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Role of HSPGs in cancer cell migration

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A chi sa prenderci, comprenderci e sorprenderci.

Ogni giorno.

ABSTRACT

Structural variations and heterogeneities associated with the amount and the position of sulfated groups of their glycosaminoglycan (GAG) chains allow heparan sulfate proteoglycans (HSPGs) to have distinct biological functions due to their ability to interact with different heparin binding ligands, such as growth factors, morphogens, chemokines, adhesion molecules and ECM components. They take part in many processes in both physiological and pathological conditions. Indeed, HSPGs are involved in multiple events of cancer progression, such as cancer cell adhesion, epithelial-mesenchymal transition, migration and invasion. Specifically, cell migration is a multistep process where the actin cytoskeleton, which is capable of reorganizing itself and forming specific structures for movement, plays a key role. Cells can move either as individual cells or collectively. Modification of cancer cell phenotype, through epithelial mesenchymal transition, allows them to leave the primary tumor, invade surrounding tissues and migrate to secondary sites, thus giving rise to metastasis.

The tetra-branched peptide NT4 can recognize HSPGs, targeting their sulfated GAGs and selectively binding to cancer cells and tissues. Based on this specific binding, the possible effects produced by the peptide on cadherins and Eph receptors were analyzed, showing no interaction in either case. By studying the localization of HSPGs in migrating cells, it was possible to note a different distribution depending on the cell line. In addition, the effects induced by NT4 and heparin on the processes of colony formation, adhesion and migration were also evaluated and compared each other, suggesting a greater efficacy of the peptide. Finally, two tumor cell lines, in particular human pancreas adenocarcinoma and human rhabdomyosarcoma cells, were transfected with a plasmid able to mark actin, making it fluorescent.

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INTRODUCTION

PROTEOGLYCANS AND GLYCOSAMINOGLYCANS

The extracellular matrix (ECM) is a complex three-dimensional network that plays important structural and functional roles in development and tissue homeostasis. In addition to providing mechanical support, it is involved in several processes, such as proliferation, adhesion, migration and differentiation of the cells with which it interacts. The ECM is a highly dynamic and non-cellular structure continuously subject to remodelling and consists of interconnected macromolecules that enable communication between the cells and their microenvironment. Generally, it is composed of water, proteins and polysaccharides; however each tissue present an ECM with unique composition generated in early embryonic stages and so its major components are tissue-specific. The most common classes of macromolecules found in the ECM include collagens, glycoproteins, such as laminins and fibronectins, proteoglycans (PGs) and glycosaminoglycans (GAGs) [Hynes, 2009; McKee et al., 2019]. In particular, PGs are glycoproteins consisting of a specific core protein to which one or more GAG chains are covalently attached [Afratis et al., 2012]. They can be classified on the basis of cellular and subcellular location, overall gene/protein homology and the presence of specific protein modules within the protein cores. According to their localization, these glycoproteins can be divided in many groups: (1) intracellular PGs; (2) cell surface PGs; (3) pericellular and basement membrane PGs; (4) extracellular PGs and (5) small leucine-rich PGs (SLRPs). They are extremely heterogeneous molecules with distinct structural characteristics that differ depending on the pathological and developmental states of the cells or tissues in which they are synthesized. They turn out to be variable in the number, type, size, and composition of GAG side chains (O-linked and N-linked oligosaccharides), but also in the primary sequence and domain arrangement of core protein, giving rise to a very high number of possible combinations. Moreover, other factors that contribute to their heterogeneity are the degree of substitution of GAG chains and their distribution along the protein core [Schwartz and Domowicz, 2018; Theocharis et al., 2010]. So GAGs represent an important distinctive structural feature amongst different PGs and

consist in long and linear polysaccharides. Their building blocks are repeating disaccharide units composed of an amino sugar (D-glucosamine that is N-acetylated, or N-sulfated, or N-acetyl-D-galactosamine) and either uronic acid (D-glucuronic acid or L-iduronic acid) or galactose. These components can be sulfated at various positions and to different extents conferring specific ligand binding sites to the carbohydrate chain [Hassan et al., 2021] and promoting enormous structural diversity and complexity. Consequently, GAGs differ from one another in the type of monosaccharide in the repeating unit, the bonds between each monomer unit, the position of sulfate groups and the degree of sulfation. All these features allow to identify various categories of GAGs (Fig. 1): heparin and heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), hyaluronan (HA) and keratan sulfate (KS) [Afratis et al., 2012; Theocharis et al., 2010; Sarrazin et al., 2011].

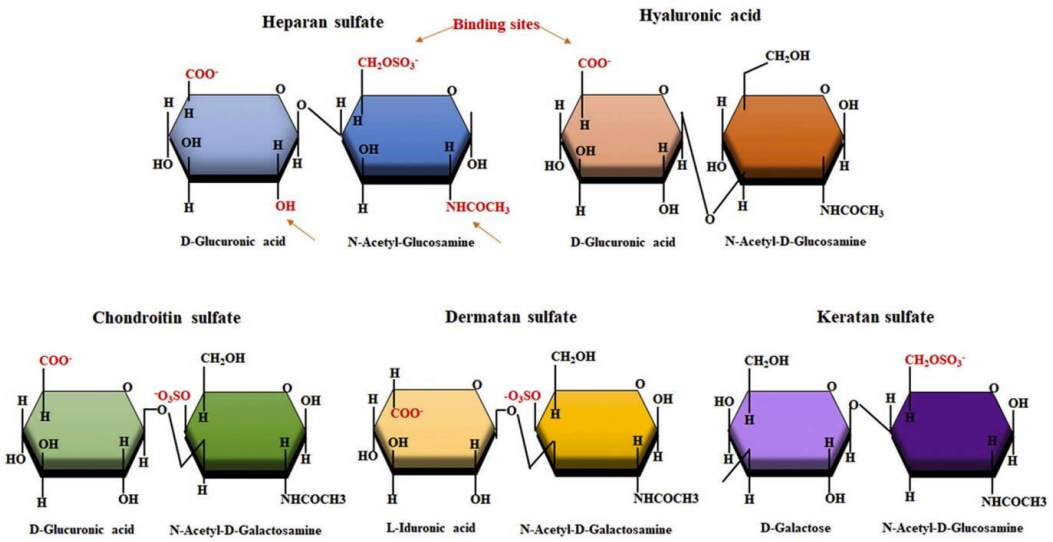


Figure 1. Glycosaminoglycan disaccharide structure [Hassan et al., 2021].

HS, CS and DS chains are covalently bound to serine residues of the PG core protein via a tetrasaccharide linkage, consisting of one xylose (Xyl), two galactose (Gal) residues and one glucuronic acid (GlcA). Keratan sulfate comprises N-acetyl-glucosamine and galactose in its disaccharide building blocks and does not contain uronic acid. In addition, unlike the tetrasaccharide linkage essential for the synthesis of HS , three possible linkers can be used for the synthesis of KS [Afratis et al., 2012; Theocharis et al., 2010; Sarrazin et al., 2011]. They are either N-linked to asparagines or O-linked to serine or threonine residues in the core protein and each

of these can be elongated with a number of other saccharides [Funderburgh, 2002; Sasarman et al., 2016]. Finally, HA, in contrast to the other GAGs, is a non-sulfated glycosaminoglycan that lacks a covalently bound protein core and it is synthesized at the intracellular surface of the plasma membrane [Faria-Ramos et al., 2021; Afratis et al., 2012; Theocharis et al., 2010; Sarrazin et al., 2011]. Thanks to their specific structural characteristics, GAGs can interact with a wide range of proteins involved in physiological and pathological processes and provide some of the structural basis for the multitude of their biological functions [Zhang, 2010; Afratis et al., 2012]. Indeed, carboxylic acid units present on the uronic acid residues and sulfate groups present on most of the units confers to GAGs a high negative charge, thus enabling interactions with the positively charged sites of amino acids in proteins. But also, the helical conformation allows them to interact with many ligands including protease inhibitors, interleukins, growth factors, morphogens and pathogen-derived proteins. Furthermore, the heterogeneous sulfation patterns form both specific and more generic binding motifs for a large variety of ligands [Karamanos et al., 2018].

HEPARAN SULFATE PROTEOGLYCANS

HSPGs are composed of a central protein carrying O-linked oligosaccharide and one or more covalently linked HS chains, together with CS chains in some of them. Their molecular mass varies according to the nature of the core protein, although it results in limited diversity, but mostly according to the number and length (50-400 disaccharide units) of the HS chains. HSPGs can be divided into three main families on the basis of their location: the secreted extracellular matrix HSPGs, including agrin, perlecan and collagen XVIII, the secretory vesicle proteoglycan, serglycin, and membrane HSPGs, the principal representatives [Esko et al., 2009; Sarrazin et al., 2011; Kirkpatrick et al., 2007; Iozzo and Schaefer, 2015].

CELL SURFACE HSPGs

Membrane HSPGs exhibit important characteristics. For example, like many surface proteins, after activating signaling, HSPGs can be internalized and lead ligands into

vesicles. Some of them are recycled to the membrane, but most end up in lysosomes where they undergo degradation by lysosomal proteases and exolytic glycosidases and sulfatases. Bound ligands are also degraded, which provides a mechanism for delivering nutrients to cells or for removal of bioactive factors from the environment [Sarrazin et al., 2011].

Moreover, HSPGs are also shed from the cell membrane and their truncated form distributed in ECM while maintaining the ability to bind ligands [Theocharis et al., 2010]. They can be enzymatically cleaved, especially by proteinases or heparanases that degrade HS-generating active glycan fragments, and released from the cell surface by ectodomain shedding [Manon-Jensen et al., 2010; Hassan et al., 2021]. Then, this ectodomain becomes responsible of an important post-translational mechanism that leads to the activation of different signaling pathways, including the MEK, CD44, TFG-β, VEGF and integrin ones, thus causing the modulation of diverse normal and pathological processes [Hassan et al., 2021].

To the family of membrane HSPGs belong syndecans, glypicans and other minor HSPGs, also called part-time PGs, because they can be present either in a non-GAG-substituted glycoprotein form or as a PG [Karamanos et al., 2018; Couchman, 2010]. HSPGs can in fact also be classified as “full-time” if their function is restricted to HS effects on cell signaling, or “part-time” if they have additional structural features and roles in multiple signaling pathways. Examples of full-time HSPGs are syndecans, glypicans and three basement membrane HSPGs (agrin, perlecan and collagen XVIII), while those part-time include CD44, betaglycan and neuropilins 1 and 2, as all of these single-pass transmembrane glycoproteins play important roles as co-receptors in additional signaling pathways independent of their HS modification (Fig. 2) [Knelson et al., 2014; Mythreye and Blobe, 2009; Kirkbride et al., 2005].

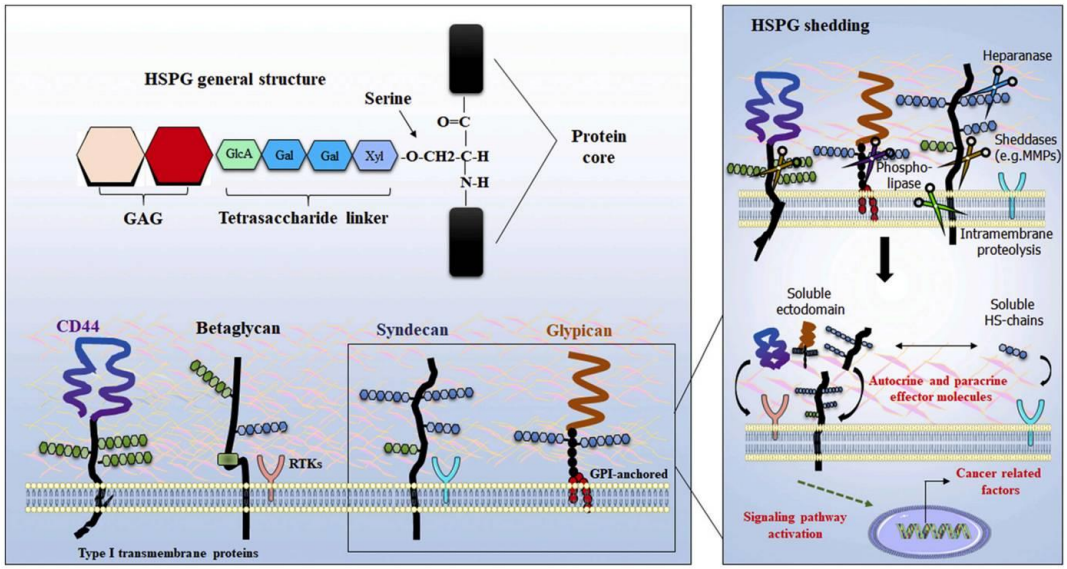


Figure 2. General structure and shedding of membrane HSPGs [Hassan et al., 2021].

CD44 is ubiquitously expressed throughout the body and consists in a HS-containing transmembrane type I protein, structurally highly heterogeneous as a result of post-translational modifications to which it undergoes. Its extracellular domain senses the external microenvironment interacting with different components of the ECM, like HA, but also collagen, laminin and fibronectin [Ponta et al., 2003]. On the other hand, its cytoplasmic tail mediates the interaction with many intracellular proteins, including the interplay with cytoskeletal ones that leads to modulation of pathways important for cell migration [Legg et al., 2002; Bourguignon et al., 1999]. CD44 can be cleaved by sheddases, like metalloproteinases (MMPs), and serine proteases, resulting in a soluble, paracrine effector molecule. However, an increase of its plasma levels can be associated with tumor aggressiveness and immune activation [Cichy and Puré., 2003].

Betaglycan, also known as transforming growth factor β receptor III (TGFβR3), is a ubiquitously-expressed cell surface proteoglycan that contains either HS or CS chains and belongs to the TGFβ superfamily of co-receptors [Hull et al., 2017; Iozzo and Schaefer, 2015]. Its extracellular domain presents several potential GAG attachment sites and protease-sensitive sequences near the plasma membrane, whereas the intracellular domain is highly enriched in Ser/Thr and contains a PDZ-binding element similar to syndecans. Instead, the membrane-proximal ectodomain is

characterized by a unique module called zona pellucida (ZP)-C that function as co-receptor for TGFβ superfamily [Iozzo and Schaefer, 2015; Lin et al., 2011].
Neuropilin-1 is also characterized by either HS or CS chains and regulate cellular responses to vascular endothelial growth factor (VEGF) and semaphorins important for angiogenesis and axonal guidance [Hull et al., 2017].
Syndecans and glypicans represent the major types of membrane HSPGs and they differ not only in their core protein structure and conformation (extended vs membrane-proximal) but also in the mode of membrane insertion [Karamanos et al., 2018].

Syndecans

Syndecans, with four members cloned in mammals (Sdc-1, -2, -3, -4), are characterized by a large ectodomain, a highly conserved transmembrane domain and an intracellular domain able to form homo- and heterodimers [Choi et al., 2015]. They contain predominantly HS chains, but sometimes also CS chains are covalently linked to the GAGs attachment sites present in the ectodomain. The latter differs among the four syndecan family in its amino acid sequence and is natively disordered, allowing syndecans to interact with many proteins and ligands [Iozzo and Schaefer, 2015] Instead, their intracellular domain consists of two regions of conserved amino acid sequence separated by a central variable sequence of amino acids, different for each family member [Choi et al., 2011]. Furthermore, the C-terminus of all the four syndecans presents a common motif (EFYA) able to bind PDZ-containing proteins that are important for proper anchoring of transmembrane proteins to the cytoskeleton [Iozzo and Schaefer, 2015]. At least, one member of the syndecans family is found in almost all cells, except erythrocytes [Kim et al., 1994]. For example, Sdc-1 can be found in epithelial and plasma cells, Sdc-2 in fibroblast and endothelial cells, Sdc-3 primarly in neuronal tissues but also in musculoskeletal tissue, whereas Sdc-4 is more heterogeneously expressed [Bernfield et al., 1999].
Syndecans can be cleaved constitutively or by exogenous stimuli such as chemokines, growth factors, trypsin and heparanase, thereby giving rise to a soluble molecule that can function as an autocrine and paracrine effector for itself or other receptors, as it can compete with intact syndecans for extracellular ligands within the ECM [Bernfield

et al.,1999; Manon-Jensen et al., 2010; Piperigkou et al., 2016]. Some syndecans also undergo intramembrane proteolysis resulting in signaling across the membrane [Schulz et al., 2003].

Glypicans

Glypicans are a family of six members (GPC-1, -2, -3, -4, -5, -6), all highly expressed during embryonic development [Tanaka et al., 2018]. They are bound to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor and contain an N-terminal secretory signal peptide and a hydrophobic domain in the C-terminal region. Their protein core turns out to be mostly unique to this family, although an amino acid tract in its ectodomain is similar to the Cys-rich domain of Frizzled proteins. In terms of their structural organization, they possess two unique features [Iozzo and Schaefer, 2015]. Unlike syndecans, in glypicans the HS chains are located near the juxtramembrane region in the C-terminus. This proximity to the cell surface allows them to present various morphogens and growth factors in an active configuration to their cognate receptors. For example, they bind to and modulate the activities of Hedgehog, Wnt and FGF [Filmus and Capurro, 2014; Capurro et al., 2014]. Another distinctive characteristic concerns how it is processed, in particular shedding is made possible by proteases and lipases. In the first case, endoproteolytic cleavage is carried out by a furin-like convertase and generates two subunits that bind to each other via disulfide bonds, in a way similar to the Met receptor. In the other case, however, the entire glypican is released from the plasma membrane through an extracellular lipase, called Notum, capable of cleaving the GPI anchor [Iozzo and Schaefer, 2015]. Additionally, most of the GPI-anchored glypicans can be found within lipid rafts on the apical side of the cell, but this is not exclusive [Taylor et al., 2009; Hull et al., 2017].

BIOSYNTHESIS

Heparan sulfate biosynthesis occurs via O-glycosylation in Golgi apparatus or at the endoplasmic reticulum (ER)-Golgi interface and can be seen as a process of “coding” and “decoding”. In the first part we have the synthesis, the elongation of the

polysaccharide chain and its modification with the addition of sulfated groups in specific positions, in order to form a pattern that represents a sort of "code". During the second part of the synthesis this code will be "decoded" by other enzymes that consequently modify the polysaccharide chain (Fig. 3) [Annaval et al., 2020; Zhang et al., 2016].

Chain initiation and polimerization

HS chain synthesis begins with the assembly of a linkage tetrasaccharide on serine residues in HSPG core proteins. This process is catalyzed by four enzymes which add individual residues sequentially to the non-reducing end of the growing chain. In particular, two xylosyltransferases XYLT1 or XYLT2 bind the xylose residue to a serine in the core protein and two galactose residues are thereafter added in sequence by the galactosyltransferases GalT-1 and GalT-2, respectively. After the addition of the first Gal residue, transient phosphorylation of the Xyl residue occurs, which is shown to be essential for subsequent assembly reactions as it increases the activity of the glycosyltransferase responsible for the addition of the second Gal residue to the nascent polysaccharide chain. The formation of the linkage region is finally completed by the addition of a glucuronic acid unit by the Glucuronyltransferase (GlcAT1) to the extremity of the chain. Simultaneously, there is dephosphorylation of the Xyl residue by the 2-Phosphoxylose phosphatase (XYLP), which turns out to be necessary for proper tetrasaccharide linker assembly [Kreuger and Kjellén, 2012; Lin, 2004; Sarrazin et al., 2011].

The above-mentioned enzymatic steps are common to the biosynthesis of heparin/HS and CS/DS GAG chains, while the following events are typical of the biosynthesis of HS. Following the formation of the linkage region, polymerization of HS chains begins with the addition of a N-acetylglucosamine (GlcNAc) residue to the non-reducing end of the acceptor linkage tetrasaccharide region by glycosyltransferases belonging to the EXTL family. After the EXTL-mediated initiation, the HS chain is extended by the action of the functional HS-polymerase complex, consisting of the EXT1 and EXT2 enzymes, which transfers alternating GlcA and GlcNAc residues to the growing polymer [Kreuger and Kjellén, 2012].

Chain modification

Once polymerized, HS chains are matured by HS modifying enzymes. Indeed, they simultaneously undergo a series of processing reactions that begins by the removal of the acetyl groups from clusters of GlcNAc residues and substitution of the free amino groups with sulfate, catalyzed by one or more N-deacetylase-N-sulfotransferases (NDSTs). Then the C5 epimerase converts many but not all GlcA units positioned next to glucosamine units into IdoA and, after epimerization, a series of O-sulfotransferases can add sulfate in different positions [Kreuger and Kjellén, 2012; Lin, 2004; Sarrazin et al., 2011]. These modifications are very important for the subsequent enzymatic reactions related to the polymerization activities of the glycosaminoglycan chains. In particular, it has been shown that the sulfation at the 3-O and 6-O sites of the GlcNAc and the sulfation at the 6-O site of GlcA are essential for the HSPGs synthesis [Knelson et al., 2014] and that, for example, the 4-O-sulfation of the second galactose molecule of the structure is associated with the synthesis of CSPGs and not of HSPGs [Kreuger and Kjellén, 2012]. Polymers can therefore undergo numerous and complex modifications, mediated by a large amount of enzymes, including different types of deacetylases, sulfotransferases, sulfatases, epimerases, peptidases and endoglycosidases, with the aim of generating structural diversity [Zhang et al. 2016].

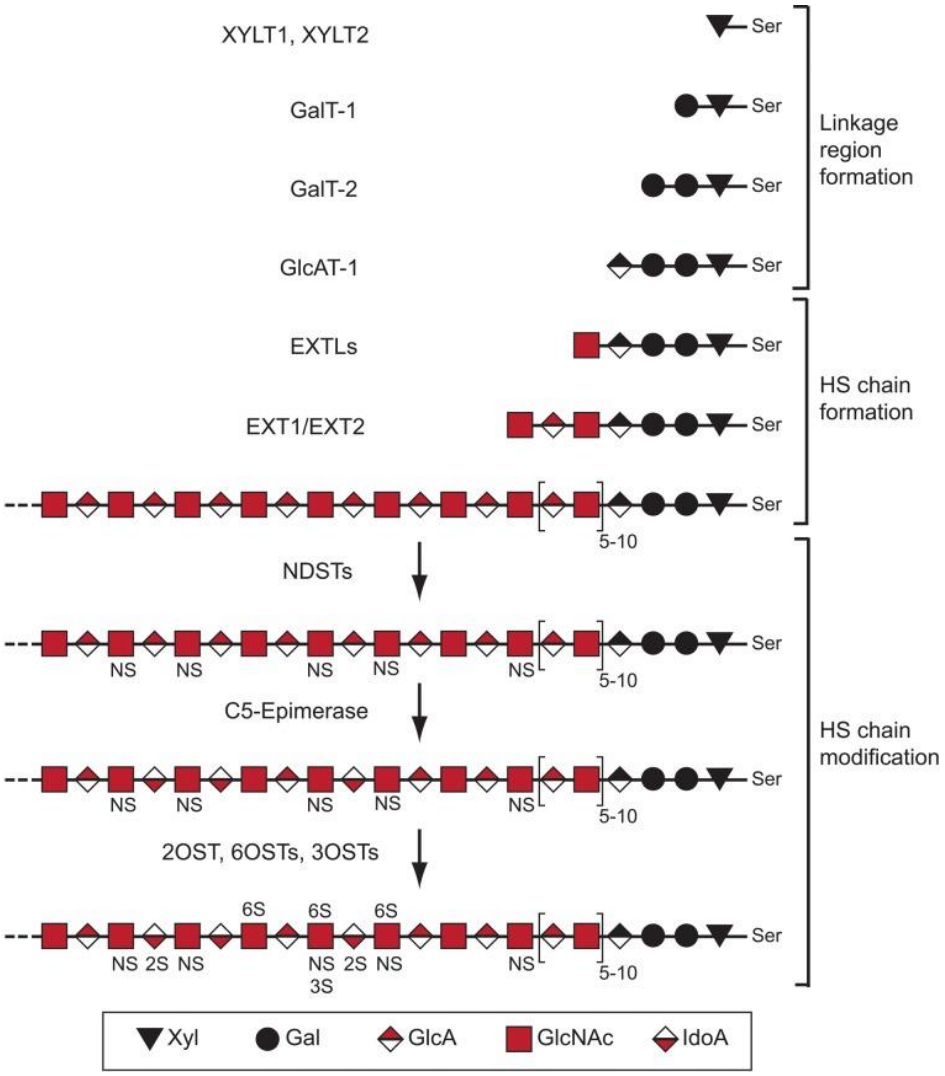


Figure 3. Steps of HS biosynthesis [Kreuger and Kjellén, 2012].

