetus (2). At the interface between the mother and fetus, the placenta plays a major role in mediating maternal-fetal communications. The placenta carries nutrients and oxygen from the maternal circulation to the fetus and simultaneously removes fetal waste products. In addition, the placenta plays a crucial role in protecting the fetus from maternal inflammatory responses (3). The placenta also senses and responds to alterations in the gestational environment and secretes numerous hormones that modulate the maternal physiology to support fetal growth (4) (5). Hence, it is not unexpected that placental dysfunction during pregnancy is linked to numerous pregnancy complications like gestational diabetes mellitus (GDM), preeclampsia (PE) and fetal growth restriction that have adverse consequences for both the mother and offspring (6) (7).

1.1.1. Glucose as an energy substrate for the placenta

For both the placenta and the fetus, glucose is the main energy substrate. Nevertheless, in fetal plasma, glucose concentration is less than that in maternal circulation (8). The main glucose transporter in human placenta is glucose transporter 1 (GLUT1) which is expressed in microvillous trophoblast membrane and the fetal-facing basement membrane (9). Another transporter GLUT3 is expressed only in the microvillous membrane suggesting that it might be responsible for taking up glucose for trophoblast cells themselves. In addition, GLUT4 is reported to be expressed in human placenta but it is not well localized. So far, there is no solid evidence that insulin regulates glucose uptake from either the maternal or the fetal circulation in mature placentas despite some previous reports showing insulin-mediated glucose uptake in perfused human placentas from insulin-treated diabetic women and in first trimester chorionic villous explants (10).

At the maternal side, during the course of pregnancy, insulin sensitivity shifts from increased sensitivity at early gestation, to enhance the uptake of glucose into adipose tissue, to a state of insulin resistance at the end of pregnancy (11). An increase in local and placental hormones including estrogen, progesterone, leptin, cortisol, placental lactogen, and placental growth hormone plays a major role in developing the state of insulin resistance (12). Consequently, blood glucose is slightly increased and made readily available to fuel fetal growth through transport across the placenta. To compensate for maternal insulin resistance and to maintain normal blood glucose, maternal pancreatic β-cell hyperplasia and hyperfunctionality are reported (13).

1.1.2. Placental mitochondria

In the placenta, numerous processes require energy in the form of adenosine triphosphate (ATP) including placental formation, substrate exchange and endocrine functions (8). Part of this energy is provided by cytoplasmic glycolysis. However, mitochondria are key orchestrators of energy production and are thus crucial for maintaining normal cellular functions. Interestingly, mitochondrial morphology differs between cell populations in placenta. While cytotrophoblast mitochondria have more classical morphology, syncytiotrophoblast mitochondria are smaller and with an irregular shape and cristae structure (14) (15). A proteomics study has unraveled that the expression levels of 29 proteins are significantly different between these mitochondrial subpopulations. These proteins included subunits of ATP synthase, carbohydrate metabolizing enzymes and others indicating that these proteins may contribute to the unique properties of these mitochondria after cellular differentiation (16). Of note, mitochondria are sensors of environmental cues and alterations in mitochondrial function could be a double edged sword either mediating or alleviating the consequences of poor environment on placental function and consequent risks of pregnancy complications like PE, GDM and fetal growth restriction (17) (18).

Mitochondrial biogenesis is an elaborate process that requires nuclear and mitochondrial (mtDNA) encoded gene expression to synchronize, ensuring the assembly of a large set of proteins comprising the electron transport chain. Mitochondrial diseases resulting from mutations in mtDNA are inherited only through the maternal line in humans (19) and women with mitochondrial dysfunctions are at increased risks of developing and showing more serious symptoms of GDM, PE and miscarriage (20). In the placenta, mitochondrial content and respiratory capacity have been linked to oxygen fluctuations over pregnancy (21) and mitochondrial responses are correlated with alterations in placental antioxidant status (22). These findings provide evidence of the ability of placental mitochondria to adapt to oxygen stimuli. Furthermore, altered mitochondrial function and suppression of complexes I and IV occur in hypoxic placental cells (23) and a role of mitochondrial reactive oxygen species (ROS) in mediating pathologies associated with placental hypoxia highlights placental mitochondria as sensors and regulators of cell stress response (24).

In the inner mitochondrial membrane, succinate dehydrogenase (SDH) complex is an integral component of both the tricarboxylic acid (TCA) cycle and electron transport chain, thus playing a major role in oxidative phosphorylation. Under physiological conditions, SDH catalyzes the conversion of succinate to fumarate. The enzyme complex is composed of four subunits: two hydrophilic subunits, SDHA and SDHB, and two hydrophobic membrane anchoring subunits, SDHC and SDHD (25). In a comparison between the placenta and fetal membranes, SDH activity is higher in the placenta than in fetal membranes implying more active TCA cycle in placenta (26). Furthermore, ATP concentrations and SDH activities are higher in placental amnion relative to reflected amnion indicating region-specific metabolic properties (27). Relevant to the current pandemic, COVID-19-affected mothers show alterations in placental mitochondrial features and oxidative balance, including reduced expression of SDHA. These observations have been attributed to impaired intrauterine environment, due to systemic viral effects (28).

1.1.3. Placental angiogenesis

During pregnancy, placental vasculature continues to expand to support the needs of the growing fetus (29). Vasculogenesis and angiogenesis are two major processes in the development of blood vessels in human placenta. Vasculogenesis refers to the formation of first blood vessels and occurs through the differentiation of undifferentiated precursor cells (angioblasts) into endothelial cells. Angiogenesis refers to the formation of new blood vessels from existing vessels (30). There are at least two different forms of angiogenesis: sprouting of capillaries and non-sprouting angiogenesis or intussusception. The process of sprouting angiogenesis includes proteolytic degradation of extracellular matrix, chemotactic migration and proliferation of endothelial cells, lumen formation and stabilization of newly formed tubes, whereas non-sprouting angiogenesis includes splitting of vessels by transcapillary pillars (31). In human placenta, vasculogenesis begins at 21 days after conception while angiogenesis expansion of first vessels continues from day 32 after conception until term (32). A complex interplay between physical and chemical factors including growth factors, oxygen and growth inhibitors regulate placental vasculature expansion (32).

Among the most prominent growth factors that regulate angiogenesis in human placenta are VEGF, placental growth factor (PlGF), fibroblast growth factor 2 (FGF2) in addition to erythropoietin (EPO), leptin, adiponectin, angiopoietins, and the insulin/insulin-like growth factor (INS/IGF) system. The expression of the receptors for all these factors is confirmed in arterial and venous endothelial cells from term placentas. Trophoblasts, Hofbauer cells and smooth muscle cells all serve as sources of proangiogenic factors and, indeed, angiogenic factors derived from the placenta and the fetus are present in fetal circulation (29). In contrast, the placenta also produces and releases plenty of anti-angiogenic factors such as soluble VEGFR1 (sFlt1) and soluble TGF-β1 receptor endoglin (33). The balance between pro- and anti- angiogenic factors in the placenta maintains fine tuning of placental angiogenesis.

Focusing on the VEGF system, the VEGF family includes several members such as VEGFA (most important and well-studied member, commonly referred to as VEGF), VEGFB, VEGFC, VEGFD and PlGF. These growth factors function by binding to a family of protein tyrosine kinase receptors including VEGFR1 (Flt1), VEGFR2 (KDR/Flk1) and Flt4 (34). There are at least seven structurally homologous isoforms of VEGF ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$), which differ in their expression patterns, biochemical and biological properties (35). These isoforms result from different splicing variants of VEGF pre-mRNA (36). $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ are preferentially produced by most cells producing VEGF while other isoforms are comparatively rare (37).

Fetoplacental endothelium expresses KDR (38) (39), which mediates mitogenic cellular responses, whereas VEGFR1 is expressed in placental macrophages and trophoblasts (38) and plays a key regulatory role in VEGFR2 signaling (40). The expression of both VEGF and KDR is more intense in early pregnancy placentas and decreases closer to term (41). In mouse embryo, VEGF, through its receptor Flt1, has been shown to be crucial for yolk sac blood island formation and early vasculogenesis (42). At early pregnancy, villous trophoblasts secrete VEGF and induce the formation of first placental vessels (43). However, as pregnancy proceeds macrophages and other cells in placental stroma such as smooth muscle cells exert more a regulatory role in placental angiogenesis (41) (44). In parallel, co-expression of VEGF and VEGF receptors in ovine fetoplacental arterial endothelial cells proposes an autocrine mechanism through which VEGF regulates endothelial functions (45). Activation of endothelial cells by VEGF can further result in production of agents that have angiogenesis regulatory effect such as nitric oxide (NO) (46). Additionally, VEGF can recruit pericytes to newly formed vessels (47) and inhibit apoptosis of nascent endothelial cells (48), both mechanisms result in maturation and stability of newly formed vessels (49).

The expression of PlGF, in contrast, is increased in placentas towards term (50). PlGF is expressed in syncytiotrophoblasts (51) and in smooth muscle cells around the fetoplacental vessels (52), where it stimulates branched capillary network formation (53) (54). In vitro data show that PlGF induces proliferation of term placental microvascular endothelial cells (55). A synergistic role between PlGF and VEGF for the formation of the vascular network with the development of the villous tree has been proposed (33).

Another important factor regulating placental vasculature development is oxygen. In early human placenta, the physiological oxygen partial pressure is low. However, this does not refer to hypoxia but rather to a need to drive placental and fetal development. At early gestation, extra-villous trophoblasts invade the decidua causing an occlusion of uterine spiral arterioles, thus preventing blood flow into the inter-villous space (56). In addition, the thickness of the villous membrane during this period is about five times higher in comparison to that in the terminal villi, which reflects higher resistance to oxygen diffusion. This low oxygen environment protects the developing embryos and early placentas from ROS since placental antioxidant defense systems are still not fully developed (22). Furthermore, low oxygen at early pregnancy enhances trophoblast cell invasion (57). Low oxygen pressure reflects on the expression levels of numerous proangiogenic factors by regulating their transcription, mRNA stability and translation. Therefore, the low oxygen environment of early placenta is accompanied by high levels of hypoxia-sensitive proangiogenic factors (29). By the end of first trimester, uteroplacental blood flow is established and is accompanied by a steep rise in oxygen levels in the placenta. Using Paratrend multiparameter probe, Jauniaux et al. established that the oxygen tension rises steeply from <20 mmHg at 8 weeks of gestation to >50 mmHg at 12 weeks (58). In term placentas, oxygen levels decrease to ~40mmHg since the needs of the rapidly growing fetus for oxygen together with its ability to extract oxygen from the placenta rise rapidly (58) (59). Hence, mechanisms regulating angiogenesis could differ between early and term placenta (32).

Notably, placental villous vascularization increases at high-altitude pregnancies in European women as compared to those living at low altitudes or sea level in the United States highlighting the significant consequences of chronic hypoxia on placental vasculature (60) (61). This enhanced vascularization in high-altitude pregnancies is accompanied with reduced oxygen pressure in uterine artery and inter-villous space, reduced antioxidant activity, and lower oxidative stress in placentas (62) (63). The contribution of genetic and epigenetic factors to greater placental efficiency in oxygen transport was demonstrated in high-altitude pregnancies from different ancestries (i.e., Andean vs. European) (64) (65).

1.1.4. Placental endothelial cells at the center of vasculature

Endothelial cells are a thin layer of cells lining all blood vessels. Resting endothelial cells are responsible for many critical functions such as maintenance of vascular integrity, prevention of blood coagulation, control of blood flow and passage of proteins from blood into tissues and control of inflammation. In addition, endothelial cells act as transducers and effectors of local stimuli (66). Unconventionally, in the placenta oxygenated nutrient-rich blood is carried in veins while low oxygen fetal blood is carried in arteries. Fetoplacental blood flow is regulated by local factors such as endothelial NO (67).

For their energy needs, endothelial cells have an absolute requirement for glucose, as they mainly use glycolysis for ATP generation. Mitochondrial content in endothelial cells is relatively low in comparison to other cells (2-6%) (68) and this suggests a role in regulating signaling responses to environmental cues rather than in energy production. Processes such as endothelial cell proliferation and migration are modified by alterations in mitochondrial biogenesis and dynamics resulting from several signals and environmental factors such as oxygen, hemodynamics and nutrients (69).

In acute inflammatory conditions, endothelial cell activation can occur in two modes of responses: Type I activation which is independent of new gene expression and type II activation which requires new gene expression. Both types of activation result in increased blood flow, leakage of plasma protein-rich exudate and local recruitment and activation of leukocytes (66). In chronic inflammation, endothelial cells respond to angiogenic factors such as VEGF to sustain vessel growth needed to support the formation of an inflammatory neo-tissue such as in rheumatoid arthritis (70). However, it is of note to mention that many inflammatory processes show features of acute inflammatory responses and chronic inflammation (66).

1.2. Gestational diabetes mellitus

GDM is the most common pregnancy complication that manifests itself in up to 18% of pregnancies (71). However, this percentage can vary considerably depending on numerous factors such as screening programs and diagnostic criteria (72). Well-established risk factors for GDM include maternal obesity, advanced maternal age, family history and previous GDM and/or PE diagnoses (2). Ethnic differences in GDM prevalence are evident (73), with Hispanic, African-Americans, Native American, South or South East Asian, Pacific Islander or Indigenous Australian being considered to be at higher risk (74). Despite several management strategies, the prevalence of GDM is rising in parallel to the rise in obesity and Western dietary practice (75). Pregnancies affected by GDM impose both mother and fetus to numerous risks such as the need for cesarean delivery, macrosomia, shoulder dystocia and neonatal hypoglycemia (76). GDM always resolves after delivery. However, longer-term programming consequences of GDM can occur and include increased risk of developing type 2 diabetes mellitus (T2DM) and cardiovascular disorders for mothers and higher risk of developing obesity, hypertension and T2DM early in life for their children (77) (78).

1.2.1. Diagnosis of gestational diabetes

According to the American Diabetes Association (ADA), GDM is diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation (79). However, so far, there has been a lack of consensus amongst health professionals regarding the exact threshold for GDM diagnosis. The Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study aimed to determine the degree of maternal glucose intolerance that is less severe than overt diabetes and associates with adverse pregnancy outcomes. The primary study outcomes showed strong continuous associations of maternal glucose levels below those used for diagnosis of diabetes with increased birth weight and increased cord-blood serum C-peptide levels. These associations held true with adjustment for potential confounders, such as maternal age, body mass index (BMI), and blood pressure (80). Currently, the International Association of Diabetes and Pregnancy Study Group (IADPSG) recommends a two-phase strategy for detection and diagnosis of hyperglycemic disorders in pregnancy. First phase aims to detect women with overt diabetes not previously diagnosed or treated outside pregnancy and the second phase is a 75-g oral glucose tolerance test (OGTT) at 24-28 weeks of gestation in all women not previously diagnosed with overt diabetes or GDM. Diagnosis of GDM occurs at any time of pregnancy if one or more of the values of a 75-g OGTT is equal or exceeds the threshold of fasting plasma glucose ≥ 5.1mmol/L (≥ 92mg/dL), 1 h ≥ 10.0mmol/L (180mg/dL), or 2 h ≥ 8.5mmol/L (153mg/dL) (81).

1.2.2. Management of gestational diabetes

A previous randomized clinical trial concluded that treatment of GDM, in the form of dietary advice, blood glucose monitoring and insulin therapy, reduces serious perinatal morbidity and may also improve the woman's health-related quality of life (82). Furthermore, another randomized control trial showed that treatment of mild GDM reduces risks of fetal overgrowth, shoulder dystocia, cesarean delivery and hypertensive disorders (83).

So far, dietary restrictions in combination with suitable physical exercise remain the primary intervention strategy for GDM management (84). Significant associations have been reported between adherence to healthful dietary patterns and lower T2DM risk among women with a history of GDM and these associations have been partly mediated by BMI. The authors recommended public health efforts to encourage women with a history of GDM to consider diets rich in whole grains, fruit and vegetables, protein sources such as white meat, seafood, nuts and legumes and reduced intake of red and processed meats and sugar-sweetened beverages (85). Similarly, a large prospective study showed that increasing physical activity may help reduce the risks of developing T2DM in women with GDM history (86).

In cases where the above-mentioned measures are not enough to achieve glycemic control, insulin therapy stands as the next line of intervention. The past few years have witnessed a remarkable increase in available insulin analogs with novel delivery systems and additional concentrations of existing insulins. Hence, data on their efficiency and safety to be used in pregnancy are crucial. Lispro is the first genetically engineered rapid-acting insulin analog to become available in the USA. The use of lispro in pregnancy has been shown to cause no adverse neonatal outcomes in comparison to regular insulin at the metabolic and immunologic levels (87). Further, a study using a placenta perfusion model reported that insulin lispro at the standard doses does not cross the placenta and only a small dose-dependent transfer of insulin lispro across term human placenta occurs at concentrations ≥ 580μU/ml suggesting that lispro is unlikely to pose a risk for the fetus (88). Insulin aspart is another rapid-acting insulin that has been found to be more effective in reducing postprandial glycemia in comparison to regular human insulin or no therapy in women with GDM (89). A recent randomized study compared the efficiency and safety of insulin detemir (IDet) as a long-acting insulin analog combined with short-acting Novoline R and Neutral protamine Hagedorn (NPH) as a commonly used intermediate-acting insulin in combination with Novoline R in pregnant women with diabetes. Results showed that compared with NPH, IDet is more efficient in controlling blood glucose and reaches the targets faster, thus reducing the number of insulin injections and the incidence of hypoglycemia in pregnant women without increasing adverse birth outcomes (90).

Since insulin therapy requires educating women in self-administration, and utility of insulin is also sometimes associated with hypoglycemia and weight gain (91), the use of oral hypoglycemic medications to control hyperglycemia in pregnancy can offer advantages above insulin. A randomized, open-label trial showed that metformin either alone or with insulin is safe and effective as a treatment option for women with GDM (91). Glyburide has also been shown to be a clinically effective alternative to insulin therapy in GDM (92). In contrast to older sulfonylurea drugs (93) and metformin (94), the quantity of glyburide crossing human placenta is minimal (95). However, more randomized controlled trials, with a long-term follow-up of mother and child, are needed to confirm long-term safety of these medications.

1.2.3. White's classification of diabetes during pregnancy

Priscilla White was a figure in the management of obstetric diabetes. She introduced a system for classification of diabetes in pregnancy based on age at onset, duration, presence of atherosclerotic vascular disease and renal complications. White's system discriminated between gestational diabetes and preexisting diabetes and aimed to assess maternal and fetal risks based on maternal risk factors (96). An evaluation of this system, taking into consideration the presence of chronic hypertension, showed that White's system remains to be a useful tool for counseling pregestational diabetic women in terms of adverse pregnancy outcomes (97). According to White's classification, GDM has two classifications: A1 in which diet control is enough to achieve euglycemic state and A2 where medications are required as diet alone fails to achieve the required glucose control (Table 1).

