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TESI DI LAUREA

Analysis of mRNA translation in endothelial cells exposed to tumour microenvironmental conditions.

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1. ABSTRACT.

Angiogenesis, the process in which new blood vessels arise from pre-existing ones, is one of the hallmarks of cancer, indispensable for the transition from a contained tumour to an invasive, metastatic disease.

Tumour microenvironment (TME), defined as the tissue context in which the tumour arises, plays a fundamental role in promoting angiogenesis, since many of its cellular components and biochemical features stimulate the production and the release in the extracellular matrix (ECM) of pro-angiogenic factors that deeply influence endothelial cells (ECs) behaviour, stimulating their transition from a quiescent, non-proliferative state, to an activated, proliferating one.

During this switch ECs (the cell type constituting the lining of the blood vessels) undergo significative metabolic rearrangements that deeply affect, among others, also their protein biosynthesis apparatus, which is redirected towards the production of proteins essential to sustain cells' proliferation, migration and reorganization in microvascular structures.

The present thesis will focus on how, in Human Umbilical Vein Endothelial Cells (HUVECs), translation (the key molecular event in which a protein-coding mRNA is converted into a functional polypeptide) is affected by the typical biochemical conditions of the TME, namely hypoxia, low glucose levels, general nutrient starvation, high lactate concentration, pronounced oxidative stress, low pH and ECM stiffness.

To assess it, useful techniques to investigate translation, among which puromycin pulse labelling and polysome profile, will be presented.

2. SUMMARY (Thesis summary in italian).

Per angiogenesi si intende l'evento attraverso il quale nuovi vasi sanguigni si formano a partire da quelli preesistenti, sia in contesti fisiologici, sia in contesti patologici, nei quali l'angiogenesi avviene in misura insufficiente oppure eccessiva. Tale processo è fondamentale ai fini del mantenimento dell'omeostasi di organi e tessuti, in quanto garantisce che essi ricevano il sufficiente apporto di nutrienti, molecole segnale ed ossigeno necessari per l'espletamento delle fisiologiche funzioni cellulari: proprio la carenza di ossigeno, definita da una concentrazione locale del gas inferiore al 5%, rappresenta lo stimolo principale per l'innesco dell'evento angiogenico, in quanto stimola le cellule in ipossia a produrre e rilasciare ingenti quantità di molecole segnale e fattori pro-angiogenici, i quali agiscono prevalentemente sulle cellule endoteliali, il tipo cellulare costituente il versante interno della parete dei vasi sanguigni, stimolandone la riattivazione da uno stato di quiescenza ad uno di proliferazione, la migrazione e la secrezione di ulteriori fattori pro-angiogenici ad azione paracrina od autocrina.

L'angiogenesi è un processo caratterizzante anche la progressione dei tumori maligni, che ad oggi rappresentano la seconda causa di morte nei Paesi industrializzati, secondi solo alle malattie cardiovascolari: il motivo principale alla base della mortalità tanto elevata di questa classe di patologie, infatti, risiede nella loro capacità di invadere tessuti distanti dal sito d'origine, durante il cosiddetto processo di metastasi. Ai fini della propria metastatizzazione, infatti, le cellule della massa tumorale stimolano la formazione di nuovi vasi sanguigni in loro diretta prossimità, da utilizzare per diffondere a siti corporei distali; anche in questo caso l'evento angiogenico è innescato da una varietà di

molecole segnale prodotte non soltanto dalle cellule tumorali propriamente intese, ma anche da quelle del cosiddetto microambiente tumorale (dall'inglese "tumour microenvironment"), rappresentante il contesto biochimico e tissutale nel quale il tumore si sviluppa ed accresce.

Tale microambiente presenta delle peculiari caratteristiche chimico-fisiche che impattano direttamente sulla crescita, la proliferazione e la migrazione delle cellule tumorali ed anche delle cosiddette "Tumour Endothelial Cells", costituenti il versante interno della parete dei vasi sanguigni irroranti il tumore. Tra le caratteristiche biochimiche del microambiente tumorale rientrano l'ipossia, dovuta ad un'insufficiente perfusione vascolare della massa tumorale, specialmente nella sua porzione più interna; la bassa concentrazione di glucosio nei fluidi interstiziali, dovuta all'incontrollata proliferazione cellulare; l'elevata concentrazione di lattato e di specie reattive dell'ossigeno, nonché il basso pH extracellulare, causati dallo switch delle cellule maligne da un metabolismo prettamente ossidativo ad uno prevalentemente anaerobico e fermentativo; la secrezione di citochine e chemochine pro-infiammatorie, come TNF-α, IL-6 e TGF-β, promuoventi l'immunoevasione e l'angiogenesi tumorale; e la rigidità della matrice extracellulare, dovuta ad un'ingente secrezione di sue componenti da parte delle cellule tumorali.

Lo scopo della presente tesi è quello di analizzare come le condizioni sopracitate influenzano il processo della traduzione, definito come la conversione di un *protein-coding* mRNA nel rispettivo polipeptide funzionale, in HUVECs ("Human Umbilical Vein Endothelial Cells"), un sottotipo di cellule endoteliali primarie isolate dalla vena del cordone ombelicale; la traduzione, infatti,

rappresenta uno step fondamentale nella regolazione della biosintesi proteica, in quanto è tutt'altro che scontato che i trascritti sintetizzati dalla cellula in un dato istante vengano automaticamente tradotti in proteine.

Per analizzare l'attività traduzionale delle cellule HUVECs quando sottoposte alle tipiche condizioni del microambiente tumorale ho principalmente sfruttato la tecnica della "Puromicin Pulse labeling", basata sull'incorporazione della puromicina, analogo strutturale del tRNA trasportante l'amminoacido tirosina, all'estremità C-terminale di un polipeptide in progressivo allungamento; utilizzando in Western Blot un anticorpo primario diretto contro la puromicina, è possibile rilevarne il segnale, la cui intensità sarà direttamente proporzionale all'attività traduzionale cellulare.

Sempre tramite Western Blot, inoltre, ho testato i livelli proteici di alcuni effettori dei più importanti pathways cellulari coinvolti nella regolazione della traduzione, ovvero l'*mTORC1 pathway* e l'*Integrated Stress Response pathway*: il primo, quando stimolato, determina la fosforilazione e la conseguente attivazione di fattori proteici che, direttamente o indirettamente, compartecipano alla formazione di ribosomi funzionalmente attivi; il secondo, al contrario, determina, tra le altre cose, la fosforilazione del fattore di inizio della traduzione eIF2α, il quale, in questo modo, non è più in grado di promuovere la formazione del complesso di pre-inizio della traduzione. Le due vie si segnale sopracitate, quindi, giocano due ruoli opposti nel controllo della traduzione. Gli esperimenti condotti hanno evidenziato come la maggior parte delle condizioni biochimiche tipiche del microambiente tumorale, tra cui l'ipossia, il pH extracellulare acidico e la presenza di stress ossidativo,

determinino una generale inibizione dell'mTORC1 pathway e la genesi di stress intracellulare, i quali si riflettono in un abbassamento del rate di traduzione cellulare, evidenziato da un calo dell'incorporazione della puromicina nelle catene polipeptidiche nascenti.

Allo stesso tempo, tramite degli esperimenti di *real-time* PCR, ho verificato che le condizioni biochimiche sopracitate non impattano sul rate biosintetico di una specifica classe di trascritti, particolarmente importanti ai fini della traduzione: i *TOP mRNAs*, codificanti, tra le altre, tutte le 79 proteine ribosomiali umane necessarie alla costituzione di ribosomi funzionalmente attivi. In condizioni stressogene, infatti, i livelli intracellulari di questi trascritti non subiscono variazioni, a differenza delle rispettive proteine da essi codificate, che, diminuendo in quantità, determinando una riduzione del numero di ribosomi funzionalmente attivi ed un calo generale del rate traduzionale della cellula; ciò evidenzia come il controllo dell'espressione dei *TOP mRNAs* non sia di tipo trascrizionale, quanto, piuttosto, traduzionale.

Per confermare questo modello e vedere come la traduzione dei *TOP mRNAs* sia più o meno attiva in funzione delle diverse condizioni ambientali cui le cellule HUVECs vengono sottoposte, un'altra tecnica che ho utilizzata è il "Polysome profile", in cui, all'interno di un gradiente 15%-50% di saccarosio e sulla base del loro peso molecolare crescente, vengono separate le seguenti frazioni di lisato cellulare: mRNA non associati ad alcun ribosoma ("free mRNAs") e, quindi, virtualmente non espressi dalla cellula; mRNA associati ad un unico ribosoma, o monosomi ("monosomes", "subpolysomes"), , virtualmente tradotti dalla cellula ad un rate relativamente basso; mRNA

associati a due o più ribosomi, o polisomi ("polysomes"), virtualmente tradotti dalla cellula ad un rate tanto più elevato, quanto maggiore è il numero di ribosomi associati ad uno stesso trascritto. Nel mio caso, ho sottoposto a *polysome profile* dei lisati cellulari provenienti da cellule HUVECs in attiva proliferazione (stato caratterizzante le cellule endoteliali tumorali durante l'angiogenesi), in quiescenza (stato fisiologico caratterizzante le cellule endoteliali di strutture vascolari stabili) e sottoposte per 24 ore ad un trattamento di ipossia (una delle principali condizioni caratterizzanti il microambiente tumorale). I risultati ottenuti hanno evidenziato come, nelle cellule endoteliali proliferanti, i *TOP mRNAs* siano concentrati nella frazione polisomica, mentre in quelle quiescenti ed ipossiche siano arricchiti nella frazione monosomica: tali osservazioni sono in linea con le aspettative, in quanto, da un lato, la proliferazione cellulare richiede una sintesi proteica massiva e costante, mentre, dall'altro, gli stati di quiescenza ed ipossia determinano uno spegnimento del processo tramite l'inibizione dell'*mTORC1 pathway*.

Infine, il trattamento di cellule HUVECs proliferanti, mimanti una condizione di *stiffness* della matrice extracellulare, con due inibitori dei pathways di *sensing* della rigidità della matrice stessa, ha evidenziato come tale parametro non impatti sulla traduzione in HUVECs. L'incorporazione di puromicina all'interno delle catene polipeptidiche nascenti, infatti, non subisce variazioni quando HUVECs proliferanti vengono trattate con Y-27632, inibitore di *Rho-associated Protein Kinase (ROCK),* o con ML7, inibitore di *Myosin Light Chain Kinase (MLCK),* due chinasi importanti per il *sensing* cellulare della rigidità della matrice extracellulare.

3. INTRODUCTION.

3.1. Angiogenesis.

3.1.1. Definition of angiogenesis and description of its main characters: vascular endothelial cells (ECs).

Angiogenesis is defined as the formation of new blood vessels from pre-existing ones, beginning in utero (where the cardiovascular system is the first organ system developed by the embryo) and continuing through adulthood. It plays an essential role in the maintenance of tissue homeostasis, since thanks to the blood flow cells are able to receive oxygen, nutrients and endocrine signalling molecules, disposing at the same time of metabolic waste products such as carbon dioxide and nitrogen; at the same time, angiogenesis characterizes also some pathological conditions, in which it happens either too little or too much: for example, the insufficient compensatory formation of blood vessels in ischaemic tissues is a major problem in coronary heart disease, or stroke; on the other hand, excessive blood vessel growth is involved in the pathogenesis of diabetic proliferative retinopathy, or solid tumour growth. Cancer will be the pathological context in which the behaviour of endothelial cells (ECs), the cell type constituting the lining of the blood vessels and regulating the exchanges between the bloodstream and the irrorated surrounding tissues, will be investigated in the present thesis.

ECs can be considered the main characters in angiogenesis: they are characterized by a spindle-like shape and can be divided into four main subtypes, according to the type of vessels (blood or lymphatic) they constitute: venous, arterial, microvascular and lymphatic. During vessels formation, they typically align and elongate following the direction of the fluid flow, interacting between each other by different types of intercellular junctions, whose tightness varies depending on the anatomical compartment in which angiogenesis takes place: for example, in the liver endothelial cells form highly-permeable, discontinuous capillaries, called “sinusoids”, in which they are separated between each other by large 100 nm to 200 nm fenestrations; in the brain, on the opposite, tightly-interacting endothelial cells form the inner part of the blood-brain barrier, which prevents solutes in the circulating blood from non-selectively crossing into the extracellular fluid of the central nervous system; continuous endothelium is found also in most arteries, veins and capillaries of the skin, lung, heart and muscle.

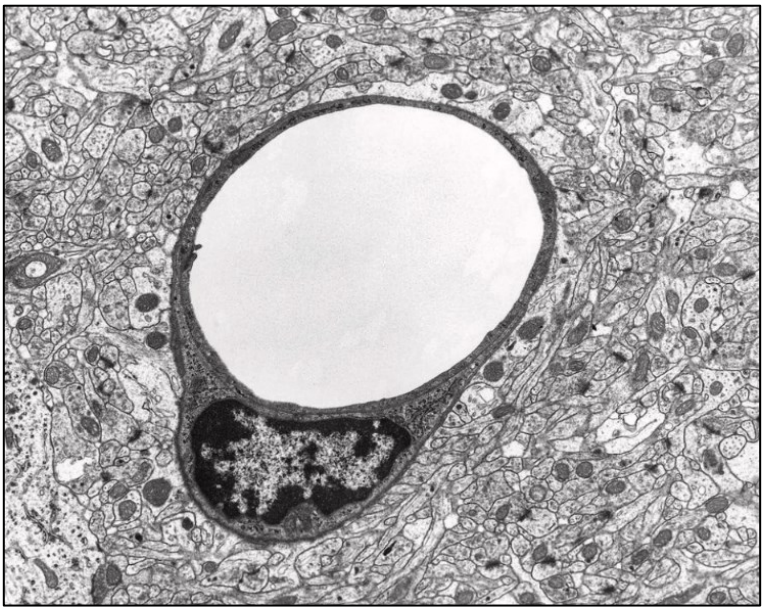


Figure 1. Capillary section analysed by transmission electron microscopy. *Endothelial cells (ECs) constitute the inner lining of the blood vessels, where they form a single cellular monolayer, characterized by a variable permeability, that regulates the exchanges between the blood and the interstitial fluids of surrounding tissues. Image taken from Dennis Kunkel Microscopy, Science Photo Library.*

In all vascular structures ECs interact with the underlying vascular basement membrane (BM), which is a specialized extracellular matrix, mainly composed by laminin, collagen IV, nidogen and heparan sulfate proteoglycans, that functions as a cellular anchorage site, a physical barrier and a signalling hub for the endothelium; for example, thanks to its collagen IV network, the vascular basement membrane ensures structural stability to the endothelium in the face of mechanical challenges; it regulates the transendothelial migration of immune cells from the bloodstream into tissues and the extravasation of cancer cells during metastasis; it engages integrin receptors on the surface of ECs, activating in this way signalling pathways involved in cell survival, differentiation, polarity and migration. For all these reasons, vascular basement membrane's stiffness, which depends on its constituents and on their relative abundance, is considered a fundamental regulator of endothelium homeostasis. Embedded in it and with a density that varies depending on the organ and vascular bed considered, pericytes constitute the mural cell type that wraps around ECs and interacts with them both trough physical contact and paracrine signalling, playing important roles in the formation of the vessel wall and the maintenance of its integrity and in the synthesis of the structural proteins constituting the vascular basement membrane.

From an embryonic point of view, vascular endothelial cells are thought to arise from the same precursors of haematopoietic cells: haemangioblasts, bipotential mesenchymal cells deriving from the splanchnopleuric mesoderm; at the beginning of vasculogenesis (which is the formation of primitive vascular structures during embryogenesis via the differentiation of endothelial precursors cells), haemangioblasts migrate towards a midline position just ventral to the notochord, where they are exposed to the gradients of several paracrine signalling molecules that address them towards an endothelial fate: among the most important ones, the secreted ligand Sonic hedgehog (Shh), the transmembrane Jagged and Delta-like ligands and Vascular Endothelial Growth Factors (VEGFs).[1]

Focusing on this last class of signalling molecules, VEGFs are secreted proteins with fundamental roles during both vasculogenesis and angiogenesis; in mammals, the family comprises 4 main members: VEGF-A, VEGF-B, VEGF-C and VEGF-D. VEGF-A, the first one to be discovered, is also the majorly involved in adult angiogenesis in the adult, both in healthy and in pathological conditions: its extracellular levels, typically low, increase during the development of the corpus luteus in pregnancy, the wound healing process and in all those diseases associated with neovascularization, including cancer.

Once produced and secreted by a variety of cell types, among which aortic vascular smooth muscle cells, keratinocytes, macrophages and many tumour cells, VEGF-A acts by binding to its tyrosine kinase receptors VEGFR1 and VEGFR2 on the surface of vascular endothelial cells;

VEGFR1 is expressed also on macrophages cell lines, facilitating their migration. VEGFR1 has a high affinity for VEGF-A (Kd = 1~10 pM), which is one order higher than that of VEGFR-2, whereas its tyrosine kinase activity is approximately 10-fold weaker than that of VEGFR2.[2] The major pro-angiogenic signal is thus generated from the ligand-activated VEGFR2, whereas the signalling cascade evocated by VEGFR1 hasn't been fully elucidated yet, but it's common thought that VEGFR1 acts as a decoy receptor, competitively reducing VEGF-A binding to VEGFR2 and therefore limiting the activity of VEGF-A pathway in the vascular endothelial cells.

Once engaged by VEGF-A, however, VEGFR2 undergoes an autophosphorylation event at the level of its cytoplasmatic domains, which creates a strong binding motif for the SH2 domain of the p85 subunit in the PI3-kinase complex, activating the PI3K/Akt/mTOR signalling pathways: the subsequent molecular events promoting angiogenesis are the stabilization of the HIF-1α subunit, the increased production of VEGF-A and the secretion of other pro-angiogenic signalling factors such as nitric oxide and angiopoietins; at the same time, survival, proliferation and migration of endothelial cells are enhanced.

Another important aspect regarding endothelial cells is their metabolism, which has only recently been recognised as a driving force of angiogenesis: ECs are characterized by a high glycolytic rate, comparable to the one in many cancer cells; in particular it has been estimated that, by converting glucose into pyruvate and then lactate, they produce the 85% of total ATP

molecules required for cellular activities, and further metabolic flux analysis revealed that, when compared to glucose oxidation (GO), fatty acid oxidation (FAO) and glutamine oxidation (QO), glycolytic flux was >200 higher.[3] On the opposite, GO doesn't seem to play a relevant role in energy production, in line with reports that endothelial cells have a smaller mitochondrial volume fraction (5%) than oxidative hepatocytes (30%) and their mitochondria serve more as signalling hubs rather than metabolic powerhouses.[4]

The fact that ECs prefer glycolysis rather than oxidative respiration to produce energy appears counterintuitive, if we think that they have easy access to high oxygen concentrations and that complete glucose oxidation leads to the production of 34 extra ATP molecules. But at the same time, fermentative glycolysis yields some advantages: first, it maximizes oxygen delivery to perivascular cells; second, when sprouting into avascular tissues, ECs become exposed to low levels of oxygen and glucose, but since glucose diffuses further away from vessels than oxygen, ECs can still rely on anaerobic glycolysis to form new vessels; third, by maintaining a low oxidative metabolism, ECs minimize the production of Reactive Oxygen Species (ROS), thereby providing protection against their high-oxygen milieu; fourth, glycolysis generates ATP more rapidly than oxidative metabolism, allowing ECs to meet the energy demands for dynamic rapid changes in motility; and finally, the glycolysis side pathways are necessary for the biosynthesis of macromolecules needed for cell mass duplication

during cell division, such as the Pentose Phosphate Pathway (PPP) and the Hexosamine Biosynthesis Pathway (HBP).