HSPGs FUNCTIONS

Considering their ability to interact with various heparin binding ligands, such as ECM macromolecules, enzymes, growth factors, cytokines, chemokines and morphogens, HSPGs are involved in multiple processes, ranging from embryonic development to ECM assembly and remodeling and regulation of cell signaling, especially that responsible for cell proliferation, differentiation and migration [Sarrazin et al., 2011; Lindahl and Li, 2009; Gama et al., 2006]. They are able to orchestrate metabolism, transport, cellular communication, support and regulation at both the systemic and cellular levels [Bishop et al., 2007]. Some HSPGs collaborate with other matrix components in order to organize basement membrane, providing a framework for epithelial support and a matrix for cell migration, regulating transport

of solutes and promoting the extravasation of cells during inflammatory responses from the vasculature into tissues, where they establish and provide cytokine gradients [Massena et al., 2010]. Those located within secretory vesicles play a role in the packaging of granular contents, maintaining proteases in the active state and regulating various activities after secretion. HSPGs modulate the morpoghen gradients distribution and other extracellular ligands signaling involved in the formation of the different tissue architectures during embryonic development [Yamaguchi, 2001]. Besides this, they are implicated in the regulation of apoptosis, cellular senescence and developmental signaling pathways, such as the Wnt, Hedgehog, transforming growth factor-β, and fibroblast growth factor pathways, all of which are HS-binding ligands [Nagarajan et al., 2018; Lin, 2004]. Another important function performed by HSPGs is to act as receptors for proteases and protease inhibitors regulating their spatial distribution and activity, like that of matrix metalloproteases within the ECM [Sarrazin et al., 2011; Bishop et al., 2007; Hull et al., 2017]. They also play a role in angiogenesis, due to their effect on angiogenic factors such as FGF, PDGF and VEGF, and in immune evasion, being able to affect innate immune response against cancer cells by modulating Natural Killer (NK) cell-mediated activity against them [Nagarajan et al., 2018]. In addition, one of the roles attributed to membrane HSPGs concerns their ability to act as co-receptors for growth factors and their receptor tyrosine kinases (present either on the same cell or on adjacent cells), lowering their activation threshold or changing the duration of signaling reactions [Bishop et al., 2007; Sarrazin et al., 2011]. Cell surface proteoglycans, for example, facilitate the formation and signaling of FGF2-FGF receptor complexes [Yayon et al., 1991; Rapraeger et al., 1991]. Then, they act as endocytic receptors, playing an important role in lipid metabolism and perhaps in the formation of morphogen and chemokine gradients, and cooperate with integrins and other cell adhesion receptors to facilitate cell motility as well as cell-cell and cell-ECM interactions and form bridges to the cytoskeleton [Sarrazin et al., 2011].

Alterations in the structure and expression of HSPGs, as well as aberrant expression of enzymes involved in regulating their biosynthesis and those responsible for their membrane shedding, could result in dysregulation of function and potentially lead to cancer [Hammond et al., 2014; Khurana et al., 2013; Gomes at al., 2013]. Indeed, several HSPGs have been shown to be upregulated in many tumors and can serve as

biomarkers for cancer diagnosis and/or prognosis [Hull et al., 2017]. Compared with non neoplastic ECM, tumor associated ECM contains higher concentrations of various growth factors and large amounts of specific proteoglycans and highly sulfated GAGs [Afratis et al., 2012; Theocharis et al., 2010]. HSPGs-ligand interactions also play multiple roles in the tumor environment, regulating, for example, cancer initiation and progression, cell proliferation, differentiation, migration and invasion [Sarrazin et al., 2011; Bishop et al., 2007; Kirkpatrick et al., 2007].

ACTIN

Actin is one of the most abundant, highly conserved and versatile proteins found in eukaryotes and is essential for the survival of most eukaryotic cells [Pollard and Borisy, 2003]. The actin monomer is a 42 kDa globular flat protein, a polypeptide chain of 375 residues characterized by two major domains, each consisting of two smaller subdomains and both presenting similar structure, but no sequence similarity, suggesting a possible gene duplication event early in evolution. According to their arrangement within the actin filament, the two major domains are known as the outer (comprising subdomains 1 and 2) and inner (comprising subdomains 3 and 4) domains. The polypeptide winds from the amino terminus in subdomain 1 to subdomains 2, 3, and 4 and back to subdomain 1 at the carboxyl terminus. It also contains a deep medial cleft in which ATP binds especially to subdomains 3 and 4, but also with residues in subdomains 1 and 2. In addition, several proteins bind in a prominent groove between subdomains 1 and 3 and, hence, some call it the "target-binding groove" [Dominguez, 2004; Pollard, 2016; Lee and Dominuez, 2010; Michelot and Drubin, 2011]. There are six different actin isoforms that are essential for a wide range of physiological functions. They can be divided into three α-actin isoforms (in muscles), one β-isoform (cytoplasmic, in non-muscle cells) and two γ-isoforms (one cytoplasmic, in non-muscle cells and one in some smooth muscles). The four muscle actins can be found in tissues with high tonic activity like striated heart muscle, skeletal muscle or smooth muscle of blood vessels, gut wall and the urogenital system [Tondeleir et al., 2009]. Cytoplasmic actins, on the other hand, are ubiquitously expressed and are much more dynamic and complex. Indeed, they can be associated

with processes such as cell motility, intracellular transport, cell shape maintenance or mitosis and include both the fairly stable cortex and a number of highly dynamic protrusions at the edges of cells. The amino acid sequence of α-actin differs from cytoplasmic actin isoforms in more than 20 residues that are spread over the entire molecule. In contrast, differences between β- and γ-actin are restricted to the N-terminus [Perrin and Ervasti, 2010; Müller et al., 2013; Dugina et al., 2009; Buracco et al, 2019]. The actin monomer represents the basic unit for building an actin filament [Holmes et al., 1990; Kabsch et al., 1990]. Thus, this protein is in a dynamic equilibrium between two states: the globular monomeric state (G-actin) and the filamentous polymeric one (F-actin) [Carlier et al., 2015]. Actin can work as ATPase and nucleotide hydrolysis by actin, together with a large number of G- and F-Actin-Binding Proteins (ABPs), plays an essential role in regulating this transition. ABPs are potentially implicated in many aspects of actin dynamics, produce higher order structures to fine-tune the mechanical properties of the cytoskeleton and carry out a wide range of functions, including actin filament nucleation, elongation, depolymerization, capping, severing, crosslinking and actin monomer sequestration [Lee et al., 2010; Pollard and Borisy, 2003].

Actin filaments are semiflexible structures having a diameter of about 7 nm and are one of the components of the cytoskeleton, along with microtubules and intermediate filaments. They consist of polymers characterized by a right-handed double-stranded helix in which the actin subunits assemble head-to-tail [Fife et al., 2014] and are all oriented toward the same end, thus giving rise to an asymmetrical filament. Therefore, these filaments exhibit inherent structural and functional polarity and consequently have two ends dinamycally different called pointed (or -) and barbed (or +) ends. The former is the preferred site of actin disassembly, while the latter is where ATP-bound G-actin is preferentially incorporated and it is much more dynamic than the previous one [Ananthakrishnan and Ehrlicher, 2007]. Thus, actin monomers join the barbed fast growing end of the filament in the ATP-bound state and leave the filament preferentially from the pointed end primarily in the ADP state, giving rise to a process known as actin filament treadmilling [Lee and Dominguez, 2010]. Multiple cycles of rapid polymerization and so elongation at the barbed end and disassembly closer to the pointed end allow the cells to remodel and rearrange the actin cytoskeleton, that represents the primary force generating machinery in the cell. It

can produce pushing (protrusive) forces through coordinated polymerization of multiple actin filaments, pulling (contractile) forces thanks to the sliding of bipolar filaments of myosin II along actin filaments and resistance (shaping) forces by forming cross-linked membrane-associated filament arrays. Actin-dependent forces are essential for cell migration, interaction with the environment and mechanosensing, shape and mechanical properties of the cell surface, polarity, cell division, endocytosis and trafficking and morphogenesis of membrane organelles [Svitkina, 2018; Mishra et al., 2014]. The actin cytoskeleton also participates in adhesion, being involved in adherens junctions.

ACTIN-BINDING PROTEINS

ABPs include several proteins. Actin-monomer-binding proteins comprise thymosin-β4 and profilin, with the latter that is necessary for the maintenance of a large pool of actin monomers, catalyzes nucleotide exchange and inhibits nucleation and pointed end elongation, but not elongation at barbed ends. Capping protein is a heterodimer of structurally similar α- and β-subunits able to bind tightly to actin filament barbed ends inhibiting their elongation, with the exception of the tropomodulin that wraps the pointed end. Cofilin, the most important member of the actin depolymerizing factor (ADF)/cofilin family, belongs to the group of severing proteins, together to large multi-domain proteins of gelsolin family [Pollard, 2016] and two formins, FRL- α [Harris et al., 2004] and INF-2 [Gurel et al., 2014]. Its main function is to sever actin filaments, leading to both the formation of free barbed ends that can be useful for actin polymerization and the disassembly of the branched actin network [Pollard, 2016; Coumans et al., 2018]. Cofilin is consitutively active and can be associated with increased filopodia [Collazo et al., 2014], whereas its phosphorylated form (p-cofilin) is inactive and seems to be important for lamellipodia-driven directional migration [Sidani et al., 2007]. Another important group of ABPs includes nucleation proteins. Cell can use the Arp2/3 complex to generate actin filament branches inducing continuous nucleation of new “daughter” actin filaments at the side of preexisting “mother”, a process called “dendritic nucleation” [Mullins et al., 1998]. This process makes it possible to create branches faster that the polymerization of linear actin filaments, which is instead entrusted to

the formins. The latter also act as actin filament polymerases, in that they can inhibit or promote elongation of the barbed ends of actin filaments by processively interacting with the ends [Paul and Pollard, 2009]. Proteins belonging to the Ena/VASP family also associate with growing actin filament barbed ends, promote elongation and inhibit capping, but, unlike formins, they do not seem to nucleate polymerization [Edwards et al., 2014; Pollard, 2016]. Cross-linking proteins allow physical connections between actin filament, promoting the formation of higher-order structures, like bundles of filaments in microvilli, filopodia, cytplasmic cables and networks of actin filaments. They include fimbrin and fascin, which are involved in the formation of actin filament bundles, but also filamin, that is important for the organization of actin filament networks, such as those at the leading edge of motile cells [Svitkina, 2018]. Finally, tropomyosin is a protein that binds along the actin filaments, protect them from severing by cofilin [Maciver et al., 1991] and affects how the motor protein myosin interacts with a filament [Pollard and Lord, 2014].

ACTIN STRUCTURES

Networks and bundles of actin filaments generate and maintain the forces that mediate cell migration. They interact with each other, with multiple signaling intermediates and with the cell exterior [Parsons et al., 2010; Small et al., 2002]. Thanks to their polymerization and their association with actin regulatory proteins, actin filaments can assemble into numerous distinct structures , built in space and time in competition with the spontaneous assembly of unorganized actin filaments and localized in distinct areas in the cell, where they can perform their functions in response to different stimuli. The main architectures produced are: branched actin networks (present in lamellipodia), crosslinked networks (present in the cell cortex), bundles of parallel actin filaments (present in filopodia) and bundles of antiparallel actin filaments (in stress fibers) (Fig. 4) [Blanchoin et al., 2014; Michelot et al., 2011].
Lamellipodia and filopodia are the main organelles driving motility while contractile structures such as traverse arcs, focal adhesion-anchored stress fibers and the cell cortex ensure mechanical integrity and coherent movement of the cells as a whole [Blanchoin et al., 2014].

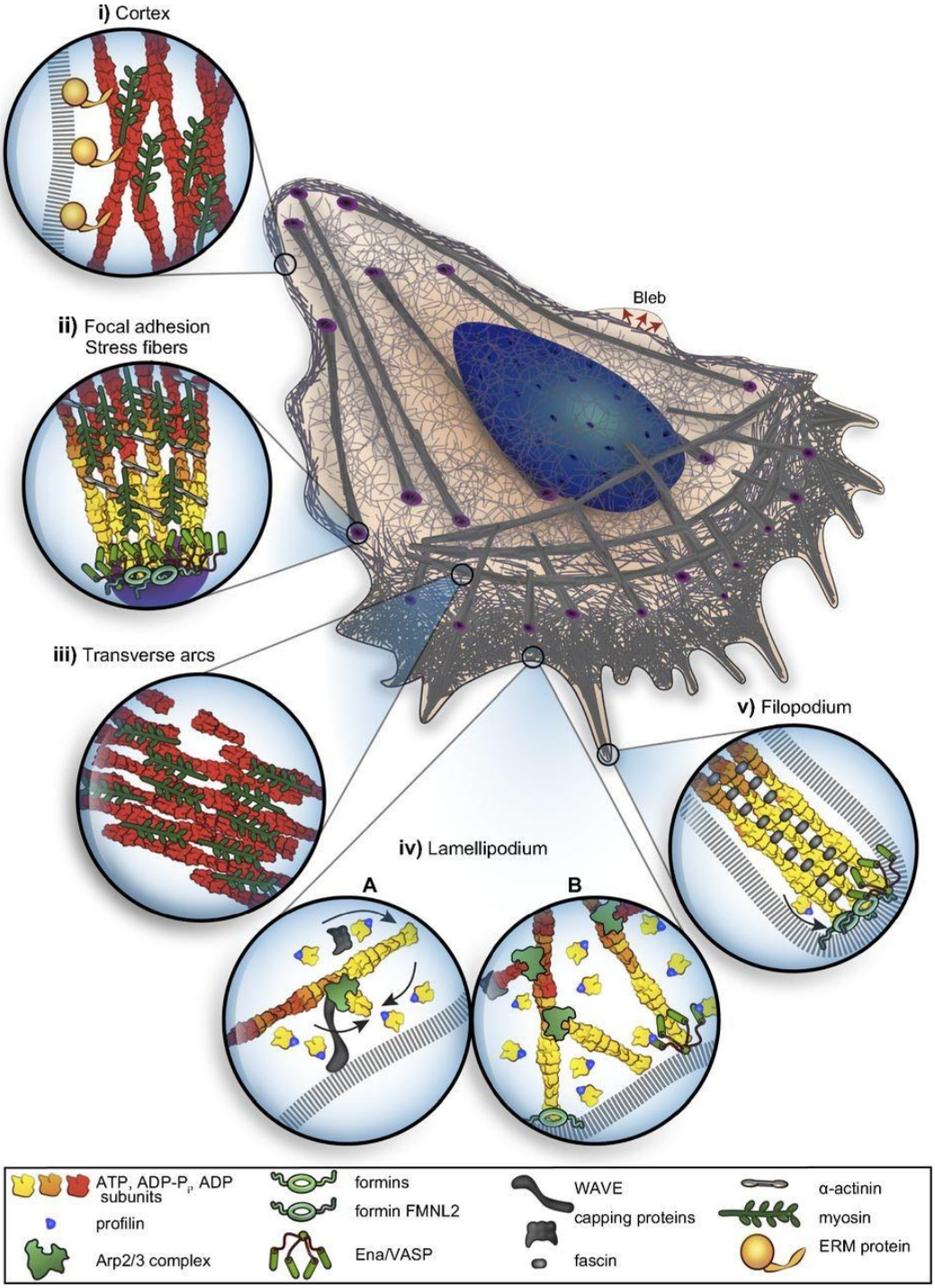


Figure 4. Specialized actin architectures in motile cells and relative proteins involved in their assembly and dynamics [Blanchoin et al., 2014].

Lamellipodia

Lamellipodia are broad, flat and sheet-like membrane protrusions present at the leading edge of migrating cells in front of the lamella [Yamaguchi et al., 2007], a region where actin is organized into contractile bundles [Vallotton et al., 2009] frequently associated with adhesion sites [Choi et al., 2008]. Lamellipodia, on the other hand, are characterized by branched (or dendritic) actin networks in which filaments are oriented with their barbed ends toward the leading edge. The width of the lamellipodium can be highly variable, but does usually not exceed 10 μm and the concentration of actin filaments normally displays a linear decay from front to back [Watanabe and Mitchison, 2002; Small et al., 1995]. However, according to cell type, the state of protrusion, the cell membrane and its surface tension, density, length and orientation of actin filaments and frequency and distribution of branches can be different. Lamellipodia containing a low density of long branched filaments protrude faster, but, at the same time, the filaments can fold upwards or backwards onto the cell, leading to ruffling and retraction of lamellipodia. By contrast, those with a high density of short filaments are more persistent, even if they protrude more slowly [Bear et al., 2002; Small et al., 2002]. Furthermore, a higher membrane surface tension led to a more oriented actin filament polymerization even in the absence of crosslinking proteins or other clustering factors, while a lower tension resulted in more protrusions, probably related to the forces generated by the lamellipodium [Houk et al., 2012]. Finally, the geometry of the available adhesive surface and a balance of intracellular signaling pathways can influence the dimensions and overall morphology of these membrane protrusions [Petrie et al., 2012; Svitkina, 2018]. Lamellipodia generate large pushing forces responsible for propelling the cell front and allow the cell to move around obstacles, sensing soluble guidance cues and probing the chemical and mechanical properties of the substratum. They are transient structures which protrude and retract, so their branched actin filaments must be assembled and disassembled rapidly and turned over. The protrusion of lamellipodia is initiated by localized polymerization of actin, so generation of free barbed ends is required at the leading edge. To achieve this purpose, there are three major mechanisms: (1) de novo nucleation by Arp2/3 complex and formins, (2) severing of pre-existing actin filaments by cofilin and (3) uncapping of barbed ends

on pre-existing actin filaments [Zigmond, 2004; Condeelis, 2001]. Lamellipodia formation is promoted by the Arp2/3 complex through the process of dendritic nucleation. This is then followed by the elongation of newly nucleated filaments, which is subsequently inhibited by capping proteins to keep short, stiff filaments as well as to concentrate polymerization to the protruding region close to the plasma membrane [Hotulainen and Lappalainen, 2006; Yamaguchi et al., 2007]. Disassembly of the network occurs through a combination of debranching and severing of actin filaments, followed by depolymerization of filament fragments. So, in this case, individual filament do not treadmill, but are "born" at a branchpoint, grow at the barbed end, become capped, and later "die" by depolymerization [Pollard and Borisy, 2003]. However, the array of branched filaments in lamellipodia undergoes treadmilling as a whole by assembling at the front and disassembling throughout its body. Many proteins are involved in lamellipodia formation, especially small GTPases of the Rho family, like Rac, and an interconnected network of WASP, Ena/VASP and formin families of actin regulators [Ridley et al., 2003; Krause and Gautreau, 2014]. Rac is primarily responsible for actin polymerization required for lamellipodia extension. Local Rac proteins are activated by Rac guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP for GTP molecules, thereby making the protein able to stimulate downstream targets. Active Rac proteins (Rac1, Rac2 and/or Rac3, depending on the cell type and conditions) interact with the WAVE-associated complex of proteins, otherwise intrinsically inactive, which in turn activates the Arp2/3 complex and so leading actin nucleation. Furthermore, active Rac interacts with lamellipodin, a scaffold protein which contributes to actin filament extension probably bringing Rac close to the WAVE complex [Ridley, 2015; Law et al., 2013; Havrylenko et al., 2015]. A precise balance between actin polymerization and adhesion is required to promote lamellipodia-based migration. Indeed, if the lamellipodium persists long enough, cells can adhere to the substrate through integrin-mediated adhesions, contract and then detach adhesions at the rear [Petrie et al., 2009].

Filopodia

Filopodia are thin, finger-like projections composed of tight parallel bundles of actin filaments which are oriented with their growing barbed ends toward the membrane due to the presence, in the filopodia tip complex, of formin and Ena/VASP proteins [Blanchoin et al., 2014]. Filopodial protrusions and retractions are governed by a dynamic balance between the rate of actin cytoskeleton assembly at the barbed ends of a filament and the retrograde flow of the actin filament bundle [Mallavarapu and Mitchison, 1999]. These protrusions are usually located at the front of a migrating or elongating cell, often found embedded in or protruding from the lamellipodial network. Very short filopodia present within the lamellipodium, but also embedded in cell cortex, are called microspikes [Small and Celis, 1978; Lewis and Bridgman, 1998; Svitkina et al., 2003; Mattila and Lappalainen, 2018]. However, dynamics, structural organization, length and position of these protrusions may vary according to the cell type, ranging from sequentially protrusive retractile filopodia to the apparently static microvilli of epithelia or the stereocilia in cochleair hair cells [Schaks et al., 2019]. Regarding the mechanism of their formation, alternative models have been proposed. In the "convergent elongation model", filopodial actin filaments seem to arise from the Arp2/3 complex-nucleated dendritic lamellipodial actin array. However, filopodia can also form in the absence of this complex, according to an alternative mechanism, the "de novo filament nucleation model". Actin filaments of filopodia, in this case, are nucleated at filopodial tips by formins and do not derive from the underlying lamellipodial network. For both models, actin filaments are then elongated via mDia2 and Ena/VASP, stabilized and bundled by cross-linkers, like fascin, thus generating "mature" filopodia [Mattila and Lappalainen, 2008].

The Rho GTPase Cdc42 is very important for filopodia formation [Batchelder et al., 2011]. In addition, molecular motors are able to transport various proteins, including transmembrane receptors, along actin filaments to the tips of filopodia to participate in maintaining and stabilizing filopodia, sensing the environment and signals, establishing cell-cell junctions and long-distance transport [Jacquemet et al., 2015]. So filopodia contain receptors for diverse signaling and ECM molecules and have several functions, both physiological and pathological processes. They can act as sites for signal transduction and play a key role in substrate tethering and environment

sensing. They act as “antennae” or “tentacles” that allow the cells to probe their microenvironment, not only sensing the ligands present in it, but possibly chemoattractant gradients as well [Jacquemet et al., 2015; Mattila and Lappalainen., 2008; Heckman and Plummer, 2013]. Furthermore, they play important roles in wound healing, adhesion to ECM, guidance towards chemo-attractants, neuronal growth-cone path finding and embryonic development, but its formation is also implicated in cancer invasion and metastasis. Abundant filopodia may be indicative of tumor, fascin results upregulated in several metastatic mouse and human contexts [Vignjevic et al., 2007; Huang et al., 2015; Li et al., 2014], as well as myosin X is upregulated by mutant p53 promoting cell invasion and metastasis [Arjonen et al., 2014].

Stress fibers

Stress fibers are bundles of 10-30 unbranched actin filaments, which are connected mainly by α-actinin and by other actin crosslinking proteins, such as fascin, espin and filamin [Cramer et al., 1997; Pellegrin and Mellor, 2007; Naumanen et al., 2008]. These structures can be detected both at the leading lamella and at the trailing edge of mesenchymal-migrating cells, while they are not evident in amoeboid ones [Friedl and Wolf, 2010]. Stress fibers are essential for adhesion, polarity and changes related to cell shape [Cramer et al.,1997; Hotulanein and Lappalainen, 2006]. According to their subcellular location, but also for their different morphology and association with focal adhesions, they can be divided in dorsal and ventral stress fibers, transverse arcs and the perinuclear actin cap [Heath, 1983; Small et al., 1998; Khatau et al., 2009]. Only three of these types (ventral stress fibers, transverse arcs and the perinuclear actin cap) are capable of contracting because they contain myosin II [Peterson et al., 2004]. Dorsal stress fibers are anchored to focal adhesions at their distal ends, which are composed of unipolar actin filaments with growing barbed ends facing the periphery of the cell. On the contrary, the more proximal parts of the bundle are characterized by actin filaments with mixed polarity [Cramer et al., 1997; Pellegrin and Mellor, 2007]. Dorsal stress fibers appear to serve as a platform for assembling other types of stress fibers and connecting them to focal adhesions [Hotulainen and Lappalainen, 2006; Tojkander et al., 2011]. Transverse arcs, instead,

are curved actin filament bundles, oriented perpendicular to the ventral fibers and characterized by a periodic α-actinin myosin pattern, typical for contractile actomyosin bundles. Arcs are not anchored in focal adhesions, but convey contractile force to the surrounding environment through their connections with dorsal stress fibers. They form beneath the dorsal surface of migrating cells just behind the lamellipodium and then head, once formed, toward the nucleus, where they disassemble [Heath, 1983; Small et al., 1998; Hotulainen and Lappalainen, 2006]. On the other hand, ventral stress fibers are often located at the posterior parts of the cell, where occasional contraction cycles promote rear constriction and facilitate cell movement [Chen, 1981; Mitchison and Cramer, 1996; Tojkander et al., 2011]. They are the most commonly observed structures and are composed of contractile actomyosin bundles that lie along the base of the cell, running approximately parallel to the direction of movement and linking to focal adhesions at each end [reviewed in Pellegrin and Mellor, 2007]. Finally, the perinuclear actin cap consists in contractile stress fibers extend above the nucleus, associates with nuclear envelope through the linker of nucleoskeleton and cytoskeleton (LINC) complex, and terminates in focal adhesion at cell rear [Maninová and Vomastek, 2016]. They play a key role in regulating the shape of the nucleus in interphase cells and might act as mechanotransducers, transmitting force from the cell environment to the nucleus [Khatau et al., 2009].
Important proteins involved in stress fibers assembly are the RhoA small GTPase and formins, especially mDia1 [Hotulainen and Lappalainen, 2006].