Class	Age of onset	Duration (years)	Vascular disease	Treatment
A1	Gestational			Diet
A2	Gestational			Medication
B	Older than 20	Fewer than 10	None	Insulin
C	10-19	10-19	None	Insulin
D	Under age 10	More than 20	Benign retinopathy	Insulin
F	Any	Any	Renal nephropathy	Insulin
R	Any	Any	Proliferative retinopathy	Insulin
H	Any	Any	Heart involvement	Insulin

Table 1: White classification of diabetes during pregnancy.

1.2.4. Pathophysiology of gestational diabetes

Several factors including genetic, epigenetic and environmental factors contribute to the development of GDM. Nevertheless, pancreatic β-cells failure to compensate for a chronic glucose abundance, eventually resulting in insulin resistance, hyperglycemia and an increased supply of glucose to the growing fetus is a common etiology in most cases. Other factors such as adipose expandability, low-grade chronic inflammation, gluconeogenesis, oxidative stress, and placental factors are thought to contribute to the pathology of GDM (2). Indeed, placental cytokine release is dysregulated in obstetric pathologies and a proinflammatory cytokine signature of IL-8 and leptin expression in placentas of GDM women has been reported (98). Placental overproduction of ROS with associated higher lipid peroxidation levels are believed to play key intermediary role in the generation of GDM (99). A mitochondrial pathway contributing to increased placental ROS has been suggested in pregnancy complications (100) (101).

Interestingly, oxidative stress can occur in pregnant women with adequate glycemic control and is explained by down regulation of the antioxidant capacity in diabetic pregnancies (102) (103).

There are numerous mechanisms by which hyperglycemia contributes to elevated oxidative stress. In summary, high glucose availability increases the formation of advanced glycation end products and activates the hexosamine biosynthetic pathway. Subsequently, glucosamine-6-phosphate competes with glucose-6-phosphate for dehydrogenase and limits the synthesis of nicotinamide adenine dinucleotide necessary for reduced glutathione (GSH) rebuilding. Eventually, activation of the polyol pathway, protein kinase C pathway, and activation of oxidases such as xanthine oxidase and nicotinamide adenine dinucleotide phosphate oxidase all contribute to increased ROS production (99). In addition, increased glucose flux through the TCA cycle in the mitochondria results in increased oxygen consumption and ROS production as well as enhanced uncoupling protein activation causing excessive proton leak and exhaustion of ATP reserves resulting in apoptotic cell death (104). In diabetic pregnancies, despite properly controlled maternal glucose levels, increased umbilical glucose concentrations together with a reduction in oxygen saturation and increased lactate concentration reflect altered fetal metabolism (105).

Furthermore, activation of transcription factors such as nuclear factor-κB (NF-KB), activator protein-1 and hypoxia inducible factor-1 (HIF1) occurs and leads to insulin resistance through distinct mechanisms. These mechanisms include inducing the expression of pro-inflammatory cytokines such as interleukin-6, tumor necrosis factor-α (TNF-α) or monocyte chemoattractant protein-1, which cause insulin resistance (106) (107) (108), or indirectly through the activation of serine/threonine kinases which interfere with key components of the insulin signaling pathway (109).

1.2.5. Placental mitochondria in gestational diabetes

Deranged placental metabolism in GDM has been proposed and reflects as enhanced mitochondrial fusion in GDM placentas (110). Further evidence shows compromised mitochondrial function in placentas from GDM A2 pregnancies relative to controls (111). Additionally, swollen or completely destroyed mitochondria have been reported in a study of ultrastructure of placental villous tissue in GDM women (112). In obese women with GDM, mtDNA is not significantly higher in comparison to normal weight controls. Nevertheless, morphological abnormalities of mitochondria indicate impaired functionality (113). In mildly diabetic rats, increased mitochondrial membrane fluidity has been observed in placental tissue, together with increased ratio of unsaturated-to-saturated fatty acids (114).

1.2.6. Placental angiogenesis in gestational diabetes

In GDM-complicated pregnancies, placental insufficiency presents as altered vascularization, villous immaturity and endothelial dysfunction (115). Unlike conditions of pregestational diabetes where the hyperglycemia affects all stages of placental and fetal development, GDM mainly affects processes that occur in later stages of pregnancy such as angiogenesis and microvascular remodeling (29). Compared to placentas from normal pregnancies, GDM placentas exhibit increased vascularization (29) (116), manifested as increased capillary branching (117) and surface area (118). In addition, longer umbilical cords are described in GDM pregnancies (119) and this is accompanied with further risk of both hypo- and hyper-coiled cords (120) (121). This phenomenon of increased placental angiogenesis in GDM reflects the increased oxygen demand of the fetus, due to insulin-induced increase in fetal aerobic metabolism (122). Indeed, chronic fetal hypoxia is a key pathological consequence of maternal hyperglycemia (29) (105) and is confirmed by measuring cord blood oxygen (105) and increased erythropoiesis (123) (124). Evidence shows that VEGF and KDR expression is increased in villous blood vessels in response to hypoxia and ischemia suggesting a significant role of VEGF family in pathological alteration in the placenta (41).

1.2.7. Endothelial dysfunction in gestational diabetes

In GDM, high glucose levels stand as the key factor inducing endothelial dysfunction in addition to other factors such as elevated insulin and leptin levels (125) (126). The combined proangiogenic force of hyperglycemia, hyperinsulinemia, hyperleptinemia as well as hypoxia induces growth factors such as VEGFA and FGF2 resulting in expansion of vascular tree and capillary volume as mentioned before (127) (128). Reduced surface expression of the adherens junctional molecules, vascular-endothelial cadherin (VE-cad) and beta-catenin in addition to tight junctional molecules, occludin and zonula occludens-1 is reported in GDM placentas denoting compromised endothelial barrier (129). Since endothelial barrier and angiogenesis are two closely related processes, these findings support dysregulated angiogenesis in GDM placentas as well. Furthermore, rupture and erosion of the endothelium of umbilical vessels with subsequent extravasation of blood within Wharton's jelly are described in GDM cords relative to controls (130).

While in healthy pregnancy endothelial cells play a major role in vascular tone homeostasis through the release of vasoactive substances including NO (131), in pathological pregnancies such as GDM (132), the synthesis and/or bioavailability of NO is dysregulated resulting in alterations in placental blood flow (133). The interplay between ROS production and NO availability has been investigated and data show that endothelial NO synthase expression and function are suppressed by ROS overproduction (134). Hence, imbalance between NO and ROS results in a shift in endothelial equilibrium and dysregulation in vascular tone and leukocyte adhesion (135). Altered infiltration, differentiation and activation of maternal immune cells in GDM placentas have been topics of research and increased neutrophil granulocyte infiltration in GDM placentas has been described (136) (137). Likewise, more macrophages accumulate in GDM placentas as compared to healthy controls (138).

1.3. Succinate between metabolism and signaling

Over the past decade, there has been an enormous increase in the knowledge and understanding of metabolic reprogramming where metabolic alterations accompanying diseases are no longer limited to energy production and biosynthesis. A role of metabolites in inducing distinct pathways that reflect on cell behavior and fate has become a key focus of immunologists and cancer biologists. As mentioned before, succinate has long been studied as an intermediate in Krebs cycle in the mitochondria. Succinate oxidation to fumarate via the enzyme SDH provides electrons to the respiratory chain. Hence, succinate plays a major role in energy production and metabolism. Recently, numerous studies have shed light on unexpected roles of succinate outside metabolism, particularly in signal transduction, post-translational modification and pseudohypoxia (139).

1.3.1. Mechanisms and relevance of succinate accumulation

Succinate accumulation in the local environment results primarily from mutations in SDH which occur in numerous cancer types such as paraganglioma/pheochromocytoma (PGL/PCC), renal carcinoma, ovarian cancer, neuroblastoma and gastrointestinal stromal tumor (140). Succinate accumulation also occurs in conditions of ischemia/hypoxia through two mechanisms: either the reverse action of SDH to reduce fumarate to succinate due to the build-up of electrons in the nicotinamide adenine dinucleotide (NADH) and Coenzyme-Q pools or the breakdown of succinyl CoA (141). Another important mechanism of succinate accumulation is through tumor necrosis factor receptor-associated protein 1 (TRAP1), a mitochondrial chaperone binding and inhibiting SDH (142).

Non-enzymatic formation of succinate from α-ketoglutarate in mitochondria under oxidative stress has been proposed in rat liver (143), while increased levels of succinate due to inverse catalysis of SDH and subsequent increased oxidative stress and neuronal damage are demonstrated in a rat model of status epilepticus (144). In cardiac ischemia, succinate accumulation occurs primarily due to canonical Krebs cycle activity, partly supported by aminotransferase anaplerosis and glycolysis from glycogen (145). In lipopolysaccharide (LPS)-stimulated macrophages, different mechanisms contribute to succinate accumulation such as GABA shunt, glyoxylate shunt and reduced SDH activity due to reduced FAD and NAD^+ cofactors (139) (146).

Circulating levels of succinate are higher in patients with head and neck squamous cell carcinoma than in the healthy controls (147), in Cowden Syndrome patients with germline mutations of PTEN, SDHB or SDHD (148). Similarly, in rodent models of hypertension, circulating succinate concentration is increased (149). Elevated succinate levels are described in kidney and urine of diabetic mice (150), as well as diabetic rat retinas (151). We also measured increased succinate concentrations in human gestational diabetic placentas relative to controls (1). Furthermore, succinate accumulation in brown adipose tissue occurs upon exposure to cold and activates thermogenic respiration in brown adipocytes (152). Elevated succinate occurs in synovial macrophages and fibroblasts subjected to LPS stimulation and low oxygen, implying that succinate accumulation provides a link between metabolic alterations and inflammatory response in rheumatoid arthritis (153). A recent publication showed a link between plasma succinate levels and visceral adipose tissue and pro-inflammatory omega-6 oxylipins, as indicators of the cardiovascular status, in young adults (154).

1.3.2. Succinate stabilization of HIF1α

HIF1 is a transcription factor first identified as a regulator of erythropoiesis through binding to erythropoietin gene enhancer (155). HIF1 is a heterodimer comprised of a regulatory oxygen-sensitive α domain and a constitutive β domain (156). As the principle regulator of hypoxic responses, HIF1α has a transactivation domain at its C-terminal and a nuclear localization signal at the N-terminal (157). During hypoxia, HIF1α becomes stabilized and translocates to the nucleus, where it forms a transcriptionally active complex with HIF1β. The activated complex binds to HIF response elements (HREs) in target genes at regulatory regions and binds transcription coactivators inducing gene expression (158). Among HIF1 targets are genes involved in different angiogenesis steps such as VEGF and metalloproteinases in addition to genes that regulate vascular tone such as NO synthase and endothelin-1, and growth factors that regulate cell proliferation and survival such as IGF-2 and transforming growth factor-α (158).

Succinate can be transported from its site of production in mitochondria to the cytosol via dicarboxylate carrier in the inner mitochondrial membrane (159), and voltage-dependent anion channel (VDAC/porin) in the outer mitochondrial membrane (160). Accumulation of succinate in the cytosol results in inhibition of prolyl hydroxylase (PHD), which is responsible for hydroxylation of HIF1α causing its degradation (160). This results in HIF1α stabilization, and consequently upregulation of target genes containing HREs (161). This phenomenon is called pseudohypoxia, where under normal oxygen levels, hypoxic responses are initiated through transcription factors such as HIF1α (162). Besides inhibiting PHD, succinate can stabilize HIF1α through ROS induction (139). Inhibition of SDH, either pharmacologically or by RNA interference of SDHB, increases ROS production and subsequently HIF1α stabilization (163). Another indirect pathway by which ROS stabilizes HIF1α is via oxidation of Fe^{2+} (an essential PHD cofactor) to Fe^{3+} thus limiting its activity (164).

The consequences of HIF1α activation have been extensively studied in immune contexts. In LPS/Interferon-γ-stimulated macrophages, HIF1α mediates the switch of the mitochondrial function from ATP to ROS production (165) and in myeloid-specific HIF1α mouse model, HIF1α induces M1 polarization in macrophages via promoting glycolysis metabolism (166). In macrophages in rheumatoid arthritis synovium, HIF1α expression is increased and is important for IL-1β production (167). In T cells, HIF1 induces TH17 development and attenuates Treg development under both normoxic and hypoxic conditions suggesting a role of metabolic modulation, possibly via succinate, in T-cell-mediated pathologies (168). In mouse dendritic cells, HIF1α plays a major role in interferon-α and -β production and HIF1α-deficient dendritic cells show hindered ability to activate T cells (169). Taken together, a role of HIF1α in innate and adaptive immune responses is evident and, hence, succinate is essential for both through its effect on HIF1α stabilization.

1.3.3. SUCNR1 is a cognate succinate receptor

SUCNR1 belongs to the superfamily of G-protein coupled receptors (GPCRs), which is one of the largest families of proteins in mammalian genome. GPCRs are currently the target of around 60% of marketed therapies (170). GPCRs are membrane proteins with conserved core structure that includes extracellular N-terminus, seven membrane-spanning α-helices and intracellular C-terminus with variable extracellular and intracellular elements (171). GPCR activation results in conformational changes and recruitment of their G-protein partners. G-proteins are heterotrimeric and dissociate into α-subunit and βγ dimer upon GPCR activation resulting in modulation of downstream effector proteins. The α-subunit of G-protein defines its basic properties and is divided into four families: Gαs, Gαi/Gαo, Gαq/Gα11 and Gα12/Gα13. Gi/o and Gs regulate cyclic adenosine monophosphate (cAMP) through adenylate cyclase modulation, Gq/11 induces $[Ca^{2+}]i$

release from intracellular stores, while Gα12/Gα13 is involved in migration, growth and cell division (172).

Since SUCNR1 shows high sequence homology to P2Y receptors, it was predicted to belong to purinergic receptors and to bind to purinergic ligands (173). However, SUCNR1 has been paired to succinate, where intravenous succinate infusion induces an increase in blood pressure, a response that is abrogated in SUCNR1-deficient animals (174). An interesting note about human SUCNR1 is that it has two possible open reading frames resulting in 334 or 330 amino acid residue protein. Human and mouse SUCNR1 orthologs show 73% homology at the nucleotide level and 68% at the protein level (173).

Succinate is usually present in blood plasma at a concentration of 2-20μM (149) (175). The half-maximal response concentration of human SUCNR1 is estimated to be 56 ± 8μM (174), denoting that only a small increase in circulating succinate results in full receptor activation. An increase in succinate concentration up to mM range occurs under pathological conditions such as in serum of patients with myocardial infarction associated with coronary artery disease (176). Altogether, it is now acknowledged that succinate triggers SUCNR1 in conditions where local stress affects cellular metabolism like hyperglycemia, ischemia and hypoxia (177).

1.3.4. Succinate and angiogenesis

As mentioned above, succinate accumulation in the cytosol results in HIF1α stabilization and subsequent expression of genes with HRE, which include angiogenesis regulatory genes such as VEGF. Succinate also can activate SUCNR1 where it mediates a wide range of responses including angiogenesis. Accumulation of succinate and increased expression of HIF1α and associated angiogenic genes were reported in tumor tissues with SDH mutations such as paragangliomas and phaeochromocytomas (178) (179) (180). Similarly, elevated succinate was measured in gastric cancer tissues compared with paracancerous tissues, where succinate-SUCNR1 axis was proposed to induce tumor angiogenesis via regulation of ERK1/2 and STAT3 signaling (181). In retinal ganglionic cells, succinate via SUCNR1 regulates angiogenesis in the settings of both normal retinal development and proliferative ischemic retinopathy through angiogenic factors such as VEGF (182). In this thesis, we show that succinate signaling via SUCNR1 induces an angiogenic phenotype in HUVECs and FpECAs, which might be increased in gestational diabetes (1).

1.4. Study questions

Within this thesis, I aimed to answer the following questions:

1. Is succinate-SUCNR1 axis dysregulated in GDM placentas?
2. Do FpECs express SUCNR1? Is it regulated in diabetic cells?
3. Does treatment with succinate induce an angiogenic response in endothelial cells?
4. Are these responses mediated via SUCNR1?
5. Can the signaling machinery downstream of SUCNR1 in endothelial cells be identified?

2. Materials and methods

Human sample selection and matching criteria were based on gestational age, BMI before pregnancy and absence of other pregnancy complications (Table 2). Informed written consents were provided by all women who participated in the study. Chemicals were obtained from Sigma (Vienna, Austria) unless specified otherwise.

	PN (n=9)	GDM (n=7)	P value
Gestational age (Weeks)	38.1 ± 0.9	38.8 ± 0.8	ns
BMI before pregnancy	22.9 ± 2.8	26.8 ± 4.8	ns
Other pregnancy complications	None	None	

Table 2. Clinical features of normal and GDM pregnancies. Samples were matched based on gestational age, BMI before pregnancy as well as the absence of other pregnancy complications. Gestational age and BMI are shown as mean ± SD. Table is adopted from Atallah et al. (1) with permission of the publisher.

Cell culture

Directly after normal delivery or caesarean-section, placentas were collected. Fetoplacental endothelial cells (FpECs) were isolated from chorionic arteries or veins as described by Lang et al. (183). Briefly, fragments of vessels were dissected and washed with 1X Hanks Balanced Salt Solution (HBSS, Thermo Fisher Scientific, Massachusetts, USA) to remove any residual blood. Using a syringe, vessel segments were cannulated and perfused with prewarmed (37°C) 0.5% Collagenase/Dispase solution (Roche, Basel, Switzerland) for 8 min (flow rate 2.5 ml/min). The detached cells were collected in a tube containing fetal calf serum (FCS, HyClone GE Healthcare, Illinois, USA), centrifuged for 7 min at 200g at room temperature (RT) and resuspended in media (Promocell MV kit, Heidelberg, Germany). During the first culture outgrowth, FCS supplement in the media was replaced with 10% human pooled and heat-inactivated serum (Atlanta Biologicals, Georgia, USA). The cell suspension isolated from one individual vessel was seeded per well in a 12-well culture plate (Corning, New York, USA) precoated with 1% porcine skin gelatin. Wells were observed daily and medium was changed after 2 days and then twice a week. After reaching confluence, FpECs were detached using trypsin/EDTA (0.04%, Gibco, Texas, USA) for 2 min at 37°C followed by trypsin inactivation using trypsin neutralizing solution (Gibco). The cells were transferred to culture flasks and human serum was replaced with FCS according to the original media composition.

Primary HUVECs were purchased from Lonza (Basel, Switzerland) and grown in EGM-2 medium. For high glucose culture, HUVECs were cultivated in EGM-2 medium with normal (5.5mM) or high (20mM) glucose for 48 h. D-mannitol was used as an osmolality control. Both HUVECs and FpECs were cultured at 21% O_2, 5% CO_2 and 37°C in a 90% humidified incubator. Experiments were performed using cells between passages 3-7.

EA.hy926 **(**ATCC, Virginia, USA) at passages (13-17) were cultured as mentioned before in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin and 1% HAT media supplement (Thermo Fisher Scientific, Massachusetts, USA).