Beside glucose, fatty acids represent another fuel source for endothelial cells, that metabolize them to acetyl-coA to sustain the Krebs cycle and dNTPs synthesis (trough the conversion of acetyl-CoA into aspartate, a nucleotide precursor); more specifically, it has been estimated that Fatty Acid Oxidation (FAO) in ECs generates 5% of the total amount of required ATP, and this percentage increases to 40% when glucose is removed from the culture medium.[5]

3.1.2. Angiogenic mechanisms in physiological conditions.

Both in utero and in the adult, physiological angiogenesis can occur through two main, different processes: sprouting angiogenesis and intussusceptive, or splitting, angiogenesis. In the first case, new sprouts composed by endothelial cells arise from pre-existing vessels and grow towards an angiogenic stimulus, represented, in the majority of cases, by VEGF-A; the leading edge of each sprout, exposed to the highest concentrations of the pro-angiogenic stimulus, is constituted by migrating tip cells, that secrete large amounts of proteolytic enzymes (especially metalloproteinases) to digest a pathway trough the extracellular matrix and that extend long filopodia endowed with VEGF-A receptors, fundamental for actin cytoskeleton contraction and for pulling tip cells towards the pro-angiogenic molecules.

Tip cells are followed by proliferating endothelial stalk cells, responsible for the elongation of the sprout and destined to become the trunk of the newly formed capillaries. The differentiation and the relative amounts of tip and stalk cells are tightly regulated by the Delta-Notch cell-to-cell signalling pathway: high VEGF-A concentrations induce the expression of Delta-like ligand on the plasma membrane of tip cells; the molecule binds to the Notch receptors expressed on adjacent stalk cells, resulting in the downregulation of VEGFR2 expression, the upregulation of VEGFR1 and the dampening of the migratory abilities of stalk cells.

Sprouting angiogenesis ends when the tip cells of two or more capillary sprouts converge at the source of VEGF-A secretion, fuse together and create a continuous lumen, or anastomosis, through which oxygenated blood can flow. Once the tissue is appropriately re-vascularized, local parenchymal cells stop to secrete VEGF-A; the maturation and the stabilization of newly formed capillaries requires the subsequent recruitment of pericytes and the deposition of new extracellular matrix, along with shear stress (defined as the tangential force of the flowing blood on the endothelial surface of the blood vessel) and other mechanical signals.[6]

However, endothelial tip and stalk cells specification doesn't constitute a permanent cell fate selection, but, on the opposite, a rather dynamic process: after the formation of an anastomosis, in fact, previous tip cells acquire a stalk cell phenotype, so that previously inhibited stalk cells are relieved from the lateral inhibition and can become new tip cells. Moreover,

endothelial cells in the extending sprouts seem to continuously compete between each other for the tip position, leading to frequent exchanges of the tip cell itself; also, this phenomenon seems to be regulated by the Delta-Notch signalling pathway.

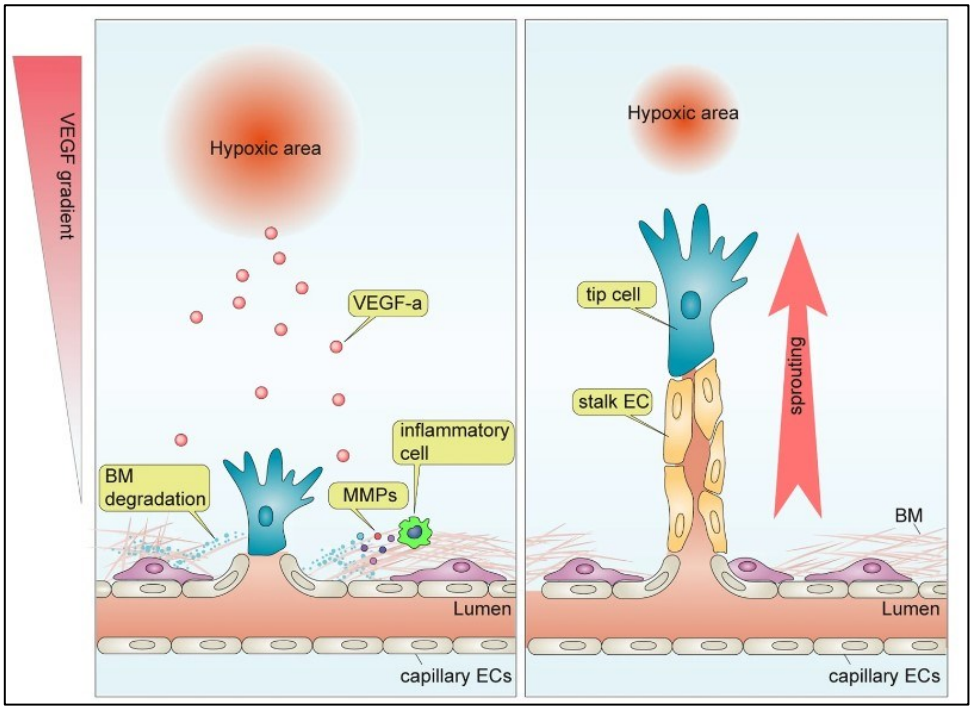


Figure 2. Sprouting angiogenesis. *During sprouting angiogenesis, the exposure to a gradient of pro-angiogenic molecules, such as VEGFs, determines the specification of endothelial cells (ECs) into different subtypes: tip cells, constituting the leading edge of each sprout and very motile; and stalk cells, sustaining vessels growth through their high proliferative abilities. Image taken from Lee HW. et al., "Flow goes forward and cells step backward: endothelial migration.", Experimental Molecular Medicine,* ***54****, 711–719 (2022).*

On the other hand, intussusceptive, or splitting, angiogenesis, is particularly important during embryonic development, where it contributes to form new capillary structures and plays a major role in the formation of arteries and veins' bifurcations. In this process the vessel wall extends towards the

internal lumen, causing a single vessel to split in two: more in details, ECs located oppositely to each other in the vessel wall protrude into the lumen and get in direct contact, initially forming an intraluminal, cylindrical tissue bridge surrounded by ECs; such structure contains elements of interstitial tissue, mainly represented by cytoplasmatic extensions of myofibroblasts, and is framed by the cytoplasmatic processes of pericytes; later on the slender tissue pillar grows into a proper intercapillary mesh, thanks to the deposition of new extracellular matrix's elements by pericytes.[7] All these events taking place during intussusceptive angiogenesis are considered faster and more efficient than sprouting angiogenesis, because, at least initially, they require only the reorganization of already existing endothelial cells, not their proliferation.

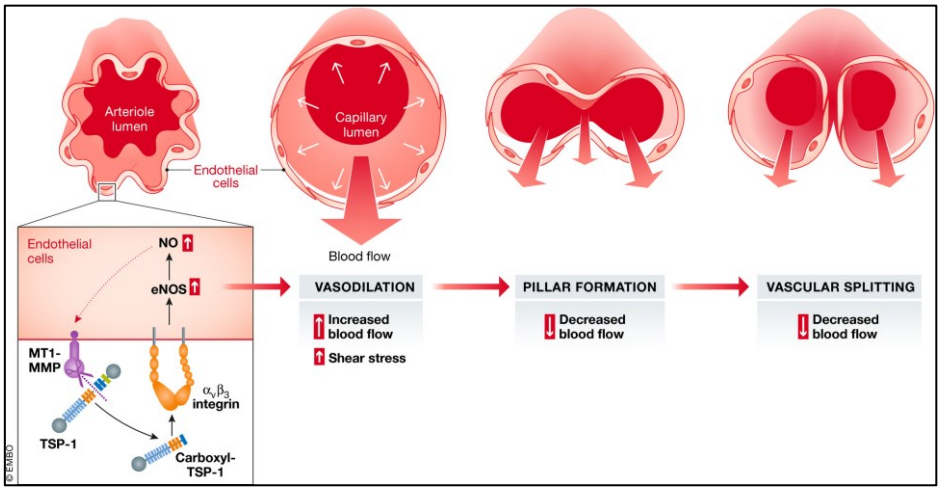


Figure 3. Splitting, or intussusceptive, angiogenesis. *During splitting angiogenesis, new blood vessels derive from the splitting of pre-existing ones: oppositely localized endothelial cells converge towards each other, forming an intraluminal pillar that progressively grows into an intercapillary mesh, thanks to the deposition of new extracellular matrix's components by pericytes. Image taken*

from D'Amico G. et al., "Splitting the matrix: intussusceptive angiogenesis meets MT1-MMP", EMBO Molecular Medicine, 2012.

3.1.3. Angiogenic mechanisms in cancer.

In cancer, the observation that rapidly growing tumours were highly vascularized, while dormant ones were not, led Judah Folkman to propose, in 1971, that initiation of tumour angiogenesis is required for tumour progression.

In general, vascular homeostasis is regulated by a variety of pro- and anti-angiogenic molecules, that, when in balance, keep endothelial cells in a quiescent and non-proliferative state; however environmental factors characterizing tumour microenvironment, such as hypoxia and chronic inflammation, stimulate the so-called event of the "angiogenic switch", in which the large amounts of pro-angiogenic molecules produced by the tumour, such as Vascular Endothelial Growth Factors, basic Fibroblast Growth Factor (bFGF), Platelet-Derived Growth Factor (PDGF), Angiopoietin 1 and 2 (ANG-1/2), activate endothelial cells proliferation, migration and reorganization, causing also important changes in their metabolism.

Once that the angiogenic switch has occurred, tumour vascularization starts, not only trough sprouting or intussusceptive angiogenesis, but also trough other mechanisms typically induced by cancer: among them, normal blood vessels co-option, the recruitment of circulating Endothelial Progenitor Cells (EPCs) and vascular mimicry.

In normal blood vessels co-option, cancer cells infiltrate the tissue space between the pre-existing vessels, ultimately leading to their incorporation into the tumour, without needing to stimulate new vessels' growth. The molecular mechanisms underlying these events are still poorly understood, even if studies from brain tumours suggest that soluble factors, such as bradykinin, CXC-chemokine receptor 4 (CXCR4)-binding cytokine and Wnt 7a/b, are critical for cancer cell co-option;[8] moreover, many cancer cells express adhesion molecules, especially integrins, that facilitate their attachment to the vascular surface.

Tumoral cells are also able to stimulate the mobilization from the bone marrow of endothelial progenitor cells, characterized by the expression of the hematopoietic marker CD34, the stem cell marker CD133 and the endothelial marker Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2), and capable of differentiating into mature endothelial cells.[9] More specifically, tumours secrete in the circulation mobilizing cytokines, such as VEGFs, Stromal Cell-Derived Factor 1 (SDF-1) and Granulocyte Colony-Stimulating Factor (G-CSF), that reach the bone marrow and stimulate the release of endothelial progenitor cells from their stem-cell niche; these molecules also work as chemoattractants to guide EPCs' migration towards tumour microvessels, their extravasation into the interstitium, terminal differentiation into mature endothelial cells and final incorporation into the neo-vessels of the tumoral mass. The recruitment of endothelial progenitor cells isn't stimulated only by tumours, but also by other pathological as well as physiological conditions, such as critical ischemia and intense physical exercise.

Finally, vasculogenic mimicry is the process through which highly-aggressive cancer cells are able to organize themselves in tubular, vascular-like structures to obtain nutrients and oxygen independently from normal blood vessels co-option and angiogenesis; the molecular mechanisms guiding vasculogenic mimicry are still largely unknown, but gene expression analysis on cancer cells undergoing this event revealed a unique and heterogenous gene profiles, characterized by the upregulation of genes typically expressed by epithelial cells, endothelial cells and fibroblasts.

3.2. The Tumour Microenvironment (TME).

3.2.1. The tumour microenvironment as an essential player in cancer onset and tumoral angiogenesis: cellular and non-cellular composition.

Cancer is a major public health problem worldwide, representing the second leading cause of death in developed countries, after cardiovascular diseases. At the beginning of January 2022, in fact, the American Cancer Society estimated that 1,918,030 new cancer cases and 609,360 cancer deaths would occur that year in the United States, with approximately 350 deaths per day caused by lung cancer, the cancer type with the overall highest mortality.[10]

For this reason, biomedical research on the histological features, molecular mechanisms and risk factors underlying tumour malignancies has extensively been made over the past century, and during the last two decades it has become clearer and clearer that, despite deriving *in primis* from

genetic and epigenetic alterations influencing the expression and activity of proto-oncogenes and tumour suppressor genes, cancer onset and progression are deeply influenced also by the surrounding tissue context in which the tumour arises: the so-called “tumour microenvironment” (TME). A tumoral mass, in fact, doesn’t comprise only transformed cells, but also a variety of other cellular and non-cellular elements, that interact with them and between each other in a paracrine or autocrine way, stimulating tumour growth and metastasis. Cellular components of the TME are immune cells, belonging both to the innate and adaptive immune system, and stromal cells, such as Cancer-Associated Fibroblast (CAFs), Tumour Endothelial Cells (TECs) and other cell types typical of the tissue or organ in which the tumour grows, such as adipocytes for breast cancer and stellate cells for liver cancer.

Focusing on the immune component of the tumour microenvironment, its cells can play both a tumour-suppressing or a tumour-promoting role, depending on the global secretome landscape of the TME itself: among the tumour-promoting immune cells that can be found here, some of the most important are Tumour-Associated Macrophages (TAMs), Myeloid-Derived Suppressor cells (MDSCs) and Regulatory T cells (Treg cells), whose action is counteracted by tumour-antagonizing immune cytotoxic $CD8^+$ T cells, $CD4^+$ T cells, and natural killer cells (NK cells).

The existence of a possible relationship between immune cells in the TME and angiogenesis has been investigated since the ’90, starting from the observation that TAMs were often found in the surrounding areas of tumoral

blood vessels and that their density was directly correlated to the perfusion levels of the tumoral mass. Further studies then revealed that macrophages are typically attracted towards the hypoxic core of a malignancy due to the secretion, by cancer cells, of hypoxia-induced chemoattractants, such as VEGF, endothelin, endothelial monocyte activating polypeptide II (EMAP II) and CCL2; such hypoxic microenvironment then promotes the metabolic switch of normal macrophages into TAMs, characterized by the stable expression of Hypoxia-Inducible Factor 1 and 2 (HIF-1, HIF-2) and by an upregulated expression of Vascular Endothelial Growth Factor A (VEGF-A), which works as a potent mitogen for endothelial cells by binding to its tyrosine kinase receptors 1 and 2 (VEGFR1, VEGFR2).[11]

Myeloid-Derived Suppressor Cells (MDSCs) are the progenitors of myeloid cells, characterized by a prominent immunosuppressive role exerted trough the inhibition of dendritic cells maturation and of T lymphocytes and natural killer cells activity; MDSCs also contribute to tumoral angiogenesis by synthetizing and releasing in the ECM metalloproteinases (in particular MMP-9) that increase the bioavailability of VEGFs in the tumour microenvironment, and the Bv8 prokineticin protein, that, by binding to its receptors EG-VEGRF/PKR-1 and EG-VEGFR/PKR-2 on the surface of endothelial cells, work as potent mitogen.

Finally, another immune cell type part of the TME and important in tumour angiogenesis is represented by N2-polarized neutrophils, particularly abundant in the invasive front of the tumoral mass and releasing in the TME large amounts of MMP-9 and of genotoxic reactive oxygen/nitrogen species

that contribute to the genetic alterations associated with tumour progression. N2-polarized macrophages (whose transdifferentiation is mainly induced by the TGFβ produced by immune and cancer cells) also sustain angiogenesis trough the release of pro-angiogenic signalling molecules, such as VEGF and the CXCL8/CXCL1 chemokines, whose production is independent from the oxygen levels in the tumoral mass (conversely from Tumour-Associated Macrophages), but rather correlates with TNF-α exposure.

On the other hand, TME is composed also by non-cellular elements that are shared between all cancer types, among which the most important two are exosomes and the extracellular matrix (ECM).

Exosomes are membranous vesicles containing specific proteins, microRNAs and other factors that cancer cells produce and address towards local and/or distant tissues' microenvironments, in order to remodel them and make them more prone in sustaining tumour growth and metastasis; in particular, exosomes' components promote tumour immunoevasion, epithelial-to-mesenchymal transition and angiogenesis. In relation to this last event, in fact, recent studies demonstrated that exosomes transport numerous pro-angiogenic signalling molecules, such as Vascular Endothelial Growth Factors (VEGFs), Fibroblast Growth Factor (FGF), annexin II (AnxII), matrix metalloproteinases (MMPs), and microRNAs that suppress the expression of HIF-1 inhibitors, such as miR-135b, miR-210,and miR-21, stimulating the production of pro-angiogenic factors by cancer cells and cells of the TME.

Moreover, recent studies demonstrated that the uptake of tumour-derived exosomes by normal endothelial cells activates pro-angiogenic signalling pathways and stimulates new blood vessels formation: in particular, *in vitro* studies evidenced that, after being released by cancer cells, exosomes are internalized by normal ECs within 2-4 hours, initially directed towards the perinuclear zone and later transported on microtubules towards cellular periphery, before being moved to adjacent endothelial or TME's cells.[12]

The extracellular matrix, or ECM, is another fundamental component of the TME, since its biological composition (made out by polymers such as collagen, fibronectin, laminins, proteoglycans and polysaccharides) and mechanical properties directly affects tumoral cells growth and behaviour. Cancer ECM is typically more abundant, dense and stiff than the one of healthy tissues, due to the fact that Cancer-Associated Fibroblasts (the cell type synthetizing the vast majority of tumoral ECM, deriving from the transdifferentiation of a variety of cell types in the TME when exposed to TGF-β, FGF-2, PDGF, EGF and TNF-α) produce more ECM proteins than normal fibroblasts.

Thanks to these features, the rigid tumour ECM acts a diffusion barrier that protects cancer cells from therapeutically effective doses of anti-cancer drugs, but it also reduces the diffusion of nutrients and oxygen to cancer cells, exacerbating their stress responses and favouring the positive selection of those ones that become able to overcome senescence and apoptosis.

Such pro-survival signalling responses are also promoted by increased cells-to-ECM interactions: an interesting example regarding this point is represented by the Hippo pathway, an evolutionarily conserved kinase cascade regulating cells survival, apoptosis, differentiation, growth and proliferation, whose activation is modulated, among others, also by mechanical cues. In mammals, the core of the Hippo pathway is represented by four serine-threonine kinases: Mammalian Sterile 20-related 1 and 2 kinases (MST1 and MST2 and Large tumour suppressor 1 and 2 kinases (LATS1 and LATS2), that, once activated trough a phosphorylation cascade, phosphorylate two oncogenic transcriptional co-activators, the Yes-associated protein (YAP) and the co-activator with PDZ-binding motif (TAZ), inhibiting their translocation from the cytosol to the nucleus. Such confinement of YAP and TAZ in the cytosol prevents them from assembling into functional nuclear transcriptional complexes with TEA domain proteins 1–4 (TEAD1–4), inhibiting the expression of target genes stimulating cell proliferation, growth, survival and migration, such as CYR61, CTGF, AREG, MYC, Gli2, Vimentin and AXL. Mutations in the Hippo pathway proteins that lead to YAP or TAZ hyperactivation cause ectopic cell proliferation; moreover, several studies have reported the deregulation of the Hippo pathway in a broad range of different human carcinomas (including lung, colorectal, ovarian, liver and prostate cancers), in which immunohistochemical analysis detected an accumulation of YAP and TAZ in the nuclei of tumour tissue, significantly correlated with poor prognosis of patients.[13]

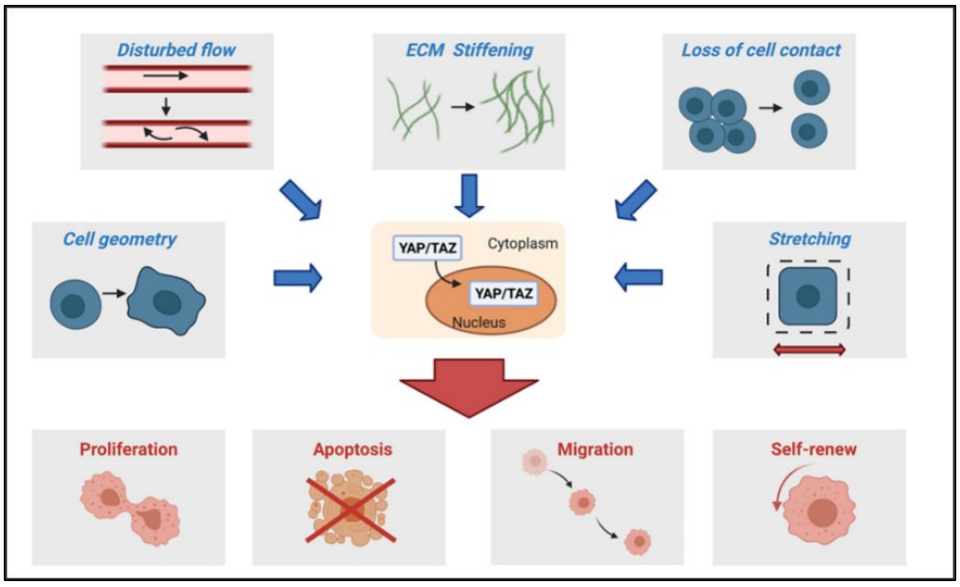


Figure 4. Main stimuli regulating the Hippo signalling cascade and biological consequences of its activation. *The Hippo signalling cascade is regulated by a variety of non-mechanical and mechanical cues: among the second ones, irregular blood flow, extracellular matrix stiffening, loss of cell-to-cell contact and changes of cellular geometry, such as cellular stress, promote the nuclear translocation of YAP/TAZ transcriptional co-activators, with the subsequent stimulation of cell survival, proliferation and migration. Image taken from Xiaomin C. et al., "Mechanoregulation of YAP and TAZ in Cellular Homeostasis and Disease Progression", Frontiers of Cellular and Developmental Biology, 2021.*

Such hyperactivation of YAP and TAZ in cancer cells has been associated, at least in part, to tumoral ECM stiffness, which causes an increase in the traction forces between cellular integrin receptors and components of the extracellular matrix, leading to the activation, among others, of Rho-associated protein kinase (ROCK) and Myosin Light Chain Kinase (MLCK).[14] Both proteins are serine/threonine-specific kinases that play fundamental roles in cell migration, adhesion and epithelial/endothelial barrier formation, by regulating actin-myosin cytoskeleton rearrangements;

they directly phosphorylate regulatory myosin light chain (MLC), fundamental for the generation of the actin/myosin-mediated contractile force required for cell migration; in addition, ROCK also interacts with specific substrates involved in reorganization of the cell cytoskeleton such as LIM kinase (LIMK), adducin, and vimentin, which can lead to actin reorganization, focal adhesion, and stress fibres formation.. For these reasons, both proteins are important for regulating cell response to ECM stiffness.