The cell cortex and blebs

The cell cortex is present in most animal cells and consists in a thin actin network which forms a contractile structure directly beneath the membrane [Hohmann and Dehghani, 2019]. It contains a mixture of bundled straight and branched filaments, but also myosin-II and several actin-binding proteins. In particular, some of actin filaments are aligned into parallel and/or anti-parallel bundles thanks to the action of proteins such as fascin and plastin, while others are organized in random branched networks by proteins like filamin. Furthermore, some proteins, for example α-actinin, appear to be able to promote both types of organization [Falzone et al., 2012]. In

addition to these crosslinkers, actin nucleation factors can be found in the cortex. In fact, cortical actin assembly appears to depend on the combined action of formins (especially mDia1), which nucleate and elongate linear actin filaments, and the Arp2/3 complex, which drives filament branch formation [Chugh and Paluch, 2018]. Capping proteins (CAPZA, CAPZB), the actin-severing and -disassembling protein cofilin (CFL1 and CFL2), and profilin 1 (PFN1), able to bind actin monomers and to promote actin polymerization, are also present [Biro et al., 2013]. Finally, proteins of the ERM (ezrin, radixin, moesin) family, essential for linking actin cytoskeleton to the plasma membrane, and signaling molecules such as Rho GTPases, Rho GEFs, and Rho GAPs (GTPase activating proteins) have been identified in the cortex [Charras et al., 2006]. Moreover, myosin-II is the major contributor to cortical contractile tension, one of the main properties of the cortex, important for the regulation of the cell shape of single cells and tissues [Lecuit and Lenne, 2007]. It depends precisely on the myosin activity and actin polymerization. Cortical tension gradients are due to local changes in cortex composition or organization and can result in local contractions and cellular deformations. Increased myosin activity and decreased actin polymerization lead to a higher cortex tension, which occurs, for example, in the posterior part of a migrating cell, promoting cell body retraction [Charras et al., 2008; Chabaud et al., 2015; Vicente-Manzanares et al., 2009]. On the other side, lower cortex tension is associated with greater protrusive activity of the cell, thus indirectly regulating cell motility [Kasza et al., 2007]. In addition to controlling morphogenesis of animal cells, the cortex is also involved in controlling cell contacts and contributes to cell polarization and division [Chugh and Paluch, 2018]. However local drops in cortex tension or cortex-membrane adhesion and local ruptures of the cortex can be the origin of so called blebs [Paluch and Raz, 2013]. They are membrane bulges, dynamically extruded at the cell surface [Charras et al., 2008; Charras et al., 2005], whose progress can be divided in three steps: initiation, growth, and retraction. Initially, the growing bleb consists in an actin free membrane protrusion but over time, when the bleb expands further, the actin cortex reassembles at the plasma membrane, stalling the bleb growth up to the point of a full restoration where the generated contractile forces retract the bleb [Hohmann and Dehghani, 2019] Recently, blebs have been shown to be a way for the cell to relax excess tension. In

other cases, blebs subsequently fill with polymerized actin and myosin, and adhere to the substratum to drive motility (ameboid motility) [Blanchoin et al., 2014].

Invadopodia

In order to invade, cells have to migrate through ECM, degrading and remodeling its structures and generating other specialized protrusions called invadopodia. Invadopodia are ventral membrane protrusions with an ECM degradation activity that allows invasive cancer cells to make holes in the extracellular matrix [Buccione et al., 2004]. They are composed of a variety of proteins, such as actin and actin regulatory proteins, adhesion molecules, membrane remodeling and signaling proteins, and matrix degradation enzymes. Other structures that are similar to invadopodia in their appearance and molecular composition are podosomes. Classic podosomes are formed by cell types of monocytic origin, such as macrophages, dendritic cells and osteoclasts [Yamaguchi et al., 2007]. So invadopodia and podosomes are dynamic actin-based molecular protrusions that form in cancer cells and normal cells respectively in response to diverse signaling pathways and extracellular matrix cues [Paterson and Courtneidge, 2018]. They establish contact with the extracellular matrix through integrins and CD44 and recruit metalloproteases in order to degrade it [Linder, 2007]. Invadopodia form in discrete temporal stages: invadopodium precursor core initiation, invadopodium precursor stabilization and invadopodium maturation [Beaty and Condeelis, 2014]. Invadopodia initiated by either growth factor or ECM signals can occur in different locations within the same tumor cell [Sharma et al., 2013] or in different tumor cells in different tissue locations [Génot and Gligorijevic, 2014]. The above stages appear to be the same for these two forms of initiation [Eddy et al., 2017].

CELL MIGRATION

Cell migration is a highly regulated phenomenon that plays a key role in many biological functions, resulting involved in both physiological and pathological processes. It turns out to be in fact essential for the development of an organism (morphogenesis), tissue homeostasis and wound healing, immune response and cancer metastasis, but it also contributes to vascular diseases, osteoporosis, chronic inflammatory diseases and mental retardation [Ridley et al., 2003; Vicente-Manzanares et al., 2005]. For example, during development neural crest cells migrate throughout the embryo and give rise to different kinds of cells such as melanocytes, vascular smooth muscle and Schwann cells [Mayor and Theveneau, 2013]. Similarly, immune cells can migrate over large distances to fight off pathogens and reach sites of inflammation [Hampton and Chtanova, 2019; Krummel et al., 2016]. However, cells can migrate not only through the ECM, but also on the top of each other, between each other and even through each other. Leukocytes, for instance, attach to and migrate on endothelial cells lining the bloodstream before crossing the endothelium either between two endothelial cells or by inducing the formation of a membrane channel through a single endothelial cell [Ridley, 2015]. In general, cell movement requires coordination of cytoskeleton dynamics and reorganization, cell adhesion and signal transduction pathways that involve lipid second messengers, small GTPases, kinases, cytoskeleton-modifying proteins and motor proteins [Devreotes and Horwitz, 2015; Welf and Haugh, 2011]. In order for cells to start moving, a signal must be generated. It may be transient, in the case of a short migration, or may involve a long-lasting change to the environment that guides cells for a prolonged period [SenGupta et al., 2021]. Directional migration can be initiated by a variety of cues: diffusible cues (chemotaxis), including growth factors and chemokines; surface-bound chemical cues (haptotaxis), such as those provided by the components of ECM (for example, fibronectin, laminin and various collagens); biophysical cues, where cells sense the topographical features of the surrounding microenvironment (topotaxis, also known as contact guidance); mechanical substrate compliance (durotaxis), with the cells able to sense differences in substrate stiffness; electric fields (galvanotaxis, also known as electrotaxis), promoting to the activation of several intracellular signaling pathways [Parsons et al., 2010; SenGupta et al., 2021].

Distinct cell types exhibit different motility styles and can even switch dynamically between states in response to intrinsic factors and changes in the environment [Caswell, 2018]. The type of cell migration and invasion can vary based on differences regarding extracellular protease activities, integrin-mediated cell-matrix adhesion, cadherin-mediated cell-cell adhesion, cell polarity and cytoskeleton arrangement. Cells can move either as single cells or in a solid multicellular component. Single-cell migration includes amoeboid and mesenchymal migration strategies, while collective migration refers to filaments, sheets, tubes and multicellular clusters [Parri and Chiarugi, 2010].

AMOEBOID MIGRATION

Amoeboid migration has some distinctive features that are typical of cells that move based on this pattern. Indeed, they move very fast, change direction frequently, and interact with the substrate through weak and short-lived connections. They exhibit a rounded but at the same time highly deformable morphology and increased cell contractility. In fact, the ability of the cortical actin cytoskeleton to reorganize allows migrating cells to expand and contract rapidly, thus leading to relocation by changing their position [Friedl and Wolf, 2003; Wu et al., 2021; Parri and Chiarugi, 2010]. Nucleus deformation also maintains the movement of amoeboid cells. Other properties related to this type of migration consist of the lack of intercellular adhesion and proteolytic degradation of the ECM, which is no longer required as the cells are able to squeeze through gaps in the surrounding matrix instead of degrading it [Holle et al., 2019]. It also turns out to be independent of cell-ECM contacts, in this case rather weak and not organized into focal adhesions. In this regard, it has been shown that integrin inhibition cannot block amoeboid movement. In addition, cells that adopt this style of motility can create cell membrane protrusions, called blebs, that allow them to perceive the microenvironment and penetrate through confined spaces [Yilmaz and Christofori, 2010; Friedl and Wolf, 2003]. Finally, amoeboid migration does not involve Rac-mediated polarization, but the protrusions and dynamics of the cortical actin cytoskeleton are mainly regulated by the small GTPase RhoA and its effector, Rho-associated kinase (ROCK) [Parri and Chiarugi, 2010]. Amoeboid strategies can be used by several cells, starting from immune cells, when to

respond quickly to an infection or damaged tissue they have to cross complex barriers, up to metastatic cells, to facilitate migration, dissemination and survival [George at al., 2023].

MESENCHYMAL MIGRATION

Mesenchymal motility consist of an elongated, fibroblast-like, cell morphology with established cell polarity and is dependent, upon proteolysis, to the degradation of the ECM [Friedl and Wolf, 2003]. In general, it is a cyclic process, characterized by multiple steps.

Polarization, actin polymerization and extension of protrusions

The initial response of a cell to a migration-promoting agent is to polarize. Indeed, upon several stimuli, generation of phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P3) at the leading edge of the cell leads to cell polarization through activation of the small GTPase Rac1, which is responsible for the organization of actin polymerization and lamellipodium formation [Ridley et al., 2003]. Activation of cell division control protein 42 homolog (Cdc42) and the recruitment of adaptor proteins can also promote actin polymerization. The directionality of cell movement is maintained by Cdc42, which coordinates actin polymerization at the front of the cell [Ridley et al., 2003; Etienne-Manneville and Hall, 2001]. Together, these events lead to the formation of the leading and trailing cell edges, and allow to extend protrusions in the direction of migration, by orienting and reorganizing (growing) the actin network at its leading edge [Ridley et al., 2003]. The presence of a distinct front and rear, in which different signaling pathways promote membrane protrusion and retraction, is a key feature of cell migration [Welf and Haugh, 2011; Devreotes and Horwitz, 2015]. Lamellipodia and filopodia are usually driven by actin polymerization and able to form stable contacts with the substrate, thus preventing the retraction of the leading edge.

Adhesion

For migration, a protrusion must form and then stabilize by attaching to the surroundings. Many different receptors are involved in the migration of different cell types; particularly, integrins represent a major family of migration-promoting receptors. Upon ligand binding, integrins recruit numerous proteins to their short cytoplasmic tails, leading to the formation of various adhesion structures that differ in morphology, subcellular localization, protein composition and mechanical properties [Schiller and Fässler, 2013]. In particular, they recruit scaffold and signaling proteins such as paxillin and the focal adhesion kinase (FAK). The first adhesion structure assembles at the leading edge forming nascent adhesions (NAs). The majority of NAs are disassembled quickly (adhesion turnover), while only a few of them grow in size along templates of actomyosin bundles becoming bigger focal adhesions (FAs). This occurs at the transition between the lamellipodium and lamella, where retrograde actin flow changes from being driven by actin polymerization to myosin-based contraction. For this reason FAs maturation is a coordinated process and requires further integrin clustering, F-actin bundling and the reinforcement of the linkage between integrin and actomyosin [Sun et al., 2016]. Behind the lamellum, the mechanical linkage between FAs and the contractile actomyosin bundles is relaxed and/or released. The result can be the clathrin-dependent FA disassembly [Ezratty et al., 2009] or the translocation of β1 integrin–containing adhesions into central, fibrillar adhesions (FBs) [Zamir et al., 2000]. Focal adhesions are elongated in morphology and can be found in both central and peripheral regions of the cell, generally at the ends of actin filament bundles [Huttenlocher et al., 2011].

Translocation of cell body

Considering their ability to connect the ECM to the intracellular cytoskeleton, adhesions serve as both traction sites over which the cell moves and as mechanosensors, transmitting information about the physical state of the ECM into the cell and altering cytoskeletal dynamics [Ridley et al., 2003]. The retraction force required for the translocation of the cell body is generally thought to derive from the sliding of myosin motors, such as myosin II, on actin filaments or actin bundles in the

cell body and rear. Since the actin filaments and bundles are connected to the cell membrane and the substrate, the force generated can be converted to traction forces that enable the cell to move forward [Ananthakrishnan et al., 2007].

Adhesion disassembly and retraction of the rear

At the end of the process adhesions at the rear of the cell must disassemble and the trailing edge retract. The tension generated by the contraction of stress fibers can be sufficient to break the linkage between integrin and the actin cytoskeleton, with the result that integrin is left behind while the rest of the cell moves on [Ridley et al., 2003]. So tail retraction requires release and/or disassembly of focal adhesions at the rear of the cell, while adhesions at the front are maintained to allow application of traction forces [Pellegrin and Mellor, 2007]. The role of RhoA turns out to be crucial in this step, regulating the contraction and the traction forces at the rear of the cell [Raftopoulou and Hall, 2004].

COLLECTIVE MIGRATION

Another type of motility is collective migration. In this case, cells maintain their cell-cell junctions and migrate in sheets, strands, tubes and cluster, either still in connection with their originating tissue or as separated, independently migrating cluster [Parri and Chiarugi, 2010]. During collective migration, signals from external cues are transmitted to the entire mass of cells through the integration of intracellular and intercellular signalling cascades as well as mechanotransduction at cell–cell junctions and cell–ECM interfaces [Ladoux and Mège, 2017; SenGupta et al., 2021]. This results in front–rear polarity at a supracellular level. A group of cells situated at the front of the supracellular unit generally becomes the leader cells. They are specialized front cells that occupy the leading edge of the collective, assume a “finger-like” structure and in response to external cues can extend stable lamellipodia or filopodia towards the substrate. Indeed, they can secrete matrix metalloproteinases that remodel the surrounding ECM, paving the way for collective migration during cancer invasion. In contrast, follower cells, which comprise the majority of the collective, are located in the cell reservoir, at the rear, and extend

small transient cryptic lamellipodia. Follower cells were long considered to be "passive passengers" that simply moved along with leader cells [SenGupta et al., 2021; De Pascalis and Etienne-Manneville, 2017]. Moreover, at the leading edge of the collective, leader cells experience asymmetric adherent connections with integrin-based focal adhesions at their extending fronts and cadherin-based adherens junctions (AJs) at their cell–cell connections with follower cells at their trailing edges. However, in the cell reservoir, follower cells experience symmetric AJ adhesions with neighboring cells [Mayor and Carmona-Fontaine, 2010; Khalil and de Rooij, 2019]. These differential focal adhesion and adherent protein distributions lead to heterogeneous contractile forces at their cell–cell junctions [Chen et al., 2018; Khalil and de Rooij, 2019], thus allowing distinct cellular activities of PI3K-Rac signaling and Rho GTPases in these two populations [Zegers and Friedl, 2014].

METASTASIS

The central role of the actin cytoskeleton and its regulation in several cell motility processes make the system vulnerable to mutations and defects. In particular, the balance between the synergistic and antagonistic activities of the proteins regulating actin dynamics can be altered as a result of mutations in the protein itself, changes affecting upstream regulatory proteins involved in signaling and changes in expression levels or actin binding proteins [Lambrechts et al., 2004]. All of these give rise to anomalies that can result in abnormal cell movement, which in turn can lead to defects during neuronal development, chronic wounds that never heal and immune deficiencies. Furthermore, an aberrant regulation of cell migration may be associated with other pathologies, such as autoimmune disease, fibrosis and especially cancer cell invasion and metastasis. The metastatic spread of cancer cells is a highly selective process characterized by a series of sequential steps that allow cells to leave primary tumors and to colonize distant anatomical sites. The efficiency with which clinically evident metastases form depends on three main factors: tumor-host interactions important for metastatic progression as tumor cells leave the primary lesion and enter a new environment; tissue structure and vasculature biomechanics that determine the pathway and distribution of circulating tumor cells, creating hypoxic

regions, oxygen and nutrient gradients as well as preferred points of vascular entry/exit; finally the acquisition of pro-metastatic molecular determinants previously absent in the primary tumor [Palmer et al., 2011]. Therefore, during this process, tumor cells acquire genetic and/or epigenetic alterations, cooperate with non-neoplastic stromal cells, have to penetrate layers of tightly connected epithelial and endothelial cells and have to breach barriers of several ECM structures [Valastyan and Weinberg, 2011; Chang et al., 2016]. Indeed, the "invasion-metastasis cascade" first involves invasion by carcinoma cells into the stroma associated with the primary tumor and then into nearby normal tissue parenchyma, all accompanied by infiltration of the basement membrane (BM) [Dongre and Weinberg, 2019]. This specialized form of ECM normally has a structural role, but, during the metastatic process, appears to be involved in alterations related to cell polarity, proliferation and survival of cancer cells [Bissell and Hines, 2011]. The loss of this barrier is mainly caused by the enhanced action of tumor cells MMPs, which release also growth factors promoting a further cancer cell proliferation [Kessembrock et al., 2010]. The next steps in the metastatic cascade consist of the intravasion of cancer cells into the microvessels and major vessels draining the tissue in which the tumor has arisen and the subsequent spread via lymphatic and hematogenous dissemination to anatomically distant sites [Dongre and Weinberg, 2019]. Usually this process is facilitated by the formation of new, immature and hyperpermeable vasculature, characterized by weak interactions between endothelial cells and the lack of perivascular coverage and BM. It causes leakage of plasma proteins, further facilitating new vessel formation, and allows tumor cells to cross their barriers and enter lymphatic or blood circulation, also stimulated by molecular changes and external impulses [Hapach et al., 2019; Valastyan and Weinberg, 2011]. At this point, circulating tumor cells (CTCs) must resist to the hemodynamic forces, immune stresses and red blood cell collisions in order to stay alive in suspension. Then, the trapping of such cells in the microvessels of distant tissues occurs through two primary mechanisms: physical occlusion and adhesion after rolling [Wirtz et al., 2011]. The final stages of the metastatic process include the extravasation of trapped cancer cells into the parenchyma of new tissues, the formation of micrometastases composed of relatively small numbers of cells and the subsequent outgrowth of macroscopic metastases from such micrometastases, which is known as colonization.

Colonization turns out to be a critical step in the metastatic cascade, as it succeeds only very rarely, probably because of the adaptive difficulties that disseminated tumor cells may encounter when they reach foreign and potentially inhospitable tissue microenvironments. In this regard, since only a very small percentage of cancer cells that emerge from a primary tumor succeed in forming macroscopic metastases, it can be said that the whole process is extraordinarily inefficient [Dongre and Weinberg, 2019].

CANCER CELL PLASTICITY

Cells from several origins and particularly cancer cells possess the ability, commonly referred to as cellular plasticity, to activate specific epigenetic programs that enable them to adapt to various environmental changes [Friedl and Wolf, 2010; Yilmaz and Christofori, 2010].

EPITHELIAL-MESENCHYMAL TRANSITION

Epithelial-mesenchymal transition (EMT) is a cellular process that occurs naturally in a wide range of tissue types and developmental stages [Zhang et al., 2018]. Through this program, cuboidal, tightly packed and non-motile epithelial cells adopt a loosely organized mesenchymal or fibroblast-like phenotype, thus determining a transition from a collective migration pathway to a detached and disseminated cell migration mechanism [Debnath et al., 2021; Friedl and Wolf, 2003]. On the basis of the biological context in which they occur, three types of EMT can be distinguished. Type I EMT relates to embryonic development, proving to be critical, for example, in promoting gastrulation and neural crest formation. Type II EMT is induced in response to inflammation and occurs during wound healing, tissue regeneration and fibrosis, in the latter case generating a significant portion of myofibroblasts. Finally, type III EMT occurs during cancer progression, allowing cells leaving the primary tumor to gain motility and invasiveness in order to spread from the site of origin to neighbouring and distant tissues [Debnath et al., 2021; Dongre and Weinberg, 2019]. In addition to this, during EMT, cells also undergo other changes regarding how they

interact with neighboring cells, their local environment and their morphology. Indeed, epithelial cells are characterized by apical–basal polarity, are normally held together by cell-cell junctions (tight junctions, adherens junctions, gap junctions and desmosomes) and interact with the underlying basement membrane via hemidesmosomes and integrins. Moreover, they also express specific cytokeratins that stabilize desmosomes to ensure the resilience of epithelial cell layers to various physical stresses [Shibue and Weinberg, 2017; Lamouille et al., 2014]. Activation of EMT results in loss of cell polarity, disruption of cell–cell junctions, and degradation of basement membrane leading cells to gain a front–rear polarity, a reorganized cytoskeleton characterized by actin stress fibers, an increased cell protrusions and motility, and, in addition, the ability to degrade ECM enabling cells to invade. EMT is induced mainly by a set of transcription factors (EMT-TFs) like ZEB1, ZEB2, SNAIL, SLUG and TWIST. They are able to regulate expression of one another and, in different combinations, allow to induce the expression of genes responsible for the mesenchymal state and repress those associated with the epithelial state. However, normal and neoplastic epithelial cells subjected to the EMT program very rarely transition to a fully mesenchymal phenotype, but usually proceed to a partially epithelial and partially mesenchymal state in which they express a mixture of features related to both [Dongre and Weinberg, 2019]. Further, EMT is a reversible process, so mesenchymal cells can revert to the epithelial state undergoing MET (Fig. 5) [Polyak and Weinberg, 2009].

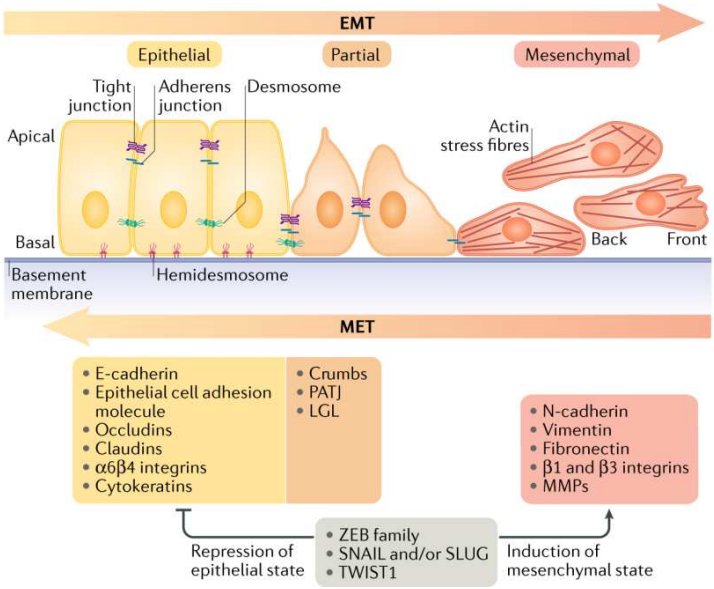


Figure 5. Outline of a typical EMT programme [Dongre and Weinberg, 2019].

MESENCHYMAL-AMOEBOID TRANSITION

Under certain circumstances, cancer cells can acquire an amoeboid-like motility, thus undergoing to what has been termed mesenchymal to amoeboid transition (MAT). This transition involves a change in cell morphology, alterations in integrin distribution and actin cytoskeleton organization and changes in molecular strategies to overcome tissue barriers. Tumor microenvironment and other several factors can lead to MAT, including abrogation of pericellular proteolysis by protease inhibitors, weakening of cell–ECM linkages and inhibition of RHO signalling pathways [Friedl and Wolf, 2003].