In situ hybridization (ISH) and immunofluorescence microscopy of tissue sections

Paraformaldehyde-fixed, paraffin embedded placental sections were deparafinized by sequential dipping in xylene followed by 100% ethanol. ISH was performed using RNAscope kit for SUCNR1 (manufactured by ACD, CA, USA) according to the manufacturer’s protocol. In summary, the slides were air-dried and incubated with hydrogen peroxide for 10 min at RT. Antigen retrieval was performed for 15 min in a pre-heated steamer. Protease plus reagent was applied to tissue sections and incubated at 40°C in HybEZ oven for 20 min. After a washing step, SUCNR1 or a negative control probe were applied to tissue sections for 2 h at 40°C. A series of 6 amplifiers was used for indicated time points. For signal detection, fast red solution was applied to tissue sections for 10 min at RT. The slides were then blocked in a blocking solution composed of 10% secondary antibody host serum + 4% bovine serum albumin (BSA) in phosphate buffer saline (PBS, Medicargo, Uppsala, Sweden) + 0.3% Triton X for 2-3 h at RT. Sections were incubated overnight in primary antibody against von Willebrand factor (VWF, Abcam, Cambridge, UK). The secondary antibody (Invitrogen, Massachusetts, USA) was added after a washing step. Sections were mounted in DAPI-containing mounting medium (Vector Laboratories, CA, USA). Sections were analyzed with a confocal laser scanning microscope (Zeiss LSM 510 META, with a Plan-Neo 63x/1.4 Oil with DIC capability lens) and processed with ZEN software (Zeiss, Jena, Germany) and Fiji (version 1.51s).

Immunohistochemistry of tissue sections

Paraformaldehyde-fixed, paraffin embedded tissue sections were immersed in xylene followed by descending alcohol concentrations for rehydration. Antigen retrieval was performed using citrate buffer (2.94g/l sodium tri-citrate and pH adjusted to 6) for 10 min in a microwave. After washing steps, the slides were incubated in 0.3% hydrogen peroxide solution in PBS for 30 min. The slides were blocked in a blocking solution as mentioned above. Primary antibody against VEGF (Abcam) was used overnight followed by biotinylated secondary antibody addition for 2-3 h. For detection, ABC reagent was used followed by DAB color development solution (both from Vector Laboratories) and the slides were counterstained with 0.5% methyl green. Sections were visually examined with an Olympus BX41 microscope (Olympus Austria GmbH, Vienna, Austria) and an Olympus U Plan Apo ×20/0.25 lens. Analysis of immunohistochemistry images was performed with Fiji (version 1.51s). by splitting the acquired RGB Images into individual components and performing a flat field correction prior to automated thresholding. The results were then normalized on the total tissue area for each image.

Succinate measurement

Succinate concentration was measured in tissue lysates (10mg) using succinate colorimetric assay kit (Abcam) following the manufacturer's instructions. In brief, succinate in samples is converted by succinyl-CoA synthetase to form an intermediate, which after a series of reactions reduced a colorless probe to a colored product. Absorbance is measured at OD=450nm.

Immunofluorescence microscopy of FpECs/HUVECs

Cells were seeded on chamber slides precoated with 1% gelatin, grown until semi-confluence and processed as previously described (184). In brief, 3.7% formaldehyde was used to fix the cells for 10 min at RT with or without a subsequent permeabilization step with 0.1% Triton X in PBS for 20 min. The cells were blocked in 10% secondary antibody host serum with 4% BSA in PBS for 1h at RT, followed by overnight incubation at 4°C with SUCNR1 antibody (Novus Biologicals, Colorado, USA). Conjugated secondary antibody (Invitrogen) was used for 2 h at RT after a washing step. For cell membrane localization, conjugated wheat germ agglutinin (WGA, Thermo Fisher Scientific) and anti-vascular endothelial cadherin antibody (VEcad, Santa Cruz Biotechnology, Texas, USA) were used. For subcellular receptor localization, CellLight Mitochondria-RFP (Invitrogen) was used to identify the mitochondria. The slides were mounted in DAPI-containing mounting medium (Vector Laboratories) and examined with confocal microscopy, z-stacks were acquired and images were processed with Fiji (version 1.51s). For cytoskeleton staining, VEcad staining was performed as mentioned before and the cells were incubated with conjugated-phalloidin (Thermo Fisher Scientific) for F-actin staining for 30 min before mounting.

Flow cytometry surface and intracellular staining of SUCNR1 in HUVECs

For surface staining, cells were blocked using human TruStain FcX (Biolegend, CA, USA) for 10 min at 4°C. For intracellular staining, cells were fixed and permeabilized using fixation and permeabilization solution and perm/wash buffer (BD Biosciences, New Jersey, USA), respectively, before blocking. Cells were incubated with primary antibody against SUCNR1 (Novus Biologicals) for 45 min at 4°C. After washing steps, the cells were incubated with conjugated secondary antibody (Invitrogen) for 45 min at 4°C. After centrifugation, the cells were suspended in PBS and the positive cells were counted using BD FACSCanto II and FlowJo (version 10).

Apoptosis assay

HUVECs were incubated with either vehicle or succinate for different time points. The cells were detached using trypsin/EDTA followed by trypsin inactivation using trypsin neutralizing solution. The cells were centrifuged at 220g for 5 min and then resuspended in a cocktail of Annexin V (Biolegend) and propidium iodide (Thermo Fisher Scientific) for 15 min at RT in the dark. Analysis was done using BD FACSCanto II and FlowJo (version 10).

JC-1 staining

HUVECs were treated with vehicle or succinate for different time points. JC-1 dye (Thermo Fisher Scientific) was used at a concentration of 5µg/ml for 10 min in the dark. Excessive dye was washed out and the cells were detached and analyzed using BD FACSCanto II and FlowJo. Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, Abcam) was used as a positive control. The ratio of JC monomers to aggregates, indicative of the mitochondrial membrane potential, was calculated.

Flow cytometric staining of phosphorylated targets

EA.hy926 cells were serum starved for 16 h before treatment with vehicle or 10mM succinate for indicated time points. The cells were detached with accutase on ice and then fixed with phosphoflow fix buffer for 10 min at 37°C and permeabilized with phosphoflow perm buffer (both from BD Biosciences) for 30 min on ice. The cells were blocked with 0.5% BSA in PBS and antibodies against pERK and pAKT (Cell signaling, Massachusetts, USA) were used for 30 min on ice. Conjugated secondary antibody was used for 30 min on ice after a washing step. 30000 cells per condition were measured by FACSCanto II and FlowJo (version10). Data were calculated as fold increase in fluorescence intensity with respect to vehicle. For some experiments, ERK inhibitor (UO-126, 10µM) was used 15 min before succinate treatment.

RT-PCR

Tissue/cells were lysed in Trizol (Thermo Fisher Scientific) and allowed to set for 5 min at RT to allow complete dissociation of nucleoproteins followed by chloroform addition and a centrifugation step. Isopropanol was added to the aqueous phase containing RNA to allow its precipitation. RNA pellet was washed twice using 75% ethanol. DNA removal was performed with Ambion DNA removal kit (Thermo Fisher Scientific). Briefly, a master mix of DNase, DNase1 buffer and DEPC treated water was used to resuspend the RNA pellet for 30 min at 37°C. DNase inactivation reagent was then added followed by a short centrifugation. Concentration of RNA in the supernatant was measured using nanodrop (Thermo Fisher Scientific). RNA was reverse transcribed using iScript cDNA Synthesis Kit (Biorad, CA, USA) according to the manufacturer's instructions using a master mix of reverse transcriptase and iScript reaction mix. RT-PCR was performed using SsoAdvanced Universal SYBR Green Supermix with PrimePCR SYBR Green Assay primers for target genes (Biorad) according to manufacturer's instructions and CDX Connect Real-Time PCR detection system with CFX Manager software (version 3.1) (Biorad).

Western blot

Tissue/cells were lysed in RIPA extraction buffer (Thermo Fisher Scientific) supplemented with 1X Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) followed by centrifugation at 14000g for 30 min. Protein concentration was measured using Pierce BCA-kit (Thermo Fisher Scientific). Sample buffer with β-mercaptoethanol was added to protein samples and then heated up at 95°C for 5 min. Proteins were separated by SDS-PAGE on a 4-20% TRIS-glycine gradient gel (Thermo Fisher Scientific) at 220V for 30 min. Afterwards, the proteins were blotted onto a polyvinylidene fluoride membrane using iBlot system (Invitrogen). Membranes were blocked with 5% skimmed milk. Target proteins were detected using specific antibodies and visualized with respective horseradish peroxidase-conjugated antibodies (Cell signaling) and Clarity Western ECL substrate (Biorad). Chemiluminescence was recorded by ChemiDoc Touch Imaging system (Biorad). After stripping the membranes using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) for 20 min, membranes were re-probed with β-Actin antibody

(Cell signaling) and further processed as described above. Densitometric analysis of protein bands was performed using Imagelab Software (Biorad).

Enzyme-linked immunosorbent assays (ELISA)

Cells were seeded in 12-well plates and treated with succinate or vehicle for 24 h. Supernatants were collected and VEGF in the media was measured using ELISA Kit (R&D Systems, Minnesota, USA) according to the manufacturer’s instructions. Each sample was tested in triplicate. Data were normalized to the cell count at the time of harvest.

EdU Proliferation assay

Cell proliferation ability after treatment with succinate or vehicle was evaluated using Click-iT EdU Cell Proliferation Kit for Imaging, Alexa Fluor 488 dye (Thermo Fisher Scientific) according to the manufacturer’s instructions. In brief, EdU (5-ethynyl-2'-deoxyuridine) as a nucleoside analog of thymidine was incorporated into DNA during active DNA synthesis. Mild aldehyde fixation using 3.7% formaldehyde in PBS and detergent permeabilization using 0.5% Triton X were applied. A copper-catalyzed detection step was used allowing the binding of an azide dye and the alkyne in EdU. The number of EdU positive cells relative to the total number of cells was counted in at least 5 different fields by an independent blinded investigator.

Transwell migration assay

The bottom chambers were filled with media with or without succinate, while Transwell inserts were seeded with endothelial cells. After 16 h of culture, the non-migrated cells on the upper side of the filter were removed using cotton swaps, and the migrated cells were fixed using 3.7% formaldehyde solution in PBS and permeabilized using 0.5% Triton X. The cells were stained with trypan blue. Images were taken from at least 5 different fields and the cell number in each field was counted.

Scratch wound healing assay

Endothelial cells were seeded into 12-well plates precoated with 1% gelatin and allowed to grow to full confluence. To eliminate any interference due to cell proliferation, cells were starved in starvation media with 0.5% FCS for 4 h and then wounded with 200µl pipette tips. The cells were subsequently cultured in starvation media with or without succinate. Time-lapse imaging was used to assess the rate of cell migration for 12 h of incubation at 37°C in a 95%:5% (v/v) mixture of air and CO_2 (Nikon TiE-2 microscope equipped with an Andor Zyla 4.2 sCMOS camera). The detailed experimental protocol was previously published (185).

Tube formation assay

The assay was performed as described (186). Fibrin (CoaChrom Diagnostica, Maria Enzersdorf, Austria) was dissolved in M199 medium (Lonza, Basel, Switzerland) in a concentration of 4mg/ml by incubation at 37°C for 1 h and then sterilized by filtration using 0.45µm filter. Thrombin IIa (CoaChrom Diagnostica) was added (0.03U/ml) and the solution was immediately pipetted (100µl/well) in a 96-well plate. The plate was incubated for 1 h at RT followed by 1 h incubation at 37°C for matrix polymerization. For thrombin inactivation, complete media additionally supplemented with 10% newborn calf serum (Thermo Fischer Scientific) and 5% human serum (Atlanta Biologicals) was added and the plate was incubated for 2 h at 37°C. Next, FpECs ($35x10^3$/well in 100µl of media) were seeded onto the fibrin matrix. After overnight incubation, medium was replaced by 100µl of media supplemented with succinate either alone or in combination with the positive control bVT; a combination of TNFα (10ng/ml; ReliaTech, Wolfenbüttel, Germany), FGF2 (10ng/ml; ReliaTech) and VEGF (25ng/ml; ReliaTech) to stimulate angiogenesis. After 48 h, the assay was terminated using warm 3.7% formaldehyde (DonauChemie, Wien, Austria) for 2 h, then replaced with PBS and stored at 4°C until imaging. Z-stacks of each well were acquired with a step size of 10µm using a Nikon TiE-2 microscope equipped with an Andor Zyla 4.2 sCMOS camera and Nikon Plan Apoλ 4x objective followed by an automated analysis in FIJI (version 1.51s).

Spheroid sprouting assay

Cells were resuspended in 0.3% methylcellulose solution and pipetted into the lids of petri-dishes to generate drops. Spheroids in hanging drops were generated by overnight incubation at 21% O_2, 37°C in hanging position. 1X HBSS with 10% FCS was used to harvest the spheroids. The spheroid suspension was centrifuged for 5 min at 300g at RT without brake. The spheroid pellet was overlaid with 1.2% methylcellulose stock solution containing 40% FCS (=0.7% methylcellulose), $NaHCO_3$ (15.6mg/mL), type-1 collagen (2mg/mL) and NaOH (1M) on ice following this order. Collagen-spheroid solution was pipetted in 24 well plate and incubated at 37°C, 21% O_2 for 2 h to allow collagen polymerization. Media containing either vehicle or succinate was added and bVT was used as a positive control. Spheroids were stimulated for 16 h. For assay termination, 3.7% formaldehyde was used for 2 h at RT and replaced with PBS. The plates were stored at 4°C until imaging. Automated z-stacks were acquired using a Nikon TiE-2 microscope equipped with an Andor Zyla 4.2 sCMOS camera and Nikon Plan Apoλ 10x objective and sprouting length measurement is performed as previously described (187).

Electric Cell-substrate Impedance Sensing (ECIS)

Biochips with gold electrodes 8W10E + (Ibidi GmbH, Gräfelfing, Germany) were treated with L-cysteine for 10 min, washed with distilled water and coated with 1% gelatin solution. The cells were seeded on the biochips and grown to confluence for two days at 37°C. On the day of the experiment, the cells were starved in basal medium for 1 h. Afterwards, the electrical resistance of the cell monolayer was measured at a frequency of 4000 Hz using ECIS Z-Theta device (Ibidi GmbH), as described previously (188). Baseline was recorded for 1 h followed by addition of treatment and electrical resistance was recorded. The ECIS chips could be recycled by washing with PBS, trypsinization overnight, washing and air-drying. The chips were sterilized with UV before use. For some experiments, UO-126 was used at a concentration of 10µM 15 min before treatment with succinate or vehicle.

Small interfering RNA (siRNA) transfection

SUCNR1 knockdown was carried out using on-target plus human SUCNR1 siRNA (mixture of 4 different siRNA) and Dharmafect 4 transfection reagent according to the manufacturer's instructions (Dharmacon, Colorado, USA). siGLO Lamin A/C siRNA DY-547 labeled and on-target plus Non-targeting Pool were used as positive and negative controls, respectively. The efficiency of transfection was determined by analyzing SUCNR1 protein using Western blot and immunofluorescence.

Statistical analysis

GraphPad Prism software (version 6.0, La Jolla, CA, USA) was used. Unpaired t-test was used to compare the means of 2 independent groups while paired t-test was used to compare the means from the same group under 2 different experimental conditions. For more than 2 groups, one and two-way ANOVA were used depending on the number of variables. Repeated measures ANOVA was used for analyzing data if the measurements were carried out more than once in the same group. P value ≤0.05 was considered statistically significant.

3. Results

Parts of the results presented in this section have been previously published (1). The reproduction of figures was performed with permission of the publisher. Wolfgang Platzer and Eva Tatzl performed experiments the data of which are presented in this section including Western blotting, PCR and ECIS. Jasmin Strutz and Sonja Rittchen assisted in establishing spheroid sprouting and tube formation assays. Jürgen Gindlhuber contributed to imaging and image analysis.

3.1. Succinate, SUCNR1 expression, SDH gene expression and VEGF are upregulated in gestational diabetic placentas

In this part, we investigated hypothesized regulation of succinate and/or SUCNR1 in GDM placental tissue lysates in comparison to matched controls. For that, we used a colorimetric kit to measure succinate concentration in placental tissue lysates from GDM and matched normal placentas and found significantly elevated succinate levels in GDM lysates (Fig 1A). Additionally, Western blot analysis for SUCNR1 in GDM and normal placental tissue lysates showed significantly higher SUCNR1 protein abundance in GDM samples (Fig 1B). Thus, these data showed regulation of succinate-SUCNR1 axis in GDM.

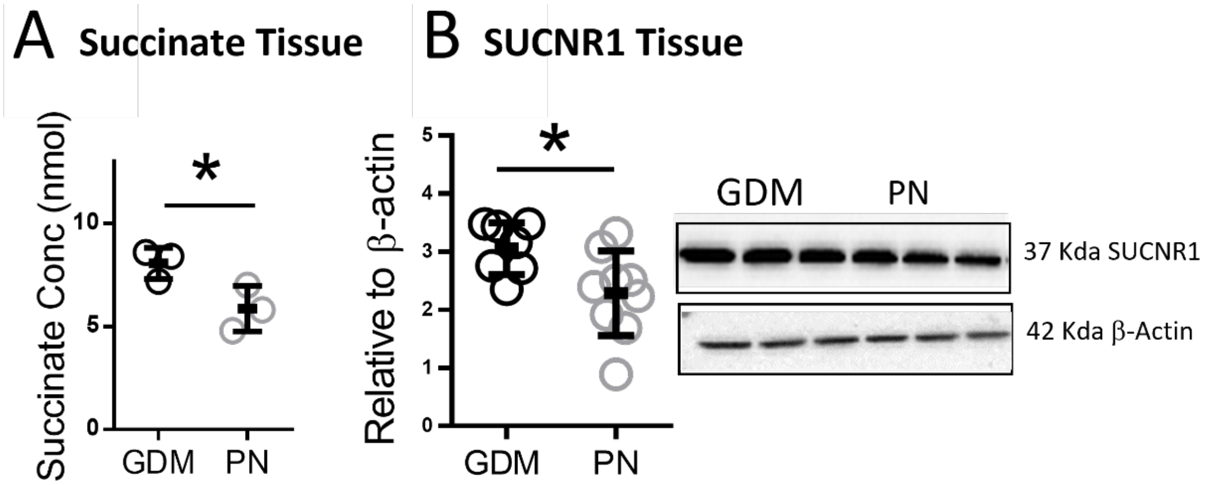

Figure 1. Succinate and SUCNR1 upregulation in gestational diabetic placentas. A) Succinate quantification in GDM and matched normal placental lysates using a spectrophotometric kit (n=3). B)

Western blot analysis of SUCNR1 in whole tissue lysates from GDM (n=7) and matched normal pregnancies (n=9). Data in A and B were analyzed by unpaired t-test, *p<0.05, data are shown as mean ± SD. Representative densitometry analysis is shown for B. Figure is adopted from Atallah et al. (1) with permission of the publisher.

To gain further insight into the metabolic landscape in GDM, we quantified the gene expression of several enzymes involved in glucose metabolism such as lactate dehydrogenase (LDH) and hexokinase1 (HK1) as well as SDH. We observed that among these enzymes only SDH gene expression was differentially expressed in GDM placentas relative to controls reinforcing the hypothesis of altered succinate metabolism in GDM placentas (Fig 2).