In the present thesis, to test if ECM stiffness impacts on endothelial cells translation in HUVECs, cells in ECM stiffness condition were treated, separately, with Y-27632 and with ML-7 inhibitors, two molecules that block, respectively, ROCK and MLCK activities by competing with ATP for their binding., preventing endothelial cells from sensing ECM stiffness and, thus, mimicking a condition of ECM softness. Subsequent analysis of translation in HUVECs was then performed, using, in parallel, YAP cytosolic or nuclear localization as a control indicator of ECM density (YAP, in fact, localizes in the nucleus when cells are exposed to ECM stiffness, whereas it accumulates in the cytosol when cells are exposed to ECM softness).

3.2.2. Biochemical features of the tumour microenvironment driving cancer progression.

Apart from its cellular and non-cellular composition, another relevant aspect regarding the Tumour Microenvironment is its unique biochemical asset, which stimulates tumour growth and is directly associated with its metabolism: important elements are, in fact, hypoxia and general nutrient starvation, due to an initially insufficient vascular perfusion, especially in the core of the tumoral mass; decreased glucose levels, due to the uncontrolled cell proliferation; high lactate levels, high radicals levels and acidity, due to the metabolic switch of cancer cells from oxidative respiration to fermentative glycolysis and to the limited removal of acidic waste products.

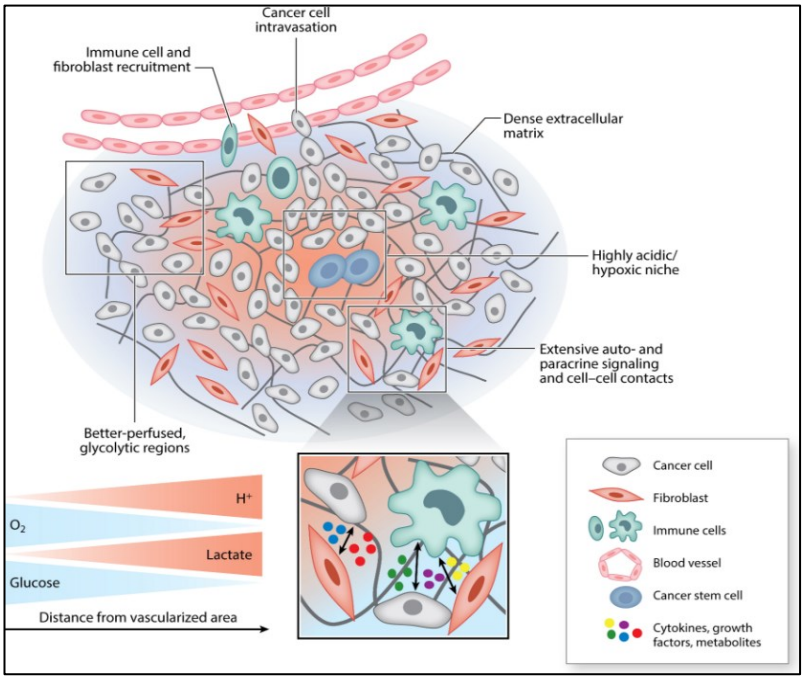


Figure 5. Biochemical features characterizing the tumour microenvironment. *The tumour microenvironment presents some unique biochemical features that directly affect cancer cells' growth, proliferation and migration. Among them, some of the most important are hypoxia, low glucose levels and general nutrients starvation, high lactate and free radical's levels, extracellular matrix stiffness and*

low extracellular pH. Image taken from Liping L. et al., "Regulation of Transcription and Translation by hypoxia", Cancer Biology and Therapy, 2020.

Hypoxia can be divided into "severe", when the local oxygen concentration is inferior to 0.02%, and "mild", when the same parameter acquires a value between 0.5% and 5%; when the diameter of a solid tumour is inferior than 1 mm, cancer cells rely on permeation to acquire nutrients from interstitial fluids, but, as the tumour grows, the rapid expansion of tumour cells exceeds the local blood supply, with the establishment of an hypoxic area mainly localized in the core part of the malignant mass. Here, cancer cells undergo a metabolic adaptation to low oxygen levels through the stabilization of HIF-1α, one of the two subunits of Hypoxia-Inducible Factor 1 (HIF-1) transcription factor, involved in the transcriptional induction of genes that participate in angiogenesis, iron and glucose metabolism, and cell proliferation/survival. HIF-1 consists of two different subunits: the nuclear, constitutively expressed HIF-1β, and the cytosolic, oxygen sensitive HIF-1α, whose stability depends on post-translational modifications such as hydroxylation, ubiquitination, acetylation and phosphorylation.

More specifically, in normoxic conditions oxygen-dependent prolyl hydroxylases enzymes 1, 2 and 3 (PHD1-3), in the presence of molecular oxygen, catalyse the hydroxylation of HIF-1α at the level of proline 402 and proline 564, allowing the association of HIF-1α subunit with the Von Hippel-Lindau (pVHL) tumour suppressor protein, part of the ubiquitin E3 ligase protein-complex; as a result of this interaction, the subsequent ubiquitination of HIF-1α leads to the proteasomal degradation of the

subunit itself. On the other hand, in hypoxic conditions the lack of oxygen, essential cofactor of PHDs, prevents these molecular events from happening, leading to HIF-1α stabilization and migration from the cytosol to the nucleus, where the protein associates with HIF-1β to form the heterodimeric, functional HIF-1 transcription factor. HIF-1 is then able to bind to target DNA sequences characterized by the presence of the so called "Hypoxia Responsive Elements", or HREs, in their promoter regions: among them, the most relevant in cancer development are genes encoding angiogenic factors (such as VEGFs, TGF-B3 and LRP1), proliferation/survival factors (such as cyclin G2 and IGFs), glucose transporters (such as GLUT1) and glycolytic enzymes (such as HK1/2 and PFKBF3). In particular, in Tumour Endothelial Cells (TECs), the cell type constituting the lining of tumoral blood vessels, hypoxia and the subsequent high levels of tumour-derived VEGFs induce an increased expression of 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3 (PFKFB3) by Tumour Endothelial Cells (TECs); this isoenzyme synthetizes fructose-2,6-bisphosphate (F2,6P_2), an allosteric activator of 6-Phosphofructo-1-Kinase (PFK-1), which catalyses a rate-limiting checkpoint of the glycolysis flux (the conversion of fructose-6-phosphate to fructose-1,6-biphosphate); for these reasons, induction of PFKFB3 in TECs determines a doubling of the glycolytic flux, particularly in tip cells. VEGF exposure also increases the expression of those genes codifying for glucose transporters (GLUT1 and GLUT3) and glycolytic enzymes such as aldolase, enolase or lactate dehydrogenase (LDH-A), determining an overall increase of glucose uptake and fermentative metabolism in tumour endothelial cells.

It's also important to know that cells' exposure to some chemical compounds can mimic the effects of hypoxia on HIF-1α stabilization: regarding this topic, one of the most widely used molecules in research is DyMethylOxalGlycine (DMOG), a glycine derivative whose structure resembles the one of 2-oxoglutarate, an essential cofactor for PDHs. When catalysing the hydroxylation of HIF-1α in normoxic conditions, in fact, these enzymes transfer one oxygen atom from O_2 to HIF-1α proline residues, whereas the second oxygen atom reacts with 2-oxoglutarate, generating succinate. By occupying the PHDs' site at the level of which 2-oxoglutarate would bind, DMOG works as a competitive inhibitor of PHDs, leading to HIF-1α stabilization even in the presence of high oxygen levels; in primary cells, in particular, such effects of DMOG can be observed after a 24 hours-long treatment when the chemical is used at a 1-2 mM concentration.[15]

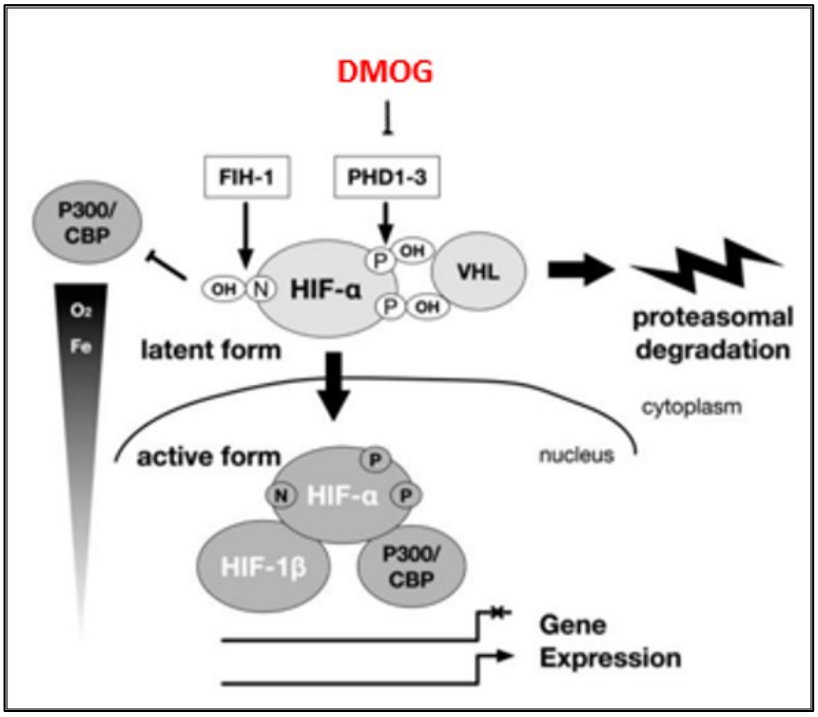


Figure 6. Mechanism of action of dimetyloxalglycine (DMOG). *Dimethyloxalglycine (DMOG) works as a competitive inhibitor of prolyl-hydroxylases (PHDs), the oxygen-dependent enzymes that catalyse the hydroxylation of HIF-1α at the level of two of its proline residues, leading to the ubiquitination and subsequent proteasomal degradation of the subunit. By inhibiting PHDs, DMOG stabilizes HIF-1α and allows its migration from the cytosol to the nucleus, where HIF-1α associates with HIF-1β subunit, forming the functional HIF-1 transcription factor. Image modified from Hirota et al., "HIF Prolyl hydroxylase inhibitors and their implications for biomedicine: a comprehensive review.", Biomedicines, 2021.*

Due to hypoxia, cancer cells adapt their metabolism from an oxidative to a mainly glycolytic one, exhibiting high levels of glucose uptake, thanks to the overexpression of transmembrane glucose transporters belonging to the GLUT family; this phenomenon has also been used for the prognostic and diagnostic of a wide range of cancers, using radio-labelled glucose analogues. For this reason, glucose concentration in the interstitial fluids of a tumoral mass spans around 0.2 mM,[16] a much lower value compared to the physiological 5.5 mM that can be found in the blood and in the interstitial fluids of healthy tissues.[17]

In contrast, the tumour microenvironment exhibits high lactate levels, spanning between 10-30 mM (whereas in healthy tissues such concentration falls in the range between 1.5-3 mM[18]): the metabolite mainly comes from the reduction of pyruvate, the final product of glycolysis, during the so called "lactic fermentation" process, in which, in

parallel, NADH is oxidized to NAD+, indispensable for glycolysis and other catabolic pathways to proceed.

Being lactate a weak acid characterized by a dissociation constant of 8.3 x 10^{-4}, its accumulation in the tumour microenvironment contributes to acidosis: the pH of the TME, in fact, spans around 6.8 units, in contrast to the physiological 7.4 of the blood and of the interstitial fluids of healthy tissues.[19] Apart from lactate production and secretion by cancer cells, such low pH value depends also on hypoxia, responsible for the switch of cancer cells' metabolism from an aerobic to an anaerobic one, and on inefficient blood perfusion, which prevents the elimination of acidic metabolic waste products from the tumoral mass. Apart from its role in the acidification of the TME, lactate also promotes tumour progression and spread through a variety of mechanisms, among which immunosuppression and stimulation of angiogenesis: for example, Fisher et al. demonstrated that lactic acid suppresses T lymphocytes proliferation and tumour-suppressing cytokines release.[20]

Moreover, lactate also works as an endogenous ligand for G Protein-coupled Receptor GPR81, overexpressed by a variety of cancer cell lines; once activated, this receptor induces the expression of Programmed Cell Death Ligand 1 (PD-L1) on the surface of cancer cells, thus inhibiting anti-tumour T lymphocytes activation and leading, on the opposite, to their apoptosis. Finally, it has been demonstrated that lactate also stimulates angiogenesis by activating the NF-KB signalling pathway, which leads to

an increase in the transcription of those genes codifying for VEGF and bFGF.[21]

High levels of superoxide, hydrogen peroxide and hydroxyl radicals are another unique feature of the tumour microenvironment, where these reactive oxygen species accumulate after being produced by both cancer cells and cellular components of the TME, especially myeloid-derived suppressor cells and tumour-associated macrophages. Hypoxia and other factors impairing oxidative respiration, such as mitochondrial DNA mutations in several genes important for the function of the electron transport chain (detected mainly at the level of complex I and III), are the main cause of high ROS production. ROS promote tumour growth when their levels reach super-physiological concentrations without leading to apoptosis and autophagy of cancer cells, and their tumour- promoting roles rely mainly on the induction of oxidative damages to DNA, the stimulation of normal fibroblasts' differentiation into CAFs and immunosuppression. ROS also have a crucial role in promoting tumoral angiogenesis trough 2 different mechanisms: the VEGF-dependent and the VEGF-independent pathways.[22] In the first case, exogenous ROS enter endothelial cells, activate the PI3K/Akt signalling pathway and increase the transcription of VEGFs genes; further, VEGFs also induces endogenous ROS production through the activation of NADP oxidase enzyme, which catalyses the transfer of electrons from NADPH to molecular oxygen. In the second case, elevated ROS levels inactivate prolyl hydroxylase enzymes (PHDs), preventing them from addressing the HIF-1α subunit to proteasomal

degradation; moreover, ROS also activate the NF-KB transcription factor, which increases the production rate of HIF-1α mRNA. Taken together, this evidence highlights how high ROS levels in the TME increase HIF-1α stability and levels, with a subsequent upregulation of its pro-angiogenic, target genes.

3.3. Translation.

3.3.1. The translation process in eukaryotes: main molecular events and factors involved.

The central dogma of molecular biology states that the genetic information flows from DNA to mRNA, during transcription, and from mRNA to proteins, during the translation process; the final result of these events is a global cellular proteome that determines cellular phenotypes and functions, and whose composition varies in response to intra- as well as extra-cellular stimuli.

Such variations, in particular, depend not only on genetic control of protein biosynthesis (which means that, in each condition and moment, the cell "chooses" which genes to express and in which amount), but also on post-transcriptional and post-translational control of protein biosynthesis: post-transcriptional modifications, such as splicing, the addition of a 5'-CAP and a 3' poly-A tail, and RNA editing, occur at the mRNA level and help to stabilize transcripts in order for them to be translated more efficiently; post-translational modifications, on the other side, modify an existing functional group or introduce a new one in an already synthetized polypeptide, in order

to regulate its activity, cellular localization and interaction with other molecules.

Focusing on translation, this process can be divided into four main stages: initiation, elongation, termination and, finally, ribosome recycling. Translation initiation happens, for most of the eukaryotic transcripts, in a CAP-dependent manner, with the assembly of a so-called "43S pre-initiation complex" (PIC), made out by the small (40S) ribosomal subunit, the initiator methyonil-tRNA(Met-$tRNA_i$, and the GTP-bound Eukaryotic Initiation Factor 2 (eIF2), near the 5′-7-methylguanosine CAP of an mRNA; such binding is favoured by the action of several Eukaryotic Initiation Factors (eIFs), among which one of the most important is eIF4F, a ternary complex made out by a CAP-binding domain for transcript binding (eIF4E), an RNA helicase domain for mRNA structure stabilization (eIF4A), and a scaffold domain (eIF4E) for the interaction with Poly-A Binding Proteins (PABP), a group of proteins that, by binding to the poly-A tail of a transcript and interacting with eIF4E at the same time, promote mRNA circularization, which favours transcript stabilization and the subsequent ribosome recycling.

After being assembled, the 43S PIC begins scanning the 5'-end of the transcript in the 5' to 3' direction, looking for a proximal starting AUG codon, located in a suitable sequence context; after finding it, the base-pairing interaction between the anticodon of Met-$tRNA_i$ and the AUG in the Peptidyl-tRNA (P) site of the 40S ribosomal subunit causes the arrest of the scanning complex and the joining of the large 60S ribosomal subunit, recruited by eIF5B; in this way, the 80S Initiation Complex (IC) is

assembled and ready to begin the elongation phase of translation. During this phase, the nascent polypeptide chain is extended of one amino acid per cycle trough repeated steps of transcript decoding, peptidyl transfer and tRNA-mRNA translocation. More specifically, translation elongation starts with the entry of a second aminoacylated-tRNA in the Aminoacyl (A) site of the 80S ribosome; if proper base-pairing between the three bases of the mRNA codon and those of the aminoacylated-tRNA (aa-tRNA) anticodon is established, structural changes stabilizing the aa-tRNA-mRNA interaction happen, followed by the formation of a peptide bond between the aminoacids in the P and in the A sites, catalysed by the Peptidyl Transfer Center (PTC) of the ribosome.

After that, the peptidyl-tRNA is moved from the A to the P site, and the deacylated tRNA in the P site is moved to the E ("exit")-site; at the same time the mRNA is ordinately translocated by one codon.

As for translation initiation, also elongation is characterized by the intervention of Eukaryotic Elongation Factors (eEFs), among which some of the most relevant are eEF1A, that mediates the entry of a new aa-tRNA in the A site of the ribosome, and eEF2, that facilitates the translocation of the tRNA-mRNA complex between the three different ribosomal sites. Termination of translation elongation happens when a stop codon (UAG, UAA and UGA in eukaryotes) enters the A site of the ribosome, where it is recognised by the eukaryotic Release Factor 1 (eRF1), part of the ternary complex eRF1/eRF3/GTP; after GTP hydrolysis by eRF3, eRF1 triggers the hydrolysis of the polypeptidyl-tRNA, releasing the completed protein

product and leaving an 80S ribosome still bound to the transcript and in need of being disassembled in order for new translation rounds to occur.

During the subsequent "ribosome recycling process", in fact, the large 60S ribosomal subunit is dissociated from the small one by ABCE-1, a protein belonging to the ATP-binding cassette family, and later on also the 40S subunit is detached from the transcript by a subset of canonical initiation factors, that, in this way, starts to re-prepare the small ribosomal subunit for a new round on transcript binding and translation initiation.