COLLECTIVE-INDIVIDUAL TRANSITION

This type of migration plasticity, including collective-mesenchymal transition (CMT) and collective-amoeboid transition (CAT), involves the conversion of collectively migrating cell groups of epithelial cancers into mesenchymal single cells or amoeboid-like cells. In the former case, the transition occurs via the EMT program leading to the loss of cell-cell connections (in particular E-cadherin) and the acquisition of mesenchymal phenotypes responsible for CMT during cancer cell invasion. In the other case, however, conversion is mediated by loss of protease function or weakening of integrin adhesion. The latter in fact turns out to be very important for cohesive cells in order to allow cell-substrate interaction and the generation of forces useful for traction, whereas it is not essential for amoeboid migration. In this regard, it has been shown that β1 integrin inhibition induces collectively migrating groups to lose cluster polarity and cohesion and to switch into amoeboid cell migration. Cells can also take on different phenotypes depending on the composition of the ECM. Additionally, collective-amoeboid transition in cancer cells is induced by hypoxia and increased HIF-1 signaling [Wu et al., 2021; Friedl and Wolf, 2003].

CADHERINS

Cadherins are a large family of proteins that mediate calcium-dependent cell-cell adhesion. This superfamily includes: classical cadherins; desmosomal cadherins (desmocolins and desmogleins); protocadherins and some other cadherin-related proteins [Saito et al., 2012]. The majority of the members are single transmembrane glycoproteins characterized by a cytoplasmic C-terminal domain and an extracellular N-terminal portion, consisting of a varying number of highly homologous domains, called extracellular cadherin (EC) domains. Each of these is formed by a repeating amino acid sequence of about 110 residues and presents a barrel-like structure that resembles Ig-domains [Ivanov et al., 2001; Shapiro et al., 1995]. Additionally, in the extracellular portion of the polypeptide chain, between neighboring extracellular repeats, are also present short highly conserved amino acid sequences that constitute binding sites for calcium, which turns out to be essential to ensure the stability of cadherin conformation [Ozawa et al., 1990]. Classical cadherins are characterized by five extracellular cadherin (EC) repeats, designated as EC1-EC5 (the former is membrane distal, the latter membrane proximal), and a cytoplasmic C-terminal domain which associates with the actin cytoskeleton thanks to catenin proteins (Fig. 6) [Ivanov et al., 2001; Colás-Algora and Millán, 2019]. The binding between cadherin to F-actin is mediated not only by α-catenin and β-catenin, but also by some essential additional adaptors such as vinculin, epilin or zona occludens protein 1 (ZO1) [Zaidel-Bar, 2013].

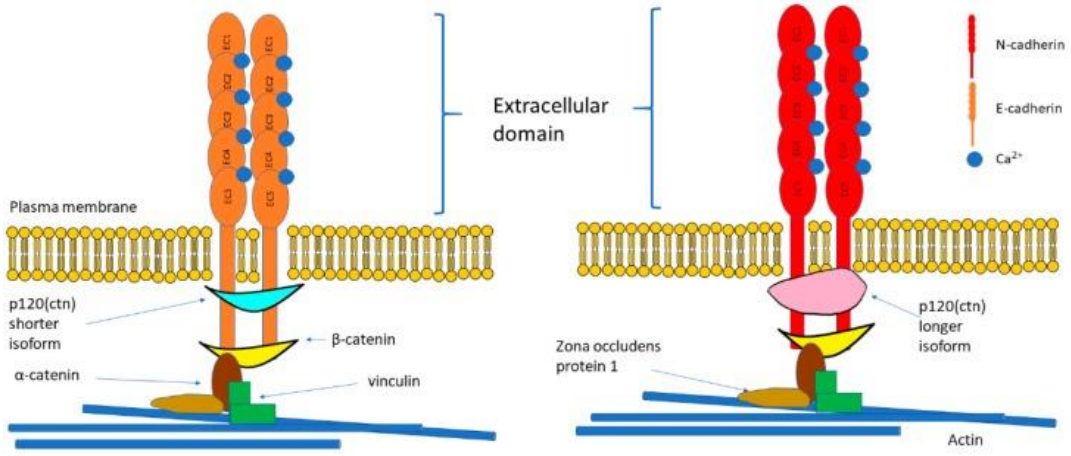


Figure 6. Structure of E-cadherin and N-cadherin [Loh et al., 2019].

Classical cadherins can be divided into type I and type II according to the molecular features of their interaction via the cadherin motif [Colás-Algora and Millán, 2019]. Type I cadherins, including E- (epithelial), N- (neural), P- (placental), R- (retinal) and M-cadherin subtypes, typically have broad distributions that are segregated by embryonic germ layer or tissue type. In contrast, type II cadherins, like VE-cadherin (vascular endothelial), show more fine-grained, and often overlapping patterns of expression, notably within the developing nervous system [Shapiro and Weis, 2009].
Classical cadherins can interact in cis to form clusters and mediate adhesion via hemophilic trans interactions with cadherins present on opposing cells. Also the highly conserved intracellular domain of classical cadherins, roughly 150 amino acids long, participates in cell adhesion. It includes a juxtamembrane domain (JMD) which mainly interacts with p120 catenin, essential in regulating the stability of cadherin-catenin complexes at areas of contact, and a catenin-binding domain (CBD), which instead binds to plakoglobin (Pg) or to β-catenin, that links cadherins to the actin-binding protein α-catenin, leading to the formation of the cadherin-catenin adhesion complex [Seddiki et al., 2018; Drees et al., 2005; Yamada et al., 2005]. Classical cadherins, together with cytoplasmic multiprotein complexes and their relative interactions, give rise to adherens junctions, specialized cell-cell adhesions able to promote cytoskeleton reorganization, intracellular signaling and transcriptional regulation and essential for morphogenetic processes in embryonic development and in adult tissue organization [Gloushankova et al, 2017]. For example, E-cadherin based AJs are apical structures that maintain structural and functional integrity of epithelia and regulate homeostasis of tissues. They are stabilized by actin-filaments-based cortical networks and are involved in the establishment of cell polarity. Their formation and remodeling are driven by many mechanisms, among which Rac and Rho signaling, for a more flexible or a more stable conformation respectively. Stable adhesions are critical, but also the plasticity of AJs is necessary for changes in cell size, shape, adhesion and relative cell movements at the basis of morphogenetic and adaptive processes of tissues [Coopman and Djiane, 2016]. In contrast, mesenchymal cells, which are more motile and less polarized than epithelial ones, express different cadherins, typically N-cadherin, which is not found in junctions. So during EMT, a “cadherin switch” occurs, resulting in the acquisition of increased migratory and invasive properties [Wheelock et al., 2008]. Finally, classical

and desmosomal cadherins are closely related to each other. They share a similar structure, but unlike the classical ones, desmosomal cadherins have an intracellular domain that associates with Pg and not β-catenin, mediate the stability of the desmosomes and bind to the cytoskeleton in a different way. Indeed, while adherens junctions connect to the actin cytoskeleton, playing an important role in the dynamics of cell contacts and the establishment of cell polarity, desmosomes are connected to the intermediate filaments and represent junctions able to resist mechanical stress [Saito et al., 2012].

EPH RECEPTORS AND EPHRINS

Erythropoietin hepatocellular carcinoma receptors (Ephs) represent the largest family of receptor tyrosine kinases (RTKs), with 16 members identified in animals, 14 of which are expressed in humans. Unlike other RTKs, whose activation occurs by soluble ligands, that of Eph receptors is mediated by the interactions they form with their membrane anchored ligands, the ephrins [Taylor et al., 2017]. Based on their sequence homology and how they interact with ephrins, Eph receptors can be divided into type A (nine receptors, EphA1-A8 and EphA10) and type B (five receptors, EphB1-B4 and EphB6) [Gale et al., 1996]. They are characterized by an extracellular side, that presents a ligand-binding domain (LBD), a Cys-rich domain, which in turn is formed by the sushi and epidermal growth factor (EGF)–like domain, and two fibronectin (FN-III) domains. Then, there is the transmembrane region (TM), consisting in a single transmembrane α-helix, thus allowing the connection between the extracellular side to the intracellular one. This is characterized by a juxtamembrane (JM) region that binds a tyrosine kinase domain (TK), which in turn is connected by a linker to a sterile-alpha motif (SAM) domain and a PDZ domain-binding motif [Lemmon and Schlessinger, 2010; Liang et al., 2019].

Ephrins can also be divided into class A (A1-A6) and B (B1-B3) depending on the way they are anchored to the membrane. In fact, class A is linked to the membrane through a glycosylphosphatidylinositol (GPI) bond, whereas class B has a transmembrane domain and an intracellular PDZ domain (Fig. 7) [Liang et al., 2019; Kania and Klein, 2016].

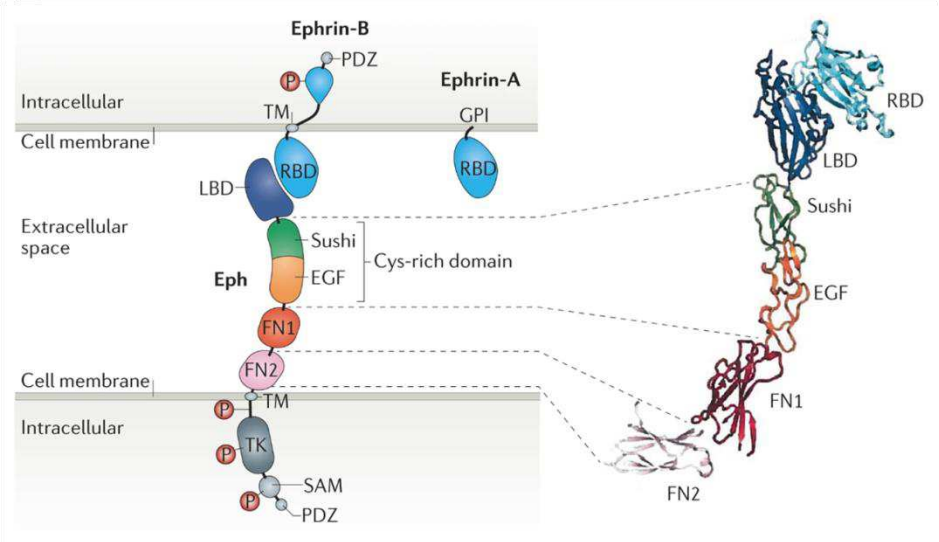


Figure 7. Structure of Eph receptors and ephrins [Kania and Klein, 2016].

EphA binds preferentially to A-type ephrins and EphB to B-type ephrins, in particular nine EphAs that interact primarily with five ephrin-As, and five EphBs that interact primarily with three ephrin-Bs [Pitulescu and Adams, 2010]. However, there are some exceptions such as EphA4, which interacts with both A-type and B-type ephrins; EphB2, which binds ephrin-A5; and EphB4, which preferentially binds ephrin-B2 [Pasquale, 2010]. Eph–ephrin interaction results in phosphorylation of Tyr residues (P), found in the juxtamembrane domain between the TK and TM domains, as well as in the TK and SAM domains. The juxtamembrane phosphorylation is required for TK function, which is crucial for many Eph signalling-induced biological responses [Liang et al., 2019; Kania and Klein, 2016]. Interactions between Ephs and ephrins mediate several cellular pathways, including proliferation, differentiation, cell-cell adhesion, migration, survival, apoptosis, tissue boundary formation and axon guidance [Gucciardo et al., 2014; Kania and Klein, 2016]. Some ephrins have also been shown to interact with HSPGs, especially ephrin-A3 and ephrin-B3. The former appears to be able to bind HS through its C-terminal juxtamembrane linker region. The latter, on the other hand, interacts with both secretory and cell associated PGs, such as agrin, collagen XVIII, perlecan and CD44. In particular, it is able to bind HSPGs on HEK-293T, HeLa and CHO cells through a binding domain, different from the receptor binding one required for EphB receptors, that could be a heparin/HS-binding domain. In fact, heparin blocks binding to HEK-293T cells independently of Eph receptors [Irie

et al., 2008; Holen et al., 2011; Prydz et al., 2018]. However, Eph and ephrin genes have been implicated in an increasing number of physiological and pathological processes in many cell types and different organs. Eph/ephrin interactions may have diverse consequences, including widespread effects on the actin cytoskeleton, cell-substrate adhesion, intercellular junctions, cell shape and movement [Egea and Klein, 2007; Pasquale, 2005]. Binding depends on cell-cell contact and the resulting signaling can occur in several ways depending on the direction of signal flow. One of the unique features of the Eph/ephrin system is the fact that both receptors and ligands are competent to transduce a signalling pathway. Forward signaling (ephrin:Eph) involves signal transduction from ephrins to Ephs; reverse signalling (Eph:ephrin) involves signalling from Ephs to ephrins; and bidirectional signalling involves the simultaneous activation of pathways downstream of ephrins and Ephs. In addition, unlike the majority of RTKs, Eph receptors and ephrin ligands may act both in trans (between two adjacent cells) and in cis (on the same cell) to promote or inhibit specific molecular cascades [Himanen et al., 2007; Arvanitis and Davy, 2008].
Their deregulation is associated with the onset of several diseases, including developmental disorders and cancer [Ireton and Chen, 2005]. However, the presence of bidirectional signaling makes these molecules both oncogenic and oncosuppressive. for example, EphA2 appears to have a suppressive function when stimulated by its ligand ephrin, whereas if overexpressed it can become oncogenic [Nikas et al., 2018].

TETRA-BRANCHED NEUROTENSIN (NT4)

NT4 is a tetra-branched peptide obtained by synthesizing four neurotensin (NT) sequences on a core of lysines (Fig. 8).

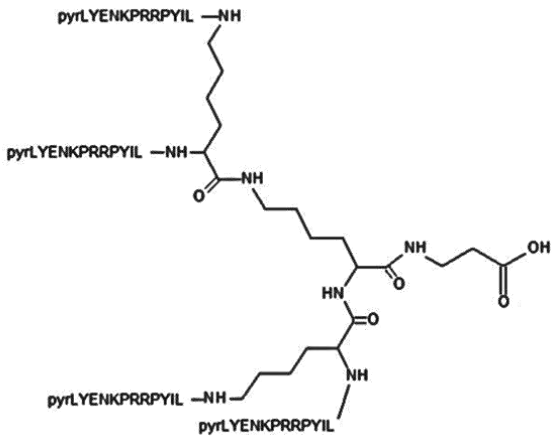


Figure 8. NT4 peptide structure.

Neurotensin is a peptide of 13 amino acids (pGlu-Leu-Tyr-Glu-AsnLys-Pro-Arg-Pro-Tyr Ile-Leu) originally isolated from bovine hypothalamus by Carraway and Leeman in 1973 [Carraway and Leeman, 1973]. It carries out different roles within the organism. In fact, it acts as both a neurotransmitter and a neuromodulator at the level of the central nervous system, while in the peripheral nervous system it functions as a paracrine and autocrine hormone, particularly of the gastrointestinal tract [Vincent et al., 1999]. Given the presence of NT receptors and their overexpression in several tumors, the possibility of using this peptide as a targeting agent was evaluated years ago. Like other endogenous peptides as well, the main problem concerning its use in the native form consists of its short half-life, due to the activity of proteolytic enzymes. Synthesis in a tetra-branched form overcomes this problem, allowing the peptide to resist degradation by peptidases and proteases and to escape proteolysis but, at the same time, maintaining its biological activity or even increasing it because of multimeric binding [Bracci et al., 2003; Falciani et al., 2007; Falciani et al., 2013]. Indeed, NT4 remains unchanged and is still detected after 24 hours in plasma and serum, while the monomeric form is completely degradated after only 2 hours [Bracci et al., 2003]. This is made possible due to the core of three lysines capable of forming

a larger molecule than the native one, whose greater steric hindrance may limit the cleavage rates of peptidases. Although binding of the peptide in the catalytic pocket of plasma peptidases may still be possible, the cleavage site may be geometrically unreachable by catalytic residues, especially in tetra-branched peptides that cannot achieve an extended conformation. For this reason the branched core can be thought of as acting like a ''knot'' in the structure, preventing the peptide from sliding the length of the pocket freely [Falciani et al., 2007].

NT4 synthesis also offers other advantages. The molecule can be modulated by adding different functional units without interfering with the part devoted to receptor recognition. While on one side of the branched structure the amine groups bind peptide sequences, on the other side the reactive groups not engaged in the binding can be coupled to liposomes and different effector molecules, such as drugs or tracers, making NT4 useful for both therapy and imaging [Falciani et al., 2007; Falciani et al., 2010; Falciani et al., 2011]. In particular, its conjugation with different chemotherapeutics provides a better selectivity and efficiency than the free drugs by transporting the chemotherapeutic moiety to the cancer cell membrane and then into the cell. Thus, in addition to being able to change the drug biodistribution by providing an enhanced tumor accumulation, the conjugation to NT4 allows to bypass drug resistance mediated by membrane transporters. One example concerns methotrexate (MTX), a folate. Considering that in cancer cells the membrane transporters of folate are mutated or down-regulated, its association with NT4 enables to overcome resistance to the drug itself [Depau et al., 2017]. Moreover, it has been demonstrated that using NT4 conjugated to methotrexate or 5-floro-2'deoxyuridine it was obtained a significant reduction of tumor growth in mice, 60% and 50% respectively, compared with mice treated with the same amount of unconjugated drug [Falciani et al., 2007; Falciani et al., 2010]. Other more recent experiments conducted on an orthotopic model of breast cancer in mice have shown the ability of NT4 conjugated to paclitaxel (PTX) to increase therapeutic activity of the drug and to induce a tumor regression which was not achieved with unconjugated paclitaxel in identical experimental conditions [Brunetti et al., 2015].

Furthermore, by conjugating NT4 to fluorophores, the tetra-branched peptide has been shown to efficiently discriminate between tumor and healthy tissues. NT4 peptides are in fact much more selective than native monomeric analogues and bind

with high selectivity different human cancer cells and tissues, such as colon adenocarcinoma, pancreas adenocarcinoma and urinary bladder cancer [Falciani et al., 2007; Falciani et al., 2010; Brunetti et al., 2015] (Fig. 9).

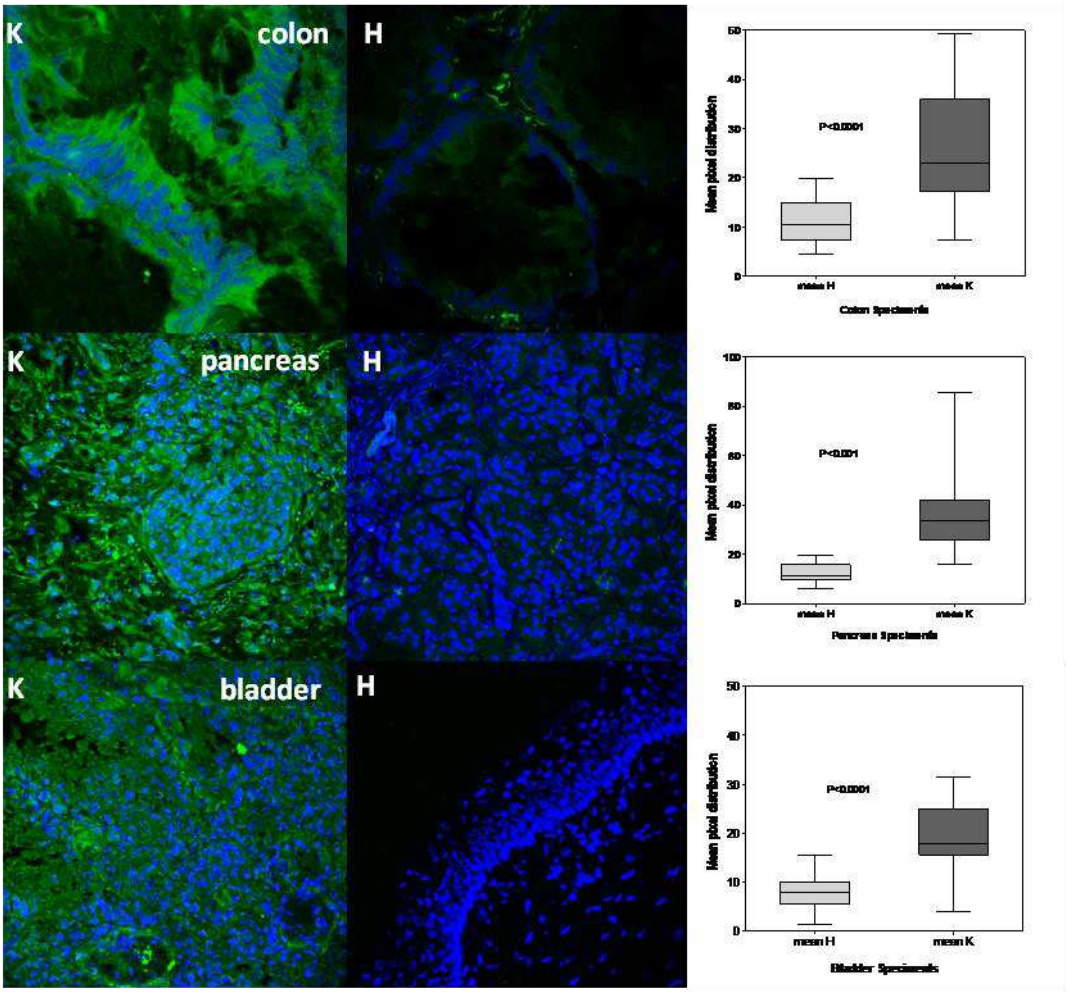


Figure 9. Confocal microscopy of human colon, pancreas [Falciani et al., 2010] and bladder cancer [Brunetti et al., 2015] biopsies. Healthy tissues (H) and the corresponding tumor tissues (K) were stained with NT4-bio, followed by streptavidin-FITC (green). Nuclei are stained with DAPI (blue). Statistic representation of mean pixel distribution on the basis of RGB system to quantify fluorescent staining in normal (light gray) and cancer (gray) tissues. The box-plot represents the interquartile range (25–75th percentile) and the horizontal line in the box is the median value. Bottom and top bars of the whiskers indicate the minimum and maximum values, respectively.

The high selectivity of NT4 toward cancer cell and tissue was demonstrated to be due to multimeric binding to sulfated GAG chains [Falciani et al., 2013].

NT4 BINDING TO GAGs

Branched structure seems to alter the receptor targets of the molecule as the C-terminal carboxyl group is missing, being engaged in coupling to the three-lysine core. This involves a change in both the way the peptide interacts with the receptor and the selectivity of binding, leading to a switch toward additional membrane receptors, which are selectively expressed by different human cancers. As a result, NT4 significantly loses affinity for its main receptors, NTR1 and NTR2, while it retains binding to the NTR3/sortilin receptor and introduces, through four-copy sequence synthesis, a typical multimeric "heparin binding" motif [Falciani et al., 2013]. It consists in a positively charged multimeric region that mediates the interaction of NT4 with sulfated GAGs and that is very similar to heparin-binding motives contained in midkine and other proteins, like Wnt, which in turn are capable of binding sulfated GAGs and LRP receptors and are overexpressed in cancer [Falciani et al., 2013; Depau et al., 2020]. Consequently, thanks to the ability to mimic "heparin-binding proteins" in their binding site, NT4 is also able to bind sulfated GAGs and endocytic receptors belonging to the low density lipoprotein (LDRL) family, including LRP1 and LRP6. Moreover, NT4 binding to different cancer cell lines and tissues is abolished by heparin and heparan sulfate, and is also inhibited by heparin-binding proteins, like midkine and ApoE [Falciani et al., 2013].

In order to verify the binding specificity of NT4 for GAGs, some tests were performed, one of which using two different cell lines, CHO-K1 and PgsA-745 cells. The latter represent a mutant form of CHO-K1 cell line and lack GAG chains because they are deficient in xylosyltransferase, the enzyme responsible for anchorage of GAG chains to the protein core. NT4 binding to these GAG-defective cells and to native CHO-K1 cells was compared. As expected, NT4 binding to PgsA-745 cells was much lower (Fig. 10) confirming that GAGs are specific NT4 targets on the cell membrane [Brunetti et al., 2016].

Nonetheless, flow cytometry analysis demonstrated that binding of NT4 to PgsA-745 cells, though much lower than to CHO-K1, was still inhibited by heparin. The ability of NT4 to bind HSPGs as well as endocytic receptors like sortilin, LRP1 and LRP6, suggests that the residual binding to PgsA-745 cells might be due to membrane receptors belonging to these families [Brunetti et al., 2016].