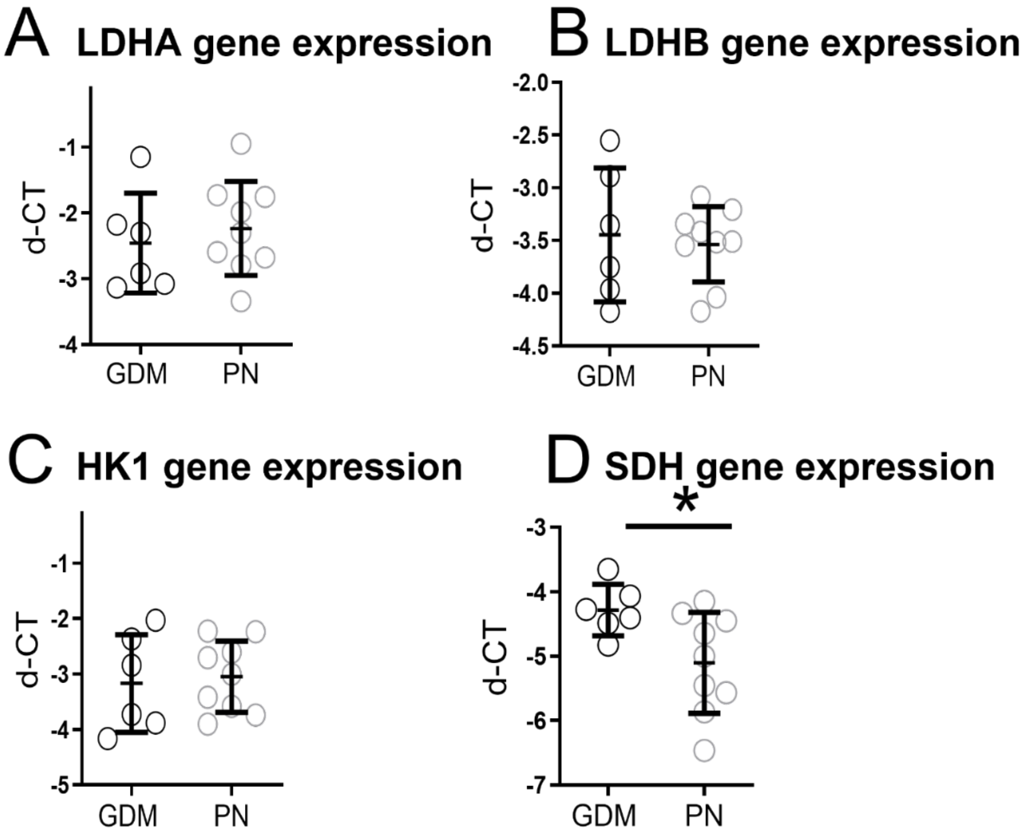

Figure 2. Upregulation of SDH gene expression in gestational diabetic placentas. RT-PCR of LDHA (A), LDHB (B), HK1 (C) and SDH (D) in placental tissue lysates from GDM (n=6) and matched controls (n=9). Data were analyzed by unpaired t-test, *p<0.05, data are shown as mean ± SD.

Since VEGF is a key angiogenic factor in the placenta (29), we quantified VEGF protein expression by Western blot of placental tissue lysates (Fig 3A), where we observed an increase in GDM samples in comparison to controls. We confirmed this finding by immunohistochemistry staining for VEGF in tissue sections that showed stronger staining intensity, particularly around the vessels, in GDM relative to normal sections (Fig 3B).

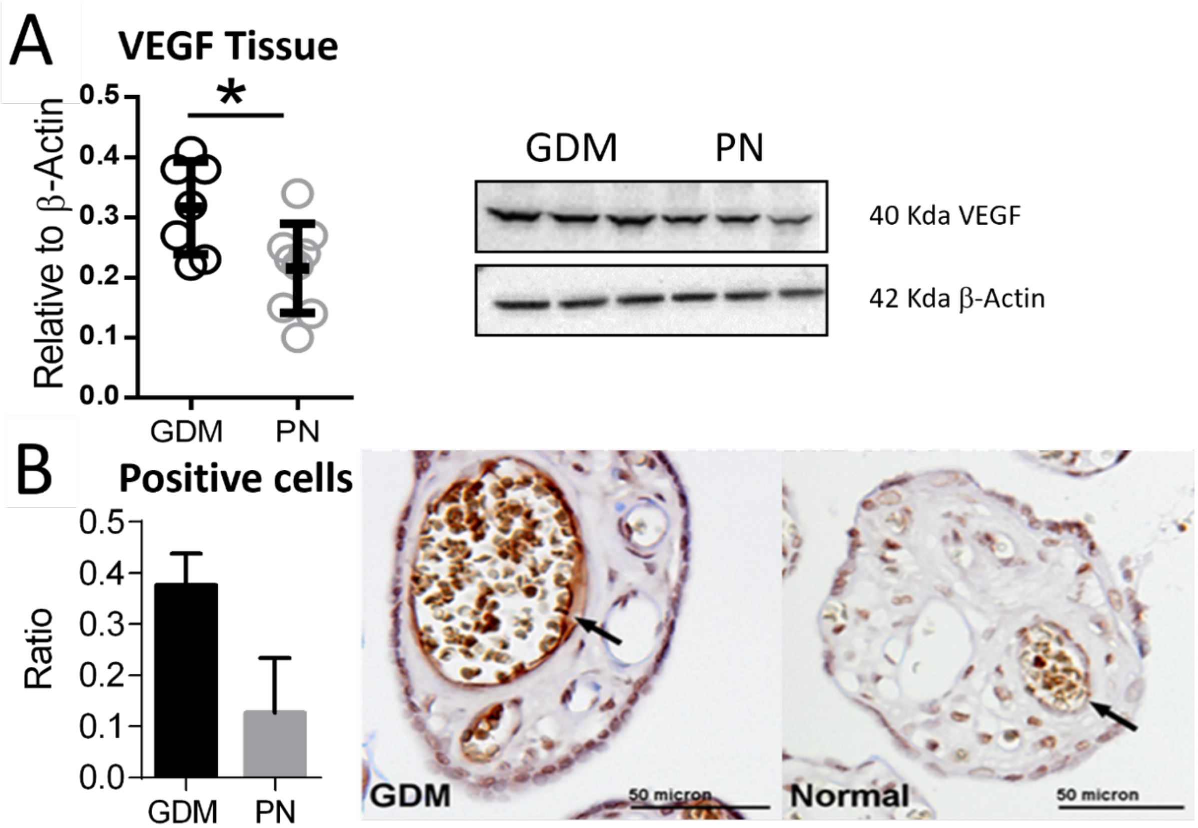

Figure 3. VEGF upregulation in gestational diabetic placentas. A) Western blot analysis of VEGF in whole tissue lysates from GDM (n=7) and matched normal pregnancies (n=9). B) Immunohistochemistry staining of VEGF in placental tissue sections from GDM and normal pregnancies. Image is representative for 4 different isolations. Quantification of the images was performed with Fiji and the ratio of positive cells was calculated (n=4). Data in A were analyzed by unpaired t-test, *p<0.05, data are shown as mean ± SD. Representative images and densitometry analysis are shown. Figure is adopted from Atallah et al. (1) with permission of the publisher.

As our data revealed an increase in SUCNR1 and VEGF protein levels in GDM placental tissue lysates, we performed a correlation analysis of the data for SUCNR1 and VEGF. A significant positive correlation implied an association between SUCNR1 and VEGF in the placenta (Fig 4).

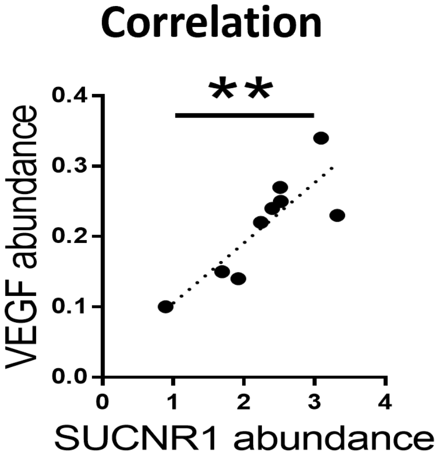

Figure 4. Positive correlation of SUCNR1 and VEGF proteins in human placentas. Pearson correlation analysis of SUCNR1 and VEGF protein abundance in normal placental tissue (n=9), $**p<0.01$, r= 0.85. Figure is adopted from Atallah et al. (1) with permission of the publisher MDPI.

Taken together, these data revealed that succinate, SUCNR1 expression as well as VEGF were increased in GDM placental tissue lysates in comparison to matched healthy controls. In addition, SUCNR1 expression was correlated with VEGF abundance in the tissue suggesting a potential link between SUCNR1 and VEGF in placental angiogenesis.

3.2. Endothelial cells in human placenta express SUCNR1

To investigate the cellular localization of SUCNR1 in human placenta, we performed ISH of SUCNR1 mRNA combined with immunofluorescence staining of endothelial cell marker vWF in human full-term placental tissue sections. We observed co-expression of SUCNR1 and vWF (Fig 5A). Furthermore, we performed immunofluorescence staining of isolated FpECAs and fetoplacental venous endothelial cells (FpECVs) using an antibody against SUCNR1. Thereby, we confirmed expression of SUCNR1 in FpECs at the protein level (Fig 5B, 5C).

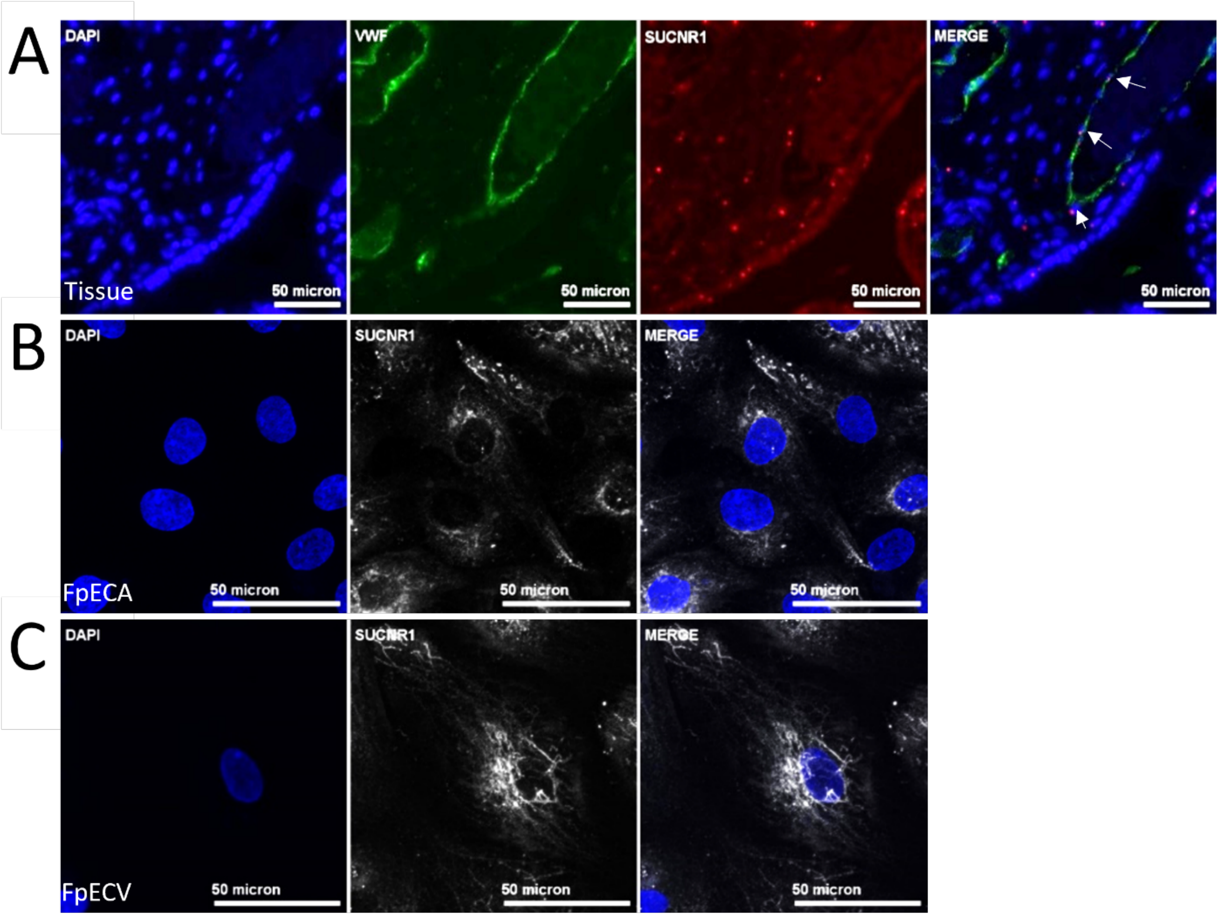

Figure 5. SUCNR1 expression in human full-term placental endothelial cells. A) ISH combined with immunofluorescence staining of human full-term placenta. Tissue sections were stained with a probe against SUCNR1 and antibody against vWF as a marker for endothelial cells. Nuclei were counterstained with DAPI. 3 sections from each placenta were examined and the shown image is representative for 5 normal placentas (n=5). B, C) Immunofluorescence staining of FpECAs and FpECVs, respectively using antibody against SUCNR1 and DAPI for counterstaining. Images are representative for 3 different isolations (n=3). Figure is adopted from Atallah et al. (1) with permission of the publisher.

To address differential SUCNR1 protein expression in normal FpECAs and FpECVs versus diabetic FpECAd and FpECVd, we used Western blot. In cells isolated from normal pregnancy, FpECVs expressed significantly higher levels of SUCNR1 relative to FpECAs. Moreover, we found that cells isolated from diabetic chorionic arteries (FpECAd) expressed significantly higher receptor protein relative to controls, while this upregulation was less pronounced in venous cells (FpECVd) (Fig 6A). Next we wanted to establish SUCNR1 expression in endothelial cells relative to other cells that are known to express the receptor, by Western blot. Our data showed that HUVECs express similar SUCNR1 levels to FpECVs but comparatively lower than human embryonic kidney (HEK293) cells and human monocytes derived macrophages (MDMs) (Fig 6B).

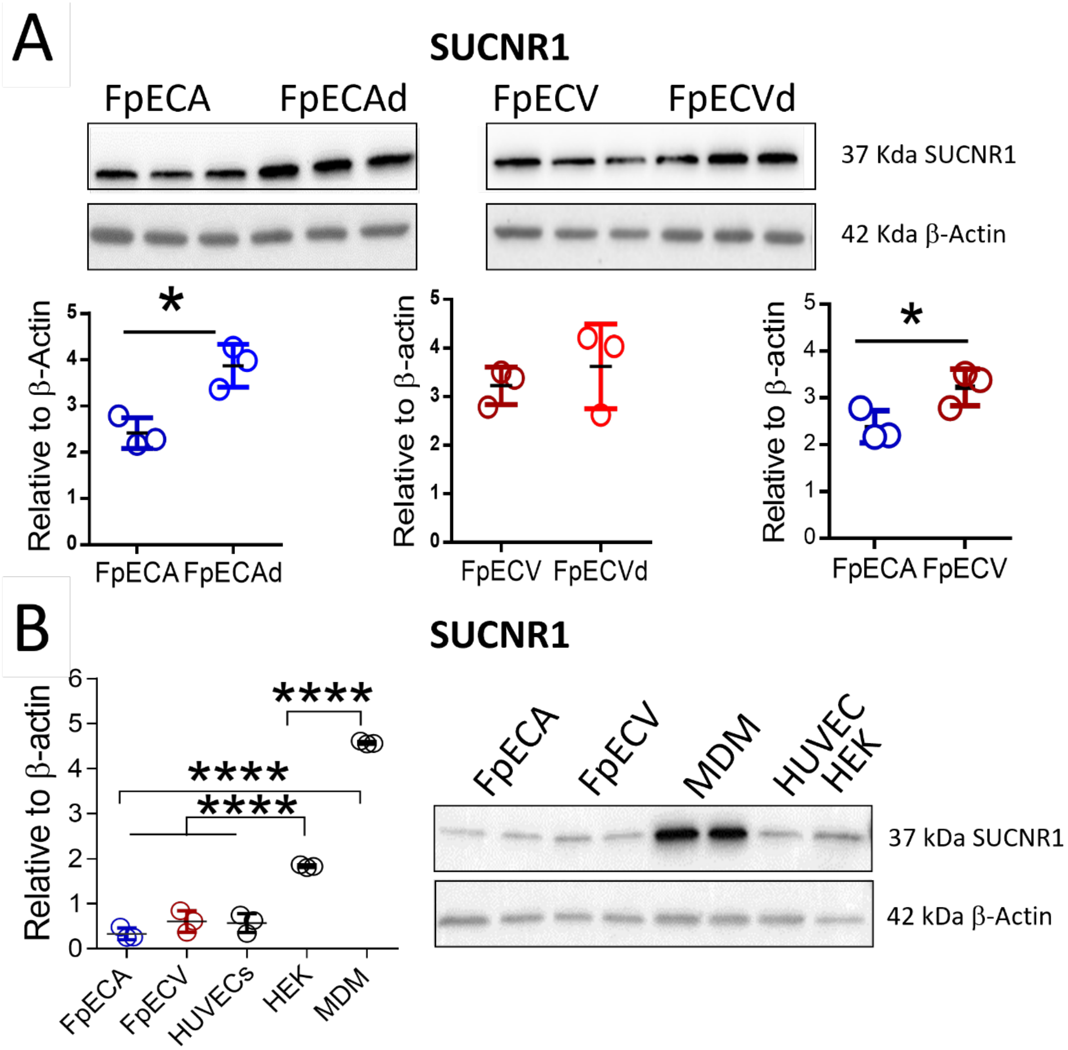

Figure 6. Differential SUCNR1 expression in human full-term placental endothelial cells. A) Western blot of SUCNR1 in isolated placental arterial and venous cells from GDM and normal pregnancies (n=3). B) Western blot analysis of SUCNR1 in different cells expressing the receptor. HEK cells (passage 16, originally from Jennifer Whistler lab (UCSF)) and MDMs (isolated and differentiated from healthy donors) used for Western blot were accessible from another running study. For A, unpaired t-test was used between groups. For B, data were analyzed by one-way ANOVA followed by Tukey's post-hoc test, $*p<0.05$, $****p<0.0001$. For A and B, data are shown as mean ± SD. Representative images and densitometry analysis are shown. A and B are independent experiments. For B, the membrane was exposed for shorter time for

SUCNR1 to avoid over-exposure of the bands since macrophages express relatively higher levels of the receptor. Figure is adopted from Atallah et al. (1) with permission of the publisher.

To investigate whether SUCNR1 expression in endothelial cells was related to prevalent glucose concentrations, we cultivated primary HUVECs, which we used for our in vitro experiments as endothelial cell model, in normal glucose (5.5mM) as well as high glucose (20mM) for 48 h. Western blot analysis for SUCNR1 revealed increased SUCNR1 expression in high glucose relative to normal glucose (Fig 7).