3.3.2. Molecular mechanisms and signalling pathways controlling translation in eukaryotes.

Translation is a key step in gene expression, and because of its sensitivity, flexibility and immediacy, it is tightly regulated by the cell through two main mechanisms, that act by modifying the rates at which mRNAs are translated into proteins: global control and mRNA-specific control of protein biosynthesis.

In the first case, as the name suggests, the translation of a large fraction of the mRNAs produced by the cell is modulated in a coordinated way, mainly through covalent modifications (especially phosphorylations) exerted on eukaryotic initiation factors (eIFs) or on their interactors.

On the other hand, mRNA-specific control of protein biosynthesis alters the translation rate of specific transcripts, usually characterized by the presence of unique sequences or of secondary/tertiary structures in their 5' or 3'-unstranslated regions, recognised by regulatory proteins or by microRNAs:

for example, the presence of an Upstream Open Reading Frame (uORF) or harpins in the 5'-UTR of mRNAs reduces or inhibits their translation rate, whereas the localization of an Internal Ribosome Entry Sequence (IRES) in the same region promotes translation initiation trough a CAP-independent mechanism, in which the translation initiation complex is assembled directly upstream of the AUG starting codon, without needing to recognize the 5'-cap and scan the 5'-leader first.

IRESs have been firstly identified in the RNA genome of poliovirus, which, after infecting host cells, causes the proteolytic cleavage of eIF4G, an essential factor for CAP-dependent translation initiation, but unrequired in IRES-mediated one; in this way, the translation of most of cellular mRNAs is impaired and host ribosomes are redirected towards the RNA viral genome. IRESs have been later identified also in eukaryotic transcripts involved in the responses to stress conditions and programmed cell death, in which CAP-dependent translation initiation may be impaired; examples of such eukaryotic transcripts are the ones codifying for Vascular Endothelial Growth Factors (VEGFs), basic Fibroblast Growth Factor (bFGF) and Hypoxia-Inducible Factor 1A (HIF-1a).

Focusing now on global control of protein biosynthesis, such regulation mostly happens at the level of translation initiation, since this phase involves a higher number of protein factors if compared to elongation or termination: eIFs, in fact, belong to a highly conserved protein superfamily comprising more than 25 polypeptides, that, being fundamental for mRNA translation, are primary targets of several signalling pathways controlling gene

expression and cell growth, such as the Phosphatidylinositol 3-Kinase (PI3K)/(AKT), the Mitogen-Activated Protein Kinase (MAPK) and Mammalian Target Of Rapamycin Complex 1 (mTORC1) pathway; their downstream effector kinases and phosphatases are then responsible for changes in eIFs' and eIFs' interactors' phosphorylation status, which is directly correlated to their activity.

Two interesting examples of this phenomenon are the eukaryotic initiation factor 2a (eIF2a) and the eukaryotic initiation factor 4E-binding protein 1 (4EBP1).

The first one is a target of the Integrated Stress Response (IRS) signalling pathway, which upregulates the expression of specific genes in response to internal and environmental stresses (such as hypoxia, nutrients deprivation, oxidative stress, and low pH), downregulating protein biosynthesis at the same time.

On the other hand, 4EBP1 is a target of the mTORC1 pathway, a serine-threonine kinases signalling pathway that promotes cell growth and protein biosynthesis in the presence of favourable extra- and intra-cellular conditions, such as nutrients availability, high oxygen levels, the presence of mitogens and growth factors, and the absence of genomic damages.

Starting from eIF2α, it is one of the three subunits (α, β and γ) of eIF2, a ternary complex that, when bound to GTP, is able to interact with the 40S ribosomal subunit in the initiation complex, favouring the localization of the Met-$tRNA_i$ in the P-site of the ribosome and, thus, translation initiation. Later on, when the start codon AUG is recognized during 5'-UTR scanning, GTP is hydrolysed, producing eIF2 in the GDP-bound state; subsequent

exchange of GDP for GTP on eIF2 happens trough the interaction between eiF2α and eIF2B, and is required to reconstitute a functional ternary complex ready for a new round of translation initiation.

In conditions of aminoacids deprivation, UV-induced DNA damages, viral infections, ER stress and haem deprivation, several effector kinases of the IRS signalling pathway, respectively GCN2 (general control nonderepressible-2), PKR (protein kinase double-stranded RNA dependent), PERK [PKR-like endoplasmic reticulum (ER) kinase] and HRI (Haem-Regulated Inhibitor), are activated and phosphorylate eIF2α subunit at the level of serine 51, blocking the GTP-exchange reaction by reducing the dissociation rate of eIF2 from eIF2B.

This event sequesters eIF2B and, therefore, GDP–GTP exchange no longer occurs, and global mRNA translation is inhibited.

For what concerns 4EBP1, this protein, when unphosphorylated, is able to interact with eukaryotic initiation factor 4E (eIF4E), that normally work as a “connecting bridge” between eIF4G, part of the pre-initiation complex 43S, and the 5’-cap of the transcript, favouring the correct localization of the first one on the latter before 5’-UTR scanning starts; for this reason, the interaction between unphosphorylated 4EBP1 and eIF4E, by sequestering the initiation factor 4E, prevents its interaction with eIF4G and, thus, translation initiation.

But in the presence of extracellular and intracellular cues that promotes cell growth and anabolism, such as the presence of nutrients, aminoacids, oxygen, mitogens, insulin and other growth factors, the mTORC1 pathway is activated and the mTORC1 complex itself directly phosphorylates

4EBP1, that, in this way, is no longer able to sequester eIF4E: the final result is the restoring of global translation initiation and of protein biosynthesis.

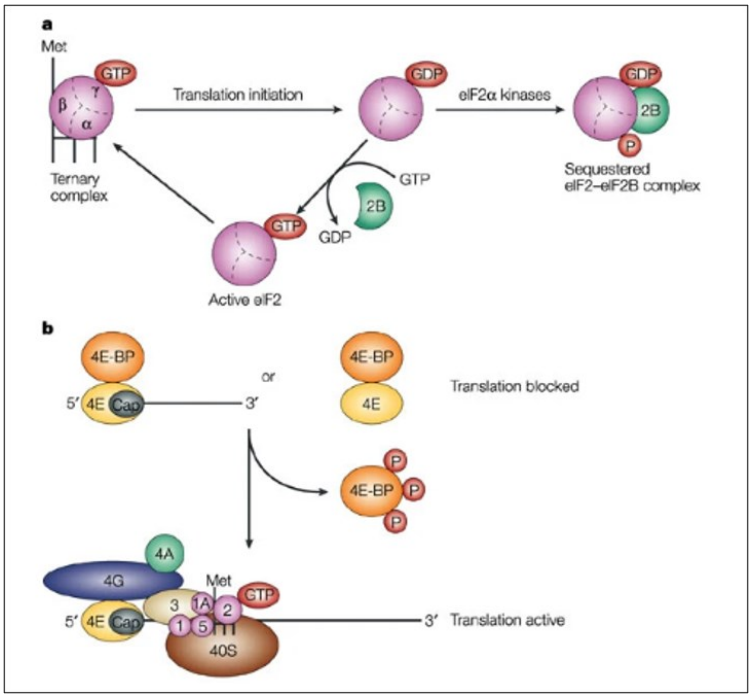


Figure 7. Schematic representation of how eukaryotic initiation factor 2α (eIF2α, figure 7a) and 4E-Binding Protein 1 (4EBP1, figure 7b) control translation in eukaryotes. *In conditions of cellular stress, effectors of the Integrated Stress Response (ISR) signalling pathway phosphorylate eIF2α, confining it in a GDP-bound, inactive state, in which the initiation factor is unable to take part to the formation of the translation initiation complex; translation is thus inhibited. In the presence of favourable intra- and extra-cellular conditions activating the mTORC1 pathway, the phosphorylation of 4EBP1 prevents the protein from sequestering the eukaryotic initiation factor 4E (eIF4E), required for the correct localization of the pre-initiation complex on the 5'-UTR region of transcripts. Images taken from Liping L. et al., "Regulation of Transcription and Translation by Hypoxia", Cancer Biology and Therapy, 2004.*

3.3.3. TOP mRNAs: another mechanism through which the mTORC1 pathway controls translation.

The direct phosphorylation of 4EBP1 and the disruption of its interaction with eIF4E, however, isn't the only mechanism trough which mTORC1 promotes translation initiation and protein biosynthesis. One of the most important effectors of this signalling pathway, in fact, is p70 S6 Kinase (p70S6K), a serine-threonine kinase existing in two different isoforms (S6K1 and S6K2), whose activation depends on multiple phosphorylation events at the level of the catalytic, linker and pseudosubstrate domains; however, phosphorylation of threonine 389 in the linker domain, directly exerted by the mTORC1 complex, most closely correlates with p70S6K activity *in vivo.*

Once activated, phospho-p70S6K (P-p70S6K) phosphorylates the Ribosomal Protein S6 (RPS6) at the level of several residues: serine 235-236, which are targeted also by other kinases, and serine 240/244, phosphorylated only by P-p70S6K.

The activation of phospho-S6RP (P-S6RP) correlates with an increase in the translation rate of a specific class of cellular mRNA, containing a Terminal OligoPyrimidine ("TOP") tract in their 5'-UTR region: for this reason, such transcripts have been defined "TOP mRNAs".

More specifically, all TOP mRNAs present a *cis-r*egulatory RNA motif that begins immediately after the m7G CAP and contains an invariant 5'-cytidine, followed by an uninterrupted tract of 4-15 pyrimidines and, often, also by a G-rich region. It has been demonstrated that the TOP motif sequence is highly conserved in TOP mRNAs, which codify for all the 79

human ribosomal proteins (among which, for example, RPL32, part of the large 60S ribosomal subunits; RPS20 and RPS6, part of the small 40S one), as well as for non-ribosomal proteins involved in translation, such as Poly-A binding proteins (PABPs), multiple subunits of eIF3, eIF4A, eEF2 and proteins involved in ribosomes biogenesis and assembly.

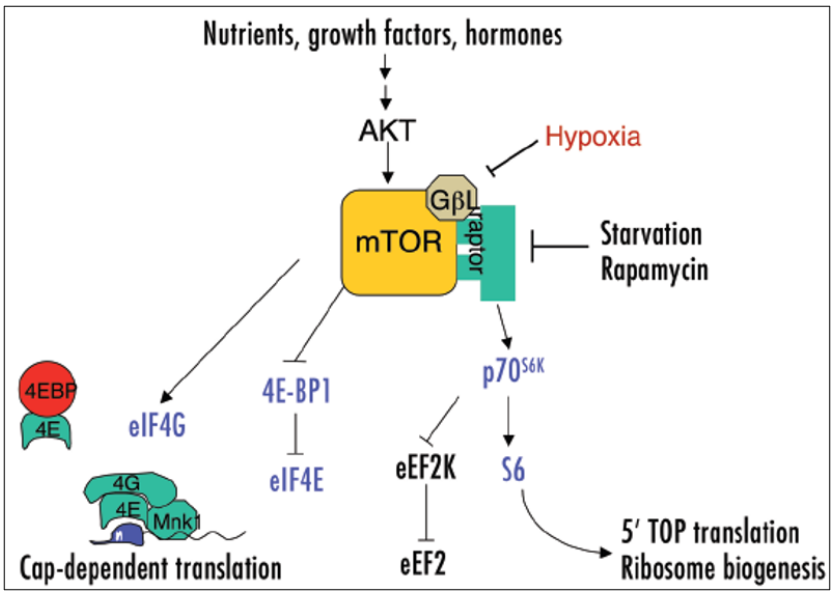


Figure 8. Schematic representation of how the mTORC1 pathway controls translation in eukaryotes. *Once stimulated by the presence of nutrients and energy supplies, the mTORC1 signalling pathway promotes translation through different mechanisms: by phosphorylating the p70S6 Kinase (p70S6K), it activates the protein, that phosphorylates the Ribosomal Protein S6 (RPS6), associated with an increase in the translation rate of a specific class of transcripts, TOP mRNAs; on the other hand, by phosphorylating 4E-Binding Protein 1 (4EBP1), it prevents the protein from sequestering eIF4E, a factor required for translation initiation. Image taken from Liping L. et al., "Regulation of Transcription and Translation by Hypoxia", Cancer Biology and Therapy, 2004.*

The regulation of TOP mRNAs' expression is highly dependent on the cellular energy state: under physiological conditions, ribosomes, translation factors and other proteins codified by TOP mRNAs are synthetized accordingly to cellular demands, but under stress conditions, such as nutrients, aminoacids and oxygen deprivation, global cellular translation must be haltered, in order to redirect the energy and resources required to support protein biosynthesis to resolve the stress-induced cellular damages; as a consequence, in such unfavourable conditions, cells downregulate TOP mRNAs' expression, acting not at the transcriptional, but at the translational level.

Scientists were also able to demonstrate the importance of the mTORC1 pathway in such translational control of TOP mRNAs expression: using ribosome profiling techniques (that will be later described also in the present thesis), Thoreen et al. showed that the disruption of mTORC1 signalling using inhibitors such as rapamycin and Torin1, led to a marked decrease in the translation rate of TOP mRNAs.[23]

The underlying molecular mechanisms have been later elucidated and are based on La-Related Protein 1 (LARP1), a nuclear and cytosolic RNA-binding protein containing a unique C-terminal DM15 domain for the selective and CAP-dependent binding to 5'-TOP motifs. LARP1 also contains a more complex "La Module" (LaM), consisting of three different subdomains: one for the binding to the Poly-A tail of the transcript (and, thus, its stabilization), one for the interaction with PABP (and, thus, for favouring transcript circularization), and another one for the CAP-independent interaction with TOP motifs.

Since it regulates the translation of TOP mRNAs, it comes with no surprise that LARP1 is a target of the mTORC1 pathway: understanding the precise role of LARP1 in the translational control of TOP mRNAs hasn't been easy: Gentilella et al. initially proposed that LARP1 interacts with PABPs and with 40S ribosomal subunit to selectively stabilize TOP mRNAs, promoting their translation;[24] in contrast, Fonseca et al., followed by several other groups, reported LARP1 as a repressor of TOP mRNAs translation regulated by the mTORC1 complex, and this second model is nowadays the commonly accepted one.[25]

More specifically, they demonstrated that the activated form of LARP1 competes with eIF4E, an essential factor for translation initiation, for the binding to the 5-CAP of TOP mRNAs, thus preventing the assembly of the 43S pre-initiation complex in this region and repressing TOP mRNA translation; in vitro biochemical RNA-binding assays, in fact, showed that LARP1's affinity for 5'-TOP motif is considerably higher than the one of eIF4E. For what concerns the regulation of LARP1 by mTORC1, it seems that the two proteins interact between each other not only functionally, but also physically: in particular, LARP1 directly interact with the RAPTOR regulatory subunit of mTORC1, that, when active, phosphorylates LARP1 at the level of serine-744 and serine-766, two residues that are located near the DM15 RNA-binding domain of LARP1. Such modification decreases the affinity of DM15 for the TOP motif, allowing eIF4E binding and translation initiation. Conditions that inhibit mTORC1 activity, on the other hand, prevent LARP1 phosphorylation and allows its binding to TOP

mRNAs: the result is the inhibition of global protein synthesis, coherently with a situation in which the mTORC1 pathway is switched off.

It's also important to remember that evidence supporting this hypothesis was obtained by studies on primary cells, whereas in cancer cells LARP1 seems to have a positive effect on overall protein synthesis and bind many mRNA, including those encoding oncogenes, in addition to TOP mRNAs. Such modifications decrease the affinity of LARP1.

3.3.4. Translational control in vascular endothelial cells and angiogenesis.

Vascular endothelial cells represent an optimal model to investigate translation and its regulation, since they are able to rapidly adapt to external stimuli, such as hypoxia and fluid shear stress, which also regulates angiogenic sprouting; they are able to do so mainly through translation control mechanisms, whose responses occur in a matter of minutes to hours, in contrast to transcriptional control mechanisms, that may require many hours or even days and a higher energy amount.

Also in endothelial cells the mTORC1 pathway is crucial to the initiation of protein biosynthesis in many circumstances: for example, fluid flow activates mTORC1 and thus p70S6K, resulting in an increased translation of TOP mRNAs; but is also induces an increase in the translation of Bcl-3, a transcription factor belonging to the Nuclear Factor Kappa B (NF-KB) family of transcription regulators. On the other hand, shear stress (due to the force applied by turbulent blood flow on the walls of blood vessels) increases the expression of E-selectin on the surface of endothelial cells,

without altering the total amount of its mRNA; E-selectin plays a crucial role leukocyte adhesion and overall endothelial cells' activation.

Also hypoxia, one of the most important driving factors of angiogenesis, deeply influence protein synthesis in endothelial cells, actin firstly at the level of translation and only secondly at the level of transcription. In particular, hypoxia inhibits the mTORC1 pathway trough two different mechanisms, depending on the entity and duration of oxygen deprivation: when it is brief (1% oxygen level for less than one hour), the Tuberous Sclerosis Complex (TSC), the main inhibitor of mTORC1, is activated trough the AMPK signalling pathway, whereas, when hypoxia is more prolonged, the stabilization of Hypoxia Inducible Factor 1 (HIF-1) transcription factor leads to the synthesis of REDD1 protein (Regulated in Development and in DNA Damages responses 1), that is able to disrupt the inhibitory interaction between TSC and the 14-3-3-sigma protein, thus activating the TSC complex and inhibiting the mTORC1 one.

Little is known about the role of translation during angiogenesis, but it's plausible to think that migratory tip cells and highly proliferating stalk cells, requiring a large set of proteins to exert their toles during the sprouting of new blood vessels, controlling gene expression and protein synthesis at both transcriptional and translational levels.

In the present thesis, apart from hypoxia, other environmental factors that influence translation in endothelial cells will be presented: among them, also low pH and glucose levels, high lactate concentrations, oxidative stress, general nutrients starvations and extracellular matrix stiffness, all conditions that can be found in the contest of the tumour microenvironment.

4. MATERIALS AND METHODS

4.1. Culturing Human Umbilical Vein Endothelial Cells (HUVECs.)

Human Umbilical Vein Endothelial Cells (HUVECs) were cultured in complete 199 Medium, prepared by adding 10% Fetal Bovine Serum (FBS), 1X Pen/Strep antibiotic (Cat.Nº.:15140122, Thermo Fisher Scientific), Heparin (0.1 mg/mL) and 1 mL of Brain Bovine Extract (BBE) (Cat.Nº.: CC4098, Lonza Bioscience) to 199 Medium base with Earle's Salts (Cat.Nº.:11150059, Thermo Fisher Scientific). Cells were seeded in plates previously coated with a 0.2% gelatin solution in PBS (Cat.Nº.: G2500, Sigma-Aldrich) and medium change was performed every two days to support and maintain HUVECs' proliferation.

4.2. Induction of cellular quiescence.

To induce cellular quiescence, $1 \cdot 10^5$ cells were seeded in 3.5 cm-diameter dishes and cultured for 7 to 10 days in SupplementPack Endothelial Cell Growth Medium 2 (Cat.Nº.: C-39211, PromoCell); medium change was performed every two days to sustain cell growth.

4.3. Non-chemical and chemical hypoxia treatment.

Non-chemical hypoxia treatment was performed by keeping HUVECs for 24 hours inside of a hypoxia chamber (New Brunswick Galaxy 48 R, Genelab), in which O_2 levels were set at 1.0%, CO_2 levels at 5% and temperature at 37.0°C. Chemical hypoxia treatment was performed by adding 1 mM Dimethyloxallyl Glycine (DMOG) (Cat.Nº.: D3695, Sigma-

Aldrich) to complete 199 Medium; cells were then incubated for 24 hours in normoxic conditions.