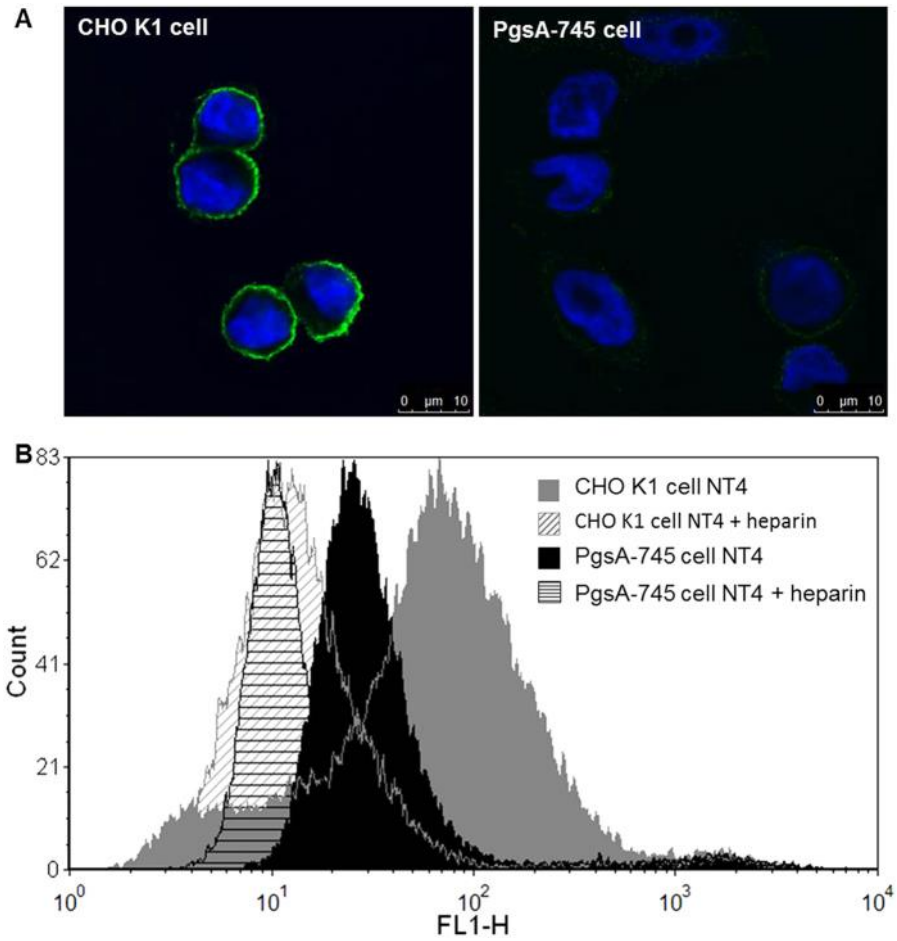
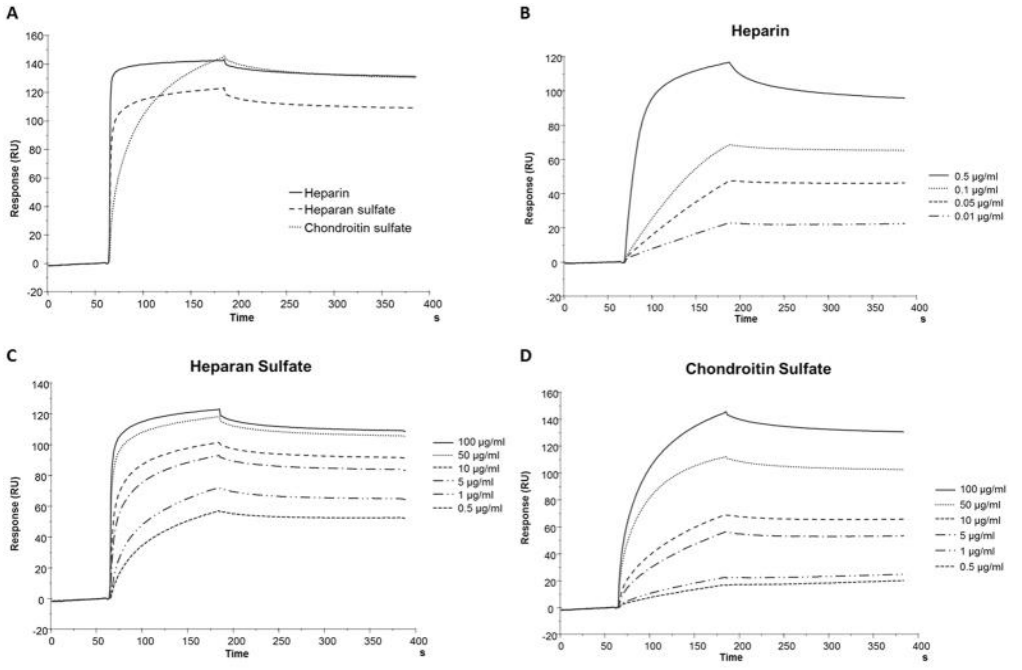


Figure 10. NT4 binding to CHO-K1 and PgsA-745 cells observed by (A) confocal microscopy and (B) flow cytometry. (A) CHO-K1 and PgsA-745 cells stained with NT4-biotin, followed by streptavidin-FITC (green). Nuclei were stained with DAPI (blue). (B) Inhibition of NT4 binding to CHO-K1 and PgsA-745 cells by heparin, analyzed by flow cytometry [Brunetti et al., 2016].

Binding specificity of NT4 to different GAGs was also examined by Surface Plasmonic Resonance (Biacore) (Fig. 11). Data confirmed the high-affinity binding to sulfated GAGs, showing preferential binding to heparin and HS compared to CS and no binding to HA, the non-sulfated glycosaminoglycan. The highest affinity was obtained with heparin, that contains a higher number of sulfated groups than both HS and CS, for which NT4 exhibited a progressively lower binding affinity. [Brunetti et al., 2016]. This is also due to the fact that HS presents some domains that are more densely sulfated than others, like the FGF binding domain and the anti-thrombin binding domain, whereas, in contrast, CS has more homogeneously sulfated patterns [Varki et al., 2009].



E	Kon (1/Ms)	Koff (1/s)	KD (M)
Heparin	1.658e6	7.63e-4	**4.603e-10**
Heparan sulfate	2.221e5	7.257e-4	**3.267e-9**
Chondroitin sulfate	1.181e4	4.095e-4	**3.468e-8**
Hyaluronic acid		no binding	

Figure 11. (A) Sensorgrams of GAGs (10 µg/mL) binding to surface immobilized NT4. (B–D) Heparin (B), heparan sulfate (C) and chondroitin sulfate (D) binding to NT4. Different concentrations of GAGs were injected over biotin-conjugated NT4, previously immobilized on a SA sensor chip. (E) Kinetics constants and affinity values of GAGs binding to NT4 peptide [Brunetti et al., 2016].

Since NT4 peptide bound heparin, HS and CS with different affinity, the inhibition activity of the three GAGs on NT4 binding to membrane receptors on the human pancreas adenocarcinoma cell line PANC-1 was measured by flow cytometry. Using identical concentrations of heparin, HS and CS, complete inhibition of NT4 binding was obtained with heparin, about 80% inhibition was obtained with HS and no inhibition was detected with the same concentration of CS. This result confirms the specificity of NT4 binding to membrane GAGs on cancer cells, with preferential binding to HS (Fig. 12) [Brunetti et al., 2016].

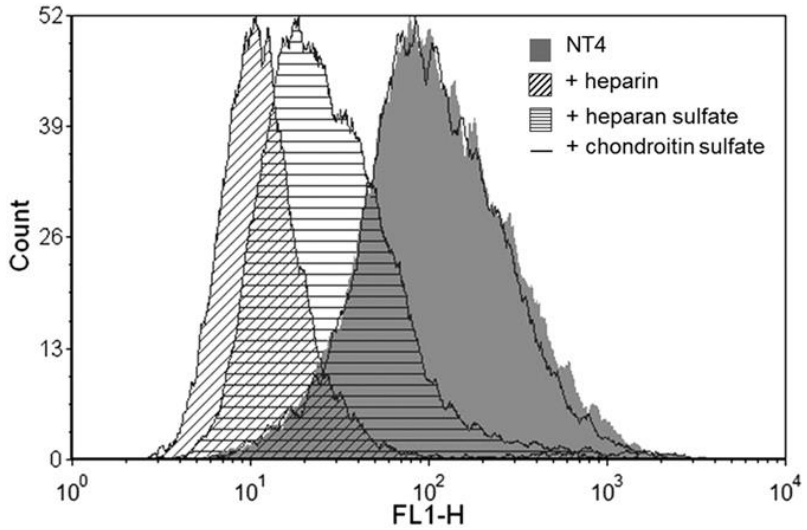


Figure 12. Flow cytometry of NT4 binding (grey) to PANC-1 cancer cells and inhibition by heparin (oblique lines), heparan sulfate (horizontal lines) and chondroitin sulfate (line) [Brunetti et al., 2016].

However, results suggest a close connection between the sulfated groups on the glycan chains and binding affinity, with the latter depending both on their number and position as also demonstrated through the use of different sulfated oligosaccharides and recombinant HSPGs. For example, NT4 has been shown to bind glypicans-3 and -4, which carry only HS chains, and syndecan-4, but with five times lower affinity than the glypicans, while it does not interact with syndecan-3, which like syndecan-1 has both HS and CS chains. [Brunetti et al., 2016; Brunetti et al., 2019]. In addition, NT4, precisely because of its ability to bind HSPGs, has been used to explore their internalization and trafficking [Mandarini et al., 2020]. Indeed exosomes, cell penetrating peptides, polycation–nucleic acid complexes, viruses, lipoproteins, growth factors and morphogens enter cells through HSPG-mediated endocytosis [Christianson et al., 2014]. In line with what is already known about HSPGs, the results revealed different internalization pathways, including caveolin- and clathrin-dependent endocytosis and macropinocytosis. Moreover, NT4 then localized in early and late endosomes in a time-dependent manner [Mandarini et al., 2020].

NT4 BINDING TO DIFFERENT CANCER CELL LINES

Binding of NT4 to different cancer cell lines was tested by flow cytometry (Fig. 13). Results highlighted the ability of the peptide to bind human bladder adeonocarcinoma (T24), human pancreas adenocarcinoma (PANC-1), human colon adenocarcinoma (HT29), human rhabdomiosarcoma (TE671), human melanoma (A375), human breast adenocarcinoma (MDA-MB-231), human breast adenocarcinoma (MCF-7) cells. Moreover, the specificity of NT4 was probed by the inhibition by heparin of the same binding [Depau et al., 2020].

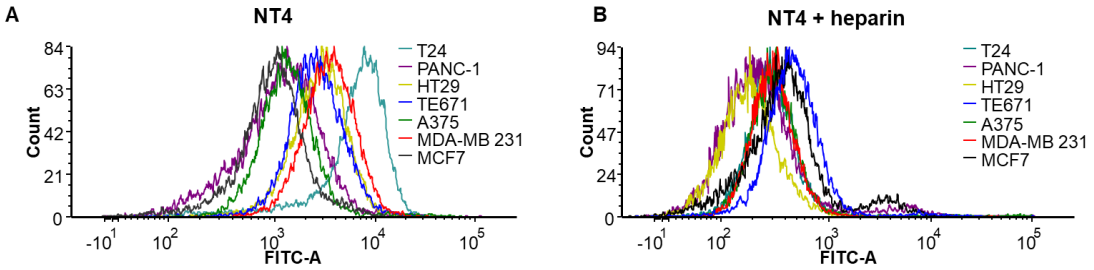


Figure 13. NT4 binding (A) and inhibition of NT4 binding by heparin (B) to PANC-1, HT29, MDA-MB-231, MCF-7, T24, TE671 and A375 cancer cells, analyzed by flow cytometry [Depau et al., 2020].

NT4 BINDING CAN BE MODULATED BY SULFATASES

Another important finding concerning the recognition by NT4 of sulfated GAG chains emerged from the analysis of Sulfs expression, especially human sulfatase 1 (hSULF-1) and human sulfatase 2 (hSULF-2). These are two extracellular enzymes characterized by four primary domains: a cleavable signal sequence, a catalytic domain (CAT), a central hydrophilic domain (HD) and a C-terminal region (C-ter). Unlike the CAT domain, which is highly homologous to the conserved catalytic domains of all eukaryotic sulfatases, the HD domain is a unique feature of Sulfs and it is essential for their anchorage to cell membrane and for recognition and endosulfatase activity on HS [Vivès et al., 2014]. In addition, SULF-1 and SULF-2 are the only two post-synthetic editing enzymes able to modify the sulfation pattern of HSPGs, especially the 6-O-sulfation of internal glucosamines of highly sulfated HSPG domains [Yang et al., 2022]. By removing 6-O-sulfate groups from HS chains,

alteration of its ligand binding properties occurs, thereby modulating a wide range of signaling pathways.

Modified expression of both Sulfs, particularly SULF-1, has been associated with different cancers [Bret et al., 2011]. Experiments on different cancer cell lines pointed out the existence of a correlation between the expression of Sulfs by tumor cells and the ability of NT4 to bind the membrane of these cells [Brunetti et al., 2019]. SULF-1 and SULF-2 gene expression and NT4 binding were assessed by RT-PCR and flow cytometry, respectively, in four different cancer cell lines: PANC-1, HT29, MDA-MB-231 and MCF-7. SULF-1 protein expression was also measured in the same cell lines using a specific anti-SULF-1 antibody (Fig. 14) [Brunetti et al., 2019].

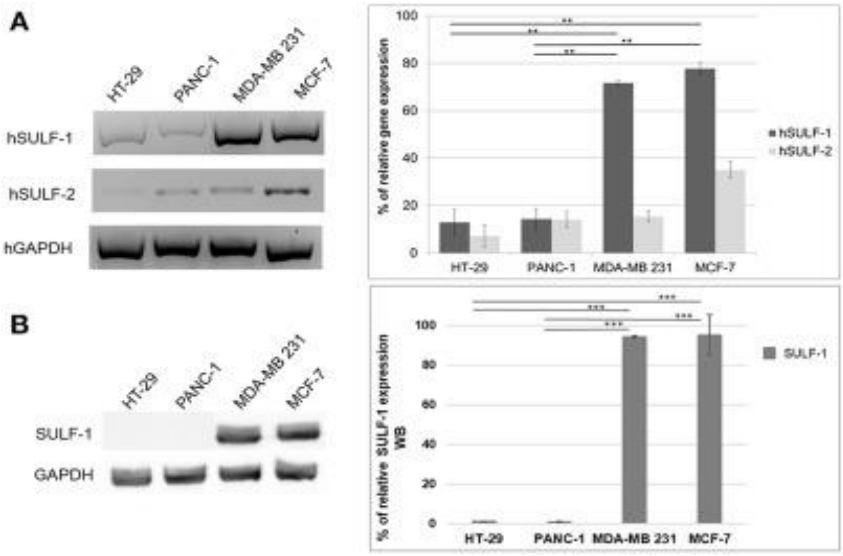


Figure 14. (A) Gene expression of hSULF-1 and hSULF-2 in HT-29, PANC-1, MCF-7, and MDA-MB-231 cell lines analyzed by RT-PCR. The GAPDH gene was tested as endogenous control. Histograms represent percentage of gene expression compared to GAPDH (GAPDH is 100%). (B) SULF-1 expression analyzed by Western blot in the same cell lines. Histograms represent percentage of SULF-1 expression compared to GAPDH (GAPDH is 100%). Significance of the differences was calculated using Student's *t*-test and GraphPad, $^{**}p < 0.01$; $^{***}p < 0.001$ [Brunetti et al., 2019].

Data demonstrated that NT4 binding to the cancer cell membrane was inversely correlated with expression of Sulfs, confirming the determinant role of 6-O-sulfate groups for recognition by the peptide. Indeed, higher expression of sulfatases resulted in lower ability of NT4 to bind tumor cells (MDA-MB-231 and MCF-7 cells); on the contrary, the peptide showed higher binding affinity for cells characterized by lower SULF-1 and SULF-2 expression (PANC-1 and TE671 cells) [Brunetti et al., 2019].

INTERACTIONS NT4-HSPGs

HSPGs and in particular their sulfated GAG chains play a key role in the cell-ECM interactions. Their altered regulation can therefore interfere with angiogenesis, cell adhesion and migration, thus causing a change in the aggressiveness of the tumor [Nagarajan et al., 2018].

EFFECT OF NT4 ON ANGIOGENESIS

Cell-associated HSPGs and those in the ECM act as co-receptors of growth factors and enhance angiogenesis, which in contrast is inhibited by heparin and soluble forms of HSPGs by neutralizing growth factors away from their receptors [Piperigkou et al., 2016; Shen et al., 2011]. In this regard, given the high-affinity binding to the GAG chains of HSPGs, the effect produced by NT4 on angiogenesis was tested in a tube formation assay on human umbilical vein endothelial cells (HUVEC), stimulated through fibroblast growth factor 2 (FGF2) and thrombin. Results pointed out that in the absence of NT4, FGF2 and thrombin increased the formation of tubes, particularly after three hours. In contrast, NT4 reduced it especially when the network was triggered by FGF2 and thrombin, but also in the control group. Instead, heparin slightly decreased the ability of HUVEC cells to form tubes, but this effect was completely nullified by NT4 (Fig. 15). This is due to the fact that by binding heparin, the peptide prevents the formation of the GAG/growth factor/growth factor receptor complex [Bracci et al., 2018].

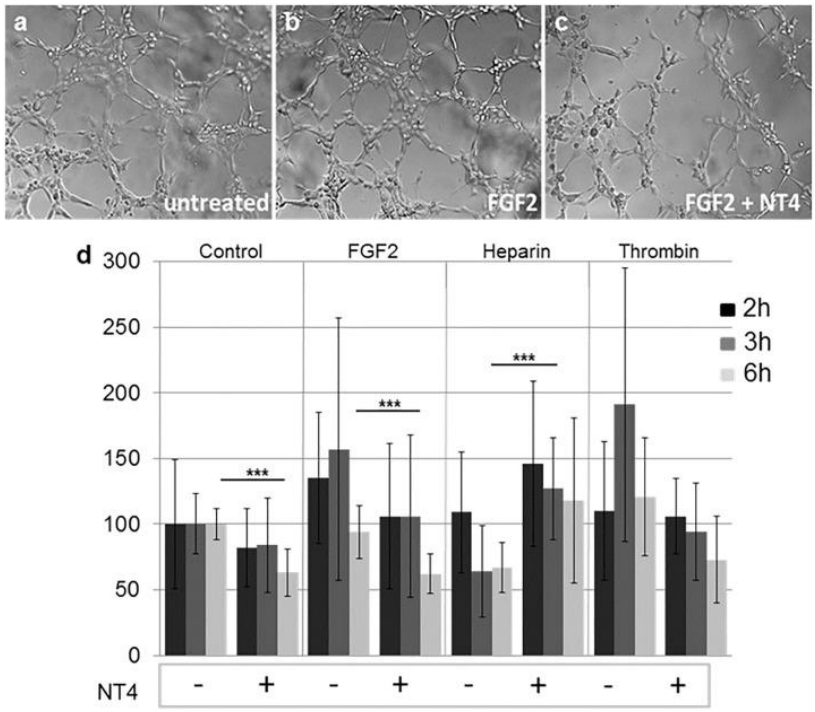


Figure 15. Effect of NT4 on HUVEC tube formation; a, b, c) representative images of tubes after 3 hours of incubation at 37°C in the presence of FGF2 and NT4; d) variation in number of branching nodes with respect to untreated cells after 2, 3 and 6 hours [Bracci et al., 2018].

EFFECT OF NT4 ON CELL ADHESION

Adhesion of several cancer cell lines was tested on different supports including cellular fibronectin, plasma fibronectin, collagen IV and plastic in the presence of NT4 (Fig. 16). The results highlighted the great variability with which the peptide interferes with each cell line, underscoring the role of sulfated GAGs as potential modulators of this process as some cells, such as PANC-1 and HT29, seem to be dependent on GAG chains, whereas others, like TE671, demonstrated to be not so dependent on them. According also to previous papers [Brunetti et al., 2016], in fact, the highest inhibitory effect was obtained on adhesion of PANC-1 cells to cellular fibronectin, collagen IV and plastic, whereas adhesion of these cells as well as the other tested cell lines to plasma fibronectin was scarcely affected and only at the highest concentration of NT4, suggesting to be sulfated-GAGs independent. This is due to the fact that unlike cellular fibronectin, plasma fibronectin is a minor component of the ECM and does not contain EDA and/or EDB segments, which are expressed in cancer tissues and whose ligands are inhibited by heparan sulfate [Klingberg et al., 2018]. Inhibition of adhesion of HT29 treated cells was also

significant, but still to a lesser extent than in PANC-1, while adhesion of the other cell lines was much less inhibited by the peptide; in particular TE671 cells, in which NT4 resulted to be almost ineffective on adhesion on all the tested supports [Depau et al., 2020].

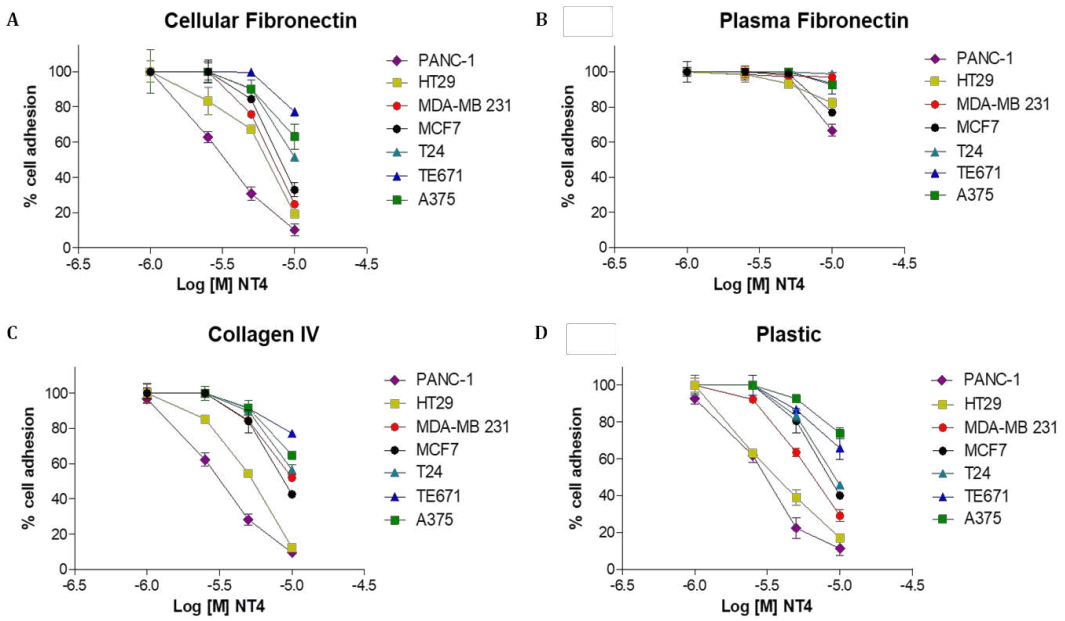


Figure 16. Adhesion of different human cancer cell lines on cellular fibronectin (A), plasma fibronectin (B),collagen IV (C) and plastic (D), .in the presence of different molar concentrations of NT4 [Depau et al., 2020].

In addition, given the importance of FAK in the formation of focal adhesions, its expression and that of its phosphorylated form were analyzed in the presence or absence of the peptide in PANC-1, TE671 and HT29 cells (Fig. 17). Despite higher expression of pFAK in HT29 than in the other two cell lines, the amount of FAK and pFAK was not modified by NT4 in all of them [Depau et al., 2020].

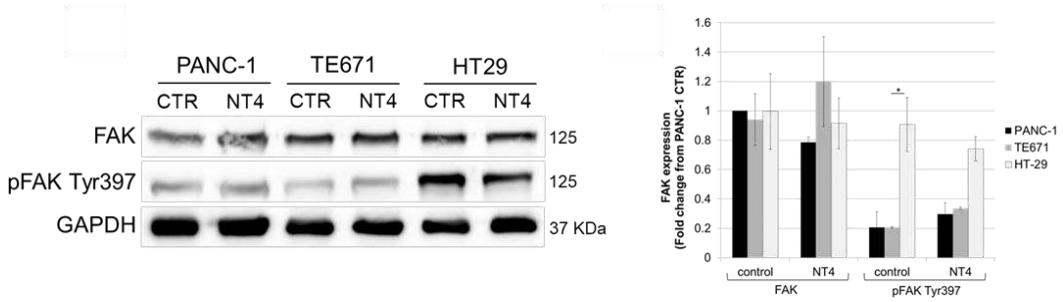


Figure 17. Western blot of FAK and pFAK Tyr397 in PANC-1, TE671 and HT29 cells treated with 10 µM NT4. GAPDH was used as an endogenous control. Densitometry analysis, carried out using ImageJ software, was normalized to PANC-1 control. Errors bars represent SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by one-tailed Student's *t*-test [Depau et al., 2020].