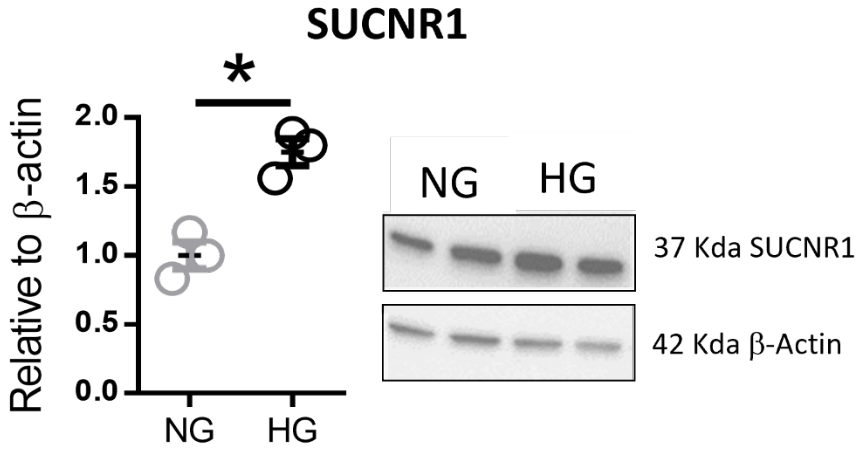

Figure 7. Upregulation of SUCNR1 expression by high glucose culture. Western blot of SUCNR1 in primary HUVECs cultured in normal or high glucose for 48 h (n=3). Paired t-test was performed, *p<0.05, data are shown mean ± SEM. Representative image and densitometry analysis are shown. Figure is adopted from Atallah et al. (1) with permission of the publisher.

In summary, our data provided evidence that SUCNR1 is expressed in placental endothelial cells and its expression is upregulated in diabetic isolations from chorionic arteries. Moreover, HUVECs, as endothelial model, upregulated SUCNR1 expression in response to elevated glucose in the culture medium.

3.3. SUCNR1 in HUVECs is located on the plasma membrane and intracellularly

Immunofluorescence staining of FpECs in Fig 5B and 5C revealed intracellular receptor expression. Keeping in mind that the subcellular distribution of GPCRs can impact their signaling, we attempted to address the localization of SUCNR1 in primary endothelial cells. We used primary HUVECs and performed flow cytometry after staining for SUCNR1 with or without permeabilization steps. We observed a shift in the signal with antibody staining relative to isotype control. This shift was more pronounced with permeabilization, indicating that receptor expression was weak on the cell surface and prominent intracellularly (Fig 8).

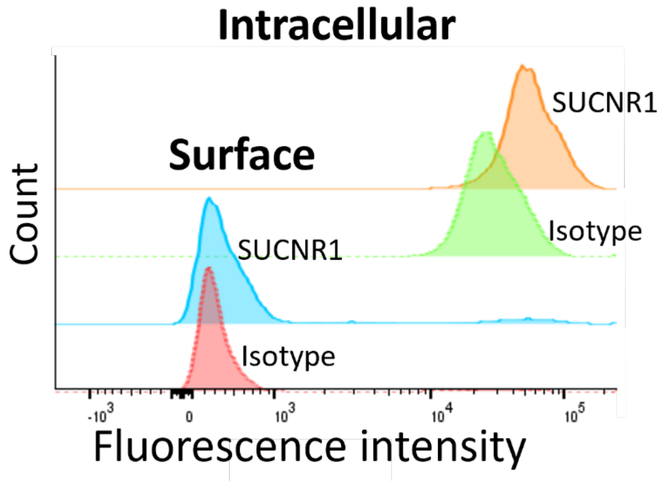

Figure 8. Surface and intracellular expression of SUCNR1 in HUVECs. Flow cytometry staining of SUCNR1 in HUVECs with or without prior permeabilization steps (n=3). Figure is adopted from Atallah et al. (1) with permission of the publisher.

In further immunofluorescence staining of SUCNR1 in non-permeabilized HUVECs, WGA and VE-cadherin were used as plasma membrane markers. SUCNR1 signal was scant and partially colocalized with WGA but not VE-cadherin indicating that the surface receptor expression was limited to the apical but not basolateral side of the cells (Fig 9A). Interestingly, a strong SUCNR1 signal was derived from within the cells. This signal colocalized with mitochondrial marker in a subsequent staining of SUCNR1 in permeabilized cells (Fig 9B).

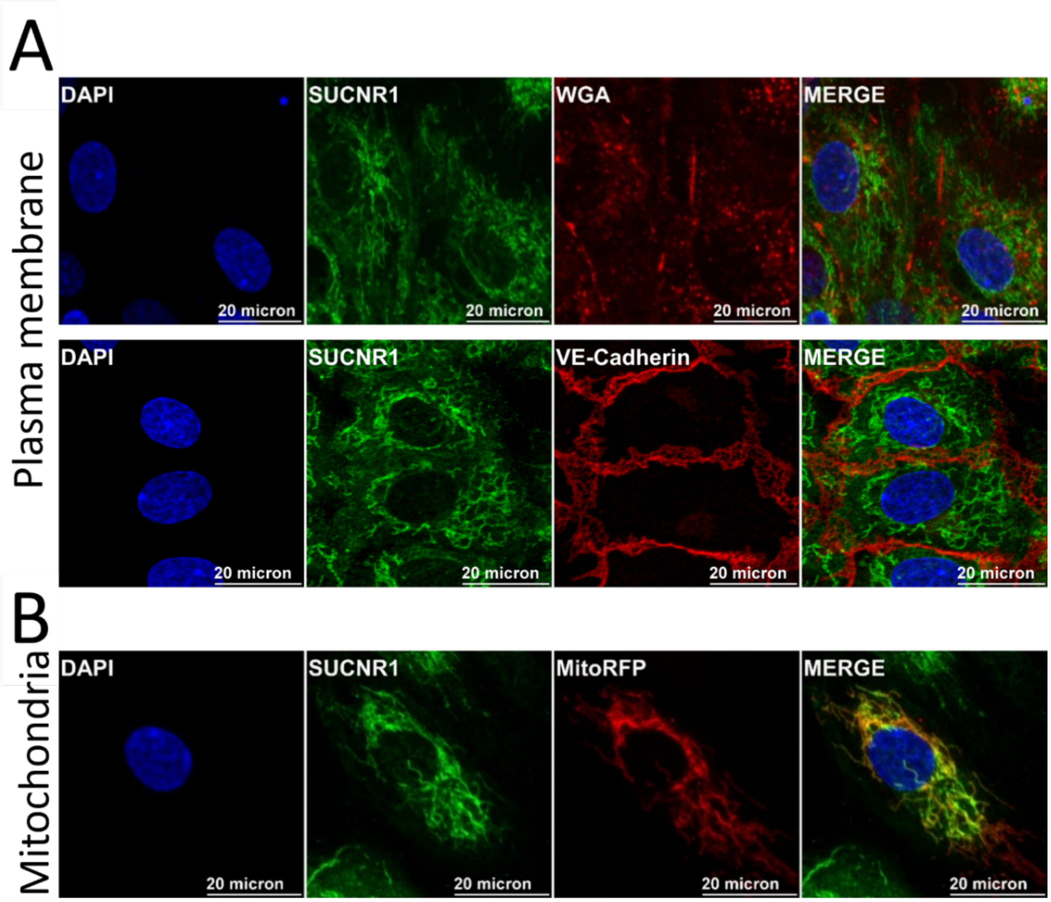

Figure 9. Subcellular localization of SUCNR1 in HUVECs. A) Immunofluorescence staining of SUCNR1 in non-permeablized HUVECs labeled with plasma membrane markers (WGA: apical; VE-cadherin: basolateral sides). B) Immunofluorescence staining of permeabilized HUVECs, using mitochondrial RFP, antibody against SUCNR1. DAPI was used for counterstaining. Images are representative for 5 different experiments (n=5). Figure is adopted from Atallah et al. (1) with permission of the publisher.

In summary, SUCNR1 was found to be only weakly expressed on the plasma membrane and was mainly found inside HUVECs, where it colocalized with mitochondria.

3.4. Succinate induces proliferation but not apoptosis of HUVECs

To address the cellular response to succinate, we treated HUVECs with the natural SUCNR1 ligand, succinate. First, to exclude any effect of the compound on cells viability, we performed apoptosis assay. Annexin V/propidium iodide staining after treatment with vehicle or succinate for 24, 48 and 72 h showed no changes in the percentages of apoptotic and live cells relative to vehicle at any time point (Fig 10).

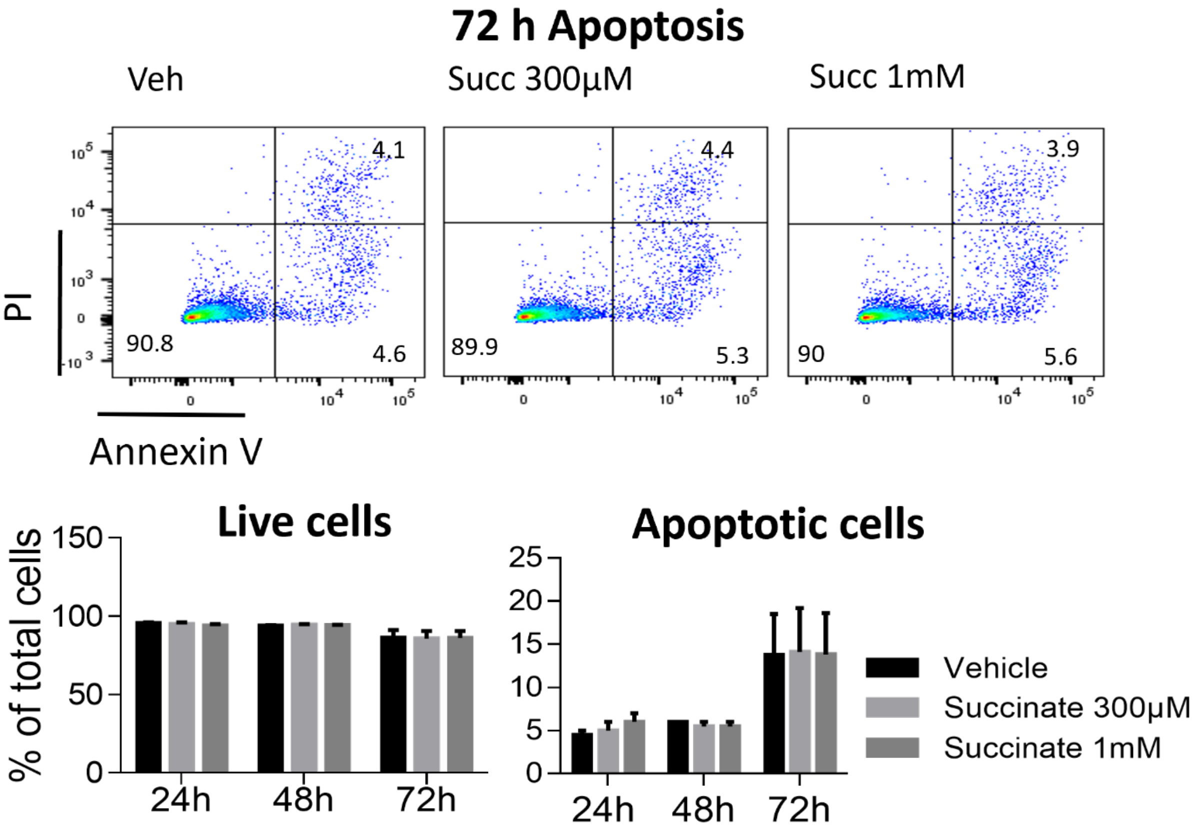

Figure 10. Succinate does not induce apoptosis of HUVECs. Annexin V/PI staining of HUVECs treated either with vehicle or succinate. The percentages of apoptotic and live cells were calculated at 24, 48 and 72 h. Representative flow cytometric dot plot is shown, data are mean ± SEM, (n=3). Figure is adopted from Atallah et al. (1) with permission of the publisher.

In parallel, we performed JC-1 staining to investigate whether succinate alters the mitochondrial membrane potential in HUVECs. The ratio of JC monomers to aggregates at 1, 4 and 24 h was calculated and showed no difference between succinate-treated cells and vehicle treatment (Fig 11).

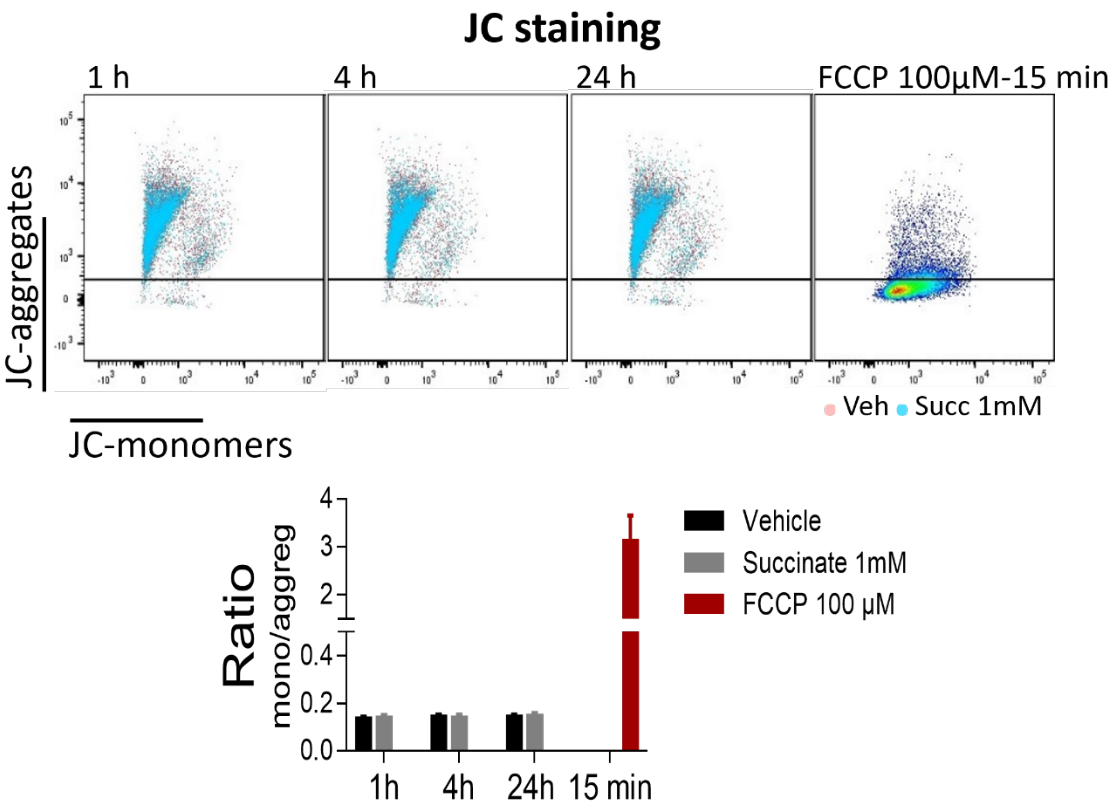

Figure 11. Succinate does not alter mitochondrial membrane potential in HUVECs. JC-1 staining of HUVECs treated with vehicle or succinate. FCCP, a mitochondrial membrane uncoupler, was used as a positive control. The ratio of JC monomers to aggregates was calculated from median fluorescence intensity at 1, 4 and 24 h. Representative flow cytometric dot plot is shown, data are mean ± SEM, (n=3). Figure is adopted from Atallah et al. (1) with permission of the publisher.

Since no apoptotic effect of succinate was observed in our cell model and knowing that endothelial cell proliferation occurs in response to angiogenic stimuli, we investigated the proliferation of HUVECs in response to succinate. We treated HUVECs either with vehicle or succinate for 24 h and performed an EdU proliferation assay. We calculated the ratio of EdU positive cells relative to the number of cells per field. Succinate induced a concentration-dependent increase in EdU positive ratio which was significant with 1mM succinate relative to vehicle (Fig 12).

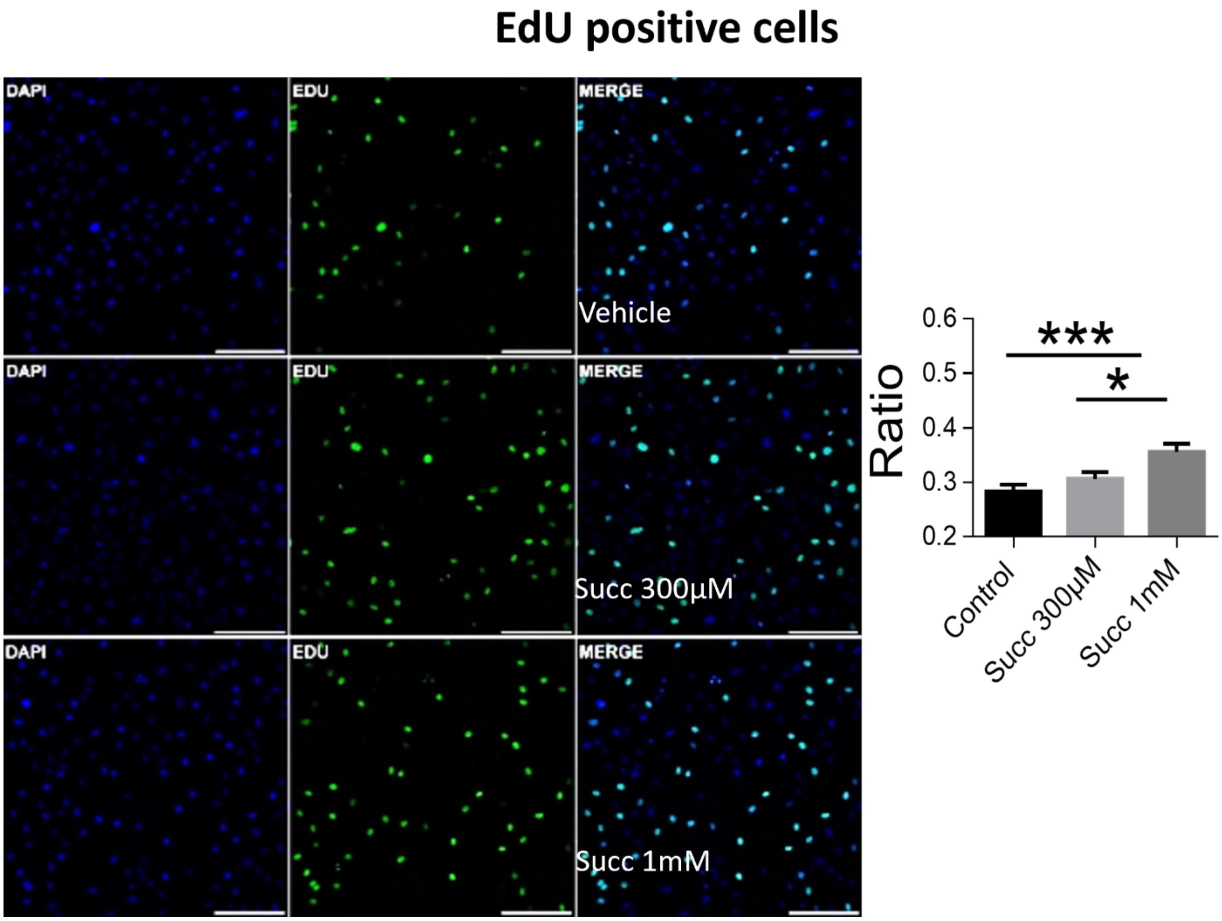

Figure 12. Succinate induces proliferation of HUVECs. EdU proliferation assay of HUVECs treated with vehicle or succinate. At least 5 different fields per slide were examined and the ratio of EdU positive cells to the total number per field was calculated. One-way ANOVA followed by Tukey's post-hoc test was used, *P<0.05, *** p<0.001. Data are shown as mean ± SEM (n=3-5). Representative images are included (scale bar 200µm). Figure is adopted from Atallah et al. (1) with permission of the publisher.