4.4. Glucose deprivation treatment.

Low glucose treatment was performed by culturing HUVECs for 24 hours in Dulbecco's Modified Eagle Medium (DMEM) with no glucose (Cat.Nº.:A1443001, ThermoFisher Scientific), supplemented with 10% FBS and 1X Pen/Strep, or in the same medium supplemented with glucose at a final concentration of 0.2 mM, comparable to the one present in the interstitial fluids of the tumour microenvironment.[15]

4.5. High lactate treatment.

High lactate treatment was performed by culturing HUVECs for 24 hours in complete 199 Medium, supplemented with 10 mM lactate, a concentration comparable to the one present in the tumour interstitial fluids.[17]

4.6. General nutrient starvation treatment.

General nutrient starvation treatment was performed by culturing proliferating HUVECs overnight in 199 Medium base with 1X Pen/Strep, without FBS, heparin and BBE as supplementations. Overnight duration treatment was chosen after observing that longer periods of starvation (24 hours) caused massive cell death in culture.

4.7. Oxidative stress treatment.

Oxidative stress treatment was performed by culturing HUVECs for 24 hours in complete 199 Medium, supplemented with 13 μM menadione.

Menadione was chosen as the chemical compound to induce oxidative stress because of the possibility to finely regulate ROS intracellular production based on its concentration: at low concentrations (starting from 2 µM), menadione induces ROS production through redox cycling, whereas, at higher concentrations (starting from 20 µM), it triggers apoptosis.[26].

4.8. Low pH treatment.

Low pH treatment was performed by culturing HUVECs for 24 hours in complete 199 Medium, in which pH had been set to 6.8 units, a value comparable to the one that can be found in the interstitial fluids of the tumour microenvironment.[18]. Firstly, medium pH was stabilized by adding 15 mM Hepes buffer (Cat.Nº.:5630080, ThermoFisher Scientific), then it was lowered from 7.4 units (the standard pH value of 199 Medium) to 6.8 units by adding HCl and measuring the pH decrease through a pH meter.

4.9. ECM stiffness treatment: treatment with Myosin-Light chain kinase ML7 inhibitor and ROCK Y-27632 inhibitor and immunohistochemistry analysis of YAP localization.

Immunofluorescence analysis of YAP localization in the nucleus or cytosol of proliferating or quiescent endothelial cells was performed by washing cells for 3 times (each time for 5 minutes) with a 1 mM Ca^{2+}/Mg^{2+} PBS solution, fixing them through a 10 minutes incubation with 3.7% paraformaldehyde in PBS and repeating the 3 washes in 1 mM Ca^{2+}/Mg^{2+} PBS. Samples were then blocked through a 1 hour-long incubation in a 5% goat-serum, 0.3 M glycine PBS solution; after that, they were incubated with a 1:100 dilution of anti-YAP primary antibody (Cat.Nº.: sc-101199, Santa

Cruz Biotechnology) in a 5% Goat serum PBS solution, and such incubation was performed overnight inside of a humidified chamber. The next day, three 1 mM Ca^{2+}/Mg^{2+} PBS washings were repeated, then samples were incubated for 1 hour at room temperature with the following solution: 5% goat serum, 1:400 dilution of goat anti-mouse Alexa Fluor 568 secondary antibody (Cat.Nº.:A-11004, Invitrogen) for YAP staining, 1:1000 dilution of DAPI (Cat.Nº.:D1306, Invitrogen) for nuclei staining, 1X Alexa Fluor 488 Phalloidin antibody (Cat.Nº.:A12379, ThermoFisher Scientific) for actin cytoskeleton staining. After incubation, 3 final washes with a 1 mM Ca^{2+}/Mg^{2+} PBS solution were performed before samples were mounted for confocal microscopy analysis. After that proliferating HUVECs were chosen as a model for ECM stiffness and in order to mimic a condition of ECM softness, cells were treated for 24 hours, separately, with ML7 inhibitor (Cat.Nº.:4310, Tocris), to a fina*l* concentration of 5 µM, and Y-27632 inhibitor (Cat.Nº.: A11001, AdooQ), to a final concentration of 10 µM: higher concentrations of the two compounds had previously resulted in elevated cellular cytotoxicity. Immunofluorescence protocol was then repeated.

4.10. Puromycin pulse labelling (SUnSET).

Puromycin (Cat.Nº.:P8833, Sigma Aldrich) was added to cellular cultures to a final concentration of 1 mg/ml; after 10 minutes of incubation, puromycin-containing medium was removed and cells were washed twice with ice-cold PBS; after complete PBS removal, cells were lysed using RIPA buffer and collected through scraping. Cellular lysates were then treated as

described in the next paragraph “Cellular lysis and Western Blot analysis”. Puromycin signal was then detected through Western Blot using a primary anti-puromycin antibody (Cat.Nº.: MABE343, Sigma Aldrich).

4.11. Cellular lysis and Western Blot analysis.

Cells were lysed using RIPA Buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 2% sodium deoxycholate, 0.2% SDS, 2% NP40, 25X protease inhibitor cocktail, 10X phosphatase inhibitor cocktail) and collected through scraping; in order to promote complete cellular lysis, samples were left in agitation on ice for 40 minutes, then cellular debris were removed through a 10 minutes-long, 20.000 RCF centrifugation at 4°C and protein extracts were quantified using the Pierce BCA Protein Assay Kit (Cat.Nº.:23225, Thermo Fisher Scientific). Western Blot analysis of target genes was performed starting from 7.5 µg of each protein extract; after transfer, Ponceau staining was performed to verify equal loading amount for each sample. Proteins of interest were detected using the following primary antibodies: anti-puromycin (Cat.Nº.:MABE343, Sigma Aldrich), anti-HIF-1α Ab (Cat.Nº.:GTX127309, Genetex), anti-VEGFR2 Ab (Cat.Nº.:2478, Cell Signalling Technology), anti-p70S6K (Cat.Nº.:2708, Cell Signalling), anti-Pp70S6K (Thr389) (Cat.Nº.: 9234, Cell Signalling Technology), anti-S6RP (Cat.Nº.: 2217, Cell Signalling), anti-PS6RP (Ser2440/244) (Cat.Nº.:BK4858S, Cell Signalling), anti-eIF2A (Cat.Nº.: MA5-38202, Invitrogen), anti-PeIF2A(Ser51) (Cat.Nº.: BK3398T, Cell Signaling), anti-4E-BP1(Cat.Nº.: 9452S, Cell Signaling), anti-P4E-BP1 (Cat.Nº.: 9452S,

Cell Signaling), anti-actin (Cat.Nº.: 691331, MP Biomedicals), anti-cyclin A1 (Cat.Nº.: C4710, Sigma Aldrich).

4.12. Quantitative PCR analysis of mRNA levels.

Cells and were lysed in Trizol and RNA purification from cellular lysates and monosomes/polysomes fractions was performed through classical phenol/chloroform extraction protocol; purified RNA was resuspended in 20 µl of RNAse-free water (Cat.Nº.: 10977015, ThermoFisher Scientific) and its purity and amount were assessed at Nanodrop. Retrotranscription was performed starting from 1000 ng of RNA for each sample, using the High Capacity cDNA Reverse Transcription kit (Cat.Nº.: 4368813, Abcam) and the following primers: actin forward (Cat.Nº.: 2-4165-11/12, Eurofins Genomics); actin reverse (Cat.Nº.: 2-4165-12/12, Eurofins Genomics); RPS6 forward (Cat.Nº.: 5-4030-5/6, Eurofins Genomics); RPS6 reverse (Cat.Nº.: 5-4030-6/6, Eurofins Genomics); RPS20 forward (Cat.Nº.: 31-4305-21/30, Eurofins Genomics); RPS20 reverse (Cat.Nº.: 31-4305-22/30, Eurofins Genomics); PABP forward (Cat.Nº.: 5-4030-3/6, Eurofins Genomics); PABP reverse (Cat.Nº.: 5-4030-4/6, Eurofins Genomics); RPL32 forward (Cat.Nº.: 5-4030-1/6, Eurofins Genomics*);* RPL32 reverse (Cat.Nº.: 5-4030-2/6, Eurofins Genomics*).* Real-time PCR analysis was performed using the 5x Hot Firepol Blend Master Mix Ready to load with 12.5mM MgCl2 (Cat.Nº.: 04-25-00125, Solis Biodine), starting from 4 µl of cDNA samples and 6 µl of reaction mix. To evaluate if the levels of tested TOP mRNAs varied significantly between controls and tested conditions,

statistical analysis was conducted by performing one sample t-test and setting a confidence level (α) of 0.05.

4.13. Polysome profile.

HUVECs were seeded in 150 mm diameter plates to reach confluence; once quiescent, a fraction of cells was splitted to obtain proliferating cells, part of which were incubated in the hypoxia chamber for 24 hours to obtain hypoxic cells. The day of the lysis, cells were pre-treated with 100 µg/ml cycloheximide, then plates were put on ice and washed with ice-cold PBS supplemented with 10 µg/ml cycloheximide, then lysis buffer was added (50 mM Tris HCl pH 7.5, 100 mM NaCl, 30 mM $MgCl_2$, 0,1% NP-40, 100 µg/ml cycloheximide, 40 U/ml RNasin, protease inhibitor cocktail) and cellular lysates were collected through scraping. Samples were left on ice for 10 minutes for efficient lysis and crude extracts were cleared through 5 minutes long, 4°C centrifugation at 14.000 RCF. RNA amount in each sample was determined at Nanodrop by reading RNA absorbance at 254 nm, then equal amount of cellular lysates, corresponding to 10 OD/ml, were loaded on a 15%-50% sucrose gradient., prepared by mixing 15% and 50% sucrose solutions dissolved in 50 mM TrisAcetate pH 7.5, 50 mM NH_4Cl, 12 mM $MgCl_2$, 1 mM DTT; mixing of the two solutions was performed through a 3 hours and 30 minutes-long ultracentrifugation at 39.000 RPM. Polysome profile was then performed by reading RNA absorbance at 254 nm using AKTA Fast Protein Liquid Chromatography UPC-900 P-920 (Amersham Pharmacia Biotech), while sucrose gradients were eluted at a 1 ml/min rate.

5. AIM OF THE STUDY.

The aim of the present study is to evaluate if and how conditions characterizing the tumour microenvironment (TME), namely hypoxia, glucose deprivation, high lactate, general nutrients starvation, oxidative stress, low pH and extracellular matrix stiffness, impact on mRNA translation in endothelial cells. The answer to such biological question may be useful to identify novel anti-cancer strategies that impair tumour vascularization (an essential step for metastasis) by acting at the level of translation in tumour endothelial cells.

6. RESULTS.

6.1. Biochemical features of the tumour microenvironment differentially regulate the mammalian Target of Rapamycin Complex 1 (mTORC1) and the Integrated Stress Response (ISR) signalling pathways in HUVECs endothelial cells.

As mentioned before, the mammalian Target of Rapamycin Complex 1 (mTORC1) and the Integrated Stress Response (ISR) signalling pathways play opposite roles in modulating translation, and both are regulated by a variety of conditions that can be found also in the TME.

A useful in vitro technique to assess the translational status of cells is represented by puromycin pulse labelling, also called “SUNSET” (“Surface Sensing of Translation”): this method is based on puromycin, an aminonucleoside antibiotic produced by the *Streptomyces alboniger*

bacterium, that blocks translation elongation and causes the premature release of the nascent polypeptide from the ribosome. These events are due to the fact that puromycin' structure resembles the 3' end of tyrosyl-tRNA, with a modified adenosine base covalently linked to a tyrosine aminoacid through a peptide bond, instead of the classic ester bond normally present in aminoacylated tRNAs. Like tyrosyl tRNA, puromycin can enter the ribosomal A site, where its free amino group accepts a nascent polypeptide chain from the P-site peptidyl-tRNA, in a reaction catalyzed by the peptidyltransferase center (PTC).

However, because the peptide bond between the two moieties of puromycin cannot be further cleaved by an incoming aa-tRNA, such incorporation into the C-terminus of elongating nascent chains prevents additional extension and results in premature termination of translation elongation. As a result, nascent polypeptides are irreversibly marked by puromycin at their C-terminus and they can be detected by Western Blot analysis using a primary anti-puromycin antibody, whose signal will be directly proportional to the translation rate of cells under the different conditions tested, in this case the ones characterizing the tumour microenvironment.

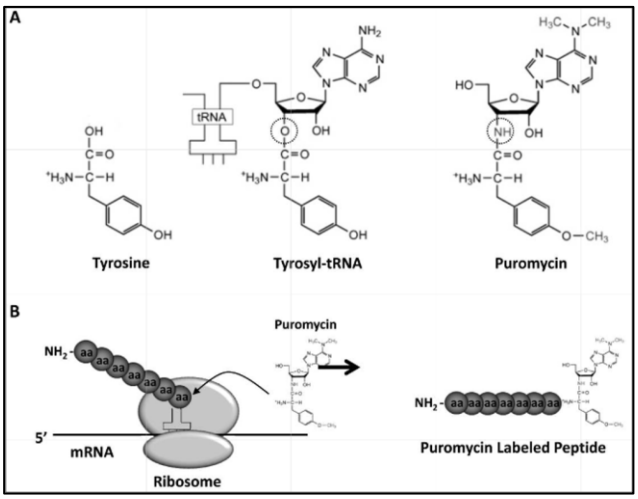


Figure 9. A. Molecular structure of puromycin. *Puromycin's structure resembles the 3' end of tyrosyl-tRNA, in which the ester bond normally present between the aminoacid and the tRNA has been substituted with a peptide bond.* ***B. Puromycin incorporation into nascent polypeptidic chains.*** *Thanks to its similarity with the tyrosil-tRNA, puromycin can enter the ribosomal A site and accept an elongating polypeptide from the P site; however, since the peptide bond present in the puromycin molecule can't be further hydrolysed by the incoming tRNA, puromycin incorporation into the C-terminus of the elongating polypeptide causes premature translation termination. Images taken from Goodman et al., "Measuring protein synthesis with SUnSET: a valid alternative to traditional techniques?", Science Review, 2013.*

6.1.1. Analysis of the mTORC1 and ISR signalling pathways in HUVECs exposed to hypoxia.

Starting from 24 hours hypoxia treatment, performed both through the use of an hypoxia chamber (1% O_2) and the addition of Dimethyloxalglycine (DMOG) 1 mM to the culture medium, I could detect the stabilization of the HIF-1α subunit only in the second case, even if both experimental conditions allowed me to see clearly the downregulation of Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) in hypoxia, in agreement with the fact that low oxygen concentration stimulates VEGF-A and VEGFR1 expression, but decreases VEGFR2 levels.[26] Compared to the control condition, represented by HUVECs cultured for 24 hours in normoxia (19% oxygen level), hypoxic cells downregulate the mTORC1 pathway, as testified by decreased protein levels of phospho-S6 Kinase

Thr389 (P-S6K(Thr389)), its target phopho-S6 Ribosomal Protein Ser240/244(P-S6RP(Ser240/244)) and phospho-4E-Binding Protein 1 (Ser65) (P-4EBP1(Ser65)); at the same time, they also activate the ISR pathway through by increasing the phosphorylation level of eukaryotic initiation factor 2α (eIF2α).

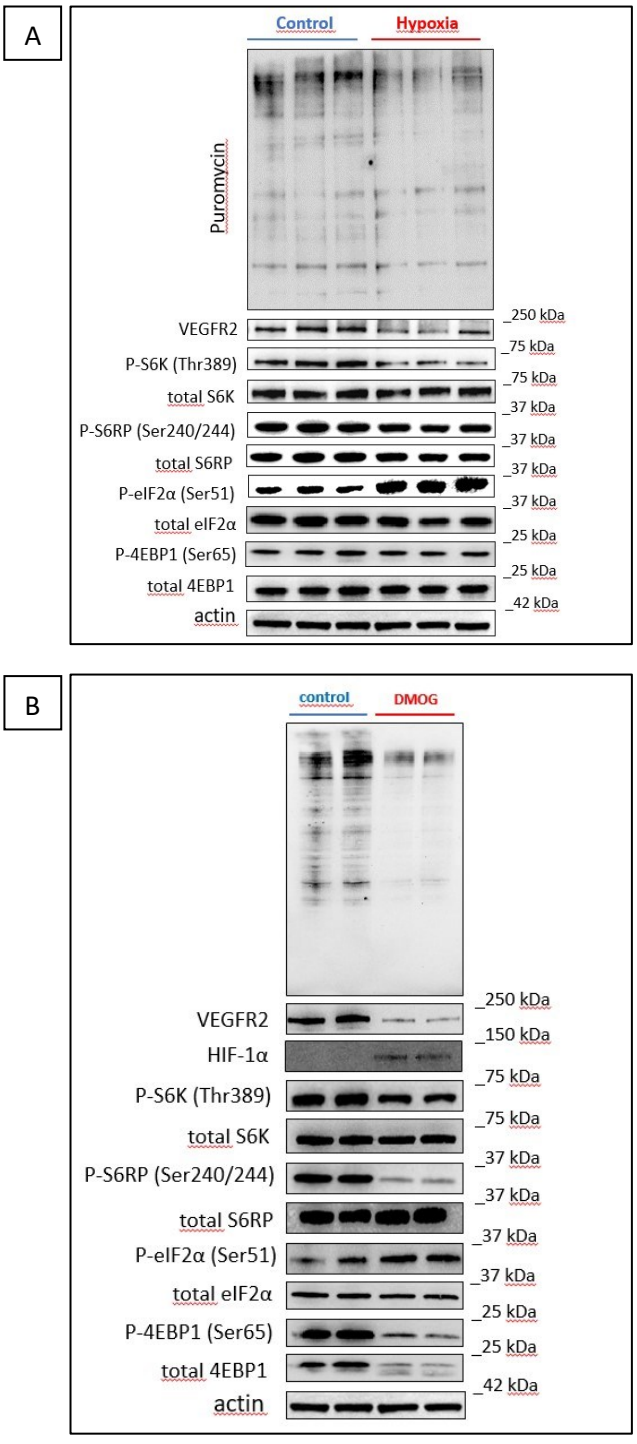


Figure 10. Puromycin pulse labelling and Western Blot analysis of the mTORC1 and IRS signalling pathways in HUVECs exposed to the hypoxia chamber (A) and to DMOG treatment (B) for 24 hours. *Coherently with the expectations, hypoxia reduces the protein levels of VEGFR2 and of the active, phosphorylated effectors of the mTORC1 pathway; at the same time, hypoxia increases P-eIF2a protein levels, indicating cellular stress.*

6.1.2. Analysis of the mTORC1 and ISR signalling pathways in HUVECs exposed to glucose deprivation.

Glucose deprivation, on the other hand, doesn't seem to affect the mTORC1 and ISR pathways: both in normoxia and in hypoxia, in fact, puromycin incorporation signal doesn't increase with glucose concentration, and the levels of the proteins tested don't change when glucose is present in the culture medium at a 0 mM concentration, 0.2 mM concentration (typically present in the interstitial fluids of the tumour microenvironment) and 5.5 mM concentration (typically present in the blood and interstitial fluids in physiological conditions); additionally, also P-eIF2α levels remain unaffected by low glucose levels, conversely from the expectations: glucose deprivation, in fact, represents one of the most important stimuli activating the ISR pathway, together with hypoxia, amino acids deprivation, viral infection and high oxidants levels: such result is thus unexpected.