EFFECT OF NT4 ON CELL MIGRATION

On the basis of their different behavior in cell adhesion, PANC-1, TE671 and HT29 cell lines were selected. The effect produced by NT4 on the migration process was analyzed in wound healing experiments using different supports such as plasma fibronectin, cellular fibronectin and plastic and again, as with adhesion, was found to vary between cell lines (Fig. 18) [Depau et al., 2020]. Data from analyses of PANC-1 cells confirmed previous results [Brunetti et al., 2016], emphasizing that NT4 was able to inhibit cell movement and drastically affect their ability to move toward the void space with oriented migration. So, in the presence of the peptide, cancer cells lost their direction of migration and the polarity of cell movement. Indeed, they did not produce an effective forward migration, but rather random circular movements. On the other hand, migration of untreated TE671 cells appeared less oriented in all the tested supports. They seemed unable to keep directional migration and migrated slower than untreated PANC-1 cells. In this case, NT4 produced an opposite effect on the cells allowing them to fill the void area in a shorter time than untreated ones. Differently from the other two cell lines, HT29 did not migrate in the presence or absence of the peptide [Depau et al., 2020].

Moreover, PANC-1 and TE671 cell migration was monitored by time lapse and single cell tracks were analyzed by measuring both distances and velocities (Fig. 19). In PANC-1 cells, they were decreased by NT4 peptide in all different (in particular in plasma fibronectin and lower on uncoated plastic wells). By contrast, in the case of TE671 cells both distances and velocities increased upon the treatment with NT4 and also the cells migrated with more oriented trajectories compared to control cells, in all the tested supports (in particular in cellular fibronectin and lower on uncoated plastic wells) [Depau et al., 2020].

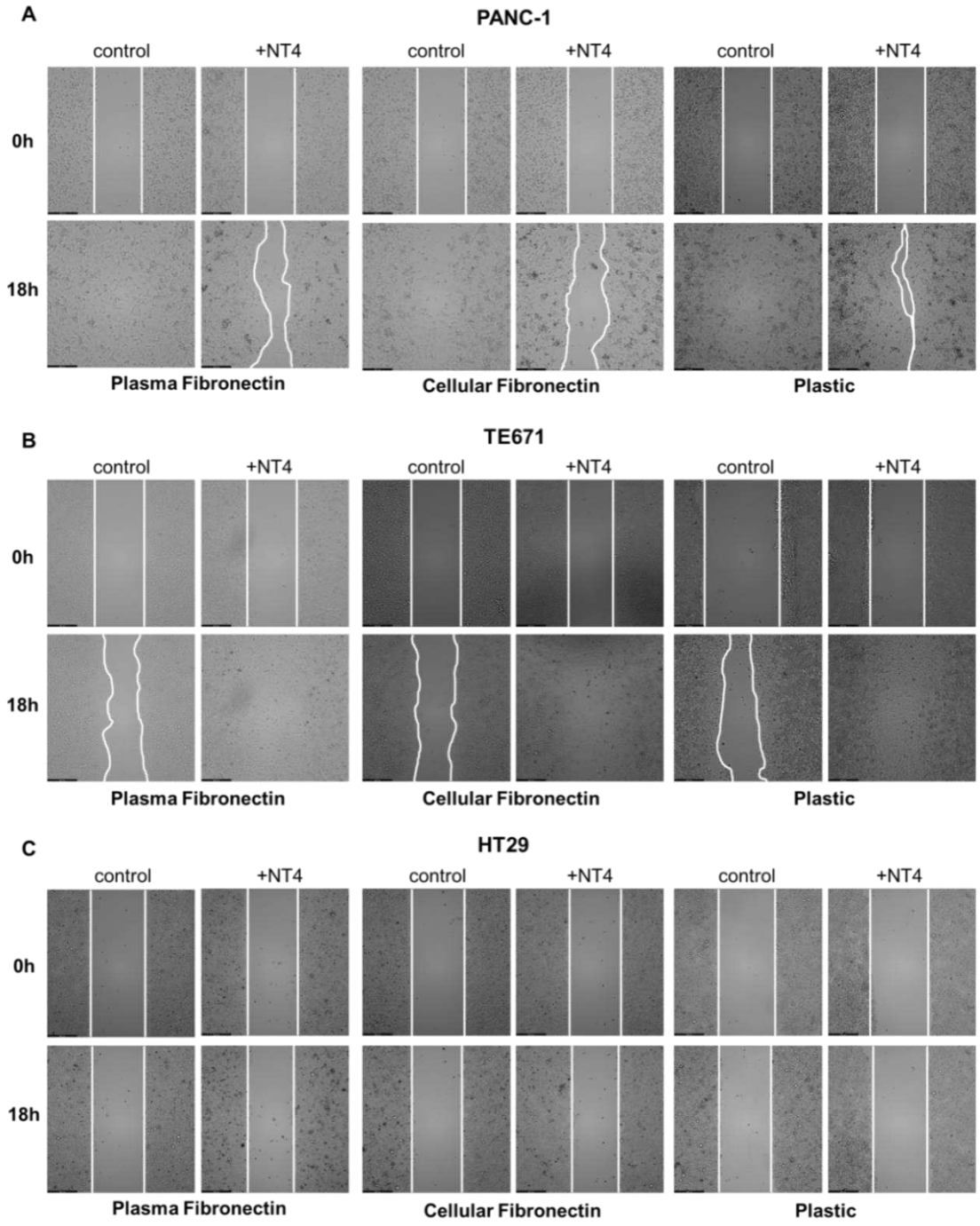


Figure 18. Wound healing assay. PANC-1 (A), TE671 (B) and HT29 (C) cancer cells were plated on wells coated with plasma fibronectin, cellular fibronectin or on uncoated wells where a silicon spacer had been placed immediately before cell plating. Once cells had reached confluence, the silicon spacer was removed and cells were treated with 10 μM NT4 peptide for 18 hours. Phase-contrast microscopy images were acquired from each well at time 0 (0h) and at 18 hours (18h) after removal of the silicon spacer [Depau et al., 2020].

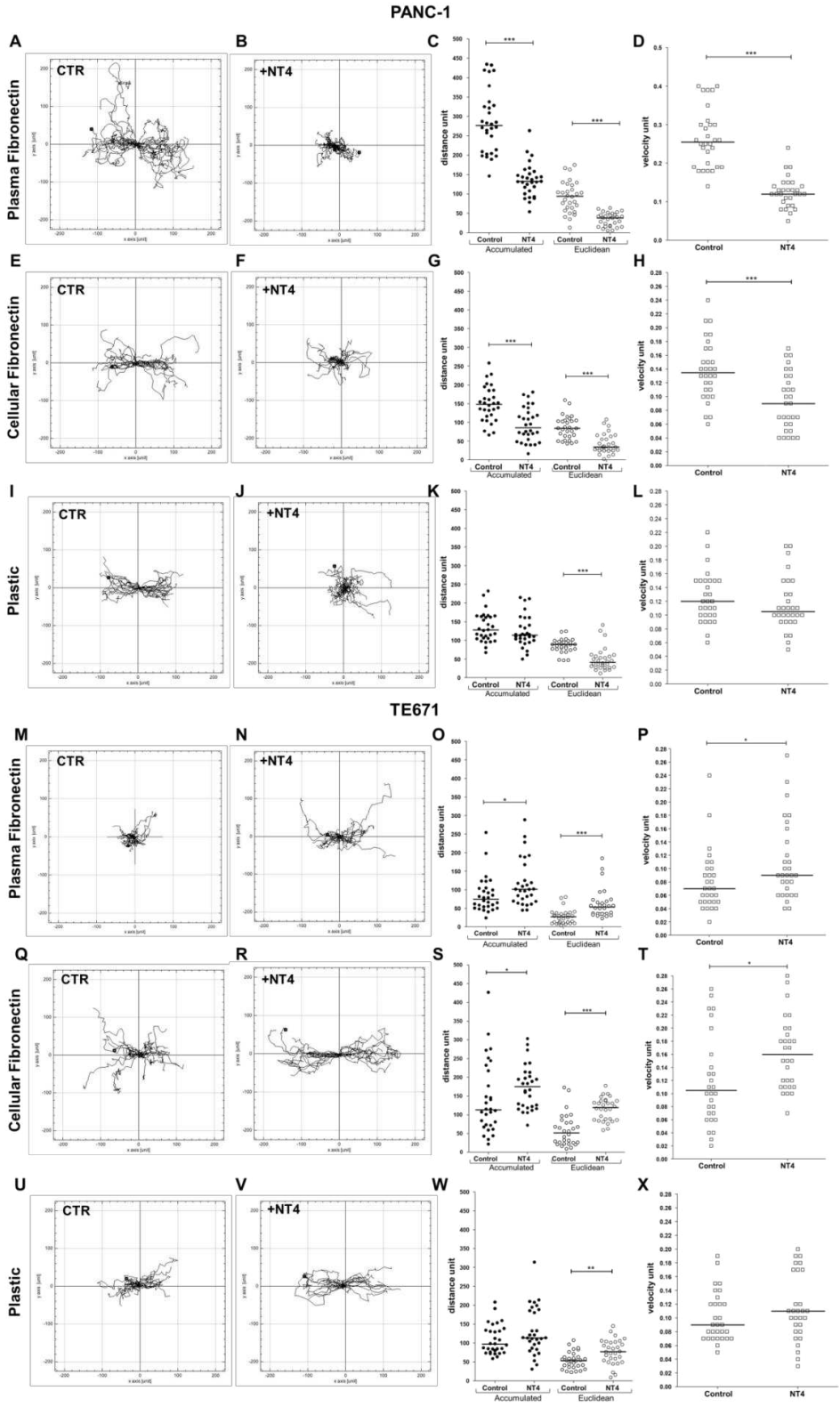


Figure 19. Directionality and velocity of PANC-1 (A-L) and TE671 (M-X) cancer cell migration analyzed by time lapse microscopy. Cancer cells, cultured on plasma fibronectin, cellular fibronectin or uncoated plastic wells were incubated with (+ NT4) and without (CTR)10 μM NT4. Cells were imaged every 10 min for 18 hours post-wounding and their paths were plotted on a polar grid. Each plot represents 30 individual cell tracks. Accumulated distance (filled circle), euclidean distance (empty circle) and velocity (empty square) of each analyzed cell track is reported in the box plot graph [Depau et al., 2020].

Results from 2D migration were confirmed by transwell invasion assays (Fig. 20). PANC-1 cells efficiently migrate through the collagen coated membrane and migration was inhibited by NT4, whereas untreated TE671 were much less effective in migrating through the membrane compared to PANC-1 untreated cells and migration was stimulated by the peptide. HT29 were not capable of migrating through the membrane in both untreated and treated conditions [Depau et al., 2020].

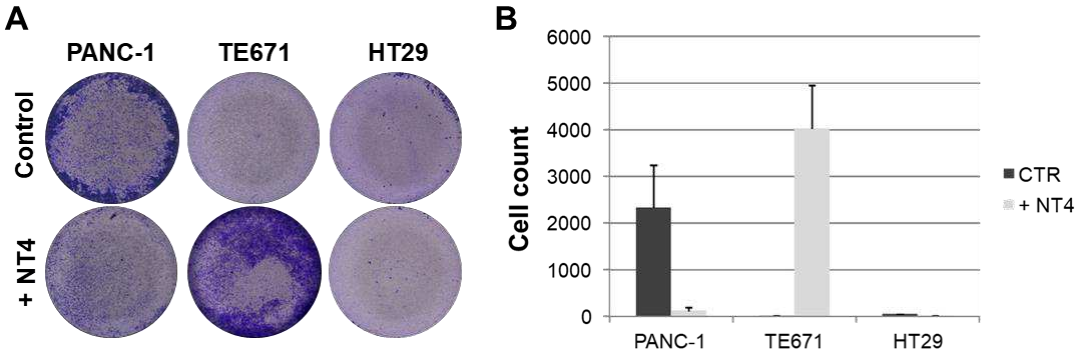


Figure 20. Invasion assay. Image of PANC-1, TE671 and HT29 cancer cells invading the lower well of Boyden chambers. Cells were seeded on the upper part of transwell inserts previously coated with collagen I and incubated for 24 h without NT4 (control) or in the presence of 10 μM NT4 (+ NT4). B) The images of migrated cells, fixed and stained with crystal violet, were obtained by confocal microscopy, on the entire well surface and were analyzed with ImageJ to count cells [Depau et al., 2020].

EFFECT OF NT4 ON ACTIN ORGANIZATION IN PANC-1 AND TE671 CELLS

Considering the key role that actin plays in migration and the effects produced by peptide in this process, modifications induced by NT4 in actin organization were analyzed by immunofluorescence, in both static (A, C, E) and migrating conditions (B, D, F) (Fig. 21). Untreated PANC-1 cells had oriented lamellipodia and organized stress fibers (A). The presence of the tetra-branched peptide caused a general disorganization of actin filaments and stress fibers and a clear increase of filopodia. So, in PANC-1 cells, treatment with NT4 lead to the loss of directionally organized stress fibers and the accumulation of actin filaments under cell membranes and in filopodia (B). Instead, staining of actin filaments in TE671 cells showed a very different organization compared to the previous cancer cell line. In contrast to untreated PANC-1, untreated TE671 cells have a high number of filopodia and a few organized actin filaments and stress fibers, with a general organization of filopodia

and actin filaments resembling that assumed by PANC-1 upon treatment with NT4 (C). In TE671 cells, no relevant modifications of cell morphology and actin organization was induced by the peptide (D). Finally, analysis was also extended to HT29 cells, characterized by a round morphology without projections unlike the other two cell lines. Despite their inability to move, they were examined in static conditions (E), not only before they reach confluence, but also in wound healing experiments (F). In the first case, actin filaments were dispersed in the cells and not organized in directional fibers. In the other case, they showed stress fibers, particularly at cell-cell contacts and actin was accumulated under cell membrane. Both these conditions were not modified by NT4. Regarding PANC-1 and TE671 cells, the effects produced by heparanase treatment were also evaluated and this enzyme was shown to behave similarly to NT4 in both cell lines [Depau et al., 2020].

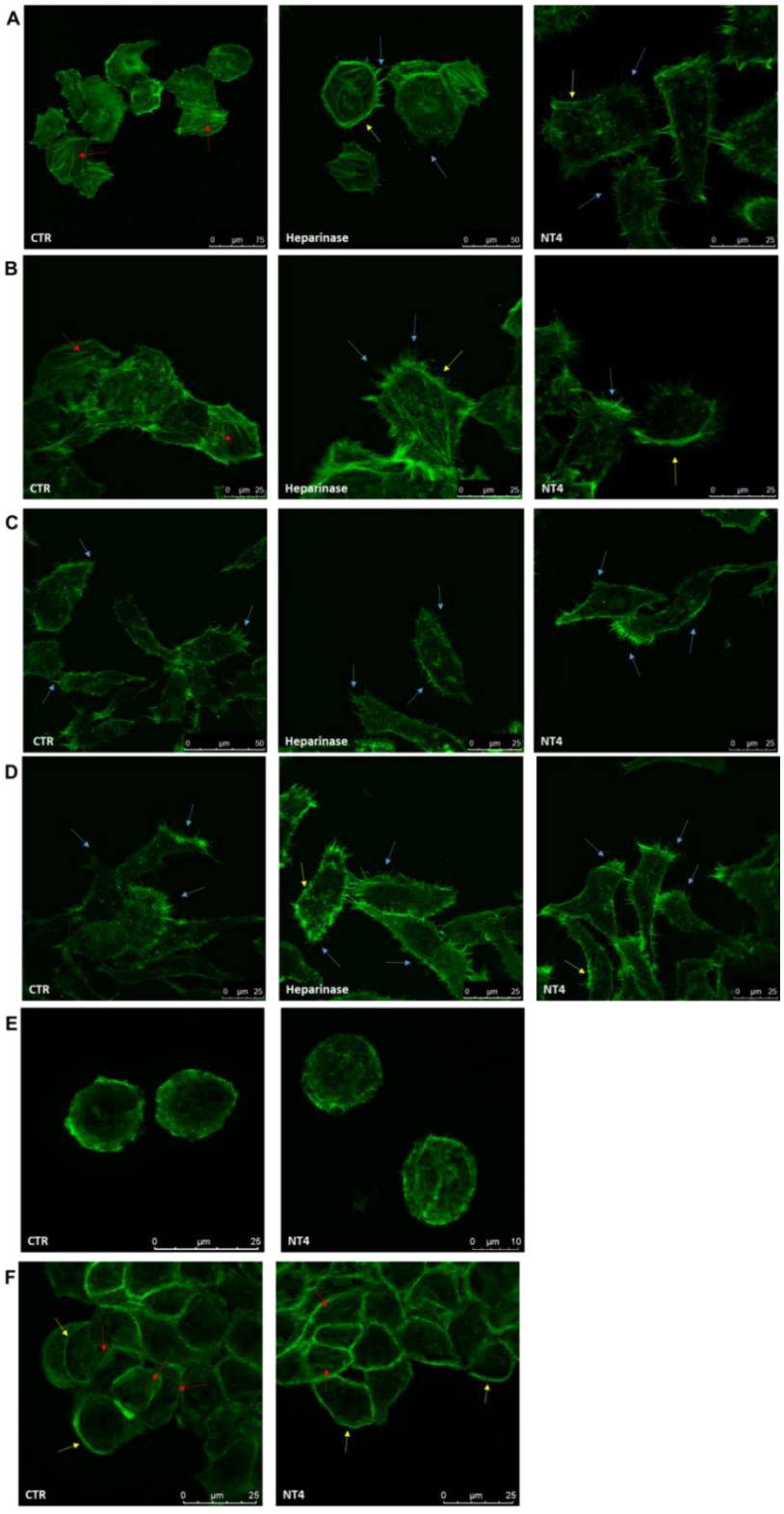


Figure 21. Effect of NT4 peptide and heparinase on actin organization in PANC-1, TE671 and HT29 cells. Confocal microscopy analysis of actin organization using phalloidin-Alexa Fluor 488 on PANC-1 cells under static (A) and migrating (B) conditions, TE671 cells under static (C) and migrating (D) conditions and HT29 cells under static (E) and migrating (F) conditions, plated on fibronectin-coated wells; in each series of images cells have no treatment (CTR), treatment with 0.03 IU/ml heparinase I/III blend (not tested in HT29) or treatment with 10 μM NT4. Red arrows indicate stress fibers, blue arrows indicate filopodia and yellow arrows indicate actin filaments accumulating under cell membranes [Depau et al., 2020].

EFFECT OF NT4 ON ACTIN REORGANIZATION SIGNALING IN CANCER CELLS WITH DIFFERENT MIGRATION PHENOTYPES

Using western blot analysis, the expression of proteins involved in signaling pathways that regulate actin dynamics was compared in the three cell lines, all of them in the presence or absence of NT4 (Fig. 22). Data showed some important differences. In particular, Rac1-GTP was evident in untreated condition and decreased by the treatment with NT4 in PANC-1 cells, while it was not detectable in untreated and treated TE671 and HT29 cells. The expression of p-cofilin in PANC-1 cells was higher than in TE671 cells, which conversely possessed lower level of p-cofilin, with respect to total cofilin, compared to PANC-1. In addition, analysis on VASP and its phosphorylated forms highlighted a higher expression of pVASP Ser 239 in TE671 compared to the other two cell lines and there were no modifications upon treatment with NT4. Finally, the expression of ERM proteins was also evaluated. They are essential for the linking of the actin cytoskeleton to the plasma membrane, thus playing a crucial role in the organization of the apical domain of polarized epithelial cells. These proteins, especially in their phosphorylated form, showed to be less expressed in TE671, as compared to PANC-1 and HT29 cells. This could be related to the chaotic migration observed in TE671 given the importance of these proteins in cell orientation. Finally, Cdc42 was less expressed in TE671 and HT29 cells compared to PANC-1 [Depau et al., 2020].

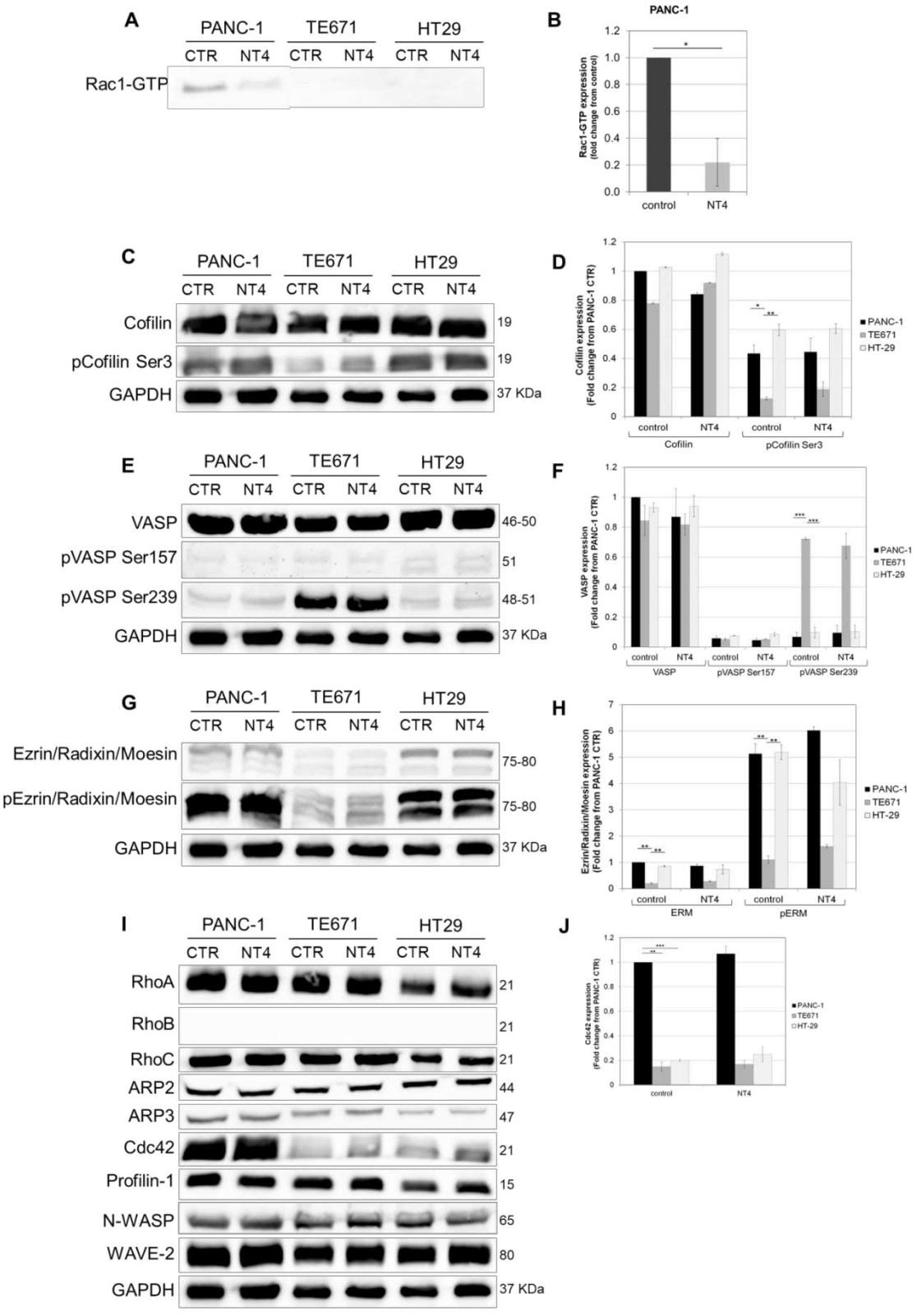


Figure 22. Effect of NT4 on Rac1 activation, actin reorganization proteins and Rho GTPase expression in PANC-1, TE671 and HT29 cells. Western blot of Rac1-GTP (A-B), cofilin (C-D), VASP (E-F), Ezrin/Radixin/Moesin (G-H), RhoA, RhoB, RhoC, Apr2, Arp3, Cdc42, Profilin-1, N-WASP and WAVE-2 (I-J) in PANC-1, TE671 and HT29 cells treated with 10 μM NT4. GAPDH was used as an endogenous control. Densitometry analysis, carried out using ImageJ software, was normalized to PANC-1 control. Errors bars represent SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by one-tailed Student's *t*-test [Depau et al., 2020].

AIM OF THE THESIS

Heparan sulfate proteoglycans interact with heparin binding proteins, which include growth factors, morphogens, many chemokines and ECM proteins. Because of these interactions, HSPGs are involved in multiple processes of cancer biology and progression, such as EMT, cell adhesion to ECM and cell migration. Consequently, HSPG interactions have been considered as potential cancer drug targets.