Taken together, our data showed that succinate neither induced HUVECs apoptosis nor impacted mitochondrial membrane potential. In contrast, succinate treatment promoted endothelial cell proliferation.

3.5. Succinate enhances the chemotactic mobility, wound healing and sprouting of HUVECs

To advance with our hypothesis that succinate induces an angiogenic phenotype in endothelial cells and since angiogenesis is a multi-step process including endothelial cell proliferation, cell migration and sprouting (189), we investigated different angiogenesis-related correlates in HUVECs in response to succinate. These included chemotactic mobility, wound healing and spheroid sprouting.

Using a Transwell migration assay, the average number of HUVECs per field that migrated towards succinate after 16 h was significantly higher than vehicle (Fig 13).

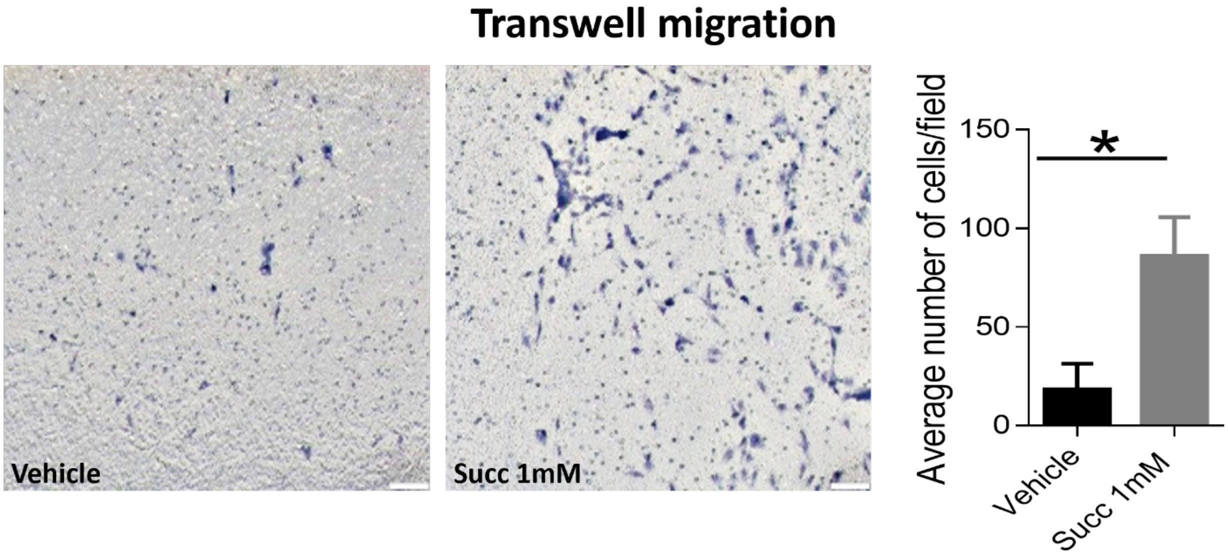

Figure 13. Succinate induces the chemotactic mobility of HUVECs. Transwell migration assay of HUVECs in response to vehicle or succinate. Migrated cells were counted after 16 h on the lower surface of the filter and average numbers per field were calculated. Paired t-test was used, *P<0.05 (n=4). Data are shown as mean ± SEM. Representative images are included (scale bar 200µm, objective 10x). Figure is adopted from Atallah et al. (1) with permission of the publisher.

In addition, the migration of the whole cell mass into cell-free areas of wounded HUVEC monolayers was investigated using 2D scratch assay. Data revealed the ability of succinate to promote wound healing of HUVECs in a concentration-dependent manner, reaching statistical significance with 1mM succinate treatment relative to vehicle (Fig 14). These data further consolidated the ability of succinate to induce collective cell migration, which is of high biological relevance in processes such as embryogenesis and angiogenesis (190) (191).

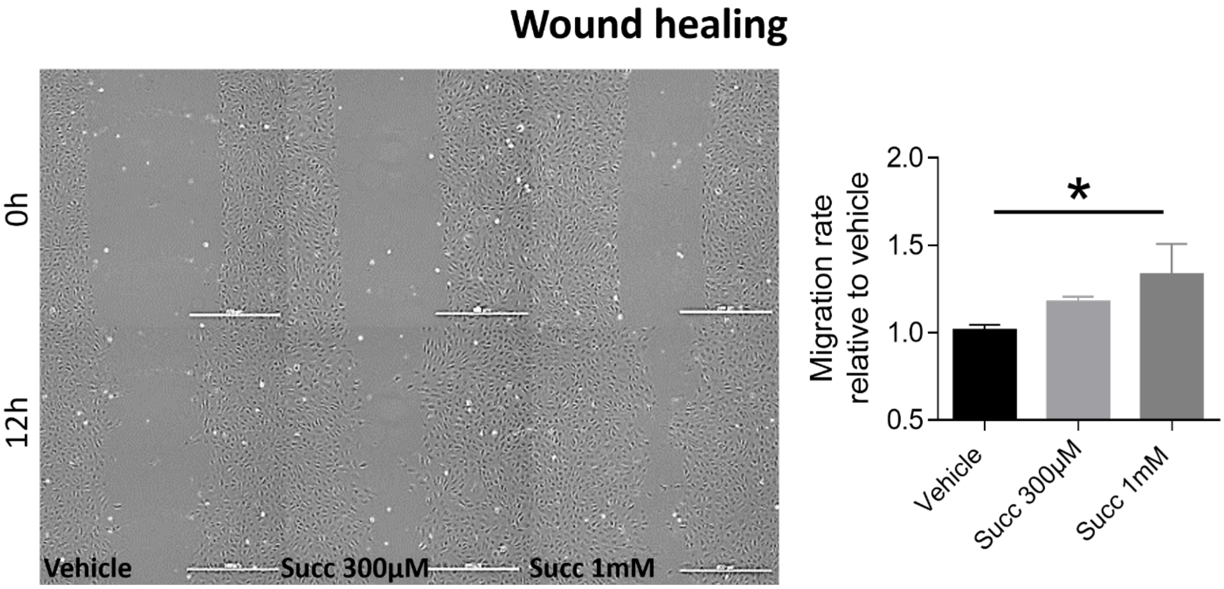

Figure 14. Succinate promotes wound healing in HUVECs. Scratch assay of HUVECs monolayer treated with vehicle or succinate. Distance migrated by cells over time was calculated and analyzed after 12 h. One-way ANOVA followed by Dunnett's post-hoc test was used, *P<0.05 (n=3). Data are shown as mean ± SEM. Representative image is included (scale bar 500µm, objective 10x). Figure is adopted from Atallah et al. (1) with permission of the publisher.

We also performed a 3D spheroid sprouting assay. Data showed that cumulative (total) sprout length as well as sprout number were higher in HUVECs treated with succinate relative to vehicle (Fig 15). This result was reproduced using a SUCNR1 agonist, cis-epoxysuccinic acid, previously described by Geubelle et al. (192).

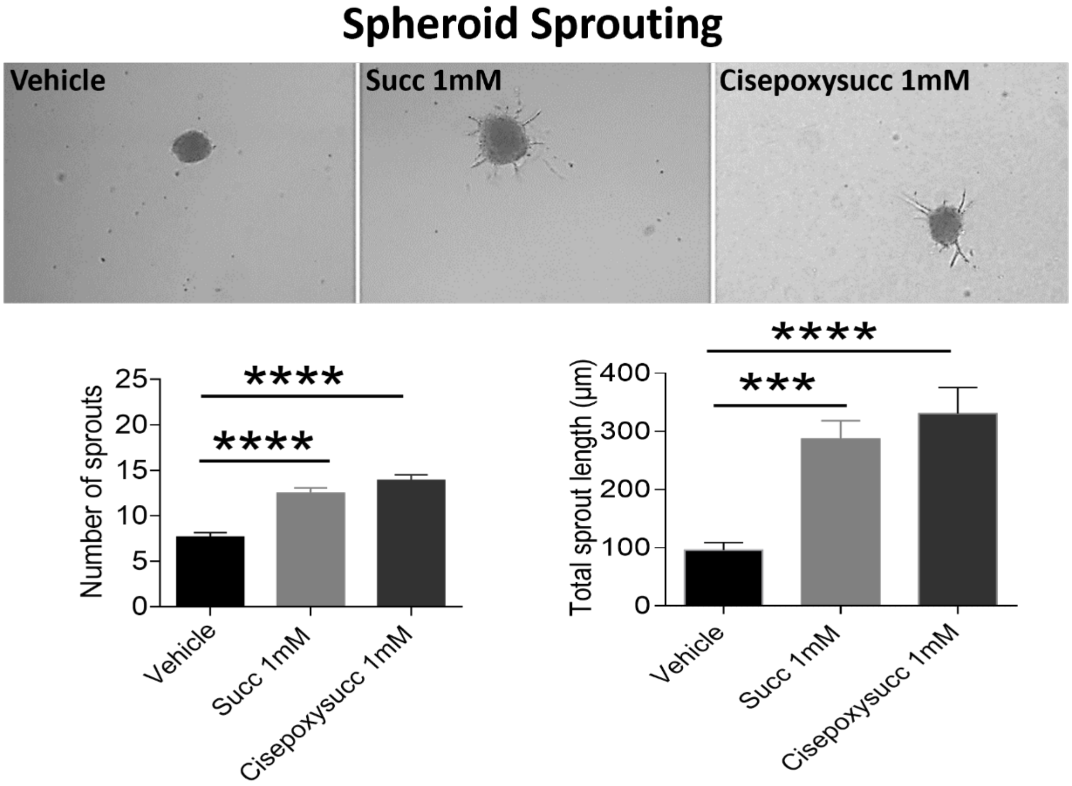

Figure 15. Succinate induces sprouting of HUVECs. Spheroid sprouting assay of HUVECs in response to succinate or cis-epoxysuccinic acid relative to vehicle indicated by total sprout length and number of sprouts. One-way ANOVA followed by Dunnett's post-hoc test was used, ***P<0.001, ****P<0.0001 (n=5). Data are shown as mean ± SEM. Representative image is included. Figure is adopted from Atallah et al. (1) with permission of the publisher.

Collectively, these data confirmed the ability of succinate to induce a proangiogenic phenotype in HUVECs which was reflected in enhanced proliferation, migration and sprouting.

3.6. Succinate disrupts the barrier function in HUVECs

Since vascular leakage and dissolution of the sub-endothelial basement membrane occur as first angiogenic responses to growth factors (193), we investigated endothelial barrier response to succinate stimulation in real-time by ECIS. Our data revealed a decrease in HUVECs barrier after treatment with succinate relative to vehicle (Fig 16A). We also stained for the cytoskeleton protein F-actin and the adherens junction protein VE-cadherin after treatment with succinate or vehicle for indicated time points. Our data showed formation of intercellular gaps and remodeling of F-actin fibers (Fig 16B).

Collectively, these data showed disruption of endothelial barrier and remodeling of cytoskeleton in HUVECs in response to succinate, thus providing further evidence of an angiogenic response to succinate.

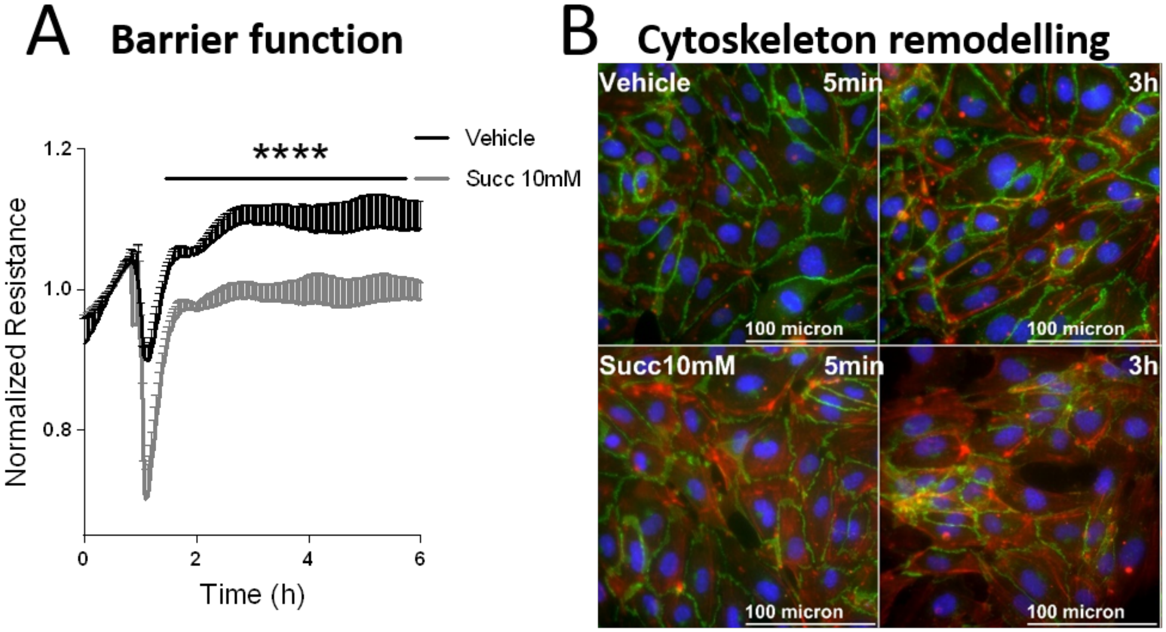

Figure 16. Succinate reduces the barrier function of HUVECs. A) Resistance of HUVECs monolayer in response to succinate or vehicle recorded with ECIS Z-Theta device. B) Immunofluorescence staining of VE-cadherin (green) and F-actin (red) in HUVECs treated with succinate or vehicle. DAPI was used for counterstaining. For A, two-way ANOVA for repeated measurements followed by Tukey's post-hoc test was used, ****P<0.0001 (n=3). Data are shown as mean ± SEM. For B, images are representative for 4 independent experiments. Unpublished data.

3.7. Succinate boosts VEGF gene expression and release in HUVECs

VEGF is a key angiogenic mediator in both physiological and pathological contexts (194). Hence, we used RT-PCR to investigate whether succinate was able to increase VEGF gene expression. Indeed, after 4 h of treatment, a significant increase in VEGF gene expression in response to 1mM succinate was observed (Fig 17A). ELISA measurements of VEGF protein in the conditioned media after 24 h of treatment showed that succinate caused an increase in VEGF secretion which reached significance with 10mM succinate (Fig 17B).

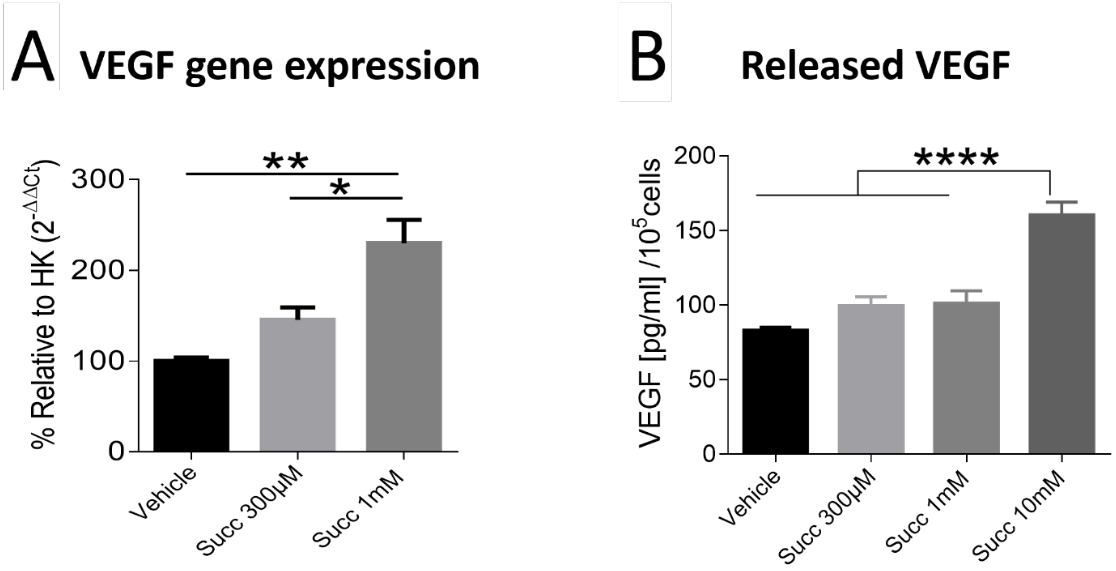

Figure 17. Succinate induces VEGF gene expression and release in HUVECs. A) RT-PCR of HUVECs treated with vehicle or succinate for 4 h. B) VEGF ELISA in culture supernatant of HUVECs treated with vehicle or succinate for 24 h. For A and B, one-way ANOVA followed by Tukey's post-hoc test was performed, *P<0.05, **P<0.01, ****P<0.0001, data are shown as mean ± SEM (n=4-5). Figure is adopted from Atallah et al. (1) with permission of the publisher.

3.8. Succinate induces capillary-like structure formation as well as VEGF gene expression in FpECAs

Discrepancies between arterial and venous cells in terms of their morphology, function, regenerative capacity, and molecular repertoire are well established (195). For this reason, we investigated whether FpECAs showed similar responses to succinate as HUVECs. We used tube formation assay and our data showed that FpECAs responded to relatively low succinate concentration (30μM). Indeed, tube length was higher upon treatment with succinate used alone or in combination with the positive control bVT (b-Fibroblast growth factor, VEGF and TNF-α) (Fig

18A). Similarly, RT-PCR showed increased VEGF gene expression in FpECAs after 4 h of treatment with succinate (300µM and 1mM) (Fig 18B).