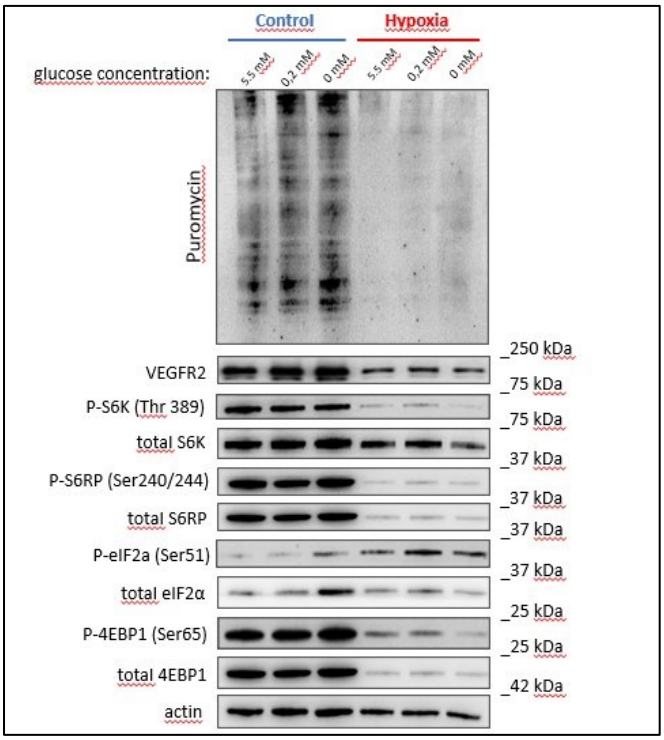


Figure 11. Puromycin pulse labelling and Western Blot analysis of the mTORC1 and ISR signalling pathways in HUVECs exposed to glucose deprivation combined with normoxia or hypoxia for 24 hours. *Complete absence or low levels of glucose don't impact significantly on protein levels of VEGFR2, mTORC1 pathway effectors and p-eIF2a, conversely from hypoxia condition, which influence tested proteins' levels as described above.*

6.1.3. Analysis of the mTORC1 and ISR signalling pathways in HUVECs exposed to high lactate treatment.

Just like glucose deprivation, also high lactate treatment doesn't impact on the mTORC1 pathway: protein levels of S6 Kinase, S6 Ribosomal Protein and 4E-Binding Protein 1 effectors, both in their active/phosphorylated and inactive/unphosphorylated forms, don't vary significantly between control

and 10 mM lactate treatment; on the opposite, high lactate increases P-eIF2α protein levels, indicating an activation of the Integrated Stress Response signalling pathway, probably due to the acidic nature of the supplement (the addition of 10 mM lactate to M199, in fact, leads to a pH decrease of the medium from 7.2 to 6.9). Taken together, these observations suggest that the general decrease of protein biosynthesis that can be observed by puromycin pulse labelling after high lactate treatment is not due to the inhibition of the mTORC1 pathway, but to the activation of the Integrated Stress Response one.

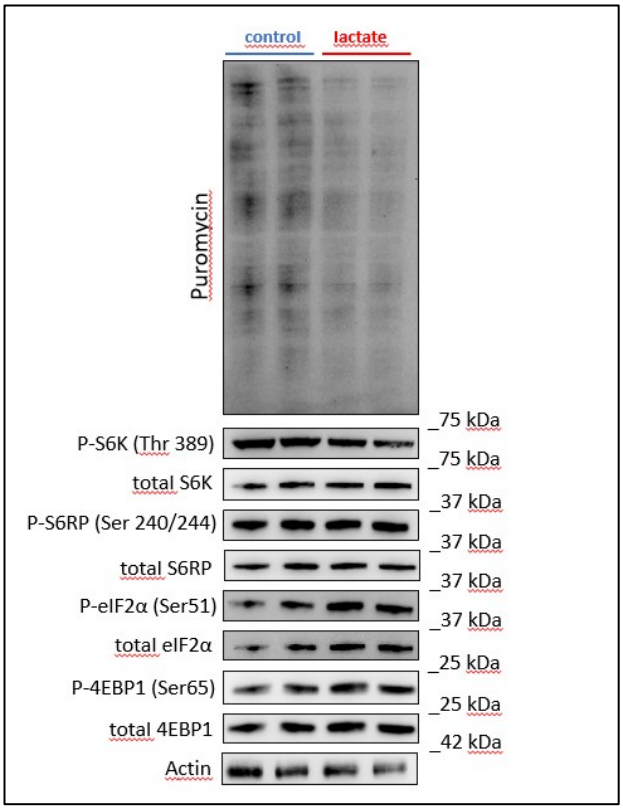


Figure 12. Puromycin pulse labelling and Western Blot analysis of the mTORC1 and ISR signalling pathways in HUVECs exposed to high lactate treatment for 24 hours. *10 mM lactate treatment leads to an increase in the protein levels of P-*

eIF2a, without significantly affecting the protein levels of active mTORC1 pathway's effectors.

6.1.4. Analysis of the mTORC1 and ISR signalling pathways in HUVECs exposed to general nutrient starvation.

General nutrient starvation inhibits protein biosynthesis, as shown by the absence of puromycin incorporation into nascent polypeptides; this event can be ascribed to the inhibition of the mTORC1 pathway, since the protein levels of all of its effectors, both in their active/phosphorylated and inactive/unphosphorylated forms, decrease when HUVECs are cultured in the absence of fetal bovine serum (source of proteins, lipids and growth factors), heparin (which stimulates endothelial cells migration and growth in the presence of serum) and bovine brain extract (working as a potent growth supplement for a variety of cell types, including endothelial cells). On the other hand, P-eIF2α protein levels don't increase significantly after general nutrient starvation, indicating that this condition doesn't stimulate the ISR pathway: such signalling, in fact, is activated by glucose and aminoacids deprivation, but both these nutrients are still present at high concentrations in the 199 Medium base in which HUVECs are starved.

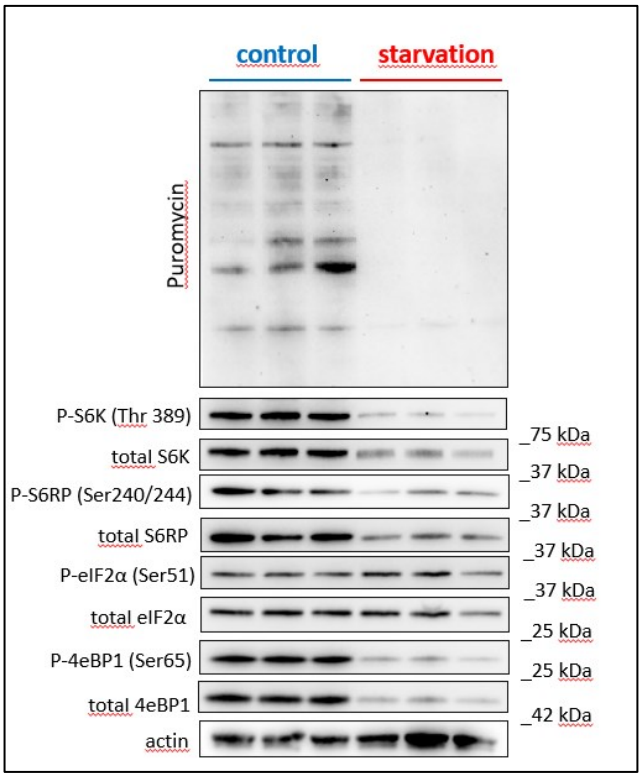


Figure 13. Puromycin pulse labelling and Western Blot analysis of the mTORC1 and ISR signalling pathways in HUVECs exposed to general nutrient starvation overnight. *General nutrient starvation leads to a general decrease of protein synthesis, as testified by reduced protein levels of both phosphorylated and total protein levels of the effectors considered.*

6.1.5. Analysis of the mTORC1 and ISR signalling pathways in HUVECs exposed to menadione-induced oxidative stress.

A different pattern can be observed in cells treated with menadione: this compound induces the intracellular production of ROS by interacting with molecular oxygen, which donates one or two electrons to generate respectively semiquinone and menadiol on the one hand, and superoxide and hydrogen peroxide on the other. Being ROS a primary source of DNA,

lipids and proteins damages, it comes with no surprise the fact they activate the ISR pathway, as illustrated by high P-eIF2α levels. Previous studies reported that short-term exposure to low doses of ROS stimulates the mTORC1 complex, while high concentrations or long-term ROS treatment inhibits its activity; here, 24 hours treatment with 13 μM menadione decrease global protein biosynthesis by turning off the mTORC1 pathway, as testified by decreased protein levels of P-S6K(Thr389), P-S6RP(Ser240/244), and P-4EBP1(Ser65) effectors; an interesting point is that, in oxidative stress conditions, not only the phosphorylated, active forms of these mTORC1 effectors decline, but also the total ones, comprehending the unphosphorylated protein fraction: this result is different from the one obtained in hypoxic conditions, where the total amount of mTORC1 effectors remained unaltered compared to control.

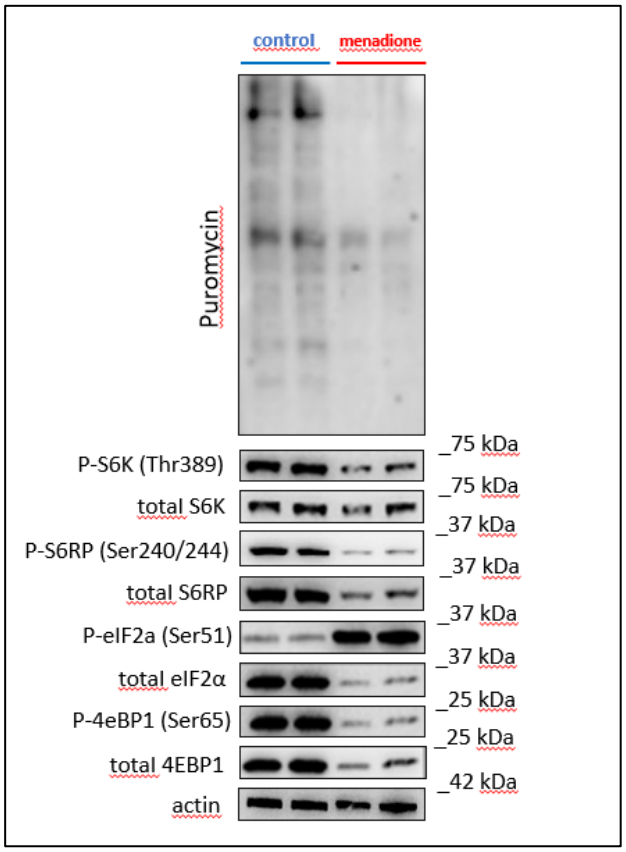


Figure 14. Puromycin pulse labelling and Western Blot analysis of the mTORC1 and ISR signalling pathways in HUVECs exposed to menadione-induced, oxidative stress treatment for 24 hours*. Menadione-induced oxidative stress treatment leads to an important decrease of both active and total mTORC1 pathway's effectors' protein levels; at the same time, it increases P-eIF2a ones.*

6.1.6. Analysis of the mTORC1 and ISR signalling pathways in HUVECs exposed to low pH treatment.

Acidic pH is another condition typically characterizing the tumour microenvironment, mainly due to the metabolism of cancer cells and to the limited removal of acidic waste products. Previous studies reported that acidic pH is able to rapidly and reversibly inhibit the activity of the mTORC1 complex, while not affecting the one of mTORC2, indicating that acidic extracellular pH doesn't directly inhibit the activity of the mTOR catalytic subunit (shared between the two complexes), but impacts on upstream mTORC1 regulatory proteins.[27] Here, 24 hours-long treatment at pH 6.8 doesn't affect the protein levels of S6 Kinase, S6 Ribosomal Protein and 4E-Binding Protein 1 effectors, probably indicating that 6.8 pH value isn't low enough to detect an inhibition of the mTORC1 pathway; on the other hand, coherently with the expectations, acidity stimulates the phosphorylation of eIF2α and the activation of the Integrated Stress Response Pathway.

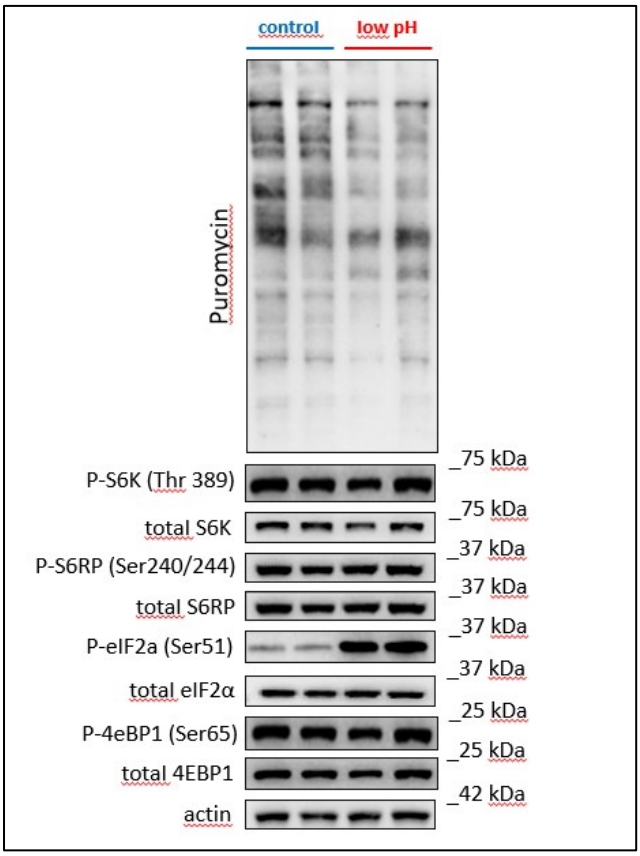


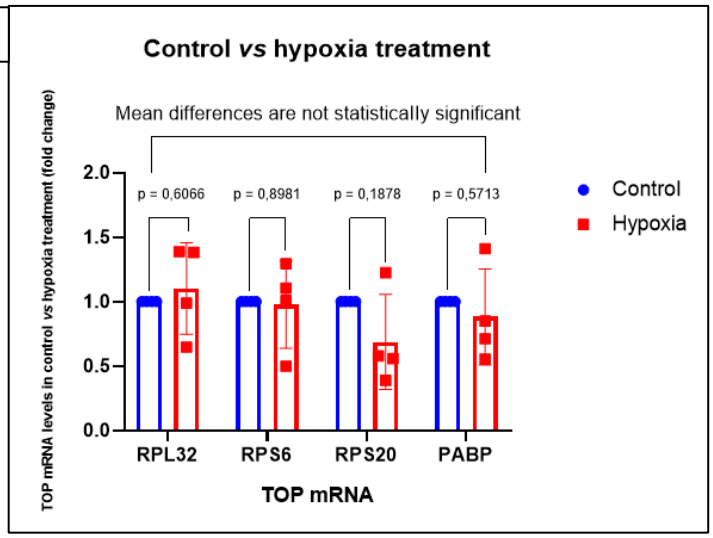
Figure 15. Puromycin pulse labelling and Western Blot analysis of the mTORC1 and ISR signalling pathways in HUVECs exposed to low pH treatment for 24 hours. *Low pH treatment, just like high lactate treatment, doesn't impact significantly on mTORC1 pathway's effectors levels. On the opposite and coherently with the expectations, it causes cellular stress, evidenced by increased P-eIF2a protein levels.*

6.2. Biochemical features of the tumour microenvironment don't influence TOP mRNAs' expression at the transcriptional level in HUVECs.

As mentioned before, TOP mRNAs are a class of transcripts codifying for proteins with fundamental roles in translation, such as factors involved in

ribosomal biogenesis and assembly. Their peculiarity relies on the fact that TOP mRNAs expression is modulated by the mTORC1 pathway not at the transcriptional, but at the translational levels, with a significant decrease of their translation initiation rate in the presence of unfavourable environmental conditions for the cell.[28] To confirm this model, the relative amounts of some TOP mRNAs were tested through quantitative PCR in HUVECs exposed to the physiological and stressful conditions of the tumour microenvironment, compared to controls. Transcripts considered were the ones codifying for Ribosomal Protein L32 (RP L32), component of the 60S large ribosomal subunit; Ribosomal Protein S6 (RPS6), component of the 30S small ribosomal subunit; Poly-A Binding Protein (PABP), fundamental for transcripts' translation and stabilization; and Ribosomal Protein S20 (RPS20), another component of the 30S small ribosomal subunit. In all the conditions tested, namely hypoxia, glucose deprivation, general nutrient starvation, high lactate treatment, low pH and oxidative stress, TOP mRNAs levels didn't decrease significantly compared to controls, with only some exceptions that should be confirmed by increasing the power of the statistical analysis that weas performed: in general, this confirms the accepted theory for which TOP mRNAs expression isn't regulate at the transcriptional level.

A



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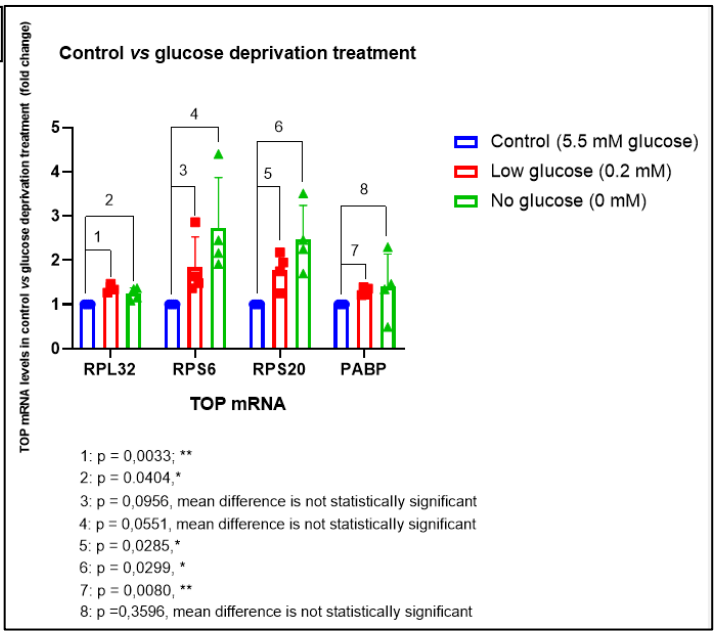
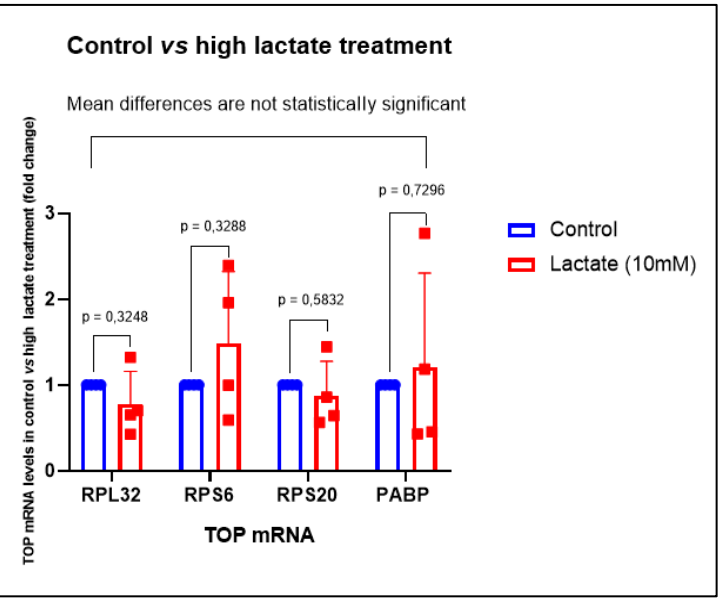
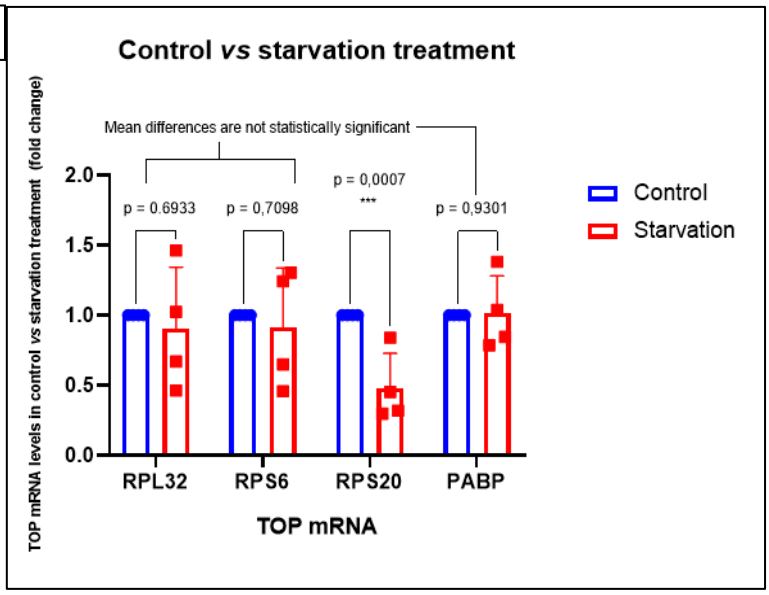


1: p = 0,0033; **
2: p = 0.0404,*
3: p = 0,0956, mean difference is not statistically significant
4: p = 0,0551, mean difference is not statistically significant
5: p = 0,0285,*
6: p = 0,0299, *
7: p = 0,0080, **
8: p =0,3596, mean difference is not statistically significant