Given its ability to recognize HSPGs and to interfere with their biological functions in tumor cells, by targeting their sulfated GAGs, the tetra-branched peptide NT4 can be used as a specific tool for decoding the role of HSPGs in cancer cell migration. Moreover, the same peptide can be tested as a potential anti-metastatic drug.

The aim of this thesis is using NT4 for decoding the role of sulfated GAGs of HSPG in adhesion and migration of cancer cells with the final goal of testing the same peptide as a potential anti-metastatic drug.

MATERIAL AND METHODS

PEPTIDE SYNTHESIS

Peptides were synthesized on an automated multiple synthesizer Syro I (MultiSynTech, Germany) by standard Fmoc chemistry. The synthesis of solid-phase peptides is based on the successive attachment of protected amino acids to an insoluble polymeric support and proceeds from the C-terminus towards the N-terminus of the peptide. NT4 was synthesized on Fmoc4-Lys2-Lys-beta-Ala-Wang resin, using protected L-amino acids (Iris Biotech), DIPEA (N,N-diisopropylethylamine) (Merck) and HBTU (O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate) (Iris Biotech) (MultiSynTech). Pyro-Glu-O-pentachlorophenylester (Bachem, Switzerland) was used for the last coupling step. NT4-biotin was synthesized on Tentagel resin with Fmoc-Lys(biotin)-OH as first coupling step, and Fmoc-PEG12-OH as second; Fmoc-Lys(Fmoc)-OH was then used to build the tetrameric core. At the end of the coupling sequence, peptides were cleaved from the resin, deprotected and lyophilized.

HPLC purification was performed on a C18 Jupiter column (Phenomenex). Water with 0.1% TFA (A) and methanol (B) were used as eluents. Linear gradients over 30 min were run at flow rates of 0.8 ml/min and 4 ml/min for analytical and preparatory procedures, respectively. All compounds were also characterized on a Bruker Ultraflex MALDI TOF/TOF mass spectrometer.

NT4 (pyELYENKPRRPYIL)4K2K-beta-Ala MS: m/z calculated for C333H519N91O81 [M+H]+ was 7094.24; detected 7095.15. HPLC RT (from 80%A to 20%A) 26.63 min. NT4-biotin (pyELYENKPRRPYIL)4K2K-PEG12-K(biotin)MS: m/z calculated for C373H594N96O95S [M+H]+ was 7976.35; detected 7978.72. HPLC RT (from 80%A to 20%A) was 26.99 min.

CELL LINES

PANC-1 human pancreas adenocarcinoma cells, HT29 human colon adenocarcinoma, TE671 human rhabdomyosarcoma, MCF-7 and MDA-MB-231 human breast adenocarcinoma cells were grown in their recommended media supplemented with 10% fetal bovine serum, 200 µg/ml glutamine, 100 µg/ml streptomycin, 60 µg/ml penicillin, and maintained at 37°C, 5% CO_2. Cell lines were purchased from ATCC and cell profiling was analyzed to authenticate human cell lines (BMR Genomics).

FLOW CYTOMETRY

2×10^5 cells were incubated in 96-well U-bottom plates with 500 nM biotinylated NT4 for 30 min at room temperature. Inhibition of NT4 binding by heparin was carried out by incubating cells with 500 nM biotinylated NT4, in presence of 10 and 20 µg/mL heparin. Cells were finally incubated with 1 µg/mL streptavidin−FITC. All dilutions were performed in phosphate-buffered saline (PBS), containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 1% bovine serum albumin (BSA). Ten thousand events were analyzed using a BD FACS Canto II (Becton Dickinson, NJ) or FACS Guava (Millipore). Results were analyzed by FCS Express 6 flow software.

CANCER CELL ADHESION ASSAY

The 96-well cell culture plates were coated with 20 µg/mL human collagen IV or with 10 µg/mL cellular fibronectin or plasma fibronectin for 2 h at 37 °C. A total of 1×10^5 cells/well were plated for 30 min at 37 °C with different concentrations of NT4 (from 1 to 10 µM) and Heparin (Sigma Aldrich) (from 1 to 200µg/ml), fixed with PBS−4% paraformaldehyde (PFA) for 15 min at room temperature and stained with 0.1% crystal violet in 200 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.0 for 1 h at room temperature. The cells were then solubilized with 10% acetic acid, and the absorbance was measured at 595 nm using a microplate reader.

WOUND HEALING

PANC-1 (2.8×10^4), TE671 (5.2×10^4), HT29 cells (7.7×10^4), MCF-7 ($1{,}05 \times 10^5$) and MDA-MB-231 (5.2×10^4) were seeded on each side of a culture insert for live cell analysis (Ibidi, Munich, Germany). Inserts were placed in wells of an uncoated or precoated 24-well plate (20 µg/mL human collagen IV for 2 h at 37 °C) and incubated at 37 °C and 5% CO_2 to allow cells to grow to confluence. Afterward, inserts were removed with sterile tweezers and the cells were treated with 10 µM NT4 peptide and 70µg/mL Heparin in complete medium. The cells were allowed to migrate in the incubator of a DMi8 (Leica Microsystems) microscope. The same instrument was used to take a picture at time zero and every 10 min. The time-lapse image stacks were analyzed using ImageJ.

CELL COLONIES

The 24-well cell culture plates were coated with 20 µg/mL human collagen IV for 2 h at 37 °C. PANC-1 (1×10^4), TE671 (1×10^3), HT29 cells (1.5×10^3), MCF-7 (1×10^3) and MDA-MB-231 (1×10^4) were plated in the presence or absence of 10 µM NT4 and 70 µg/mL Heparin. Cell colonies were analyzed after 6 days of incubation at 37°C in a humidified atmosphere with 5% CO_2, fixed with 4% PFA in PBS and stained with 0.1% crystal violet in 200 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.0. The entire image of stained cells was observed with an SP8 confocal microscope (Leica Microsystems) and analyzed by ImageJ to count cells.

IMMUNOFLUORESCENCE

PANC-1 (2.8×10^4), TE671 (5.2×10^4), HT29 (7.7×10^4), MCF-7 ($1{,}05 \times 10^5$) and MDA-MB-231 (5.2×10^4) cells were seeded on each side of a culture insert for live cell analysis (Ibidi, Munich, Germany). Inserts were placed in wells of an uncoated or precoated 24-well plate (20 µg/mL human collagen IV for 2 h at 37 °C) and incubated at 37 °C and 5% CO_2 to allow cells to grow to confluence. Afterward, inserts were

removed with sterile tweezers and the cells were treated with 10 µM NT4 peptide at 37°C and 5% CO_2. After 3 h, cells were fixed with PBS-4% paraformaldehyde (PFA)without Methanol, saturated with PBS–5% bovine serum albumin (BSA) for 60 min, then incubated with Alexa Fluor 488 phalloidin 1:40 in PBS–1% BSA for 30 min.

NT4 binding

Cells were incubated with NT4-biotin for 30 min at 37°C (2 µM in PBS-1% BSA), followed by incubation for 30 minutes with 0.5 µg/ml Streptavidin-Atto 550 at room temperature

HS staining

HS was stained using 4 µg/mL anti-heparan sulfate (10E4 epitope) in PBS-1% BSA for 1 h, followed by incubation with 4 µg/mL goat anti-Mouse IgM anti-mouse Alexa Fluor 546 (Thermo Fisher Scientific, Waltham, MA, USA).

Detection of Cadherins, Eph receptors and Ephrin B2

For the analysis of E and N-cadherins, Eph receptors and Ephrin B2, cells incubated with anti E-cadherin (1:500) and N-cadherin (1:200), anti-Eph receptor A2+A3+A4 (1:500), anti-Eph receptor A2+A3+A4 (phospho Y588 + Y596) (1:1000), anti-Eph receptor B1 + Eph receptor B2 (1:1000), anti-Eph receptor B1 + Eph receptor B2 (phospho Y594 + Y604) (1:1000), anti-Eph receptor EphB4 (D1C7N) (1:800) and anti-Ephrin B2 (1:200) in PBS–1%BSA for 2 h at room temperature. Cells were finally incubated for 2 h at room temperature with an anti-rabbit IgG Alexa Fluor 546 antibody diluted 1:1000.

Nuclei were stained with DAPI (0.5 µg/mL in PBS-1% BSA). Each step was followed by three washes in PBS. Peptide binding was analyzed by confocal laser microscope (Leica TCS SP8).

WESTERN BLOTTING

Cells were seeded in six-well plates (1.5×10^6cells per well), previously coated with 10 μg/mL plasma fibronectin and maintained overnight in a CO_2 incubator. Cells were treated with 10μM NT4 in DMEM for 3 h at 37 °C and lysed according to the antibody supplier's instructions.

In total, 20 μL/lane total proteins was separated by 12% sodium dodecylsulfate-polyacrylamidegel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% w/v BSA in TBS containing 0.1% Tween 20 for 1 h at room temperature and then incubated overnight at 4 °C with specific antibodies diluted 1:1000 in 5% w/v BSA or nonfat dry milk in TBS containing 0.1% Tween 20, according to the antibody supplier's instructions:

- Anti-E-cadherin antibody
- Anti-N-cadherin antibody
- Anti-Eph receptor B1 + Eph receptor B2 antibody
- Anti-Eph receptor B1 + Eph receptor B2 (phospho Y594 +Y604) antibody
- Anti-Eph receptor A2+A3+A4 antibody
- Anti-Eph receptor A2+A3+A4 (phospho Y588 + Y596) antibody
- Anti-EphB4 (D1C7N) antibody
- Anti-Ephrin B2 Recombinant Rabbit Monoclonal Antibody (JM53-21) antibody.

After washing with TBS containing 0.1% Tween 20, the membrane was incubated for 1 h with horse radish peroxidase-conjugated anti-rabbit and anti-mouse IgG antibodies diluted 1:2000 in 5% w/v non fat dry milk in TBS containing 0.1% Tween 20. Signals were detected using Image LAS4010 (GE Healthcare) and analyzed by ImageJ.

TRANSFECTION FOR F-ACTIN LABELING AND SELECTION OF FLUORESCENT COLONIES

2 x 10^5 PANC-1 cells and 1.1 x 10^5 TE671 cells were plated in 24-well cell culture plates, in the specific culture medium without antibiotics and glutamine, and incubated at 37°C with 5% CO_2 supply for 24 h.

The following components were used for transfection:

- Opti-MEM medium without phenol red (Gibco);
- Lipofectamine 3000 transfection agent (Invitrogen);
- Plasmid DNA containing a molecule capable of marking the F-actin strands in the cells. The plasmid used is mAppleLifeact-7 (Addgene), which is capable, in addition, of conferring to the cell lines transformed resistance to neomycin. The plasmid DNA was previously maintained and amplified in DH5α E. Coli.

Cells were transfected using 2,5 µl/well of DNA plasmid, diluted in Opti-MEM medium without phenol red, and 1,5 µl/well of transfection agent Lipofectamine 3000. After 24 h, cells were treated using DMEM medium with 10% FBS, antibiotics, glutamine and, in addition, geneticin (G418) able to select and maintain in culture cancer cell lines transfected with the plasmid as it contains within it the gene for neomycin resistance. This procedure was repeated several times. The transfected cell cultures were expanded and subsequently, plated at low concentrations (2/3 cells/well in black 96-well plates), in order to isolate colonies that were fluorescent. Once confluence was reached, the cells were analyzed under a confocal microscope (Leica TCS SP8) to verify their transfection, with excitation λ of 568 nm and emission λ at 592 nm specific to mApple.

WOUND HEALING FOR LIVE-CELL IMAGING

Transfected PANC-1 (3.1×10^4) cells were seeded on each side of a culture insert for live cell analysis (Ibidi, Munich, Germany). Inserts were placed in wells of a black uncoated 24-well plate and incubated at 37 °C and 5% CO_2 to allow cells to grow to confluence. After 24 h, inserts were removed with sterile tweezers. The treated wells were subsequently incubated with NT4 10 µM, diluted in culture medium with G418,

while in those used as controls, medium was added to the culture medium with G418. For live observation of the cells, a DMi8 fluorescence microscope (Leica Microsystems) and a microscope confocal microscope (Leica TCS SP8). In both experiments, the plates were placed inside the incubator of each microscope at 37° C, with 5% CO_2, to allow the cells to migrate. Images were acquired every 15 min for a total of 18 h and then analyzed with ImageJ software (NIH).

STATISTICAL ANALYSIS

Data were presented as mean ± SD. The significance of difference was analyzed by one-tailed Student's t-test using GraphPad Prism 5.03 software. *p* values are reported in figure legends.

RESULTS

EXPRESSION AND DISTRIBUTION OF N- AND E-CADHERIN IN DIFFERENT CANCER CELL LINES

Cadherins play a very important role in cell-cell adhesions and may be associated with different migration phenotypes. The expression of N-cadherin and E-cadherin was measured in PANC-1, TE671 and HT29 cells by western blotting (Fig. 23). The results showed a different cadherin profile in the three cell lines. PANC-1 cells expressed both N- and E-cadherin, TE671 cells expressed N-cadherin and not E-cadherin, and HT29 cells expressed E-cadherin and not N-cadherin. However, in all these cases, treatment with NT4 (10 μM) did not affect the expression of either cadherin [Depau et al., 2020].

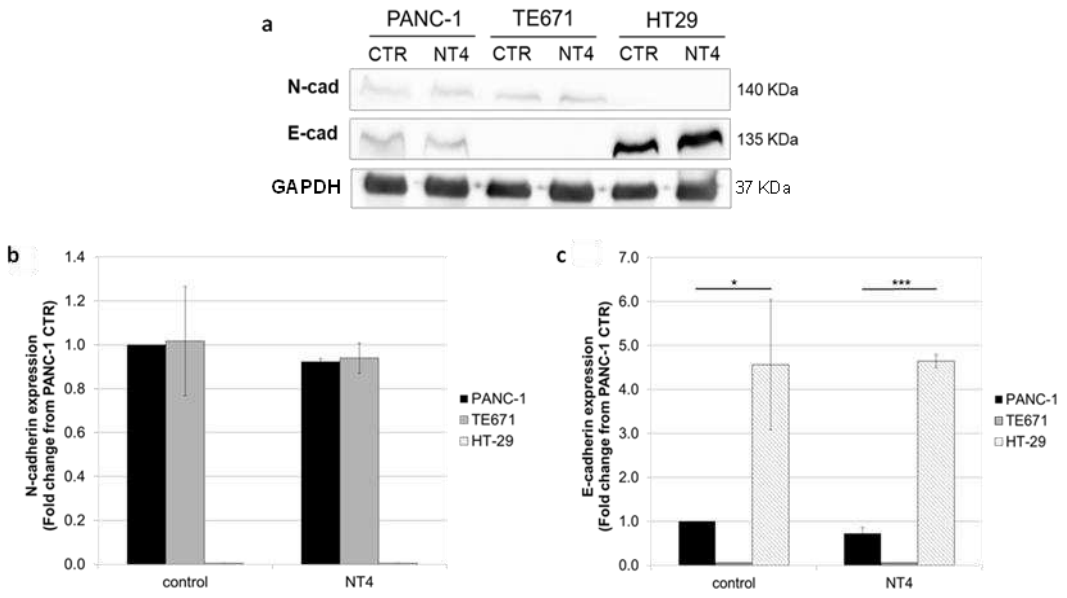


Figure 23. Western blotting of N- and E-cadherin expression in PANC-1, TE671 and HT29 cells (a). GAPDH was used as an endogenous control. Densitometry analysis, carried out using ImageJ software, was normalized to PANC-1 control (b, c). Errors bars represent SD. * $p < 0.05$ and *** $p < 0.001$ by one-tailed Student's *t*-test [Depau et al., 2020].

According to protein expression, also confocal microscopy confirmed these results (Fig. 24). Moreover, a different distribution of cadherins in migrating cells was observed [Depau et al., 2020].

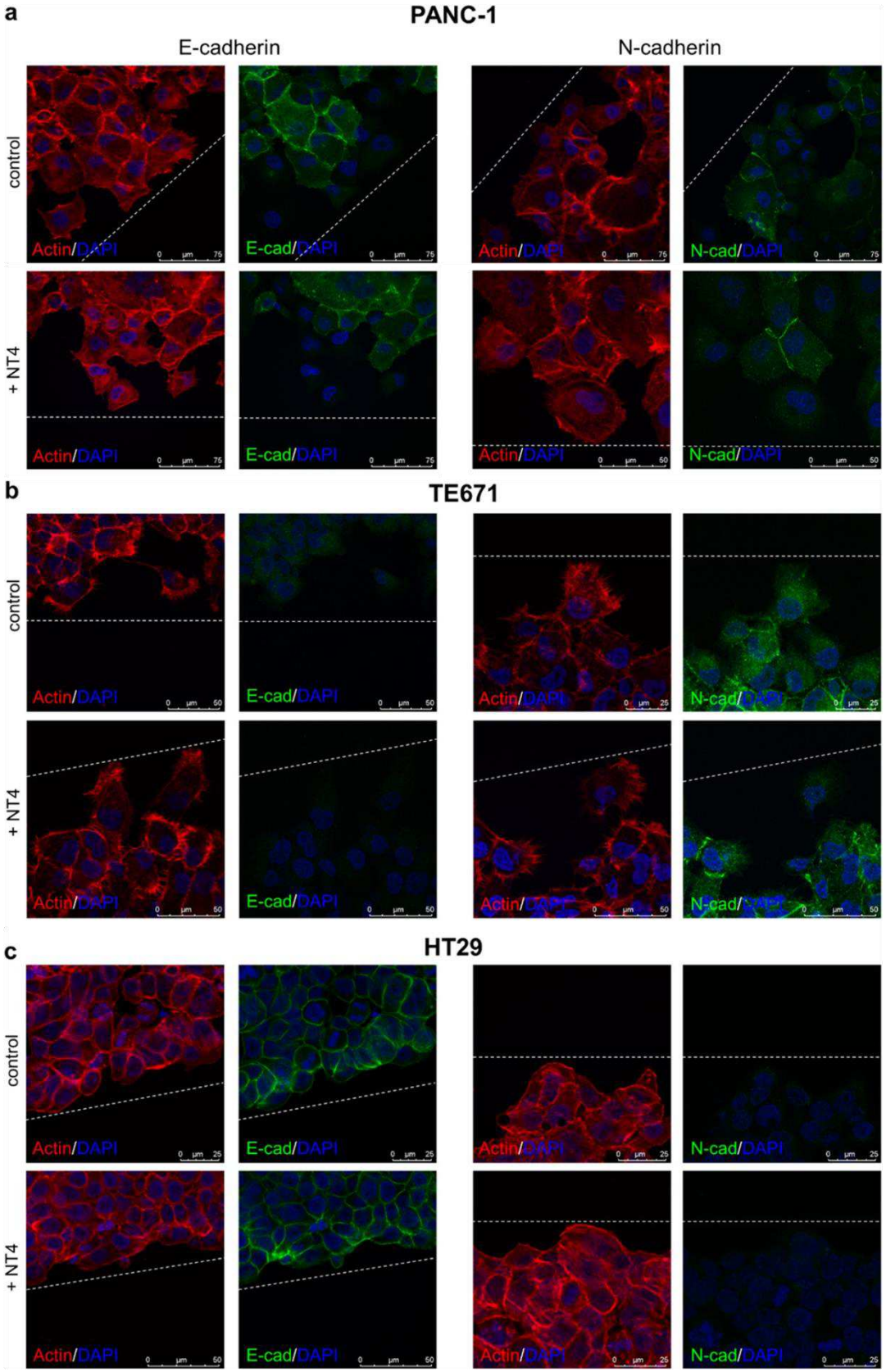


Figure 24. Effect of NT4 peptide on E- and N-cadherin distribution in migrating PANC-1 (a), TE671 (b) and HT29 (c) cells. Confocal microscopy analysis of E- and N-cadherin (green signal) and actin (red signal). Each series of images shows cells under not treatment (control) or treatment with 10 μm NT4 (+NT4). Nuclei are stained with DAPI (blue signal). The dotted lines indicate the cell migration front [Depau et al., 2020].

In PANC-1 cells, both N-cadherin and E-cadherin were clearly evident at contacts among cells and were less expressed in cells on the migration front. In TE671 cells, while E-cadherin was not detected, N-cadherin was clearly evident at cell-cell contacts and similarly to PANC-1 cells, it was less expressed in cells on the migration front and was not evident in isolated migrating cells. In contrast, HT29 cells expressed only E-cadherin, which was clearly evident at cell-cell contacts and all around the cell membrane, even on the migration front. In all the three cases, treatment with NT4 did not modify their distribution [Depau et al., 2020].

Subsequently, these analyses were also extended to two other cell lines. These are two types of human breast adenocarcinoma cells presenting two different ways of moving: MCF-7 cells, which are characterized by collective migration, and the highly aggressive MDA-MB-231 cells, which instead migrate individually, like PANC-1 and TE671 cells. As western blotting analyses showed, MCF-7 cells expressed large amounts of E-cadherin but not N-cadherin. In MDA-MB-231 cells, both cadherins were not present. No modifications on their expression were induced by incubation of cells with NT4 peptide (Fig. 25).

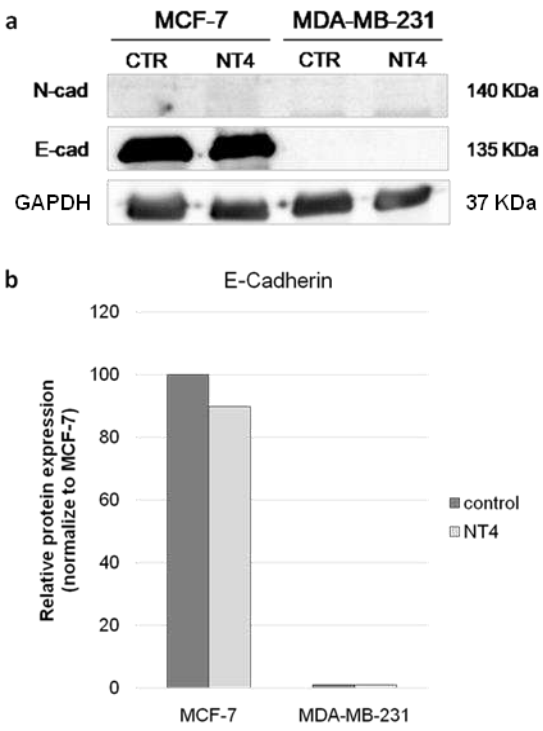


Figure 25. Western blotting of N- and E-cadherin expression in MCF-7 and MDA-MB-231 cells (a). GAPDH was used as an endogenous control. Densitometry analysis, carried out using ImageJ software, was normalized to MCF-7 control (b).

Again, the results obtained were confirmed by confocal microscopy (Fig. 26). Only in MCF-7 cells was it possible to observe the presence of E-cadherin, distributed mainly at the points of contact between cells and less evident in the leader cells that drive migration. As in the case of the other cells considered, treatment with NT4 induced no change in cadherin distribution.