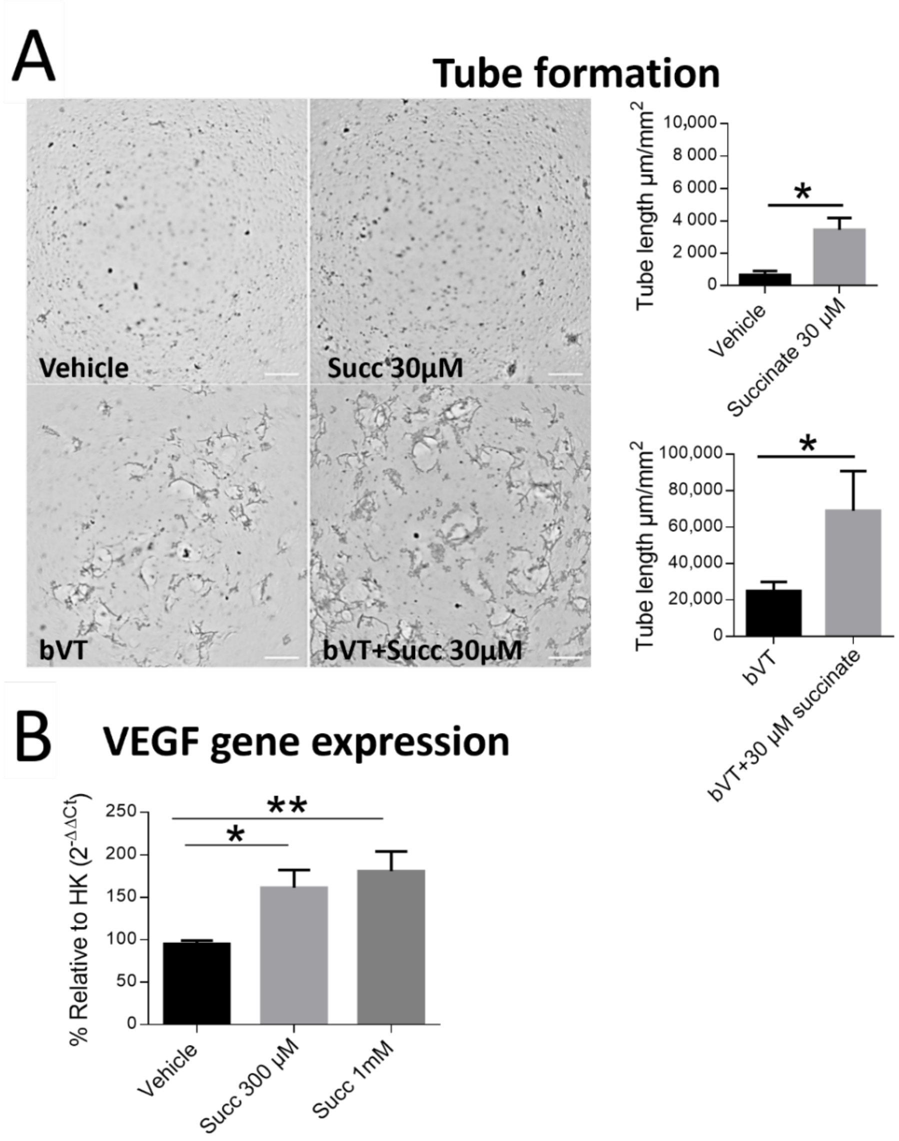

Figure 18. Succinate induces capillary structure formation as well as VEGF gene expression in FpECAs. A) Tube formation assay in FpECAs treated with vehicle or succinate either alone or in combination with the positive control bVT. Tube length was calculated as µm per mm^2. Paired t-test was used, *$p<0.05$, data are mean ± SEM (n=4). Images are representative of 4 independent experiments. B) RT-PCR of FpECAs treated with vehicle or succinate for 4 h. One-way ANOVA followed by Dunnett's post-hoc test was used,

*P<0.05, **P<0.01, data are shown as mean ± SEM (n=6). Figure is adopted from Atallah et al. (1) with permission of the publisher.

3.9. Knockdown of SUCNR1 suppresses the proangiogenic phenotype of HUVECs in response to succinate

Our data so far suggested that succinate stimulates angiogenic responses in placental and umibilical cord endothelial cells. To examine the role of SUCNR1 in these succinate-mediated responses in HUVECs, we targeted SUCNR1 using an SiRNA approach. Flow cytometric analysis of DY-547 labeled positive control SiRNA showed the presence of SiRNA inside the cells at 24, 48 and 72 h (Fig 19A). The knockdown efficacy was confirmed at the protein level by Western blot and immunofluorescence staining of the receptor in control and SUCNR1 SiRNA-transfected cells at 72 h (Fig 19B, 19C).

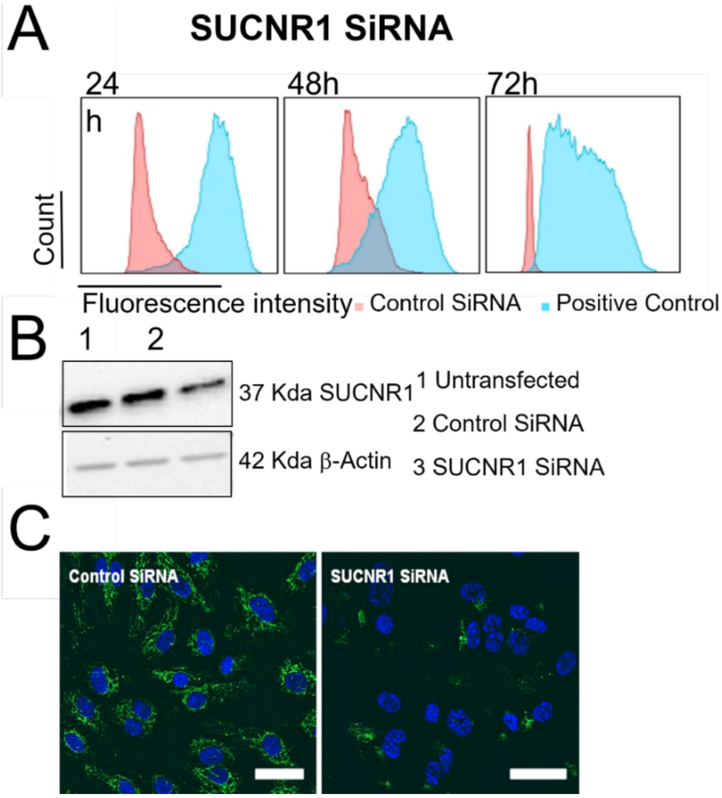

Figure 19. Knockdown of SUCNR1 in HUVECs. A) Representative histogram of flow cytometric analysis of DY-547 labeled positive control SiRNA at 24, 48 and 72 h. B) Representative Western blot of SUCNR1

in non-transfected, control or SUCNR1 SiRNA-transfected HUVECs at 72 h post transfection. C) Representative images for immunofluorescence staining of SUCNR1 in HUVECs transfected with either control or SUCNR1 SiRNA for 72 h (scale bar 10µm, objective 20x). DAPI was used for counterstaining. Figure is adopted from Atallah et al. (1) with permission of the publisher.

Our data demonstrated that the wound healing capacity of SUCNR1 SiRNA-transfected HUVECs in response to succinate was significantly hampered relative to control transfected cells (Fig 20A). In a Transwell migration assay, the number of migrated cells after 16 h towards 1mM succinate was significantly lower in SUCNR1 SiRNA-transfected HUVECs relative to control SiRNA (Fig 20B). Similarly, knockdown of SUCNR1 resulted in reduced VEGF gene expression in response to 1mM succinate as compared to control SiRNA-transfected cells after 4 h of treatment (Fig 20C). Finally, the barrier response of SUCNR1-transfected HUVECs to treatment with 10mM succinate was also hindered (Fig 20D).

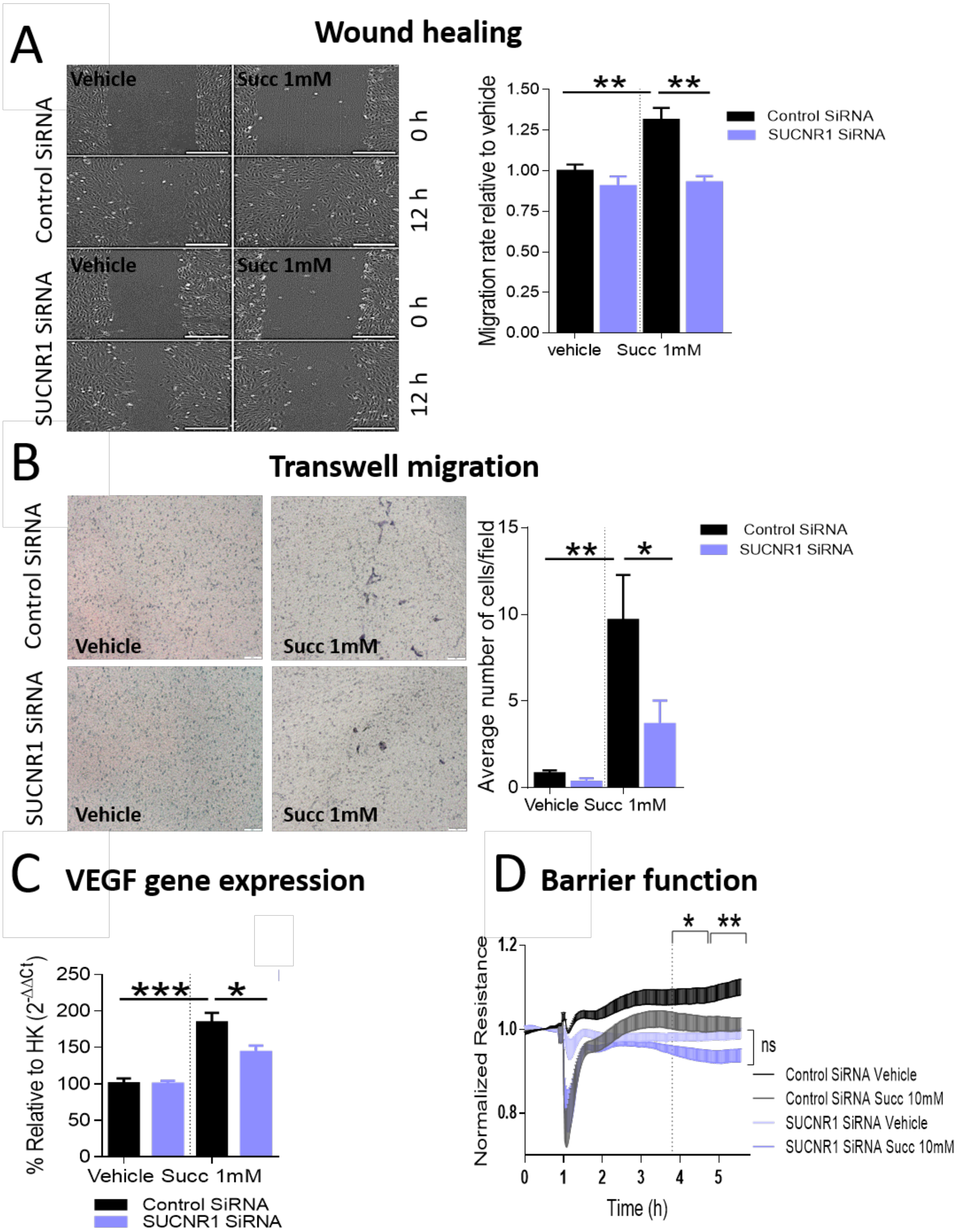

Figure 20. Knockdown of SUCNR1 suppresses the proangiogenic phenotype of HUVECs in response to succinate. A) Scratch assay in control or SUCNR1 SiRNA-transfected HUVECs treated with vehicle or

succinate for 12 h. Representative images are shown (scale bar 500µm, objective 10x). B) Transwell migration assay of control or SUCNR1 SiRNA-transfected HUVECs in response to vehicle or succinate after 16 h. Representative images are shown (scale bar 200µm, objective 10x). C) RT-PCR of VEGF gene expression in control or SUCNR1 SiRNA-transfected HUVECs treated with either vehicle or succinate for 4 h. D) Resistance of control or SUCNR1 SiRNA-transfected HUVECs in response to succinate or vehicle recorded with ECIS Z-Theta device. A, B and C were analyzed by two-way ANOVA followed by Tukey's post-hoc test. D was analyzed by two-way ANOVA for repeated measurements followed by Tukey's post-hoc test. *P<0.05, **P<0.01, ***P<0.001, data are shown as mean ± SEM (n=3-5). Part of the figure is adopted from Atallah et al. (1) with permission of the publisher.

Collectively, knockdown of SUCNR1 hampered succinate induced wound closure of endothelial monolayers, migration, VEGF gene expression and barrier disruption in HUVECs implying that succinate-induced responses were, at least partly, mediated via SUCNR1.

3.10. Signaling downstream of SUCNR1

To unravel the signaling machinery downstream of SUCNR1, we used the HUVEC cell line EA.hy926 and examined EKR1/2 and AKT phosphorylation upon stimulation with 10mM succinate. Significant increase in ERK1/2 phosphorylation was observed 15 min after treatment (Fig 21A). However, no effect on AKT phosphorylation was observed (Fig 21B). Pretreatment with MEK/ERK inhibitor (UO-126, 10µM) resulted in inhibition of succinate induced phosphorylation although the inhibitor alone seemed to inhibit some constitutive phosphorylation in our cell line (Fig 21C). We further performed ECIS in primary HUVECs including UO-126 to our conditions. Although the inhibitor alone initially increased the barrier of the cells, it was capable of inhibiting succinate induced drop in barrier. This drop was completely abolished after 5 h (Fig 21D).

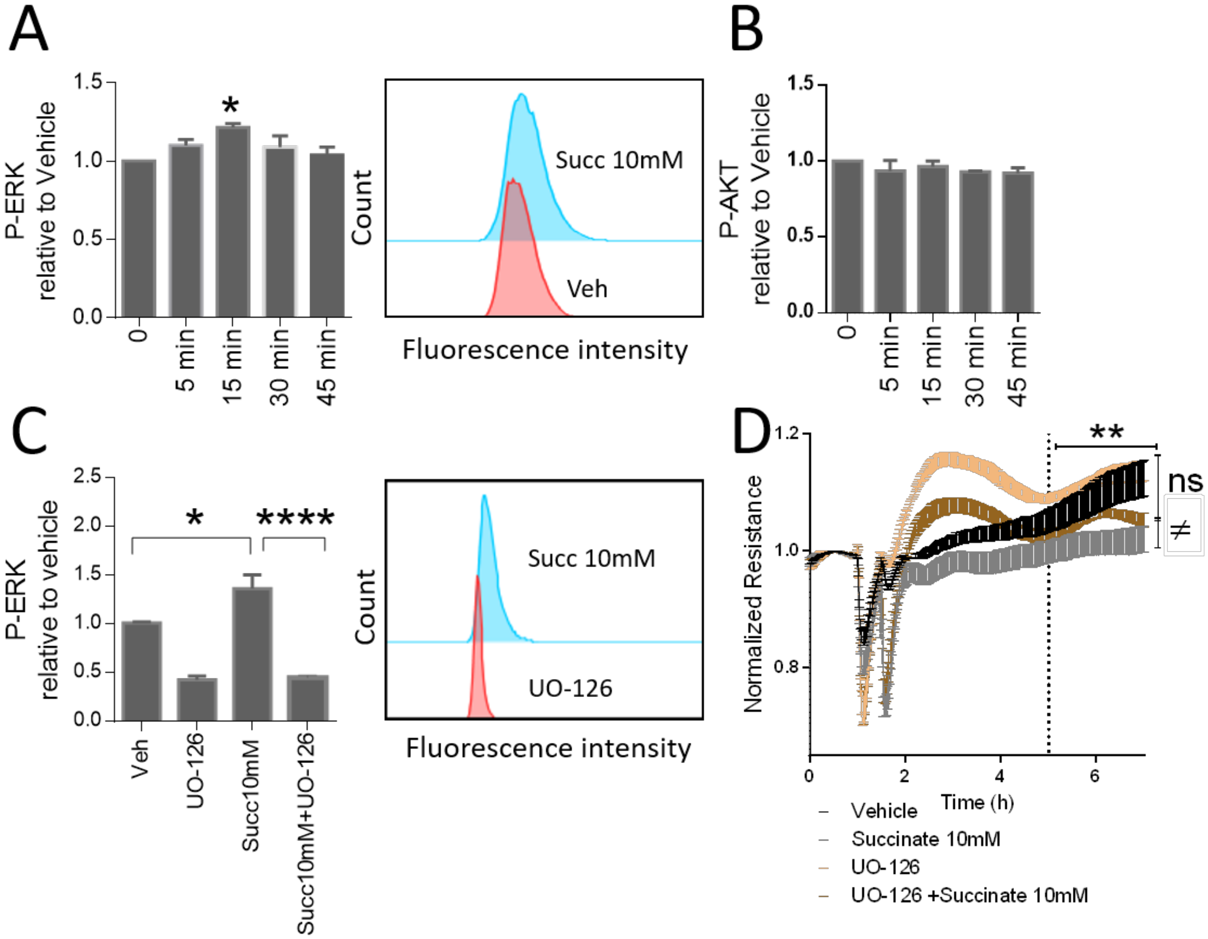

Figure 21. Succinate regulates ERK1/2 signaling pathway. A) Time curve of ERK1/2 phosphorylation after treatment with succinate or vehicle in EA.hy926. B) Time curve of AKT phosphorylation in EA.hy926 cells after treatment with succinate or vehicle. C) Pretreatment with UO-126 inhibited succinate-induced ERK1/2 phosphorylation in EA.hy926 cells. D) Pretreatment with UO-126 inhibited succinate-induced drop in HUVECs barrier. For A, B and C, data are expressed as fold change in fluorescence intensity relative to vehicle, data were analyzed by one-way ANOVA followed by Tukey's post-hoc test, $*P<0.05$, $****$ $p<0.0001$. D was analyzed by two-way ANOVA for repeated measurements followed by Tukey's post-hoc test. $**P<0.01$ between vehicle and succinate, # $P<0.05$ between succinate and UO-126 + succinate, ns denotes no statistical difference between vehicle and UO-126 or vehicle and UO-126 + succinate. For A and B, representative histograms are shown. Data are shown as mean ± SEM (n=3-5). Part of the figure is adopted from Atallah et al. (1) with permission of the publisher.

4. Discussion

In this thesis, my focus was placental angiogenesis which is a tightly regulated process in normal pregnancies and deranged angiogenesis is by far the most commonly identified placental anomaly in complicated pregnancies (196), (197). We investigated a metabolite axis, comprised of succinate and its GPCR partner (SUCNR1) in human placenta, as possible regulator of placental angiogenesis. We hypothesized that altered placental/fetal metabolism in pregnancy complications such as GDM presented as dysregulation of this axis and resulted in dysregulated placental angiogenesis.

Our data showed that succinate, SDH gene expression and SUCNR1 protein abundance were upregulated in placental tissue lysates from GDM pregnancies as compared to matched controls in parallel to an increase in VEGF protein expression. A correlation analysis showed a significant positive correlation between SUCNR1 and VEGF protein expression in the tissue. At the cellular level, we confirmed the expression of SUCNR1 in placental endothelial cells. Arterial cells from diabetic pregnancies expressed more SUCNR1 relative to matched controls. We performed a variety of angiogenesis related assays in primary HUVECs and we showed that succinate was able to induce an angiogenic response in these cells. We also confirmed that FpECAs respond in a comparable manner to succinate in a tube formation assay and by upregulating VEGF gene expression. Furthermore, we targeted SUCNR1 in HUVECs using SiRNA knockdown and this resulted in abrogation of the angiogenic response to succinate. Looking into the downstream signaling machinery, ERK1/2 signaling pathway was induced with succinate treatment in EA.hy926 cells and hence is a valid target to moderate SUCNR1 mediated responses in endothelial cells.