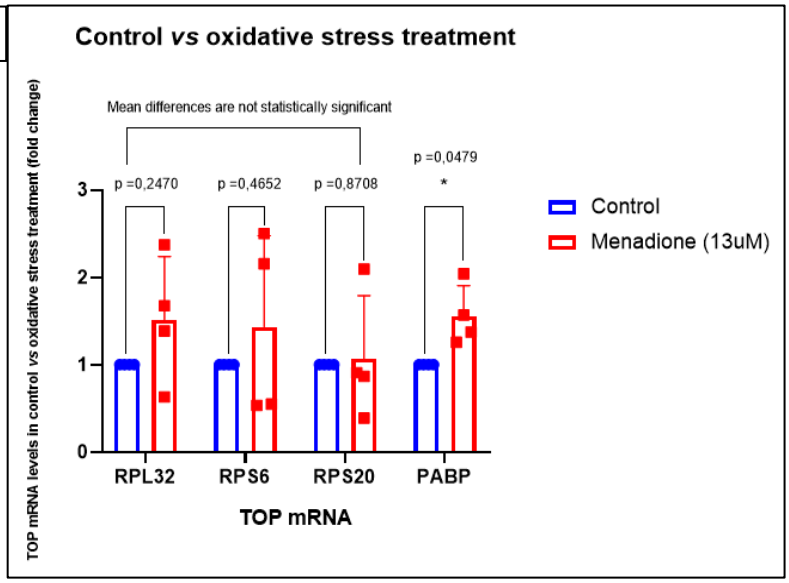
C



D


Control vs starvation treatment
Mean differences are not statistically significant
p = 0.6933
p = 0,7098
p = 0,0007
***
p = 0,9301
Control
Starvation
TOP mRNA levels in control vs starvation treatment (fold change)
2.0
1.5
1.0
0.5
0.0
RPL32
RPS6
RPS20
PABP
TOP mRNA

E


Control vs oxidative stress treatment
Mean differences are not statistically significant
p =0,2470
p =0,4652
p =0,8708
p =0,0479
*
Control
Menadione (13uM)
TOP mRNA levels in control vs oxidative stress treatment (fold change)
3
2
1
0
RPL32
RPS6
RPS20
PABP
TOP mRNA

F

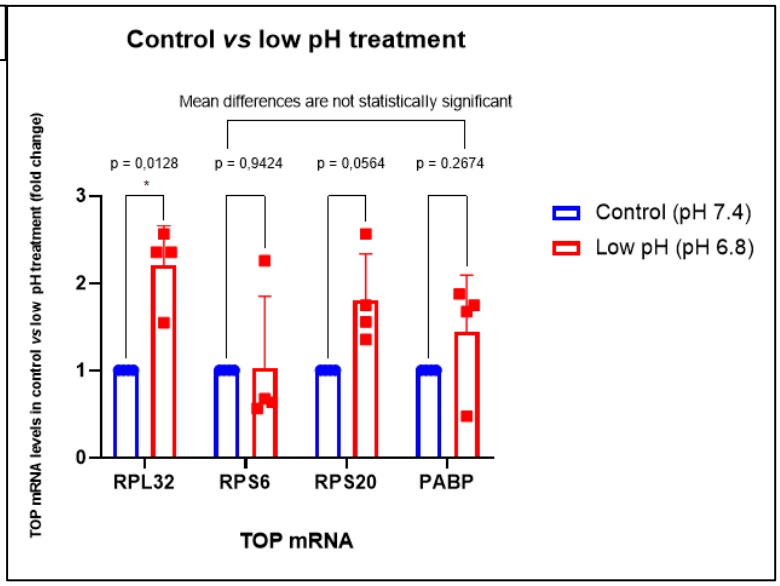

Control vs low pH treatment
Mean differences are not statistically significant
p = 0,0128
*
p = 0,9424
p = 0,0564
p = 0.2674
Control (pH 7.4)
Low pH (pH 6.8)
TOP mRNA levels in control vs low pH treatment (fold change)
3
2
1
0
RPL32
RPS6
RPS20
PABP
TOP mRNA

Figure 16. Quantitative PCR analysis of RPL32, RPS6, RPS20 and PABP TOP mRNAs in HUVECs exposed to the typical conditions of the tumour microenvironment: hypoxia (A), glucose deprivation (B), high lactate treatment (C), general nutrient starvation (D), oxidative stress treatment (E) and low pH treatment (F). *Regulation of TOP mRNAs' expression happens at the translational level, not at the transcriptional one, hence transcripts amount isn't influenced by the typical conditions of the tumour microenvironment, which, on the opposite, impact on the levels of those proteins codified by TOP mRNAs, as testified by previous Western Blot analysis. Statistical analysis was performed through one-sample t test, setting the following significance levels: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.*

6.3. Polysome profile analysis of proliferating, quiescent and hypoxic endothelial cells revealed clearly distinct translational patterns.

Polysome profile is a useful *in vitro* technique to assess the entire cellular translational state or just the one of a specific mRNA, when cells are exposed to specific environmental conditions (in this case, quiescence and hypoxia). The technique is based on the fact that mRNA transcripts can be divided in three main fractions, depending on the frequency at which they are translated by cellular ribosomes: free mRNAs, monosomes (or subpolysomes) and polysomes.

Free mRNAs are transcripts that aren't bound by any ribosome, so, despite the fact they are produced by the cell through transcription, the protein they codify isn't synthetized: expression of protein-coding genes, in fact, depends on both transcriptional and translational control of protein biosynthesis, two different mechanisms that, when combined, allow a more

precise regulation of global proteome composition. Monosomes, or subpolysomes, fraction, is composed by mRNAs bound by single 40S or 60S ribosomal subunits (usually present at the level of their untranslated regions), or by a single 80S complete ribosome: these transcripts are presumed to be translated by the cell with a low rate. Finally, the polysome fraction is composed by mRNAs that are associated with two or more 80S ribosomes, and that, for this reason, are considered highly expressed by the cell. An important point to consider is that transcriptome-wide, relative polysome and monosome occupancy is a function of translation initiation versus total elongation time: if initiation is faster than elongation, a mRNA will be predominantly polysome-associated; on the opposite, if initiation is much slower than elongation, a mRNA will be predominantly monosome-associated. The typical outcome of polysome profile is a curve that correlates each elution fractions of the sucrose gradient in which free mRNAs, monosomes and polysomes have been separated according to their molecular weight, to UV absorption at 245 nm: in this way it is possible to quantify relative transcripts' amount in each fraction (such amount will be directly proportional to the height of absorption peaks) and to assess if, globally, cellular translation is more or less active under specific conditions.

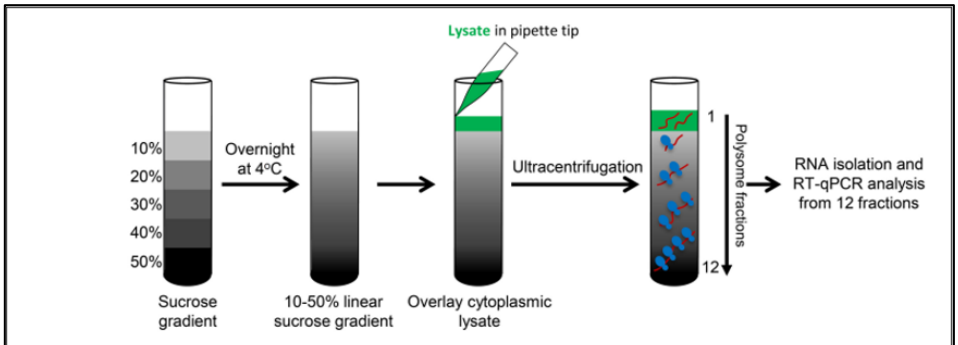


Figure 2. Schematic of polysome fractionation

Figure 17. Schematic representation of polysome profile workflow. *Polysome profile is a useful in vitro technique to assess the global translation status of cells: total cellular lysates are loaded on top of a 15%-50% sucrose gradient, in which free mRNAs, monosomes and polysomes are separated according to their molecular weights, following a 39.000 RPM, 3:30 hours long ultracentrifugation; RNA extraction from each fraction is then performed, in order to assess the enrichment of specific mRNAs (in this case, TOP mRNAs) in the free mRNAs, monosomes or polysomes fractions. Image taken from Panda et al., "Polysome fractionation to analyse mRNA distribution profiles", Bio-Protocol, 2017.*

Polysome profile analysis of proliferating, quiescent and cultured-in-hypoxia HUVECs clearly revealed distinct translation patterns: starting from proliferating HUVECs, they are characterized by the presence of three distinct, evident peaks corresponding to the subpolysomes fraction (the first peak indicating mRNAs bound by the 40S ribosomal subunit, the second indicating transcripts bound by the 60S ribosomal subunit and the third indicating transcripts bound by the complete 80S ribosome); such subpolysomes peaks indicate that a significative fraction of transcripts are actively translated by proliferating cells at a modest pace. But what characterizes PECs' curves the most is their final trait, in which a slowly but progressively increasing profile, indicating the polysomes fraction, can be detected: the peculiar shape of this "peak" is due to the fact that polysomes' molecular weight is highly heterogenous, depending on the number of ribosomes that, in a certain moment, are associated to actively translated transcripts. The presence of the polysome peak in PECs indicates that, in

these cells, translation is globally active: the result is compliant with the expectations, since PECs needs to synthetize large amounts of proteins to maintain their active metabolism, sustain cell cycle progression and perform cellular division.

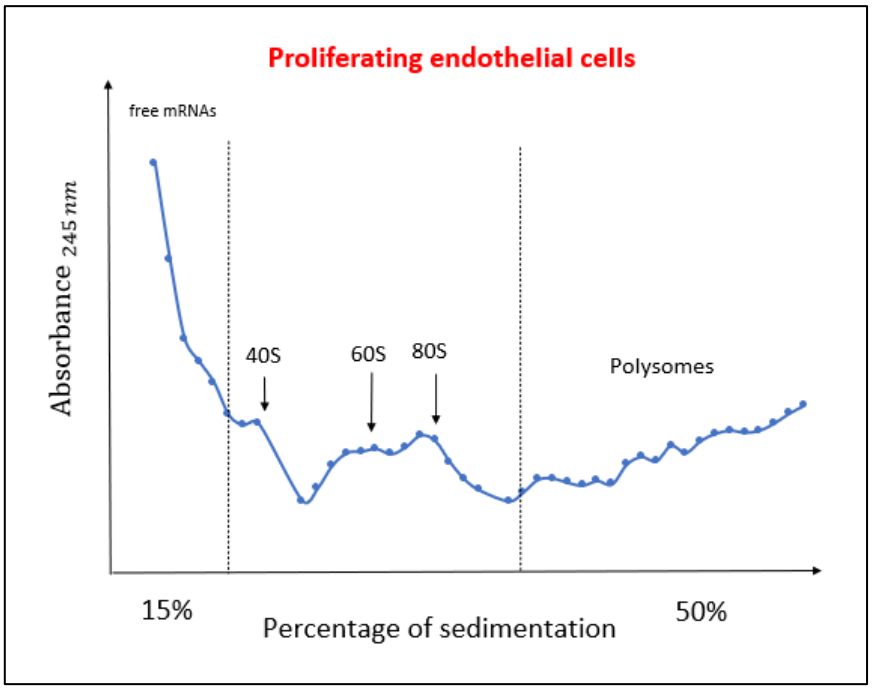
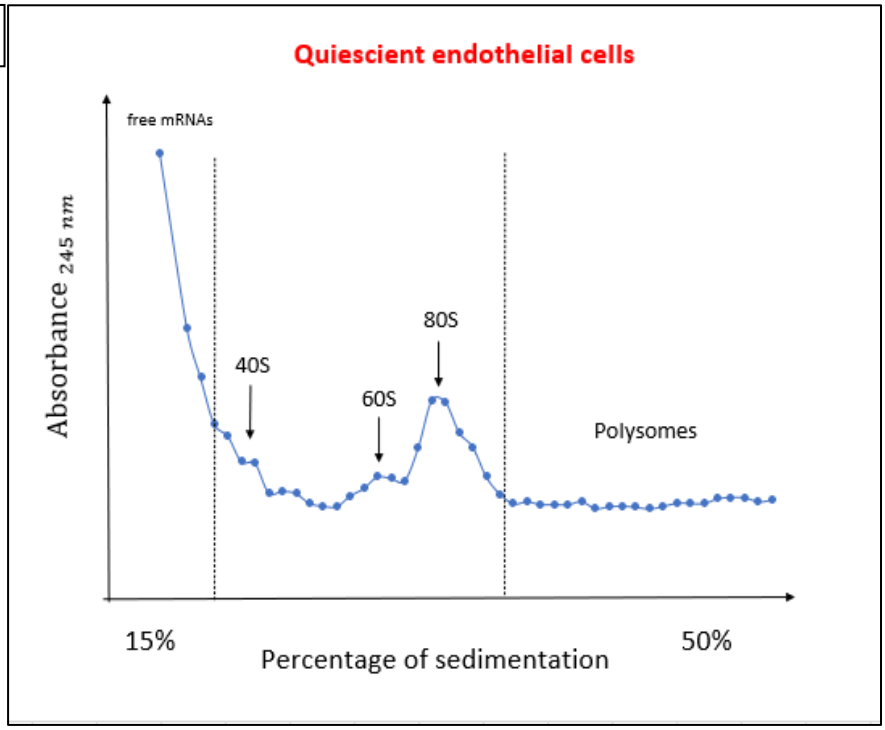


***Figure 18. Polysome profile analysis of proliferating endothelial cells**. Polysome profile analysis of proliferating endothelial cells revealed a relative mRNAs enrichment in correspondence of the polysome peak, indicating an intense translational activity.*

QECs profile, on the other side, are characterized by the presence of the three evident monosomes peaks, but by the absence of the polysome one, suggesting that the quiescence state significantly decreases cellular translation rate, without totally inhibiting it: this effect is mainly achieved through lateral inhibition mechanisms. Moreover, Western Blot analysis of

quiescent endothelial cells revealed the inhibition of the mTORC1 pathway and the activation of the ISR one in quiescent HUVECs.

A



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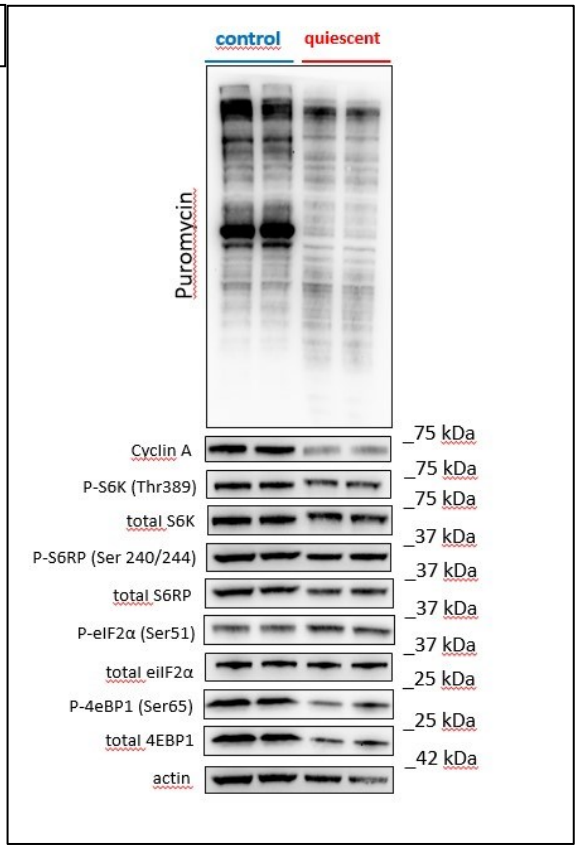

Figure 19. A. Polysome profile analysis of quiescent endothelial cells. *Polysome profile analysis of quiescent endothelial cells revealed a relative mRNAs enrichment in correspondence of the monosome peaks, indicating a basal translational activity.* ***B. Global translation inhibition observed in quiescent endothelial cells is due to the inhibition of the mTORC1 pathway.*** *Western Blot analysis of quiescent endothelial cells revealed the inhibition of the Mammalian Target of Rapamycin Complex 1 (mTORC1) pathway and the activation of the Integrated Stress Response (ISR) one in quiescent HUVECs.*

Finally, hypoxic cells profile lacks both monosomes' and polysomes' peaks, with most of the transcripts accumulating in the free-mRNAs, untranslated fraction: this effect is due, at least in part, to the inhibition of the mTORC1 pathway, as demonstrated through Western Blot analysis.

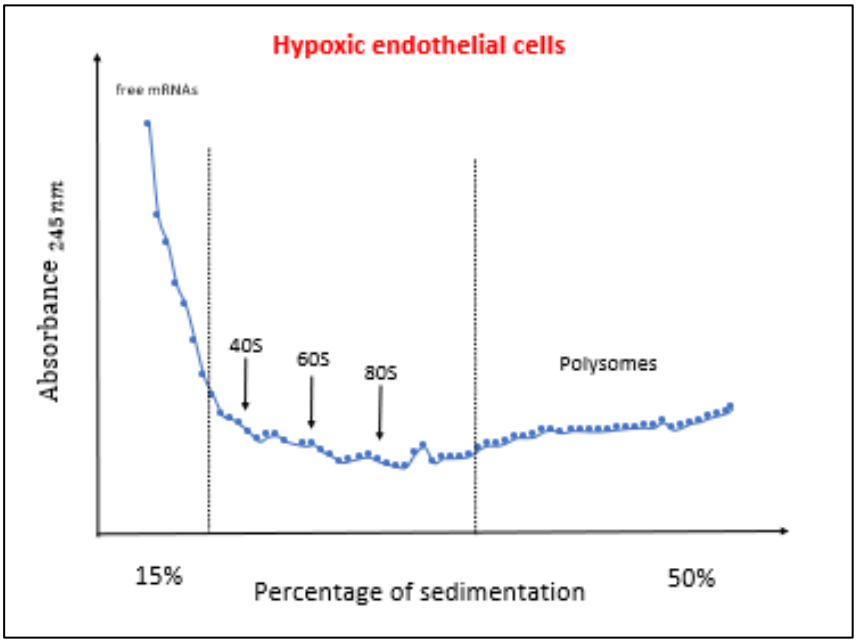

Figure 20. Polysome profile analysis of hypoxic endothelial cells. *Polysome profile analysis of hypoxic endothelial cells revealed no relative mRNAs enrichment in correspondence of the monosome or polysome peak, indicating the absence of s significant translational activity.*

Another important point to consider is that, in all the three conditions analysed, the vast majority of transcripts accumulate in the free-mRNA, untranslated fraction, meaning that both quiescent, proliferating and hypoxic endothelial cells produce a consistent "transcripts reservoir" that they don't convert into proteins.

6.4. Quantitative PCR analysis of TOP mRNAs associated with monosomes and polysomes fractions in proliferating, quiescent and hypoxic endothelial cells confirmed the different translation patterns identified by polysome profile analysis.

TOP mRNAs are a category of transcripts with fundamental roles in eukaryotic translation, since they codify proteins involved in ribosomal biogenesis and assembly. The peculiarity of this class of transcripts is that their expression is regulated not at the transcriptional level, but at the translational one: this means that, even in the presence of stimuli that inhibit translation, such as cellular quiescence and hypoxia for HUVECs, TOP mRNAs are still produced by the cell, but their translation is strongly decreased. In accordance with this theory, real-time PCR of TOP mRNAs associated with monosomes' or polysomes' fractions obtained from proliferating, quiescent and hypoxic HUVECs revealed an enrichment of these transcripts in the monosome fraction of quiescent and hypoxic endothelial cells, whereas, in proliferating ones, TOP mRNAs accumulated in the polysome fraction.

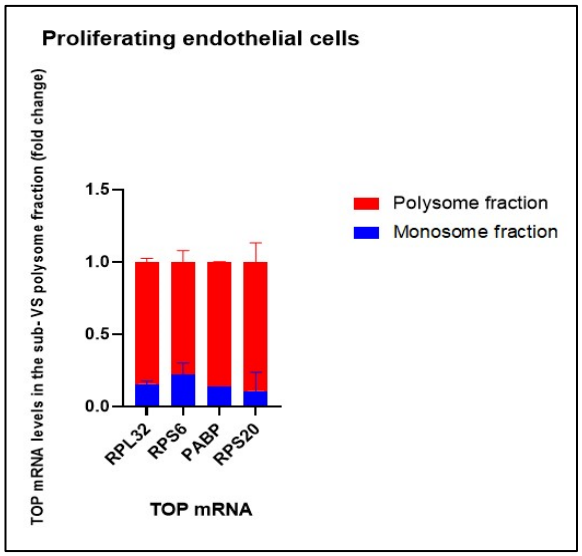

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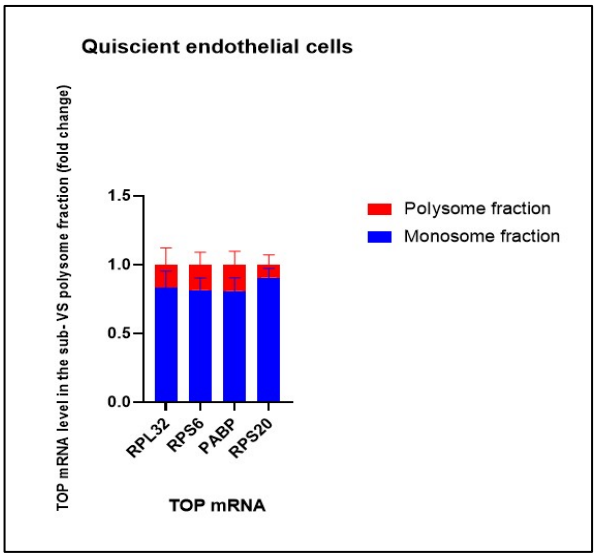
Figure 21. TOP mRNAs enrichment in the proliferating endothelial cells. *Polysome profile analysis revealed that, in proliferating endothelial cells, TOP mRNAs accumulate in the polysome fraction, indicating a high translational activity.*



22. TOP mRNAs enrichment in quiescent endothelial cells. *Polysome profile analysis revealed that, in quiescent endothelial cells, TOP mRNAs accumulate in the monosome fraction, indicating a basal translational activity.*

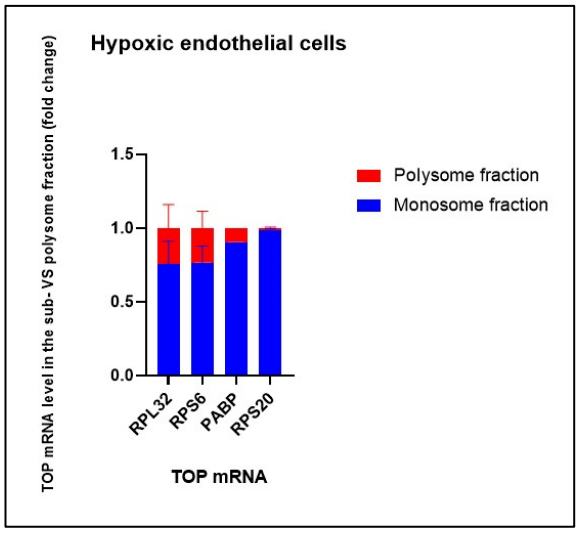


Figure 23. TOP mRNAs enrichment in hypoxic endothelial cells. *Polysome profile analysis of hypoxic endothelial cells revealed an enrichment of TOP mRNAs in the monosomes fraction, indicating a low translational activity.*

These results are coherent with polysome profile curves: the inhibition of global translation that can be observed in quiescent and hypoxic conditions is due, at least in part, to a decrease in the translation rates of TOP mRNAs, enriched in the monosome fraction; at the same time, active global translation characterizing proliferating cells is presumably due to the fact that, in these conditions, TOP mRNAs accumulate in the polysomes, actively-translated fraction.

6.5. Immunofluorescence and Western Blot analysis of proliferating endothelial cells treated with ECM stiffness inhibitors myosin light chain kinase (MLCK) ML7 and Rho-associated protein kinase (ROCK) Y-27632 revealed no impact of ECM stiffness on cellular translation.

Extracellular matrix (ECM) stiffness characterizes most solid tumours, such as breast and liver cancer, where it serves as a physical support, a deposit for growth factors and pro-angiogenic molecules, and a signalling hub to promote cancer cells proliferation, motility and invasion. Cancer cells can sense ECM stiffness and respond to it through cytoskeletal rearrangements and downstream mechanotransducing signalling pathways: among them, the Hippo pathway controls the transcriptional co-activator Yes-associated Protein (YAP), that, in conditions of ECM stiffness, migrates from the cytosol to the nucleus. To test if ECM stiffness impacts on endothelial cells translation, cells were treated, separately, with Y-27632 and with ML-7 inhibitors: the first molecule competes with ATP for the binding to Rho-associated Protein Kinase (ROCK), while the second, through the same modality, inhibits Myosin Light Chain Kinase (MLCK). Both proteins mediate actin-myosin cytoskeleton rearrangements in response to ECM stiffening, which represent the main activating stimulus for them.

Since matrix stiffness is a mechanical feature that can be found in many solid tumours, I wanted to test if it influences endothelial cells translation machinery: first, YAP intracellular localization was assessed through immunofluorescence in proliferating and quiescent endothelial cells, to test if they could be used as models to mimic different stiffness of the ECM. In parallel, also actin cytoskeleton was visualized, since the two kinases inhibited by ML7 and Y-27632 (respectively, MLCK and ROCK) are important for cytoskeletal rearrangements in response to ECM stiffening. Immunofluorescence analysis revealed that, in proliferating endothelial cells (PECs), YAP accumulates in the nucleus, whereas, in quiescent

endothelial cells (QECs), YAP is mainly located in the cytoplasm, even if, in a consistent fraction of QECs, YAP is still present in the nucleus. For these reasons PECs were chosen as a model for mimicking ECM stiffness, since also in this condition YAP localization is nuclear.

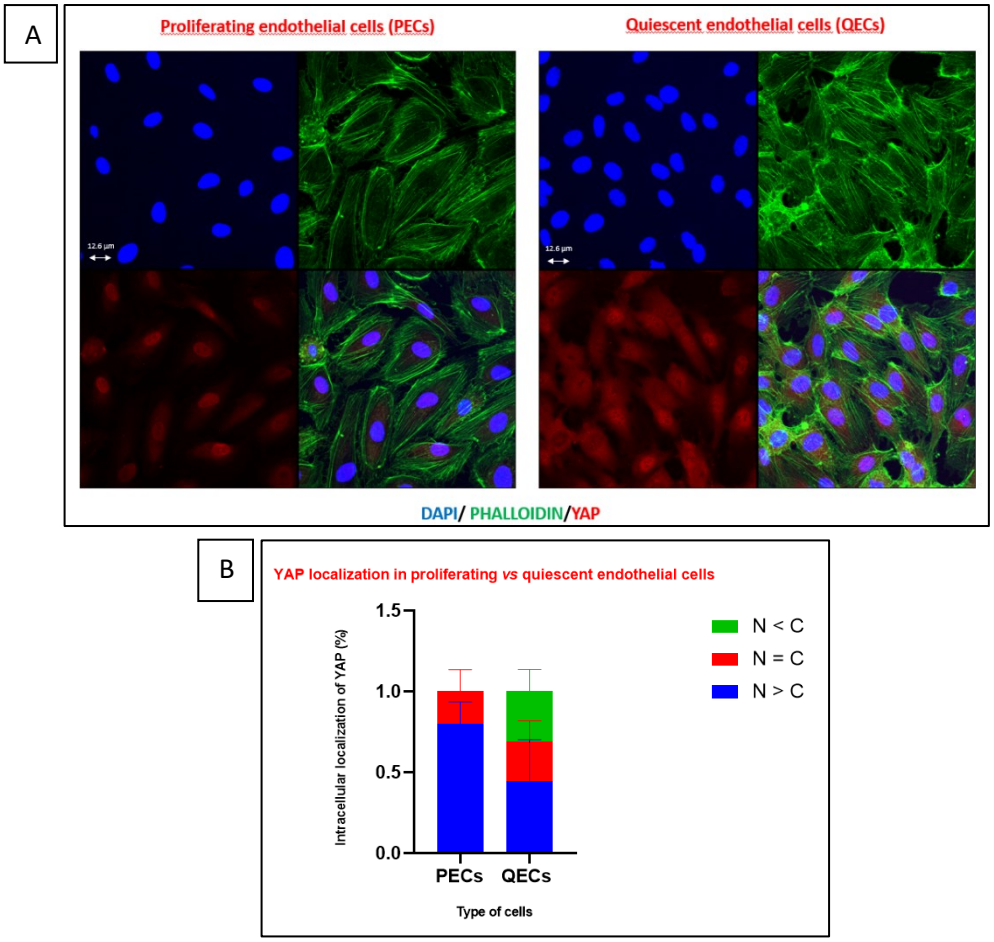
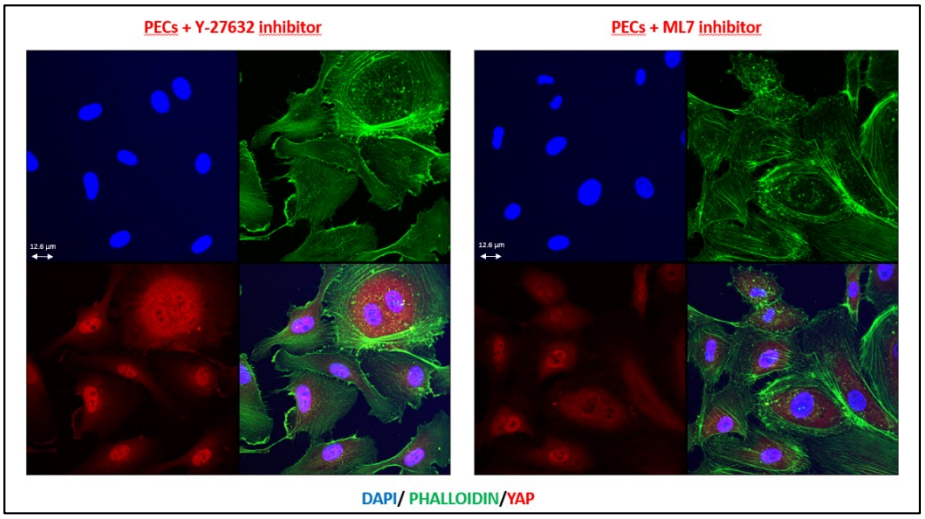


Figure 24. Immunofluorescence analysis of YAP localization in proliferating and quiescent endothelial cells (figure A) and relative nuclear/cytosolic enrichment quantification (figure B). *In proliferating endothelial cells, activated YAP localizes in the nucleus, whereas in quiescent endothelial cells, a significant fraction of cells presents the transcriptional co-activator in the cytoplasm. Since also ECM stiffness induces YAP translocation into the nucleus, proliferating endothelial cells were chosen as a model to mimic ECM stiffness and to evaluate its possible impact on mRNA translation. In figure 24.A, blue (DAPI) stains for the nuclei, green (phalloidins) stains for actin cytoskeleton and red stains for YAP; scale bar used: 1 cm = 12.6 µm. In the legend of figure 24.B, "N"*

stands for nucleus and "C" stands for cytosol, the two cellular compartments in which YAP can be found, respectively in its active and inactive forms; "N < C" indicates that YAP is less present in the nucleus than in the cytosol; "N = C" indicates that YAP is present in the nucleus as much as in the cytosol; "N > C" indicates that YAP is more present in the nucleus that in the cytosol.

Once that PECs were chosen as a model for ECM stiffness, they were treated separately with Y-27632 and ML7 inhibitors, which prevented them from sensing matrix-stiffness, mimicking in this way a condition of ECM softness, in which YAP is expected to accumulate in the cytosol. Thus, after 24 hours-long inhibitors' treatment, YAP localization was tested again by immunofluorescence; however, no significant enrichment of YAP in the cytosol of PECs treated with Y-27632 or ML7 could be observed, indicating that either treatment duration was too short, or inhibitors should be used at higher concentrations, for YAP nuclear translocation to be blocked by ECM softness.

A



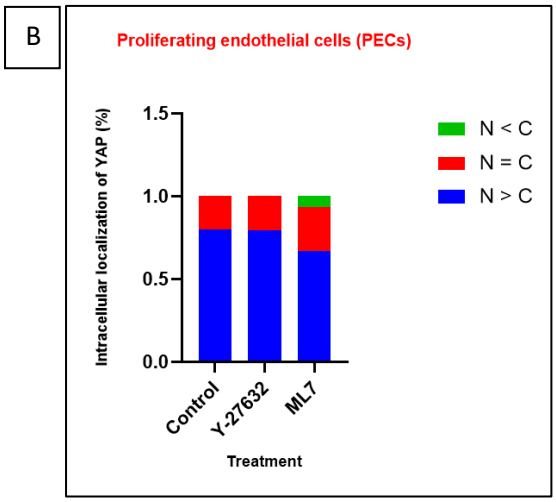


Figure 25. Immunofluorescence analysis of YAP localization in proliferating endothelial cells untreated or treated with Y-27632 and ML7 inhibitors (figure A) and relative nuclear/cytosolic enrichment quantification (figure B). *After the treatment with ECM stiffness inhibitors Y-27632 and ML7, proliferating endothelial cells, used as a model to mimic ECM stiffness, still present YAP localized mainly in the nucleus; this result is unexpected, since the two inhibitors should mimic a condition of ECM softness, which induces YAP cytosolic localization. In figure 25.A, blue (DAPI) stains for the nuclei, green (phalloidins) stains for actin cytoskeleton and red stains for YAP; scale bar used: 1 cm = 12.6 µm. In the legend of figure 25.B, "N" stands for nucleus and "C" stands for cytosol, the two cellular compartments in which YAP can be found, respectively in its active and inactive forms. "N < C" indicates that YAP is less present in the nucleus than in the cytosol; "N = C" indicates that YAP is present in the nucleus as much as in the cytosol; "N > C" indicates that YAP is more present in the nucleus that in the cytosol.*

Puromycin pulse labelling and Western Blot analysis of the mTORC1 and ISR pathways were then performed, to test if ECM stiffness and softness have different impacts on mRNA translation: the experiments led to the observation of no significant difference in the translational rates between control PECs (modelling ECM stiffness) and PECs treated with the two

ECM stiffness inhibitors (modelling ECM softness); thus, the final conclusion is that ECM stiffness has no influence on cellular mRNA translation.

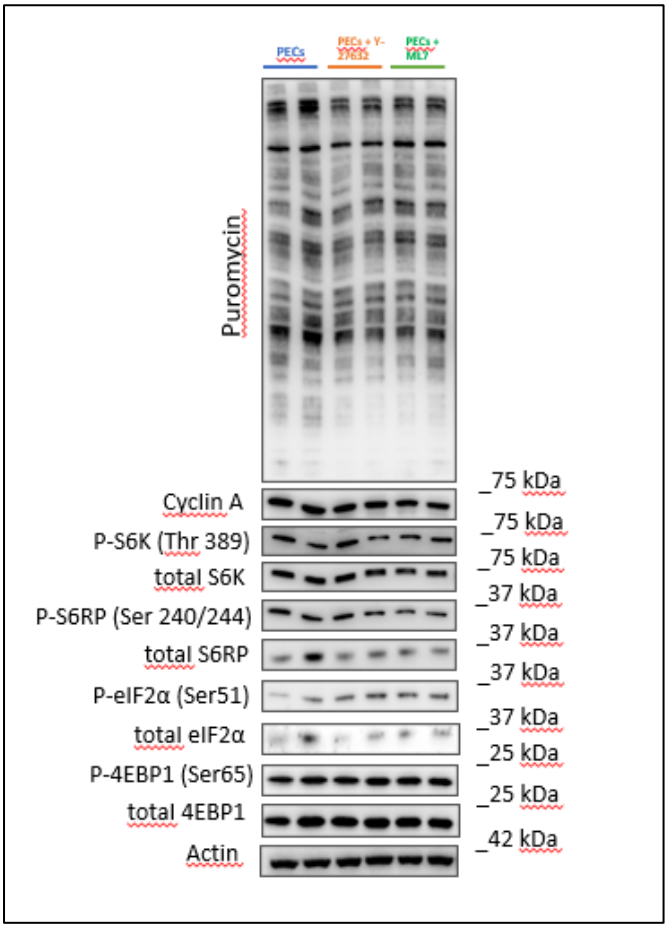


Figure 26. Puromycin pulse labelling and analysis of the mTORC1 and ISR signalling pathways in Proliferating Endothelial Cells (PECs) untreated or treated with Y-27632 or ML7 extracellular matrix stiffness inhibitors. *Treatment with ECM stiffness inhibitors didn't affect mRNA translation in proliferating endothelial cell (chosen as a model for ECM stiffness), as indicated by the fact that the protein levels of mTORC1 and ISR pathways' effectors don't change between untreated and treated samples. This result indicates that either ECM density has no influence on cellular mRNA translation, or that Y-27632 and ML7 did not work efficiently in mimicking ECM softness, due to insufficient concentration of use of the inhibitors or short treatment's duration.*

7. DISCUSSION.

The present thesis aims to investigate how the physiological biochemical conditions of the tumour microenvironment influence translation in HUVECs endothelial cells: Western Blot analysis of the mTORC1 and ISR signalling pathways, puromycin pulse labelling and polysome profile revealed that all the conditions tested, apart from glucose deprivation and ECM stiffness, determine a significant reduction of cellular translational activity. This observation may appear counterintuitive, if we think that cancer, by promoting the angiogenic switch event that marks the beginning of tumour angiogenesis, stimulates endothelial cells' growth, proliferation and migration, which are cellular activities that require the constant production of a wide set of proteins.

However, two important considerations need to be made: the first one is that I performed my experiments on HUVECs primary cultures, which harbour none of those genetic and/or genomic alterations, typically selected by the tumour microenvironment, that may confer to them the ability to maintain high translational rates despite the unfavourable biochemical features of the TME itself. If compared with Normal Endothelial Cells (NECs), in fact, Tumour Endothelial Cells (TECs) show chromosomal instabilities, proangiogenic properties and different gene expression profiles; furthermore, they exhibit resistance to antineoplastic drugs including paclitaxel and 5-fluorouracil, with upregulated expression of multidrug resistance 1 (MDR1) and aldehyde dehydrogenase (ALDH) genes.[29]

For these reasons, it would be interesting to expose not HUVECs, but TECs, to the typical biochemical features of the tumour microenvironment, to test if they impact on cellular translation in the same way or differently, compared to NECs; unfortunately, tumour endothelial cells are not commercially available, but they can be obtained in small quantities from surgical specimens, even if, also in this case, they present a short life span *in vitro* due to their cellular senescence.[29]

A second, important point to consider is that, in my experiments, I wasn't able to reproduce the secretome landscape characterizing tumour microenvironments, in which cytokines, growth factors and chemokines maintain a sustained proliferative rate, cell survival signals avoid apoptosis, and proangiogenic-factors and matrix-modifying enzymes promote angiogenesis and epithelial-to-mesenchymal transition; such signalling molecules also enhance protein biosynthesis and translation, thus they should be definitely taken into account to investigate how the tumour microenvironment affects endothelial cells behaviour.

In order to answer this question more precisely, in fact, cells exposed to the typical biochemical features of the TME (such as hypoxia, glucose deprivation, high lactate levels, general nutrient starvation, oxidative stress, low pH and ECM stiffness) should be cultured in cancer-conditioned media, containing factors secreted by cultured cancer cells, capable of affecting phenotypes and behaviours of normal cells, in this case endothelial ones. By using cancer-conditioned media, in fact, scientists have already selected and characterized several elements produced by the tumour that affect

endothelial cells, making them potential effectors useful in anti-cancer treatments; Saladino et al., for example, demonstrated that the co-culturing of endothelial cells with MDA-MB-231 and 8701BC breast cancer cell lines led to an increase in the production of pro-MMP9 (the inactive precursor of matrix metalloproteinase 9, essential for ECM remodelling), VEGF-A (a potent pro-angiogenic molecule) and β3-integrin (marker of epithelial-to-mesenchymal transition) by endothelial cells.[30]

In conclusion, the biochemical features of the tumour microenvironment alone do not promote translation in HUVECs endothelial cells, but, on the opposite, they repress it through the inhibition of the mTORC1 pathway and/or the activation of the ISR one (except for glucose deprivation and ECM stiffness). Nonetheless, in order to overcome such suppressive effects induced by TME, tumours secrete large amounts of mitogens, growth factors, cytokines and pro-angiogenic signalling molecules, that, by reactivating endothelial cells from their quiescent state and stimulating their proliferation and migration, mark the beginning of tumour angiogenesis.

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