Succinate form and concentration

In this study, we used succinate as sodium salt which was readily soluble in water. The sodium ions and succinate dissociate in solution leading to the release of both carboxylic groups which are necessary for receptor activation. Majority of mammalian cell membranes are believed to be impermeable to succinate and exogenously supplemented succinate has limited uptake into the cells (198) (199). We used cis-epoxy succinate (specific SUCNR1 agonist) in spheroid sprouting assay and observed similar results as with sodium succinate. However, the agonist had an impact on the pH of the media. To avoid any influence on cell viability, we decided to use the sodium salt for all experiments.

The concentrations used in our study are in the pathophysiological range (µM to mM). Succinate is measured in human systemic circulation in the low µM range (175). However, the concentration can vary extensively depending on the tissue and the extent of metabolic stress. Local concentrations of succinate are reported in mM concentration range in human gastric cancer tissues (181) and in cases of ischemia or hypoxia (200) (182). Preliminary measurements of succinate concentrations in cord plasma from normal and GDM pregnancies showed a clear trend of succinate accumulation in the diabetic samples which was up to 100µM in diabetic plasma (unpublished data).

Succinate accumulation in gestational diabetic placentas

In this study, we measured higher succinate concentration in GDM placentas relative to matched controls. In the context of gestational complications, a recent study linked low succinate levels in chorionic villi to recurrent spontaneous deliveries. This reduction was accompanied with reduced SDHB DNA methylation and elevated SDHB expression (201). In contrast, succinate, among other metabolites, was higher in placental extracts from PE women relative to controls (202).

As mentioned before, accumulation of succinate was similarly described in numerous tissues where imbalance between energy demand and oxygen supply occurred and had multiple implications. For instance, in brown adipose tissue, accumulation of succinate resulted in elevation of thermogenic respiration (152) and in synovial joints of rheumatoid arthritis patients elevated succinate provided a link between metabolism and inflammation (203). In tumors with SDH mutations, increased succinate suggested a pro-oncogenic role of succinate (204). Succinate was also elevated in tissue and circulation of models of diet-induced obesity (149) and higher plasma succinate concentrations were measured in patients with T2DM (205). Higher succinate concentrations in plasma and intestinal mucosa of Crohn's disease patients implied a role of succinate in intestinal inflammation (206). In ischemic hearts across species succinate accumulation was a common feature and played a role in tissue damage upon reperfusion (207). All these findings implied crucial extra-metabolic roles of succinate in numerous tissues and contexts.

Upregulation of SUCNR1 and SDH in gestational diabetic placentas

Our Western blot data showed increased SUCNR1 expression in GDM placental tissue lysates, which was in agreement with previous publications that showed upregulated SUCNR1 expression in intestinal surgical resections of Crohn's disease patients relative to controls (206) as well as in ovarian cancer (208). In the context of diabetes, succinate-SUCNR1 signaling was proposed as a molecular mechanism in diabetic neuroretinal angiopathy (182) as well as nephropathy (150).

Interestingly, SDH gene expression was upregulated in GDM placental tissue lysates in comparison to matched controls. On the one hand, this denoted dysregulated mitochondrial function in GDM A1. On the other hand, this upregulation in gene expression, rather than downregulation, could be a compensatory mechanism in the placenta to reduced SDH protein level previously reported in GDM placentas. Indeed, a previous study showed that mitochondrial electron transfer complex subunits I, II, III and IV were significantly reduced in placentas from GDM A2 relative to GDM A1 and controls (209). Another possible explanation, which remains as a hypothesis in GDM, is that SDH is functioning in the opposite direction converting fumarate to succinate. Under most physiological condition, SDH catalyzes the conversion of succinate to fumarate. However, in conditions of hypoxia or ischemia SDH can function in reverse mode (210) (211) (212). This was proposed in lung fibrosis where SDH promotes the production of succinate (213) and in liver fibrosis in mice fed a high-fat diet (214). Nevertheless, in hepatic stellate cells, inhibition of SDH resulted in increased succinate levels and had pro-fibrotic effect (215). All these observations suggest that the function of SDH is very context specific and further research is needed to unravel how this enzyme functions in human placenta under normal and pathological contexts. In agreement with our data at the mRNA level, Muralimanoharan et al. showed no significant differences in LDH between normal and GDM A1 (111).

SUCNR1 expression in placental endothelial cells

We confirmed expression of SUCNR1 in placental endothelial cells by ISH of tissue sections where co-expression of SUCNR1 mRNA and VWF was noted and using isolated cells by Western blot and immunofluorescence. Previously, Toma et al. (150) and Zhang et al. (216) described SUCNR1 expression in endothelial cells of the afferent arterioles of rabbit and mice kidney as well as human venous and arterial endothelial cells from the umbilical cord. However, the placental endothelium is distinct in structure and function as veins carry oxygenated nutrient-rich blood from the mother, whereas arteries carry deoxygenated blood from the fetus (217).

Interestingly, SUCNR1 expression was higher in venous cells relative to arterial cells in normal pregnancies. Since placental arterial cells are more sensitive to angiogenic signals than venous cells (217), we hypothesize that the reason behind this observation is that our endothelial cells were isolated from term placentas and not earlier in pregnancy when angiogenesis mainly occurs. In human placenta, both FpECAs and FpECVs express classical endothelial markers. However, they differ in their phenotypic, genotypic, and functional characteristics. For instance, in response to different VEGF isoforms FpECAs showed more proliferative capacity in comparison to FpECVs which were more sensitive to PlGF (183). Further, microarray and gene ontology analyses identified genes related to GPCR signaling pathways, angiogenesis, and blood vessel remodeling to be differentially expressed in HUVECs and placental microvascular cells (218).

In contrast, SUCNR1 expression was increased in diabetic arterial endothelial cells relative to cells from normal placentas although this increase was not significant in venous cells. A previous study by Cvitic et al. showed that GDM programs atypical morphology and barrier function in FpECs by DNA methylation and gene expression alteration. They highlighted that these cellular responses differed between arterial and venous cells denoting cell-specific sensitivity to adverse exposures (219).

To examine the hypothesis that high glucose in the environment may explain this upregulation in SUCNR1 expression, we cultured our cell model in high glucose and – indeed - after 48 h Western blot showed increased SUCNR1 protein abundance. The regulation of SUCNR1 expression by stressors was previously shown where hindlimb ischemia induced SUCNR1 upregulation in ischemic mouse muscles after 4 days (220).

Intracellular SUCNR1 expression

Our flow cytometric data that were corroborated with confocal microscopy revealed weak expression of SUCNR1 on the cell membrane, which is typical for most GPCRs. Classically it was established that signal transduction from GPCRs starts from the cell surface where interaction of the receptor with external stimuli can be transformed into a wide range of cellular responses (221).

Interestingly, we observed that SUCNR1 in non-stimulated cells was also expressed intracellularly and was coupled to the mitochondria. Similarly, the cannabinoid receptor CB(1) was detected in mouse brain mitochondrial membranes of neurons (222) and expression of angiotensin type 1 and 2 receptors in human mitochondrial membranes was shown and coupled to mitochondrial NO production (223). To discriminate between signaling events at the cell surface and intracellularly, Gbahou et al. used cell-impermeable melatonin receptor agonist and they confirmed expression of mitochondrial MT1 receptors in HEK293 cells (224).

Despite lack of comprehensive insight, activation and/or inhibition of mitochondrial GPCRs seems to regulate processes such as mitochondrial Ca^{2+} uptake, ATP production, ROS production and apoptosis (221). The purinergic receptors P2Y(1)-like and P2Y(2)-like in rat hepatocyte mitochondria were suggested to regulate mitochondrial calcium uptake (225). Activated CB_1 receptors induced intra-mitochondrial $G_{\alpha i}$, thus inhibiting soluble adenylate cyclase leading to decreased cAMP and decreased PKA phosphorylation of various complex 1 proteins. Subsequently, a reduction in mitochondrial activity occurred (222) (226). While the short-term consequences of mitochondrial CB_1 receptor activation included loss of mitochondrial mobility and synaptic depression, long-term consequences included memory loss (226) as well as metabolic defects and apoptosis (227).

Through their life-cycle, GPCRs can be detected on the endoplasmic reticulum (the site of their synthesis, folding, modification and assembly) in addition to sorting vesicles on their way to the cell surface, and on endosomes just having been internalized (221). However, numerous publications in the last few years indicated that certain intracellular membranes may serve as alternate destinations or even preferred localization sites for a number of GPCRs (228) (229) (230). In-situ activation of many GPCRs in different intracellular compartments was demonstrated (221) (231) (232).

As mentioned before, for our assays we used a non-permeable form of succinate, so the chances of ligand diffusion through the plasma membrane are minimal. Nevertheless, the possibilities of this receptor fraction activation by de-novo succinate synthesis in the mitochondria or by active transport of succinate from the extracellular space require detailed investigation. Further questions arise including the signals that regulate the trafficking or retaining of this receptor fraction in this unique location and the functional or even pathophysiological consequences of its activation.

Succinate-SUCNR1 axis and placental angiogenesis

Developing a highly efficient network of capillaries within the placental villi is required for successful exchange between the mother and the fetus. Hence, placental vasculogenesis and subsequent angiogenesis are crucial processes to secure the enlargement of the placental vascular tree and placental growth (197). Angiogenesis is a complex process that starts with an increase in angiogenic factor concentration in the environment followed by breakdown of basement membrane. Subsequently, endothelial cell migration, proliferation and sprouting to initiate the formation of tube-structures occur (233). These tubes next stabilize into mature vessels with pericytes or smooth muscle cells (234).

In our in-vitro experiments, we addressed different endothelial cell angiogenesis correlates in response to succinate. Our data confirmed the angiogenic potential of succinate by inducing endothelial proliferation, chemotactic mobility, wound healing and spheroid sprouting. Similar angiogenic response was previously shown in other cell models such as retinal ganglion neurons

(182), HUVECs (181), and rat aortic endothelial cells (153). In addition, succinate injection was suggested to promote earlier angiogenesis after ischemia in mice, subsequently inducing more effective revascularization and enhanced functional recovery (220).

A recent study showed that proliferating ECs both in cancer and wet, age-related macular degeneration upregulated the expression of metabolic genes involved in one-carbon metabolism, nucleotide synthesis, TCA cycle and oxidative phosphorylation (235). This emphasizes the role of endothelial cell metabolism in cellular functions. The authors showed that in hypoxic conditions oxidative phosphorylation genes were upregulated, despite previous reports demonstrating that hypoxia suppresses oxidative phosphorylation (236) (237). The role of SUCNR1 in the proangiogenic phenotype induced by succinate in HUVECs was confirmed since knock-down of SUCNR1 suppressed the cellular response to succinate.

Since endothelial cell migration is essential for angiogenesis and this process requires cytoskeleton remodeling, we investigated the barrier function of HUVECs in response to succinate stimulation in real-time using ECIS. Indeed, our data showed the high concentration of succinate (10mM) reduced HUVECs barrier resistance. Staining for VE-cadherin and F-actin showed formation of intercellular gaps and alterations in F-actin organization. In endothelial cells, actin is a major cytoskeletal component and is composed of 43-kDa monomeric globular subunits (G-actin) that polymerize into helical filaments (F-actin). Remodeling of the actin cytoskeleton is crucial for cell migration (238). In addition, endothelial junction integrity is regulated by proteins that form adherens junctions and tight junctions. Focal alterations in junctions result in endothelial gaps and leakage. Among the proteins that play a critical role in adherens junction is VE-cadherin that joins adjacent endothelial cells through homophilic interactions (239). In placenta, both the outer syncytial lining and fetal endothelium provide a barrier controlling the transfer of solutes from maternal to fetal blood (240) and in placental capillaries, adherens junctions are major regulators of paracellular permeability. The transmembrane adhesion molecule VEcad is the key player maintaining the architecture of placental endothelial cell-cell junctions (241) (242). Upon phosphorylation of VE-cadherin, breakage of homophilic binding, loss of anchorage to peri-junctional actin and increased paracellular permeability occur (243). Previously, VEGF-A was demonstrated to increase phosphorylation of VE-cadherin resulting in disrupted barrier and subsequently extravasation of cells (244). In pregnancies complicated with maternal type 1 diabetes, phosphorylation and loss of VE-cadherin and beta-catenin were partly attributed to elevated levels of VEGF in the placenta (245). Similarly, in GDM complicated pregnancies altered surface expression of placental junctional proteins suggested impaired barrier function (129).

Furthermore, our data demonstrated that VEGF gene expression was upregulated in both HUVECs and FpECAs in response to succinate treatment. In human placenta, VEGF acts as a major regulator of placental vasculogenesis and angiogenesis sequentially (37). In heterozygous VEGF-deficient (VEGF+/-) embryos, abnormal vessel development and lethality occurred (246) (247). Similarly, disruption of genes encoding VEGF receptors such as VEGFR1 (248) and VEGFR2 (42) resulted in embryonic lethality due to malformation of blood vessels. These findings confirmed a crucial role of VEGF/VEGFRs in vasculogenesis. In addition, VEGF was shown to regulate all steps of angiogenesis. In vitro treatment of endothelial cells with VEGF induced release of proteases such as collagenase to break down extracellular matrix and thus allowing the cells to proliferate and migrate (249). Furthermore, VEGF induced placental endothelial cell proliferation and migration as well as the formation of tube-like structures on matrigel (250) (251).

In tube formation assay, FpECAs readily responded to lower succinate concentrations (30μM). Previously, Hiden et al. attributed the sensitivity of placental arterial endothelial cells to angiogenic stimuli to the need to expand fetal vasculature in proximity to the higher oxygen levels in the maternal blood, ensuring adequate supply to the fetus (217). However, we are not aware of the exact mechanisms behind this discrepancy. A recent study highlighted different metabolic signatures between different endothelial cell phenotypes. While venous tumor endothelial cells upregulated transcripts of genes involved in prostaglandin metabolism, suggesting a role in vasoregulation, sprouting or vascular inflammation, breach tumor endothelial cells (which express both tip cell and podosome rosette markers) mostly upregulated genes involved in extracellular matrix production, in line with their presumed role in vessel sprouting initiation (235). Hence, we hypothesize that there might be a metabolic basis for this sensitivity.

SUCNR1 downstream signaling

The mitogen-activated protein kinase (MAPK) signaling pathways are conserved signal transduction cascades which are crucial for transmitting extracellular signals from growth factors, hormones, chemokines and others to intracellular targets. In vertebrates, the MAPK family has been categorized into three subfamilies, including ERKs, p38, and Jun N-terminal kinases. The MAPK signaling is important for nearly all fundamental cellular processes (37) and ERK1/2 regulates numerous cellular processes such as proliferation, differentiation and cell responses to stress (252).

We demonstrated that succinate induced ERK1/2 phosphorylation in EA.hy926 cells which is in agreement with previous studies in macula densa cells in the kidney (253), retinal ganglion cell line (RGC-5) (254), human erythroleukaemia cell line (TF-1) (255) and cardiomyocytes (176).

In placenta, ERK2 was shown to be indispensable for normal placental development and ERK2-deficient mouse embryos developed severe impairment in the labyrinthine layer of the placenta (256). In placental endothelial cells, MAPK signaling pathways were shown to be crucial for cellular responses to VEGF and FGF2 stimulation such as proliferation, migration and tube formation (250) (257) (258).

Our data demonstrated that ERK inhibitor was able to inhibit succinate induced ERK1/2 phosphorylation in EA.hy926 and to concomitantly inhibit succinate-induced disruption in HUVECs barrier. Similarly, inhibition of ERK1/2 pathway completely blocked VEGF-induced cell proliferation in addition to VEGF- and FGF2-stimulated cell migration, while it partially attenuated FGF2-induced cell proliferation (250) (251) (257) (259).

Conclusions

Our study identified succinate-SUCNR1 signaling axis in placental endothelial cells as a novel target regulating placental angiogenesis. We provided evidence that succinate signaling through its receptor triggers a proangiogenic phenotype in endothelial cells, a phenomenon which might be aggravated in GDM.

Study limitations

- We acknowledge that the small sample size of our cohorts is a limitation of the study and reproducing the study findings with bigger sample size is a mandate to be able to advance with these data.
- We used primary HUVECs for in vitro functional assays due to limited access to sufficient number of FpECs isolations. We validated our observations in FpECAs. However, we propose that using placental cells for further assays would be of great advantage.
- FpECAs in vivo are in contact with less oxygenated blood and it is known that oxygen has profound effects on the functionality of endothelial cells. We cultured our cells at atmospheric oxygen levels (21% O_2). However, we suggest that culturing these cells in physoxia (~8% O_2) would be more relevant and closer to reflect their state in vivo.
- We also believe that the inclusion of a second subgroup of diabetic pregnancies where women have received treatment (GDM A2) will provide a further insight on how succinate-SUCNR1 axis is regulated in this case.
- Eventually, a comprehensive metabolomics investigation of arterial and venous endothelial cells from normal and pathological pregnancies will give further information on how the metabolism in these cells might be altered under stress conditions. Additionally, this approach will enable identifying other metabolic targets which could be playing major roles in the pathogenesis of pregnancy complications.

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6. Appendix

Buffer recipes/preparations

Western blot 6x Sample Buffer:

375mM Tris HCL (pH 6.8)

12% SDS

50% Glycerol

0.03% Bromophenol Blue

Western blot 10x RUN Buffer:

30.3g TRIS

150.1g Glycine

10g SDS

Adjust volume to 1000ml with distilled water and store at RT

Western blot 10x Wash Buffer:

5g Tween 20

90g NaCl

100ml 1M TRIS (pH 7.4)

Adjust volume to 1000ml with destilled water and store at 4°C

1M TRIS pH7.4:

60.57g TRIS/HCl in 500ml destilled water, adjust pH and store at 4°C

1.2% Methylcellulose stock solution for spheroids assay:

For a stock solution of 250ml, weigh 3g of methylcellulose in a 500ml glass bottle

Add a magnetic stirrer and autoclave dry

Preheat 125ml M199 media at 60°C in a water bath for 1-2 h

Add prewarmed M199 media to the autoclaved methylcellulose

Stir using a magnetic stirrer for ~ 20 min

Add another 125ml of RT M199 media

Stir over night at RT

Make 50ml aliquots

Centrifuge at 3700rpm for 4 h

Transfer dissolved and clear methylcellulose solution into fresh 50ml falcon tubes

Store at +4°C for ~ 2 years

2% Gelatin stock solution:

Weigh in 10g of gelatin

Add 500ml of 1X HBSS

Use a magnetic stirrer at 70°C to dissolve the gelatin

Sterile filter the solution

Add 10ml gentamicin (Thermo Fisher Scientific) to achieve a concentration of 2%

Store at 4°C and dilute 1:2 in 1X HBSS before use

Table 3: List of primary antibodies used in the study and their catalogue numbers:

Antibody	Company	Cat #
SUCNR1 (WB)	Novus Biologicals	NBP2-82350
SUCNR1 (IF, Flow)	Novus Biologicals	NLS3476
VEGF	Abcam	ab51745
VE-Cad	Santa Cruz	sc-9989
VWF	Abcam	ab6994
β-Actin	Cell Signaling	mAB3700
P-ERK	Cell signaling	4370S
P-AKT	Cell signaling	4060S

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