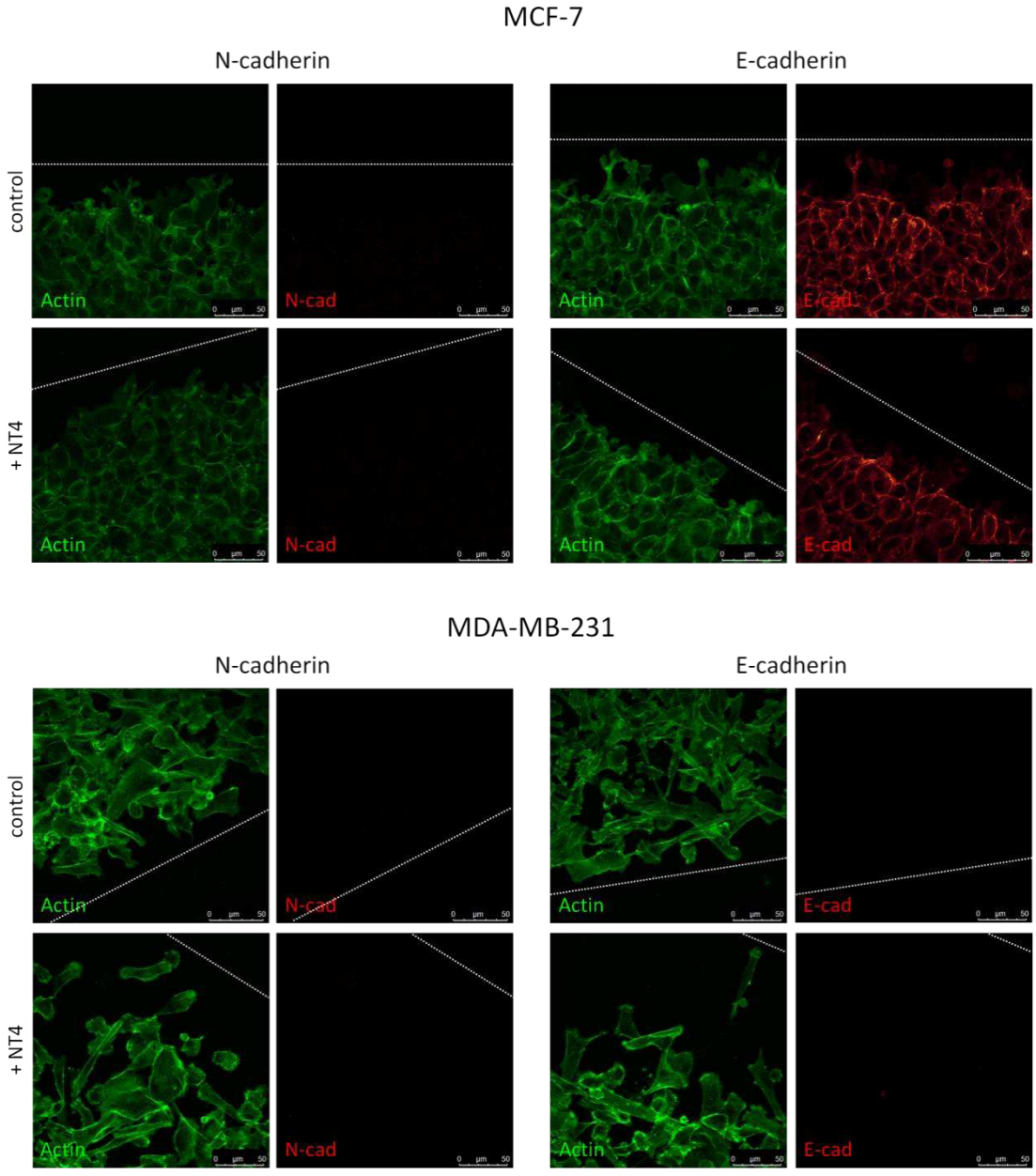


Figure 26. Effect of NT4 peptide on N- and E-cadherin distribution in migrating MCF-7 (a) and MDA-MB-231 (b) cells. Confocal microscopy analysis of N- and E-cadherin (red signal) and actin (green signal). Each series of images shows cells under not treatment (control) or treatment with 10 μm NT4 (+NT4). The dotted lines indicate the cell migration front.

DISTRIBUTION OF HSPGs IN MIGRATING CELLS

In order to study the localization of HSPGs during cancer cell migration, both binding of NT4 and that of anti-heparan sulfate 10E4 monoclonal antibody were analyzed by immunofluorescence (Fig. 27).

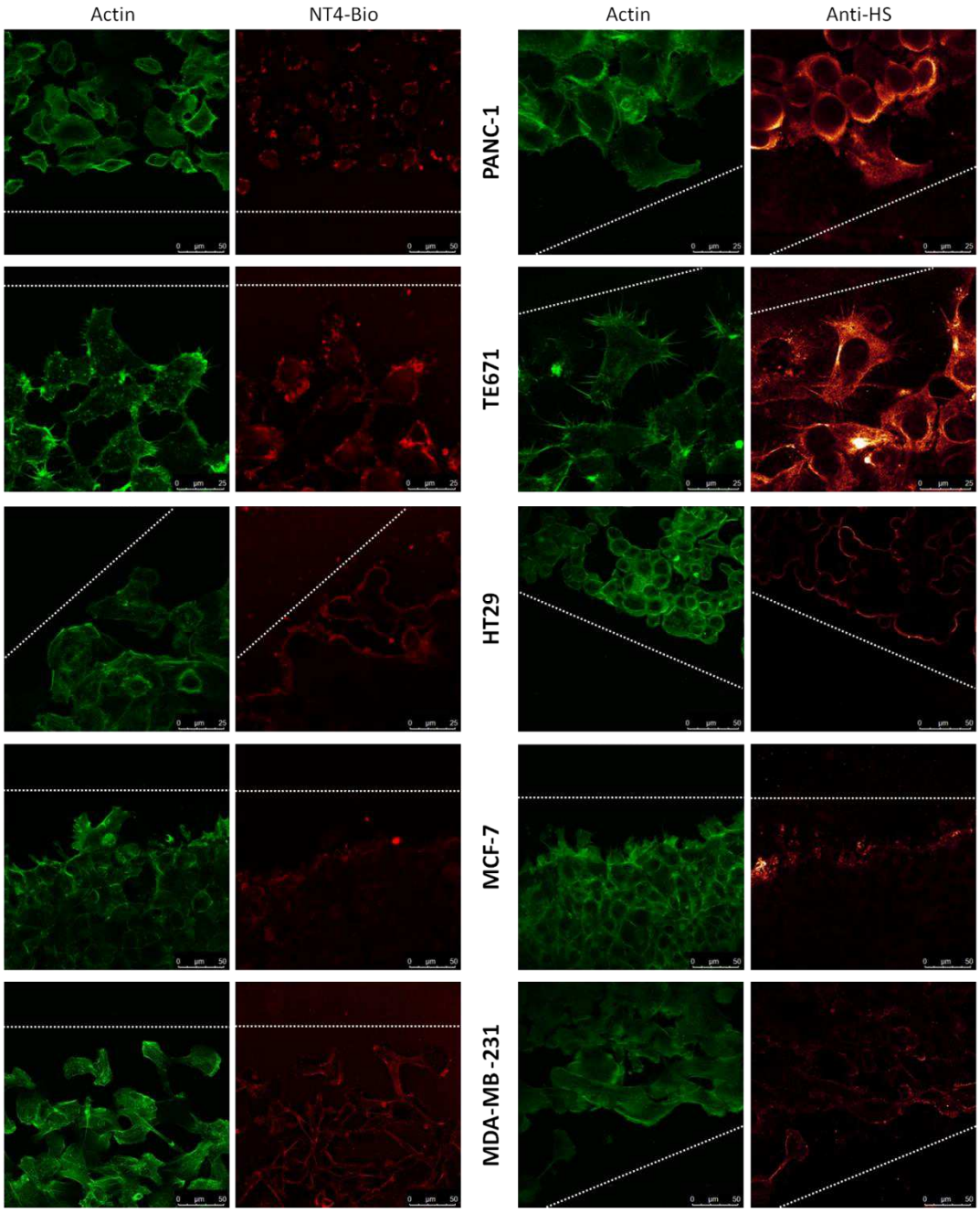


Figure 27. Binding of NT4 (left panel) and binding of anti-heparan sulfate mAb 10E4 (right panel) in migrating PANC-1, TE671, HT29, MCF-7 and MDA-MB-231 cells. Confocal microscopy analysis of actin organization (green signal), NT4-Bio (red signal in the left panel) and anti-HS 10E4 mAb (red signal in the right panel). The dotted lines indicate the cell migration front.

Different cancer cell lines with different migration phenotypes were taken into account: PANC-1, TE671, HT29, MCF-7 and MDA-MB 231 cells. In all of them, results showed a similar distribution between the peptide, conjugated to biotin to allow its staining by using streptavidin, and the anti-HS monoclonal antibody. In PANC-1, TE671 and MDA-MB-231 cells HSPGs are located along the entire cell membrane, while in MCF-7 cells, these glycoproteins are found at the exposed front of leader cells. Also in HT29 cells, which are not able to migrate, HSPGs are present only on the edge exposed to the void space and the signal clearly decreased at cell-cell contacts.

EXPRESSION AND DISTRIBUTION OF EPH RECEPTORS IN DIFFERENT CANCER CELL LINES

Using western blot analysis, the expression of Eph receptors and that of ephrin B2 were compared in PANC-1, TE671 and HT29 cells (Fig. 28), but also in MCF-7 and MDA-MB-231 cells (Fig. 29), all of them in the presence or absence of NT4 (10 μM).
Data showed some differences. TE671 cells were characterized by a lower expression of the Eph receptors, in particular EphA and its phosphorylated form, the phosphorylated form of EphB and EphB4, and a higher expression of ephrin B2 compared with PANC-1 and HT29 cells. In addition, EphA was found to be more highly expressed in PANC-1 cells than in TE671 and HT29 cells, while EphB4 was more present in HT29 cells compared with PANC-1 and TE671 cells. Finally, the expression of EphB4 in MCF-7 cells was higher than in MDA-MB-231 cells, in contrast to the other Eph receptors analyzed and ephrin B2 all found to be more highly expressed in MDA-MB-231 than in MCF-7 cells. However, NT4 treatment induced no significant change in Eph receptors expression and ephrin B2 expression in any cell line, with the exception of the expression of EphA, which was slightly inhibited in PANC-1 cells, and that of the two phosphorylated forms Eph pA and Eph pB, both of which slightly increased after NT4 treatment, the former in HT29 cells and the latter in both HT29 and MDA-MB-231 cells.

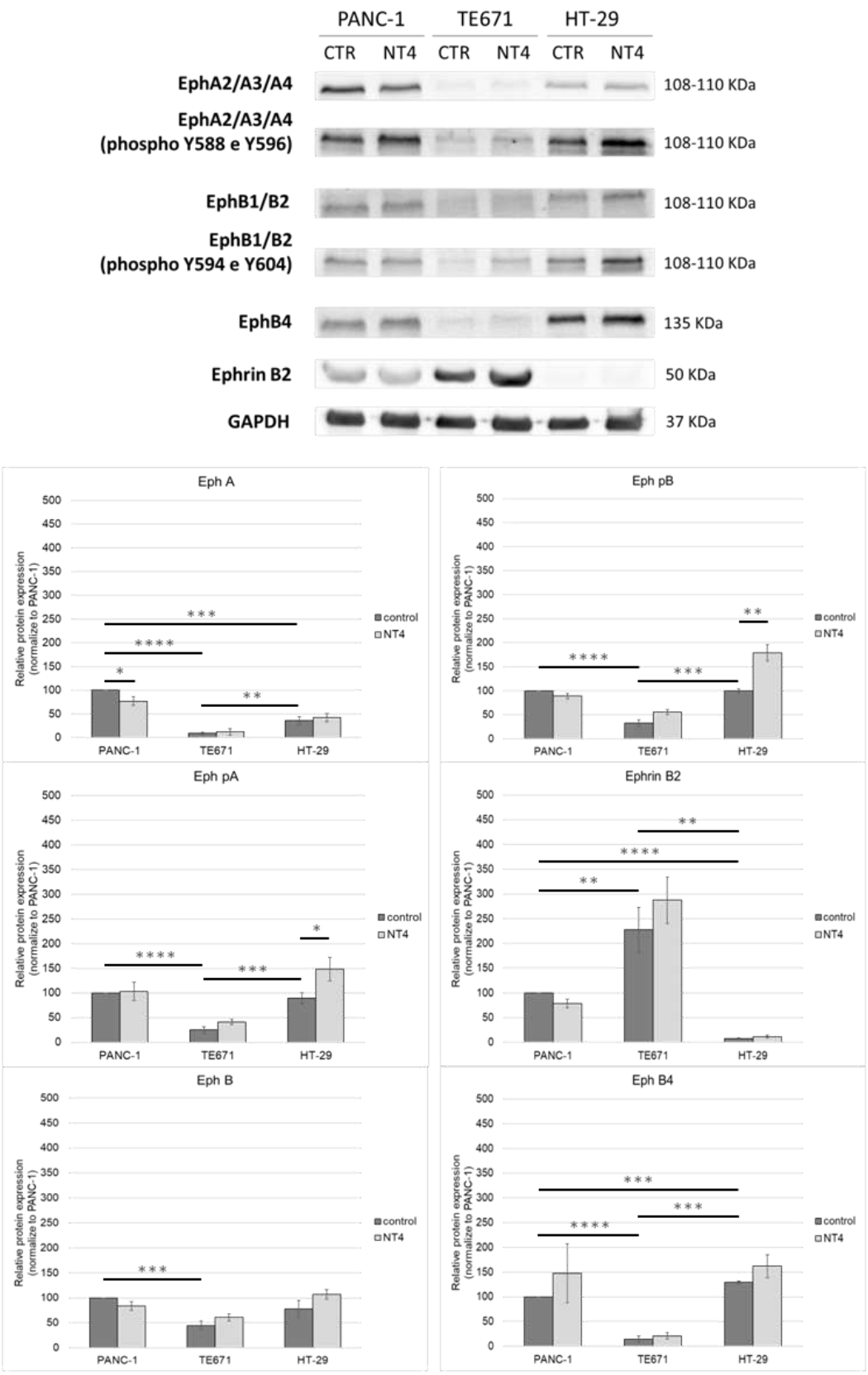


Figure 28. Western blotting of Eph receptors and ephrin B2 expression in PANC-1, TE671, HT29 cells treated with 10 μM NT4. GAPDH was used as an endogenous control. Densitometry analysis, carried out using ImageJ software, was normalized to PANC-1 control. Errors bars represent SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by one-tailed Student's *t*-test.

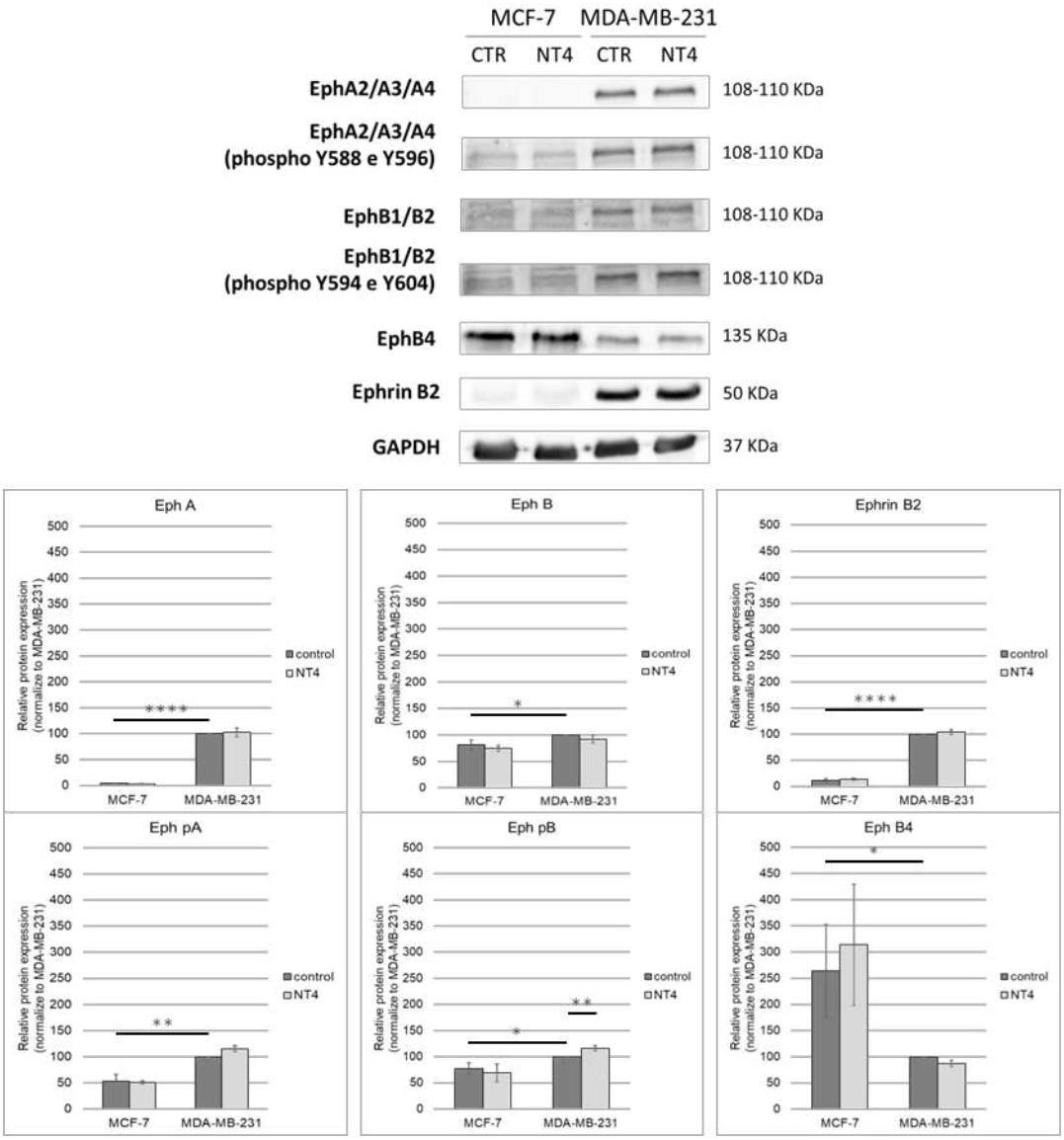


Figure 29. Western blotting of Eph receptors and ephrin B2 expression in MCF-7 and MDA-MB-231 cells. treated with 10 μM NT4. GAPDH was used as an endogenous control. Densitometry analysis, carried out using ImageJ software, was normalized to MDA-MB-231 control. Errors bars represent SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by one-tailed Student's *t*-test.

In addition, distribution of Eph receptors was analyzed in PANC-1 (Fig. 30), TE671 (Fig. 31) and HT29 (Fig. 32) cells by immunofluorescence under migrating conditions, but no significant differences emerged in all cells tested, both in the presence and absence of NT4. Only with regard to Eph B4 in PANC-1 and TE671 could lower expression be seen in cells at the migrating front compared with ones on the inside.

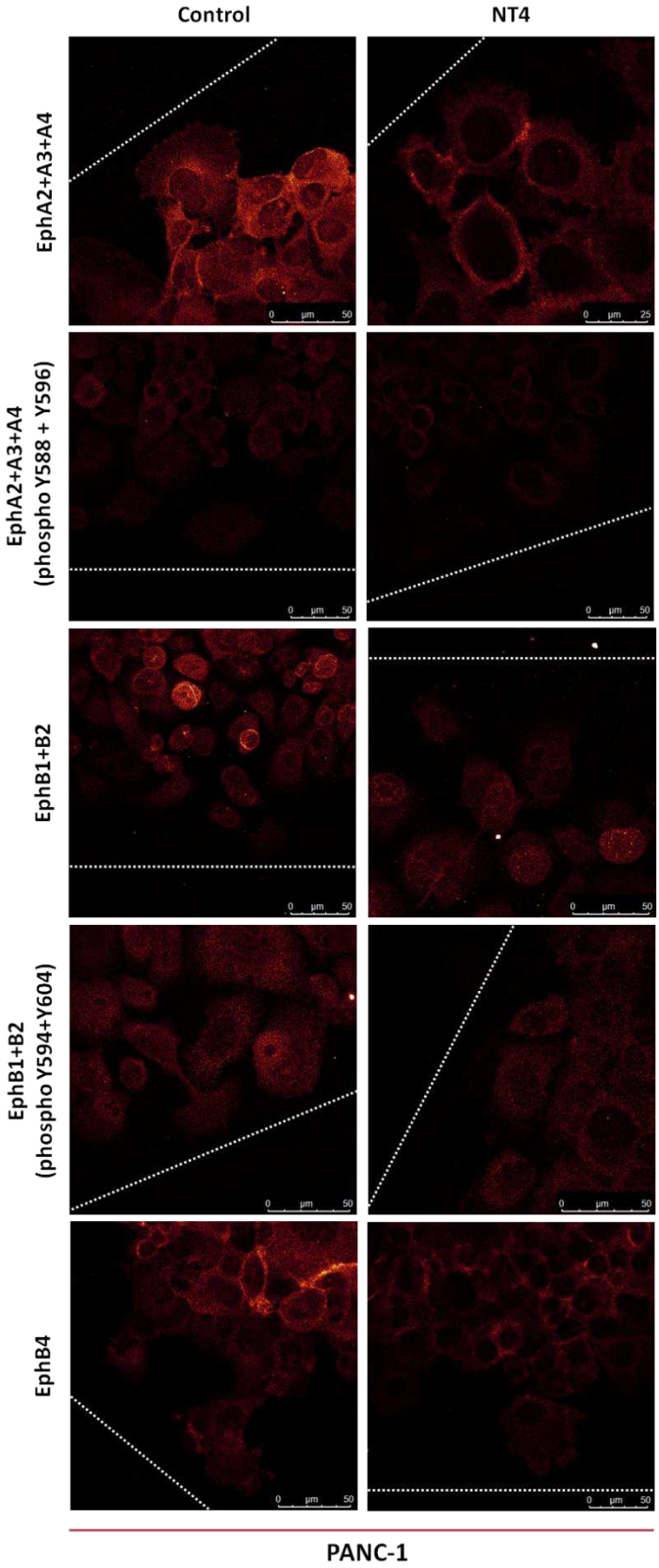


Figure 30. Effect of NT4 peptide on Eph receptors distribution. Confocal microscopy analysis of Eph receptors in PANC-1 cells under migrating conditions. Each series of images shows cells under not treatment (control) or treatment with 10 μm NT4. The dotted lines indicate the cell migration front.

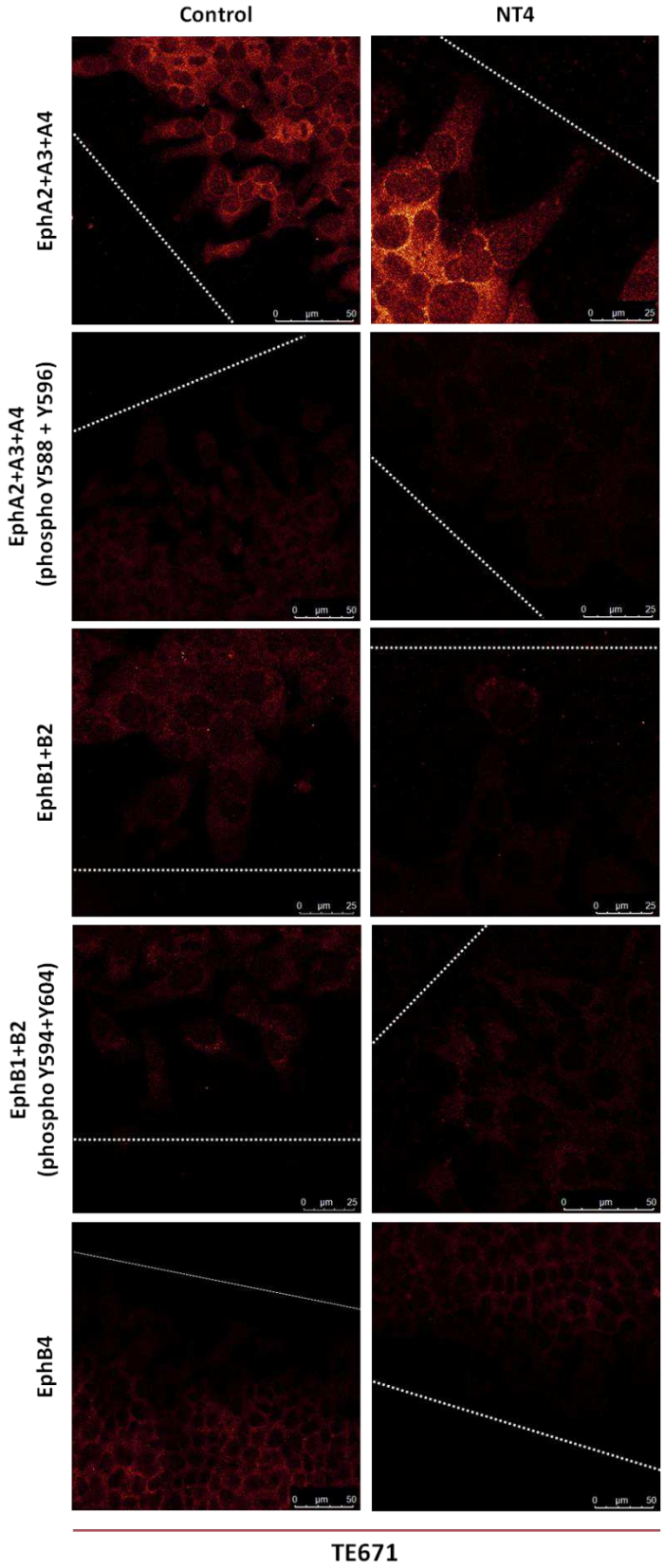


Figure 31. Effect of NT4 peptide on Eph receptors distribution. Confocal microscopy analysis of Eph receptors in TE671 cells under migrating conditions. Each series of images shows cells under not treatment (control) or treatment with 10 µm NT4. The dotted lines indicate the cell migration front.

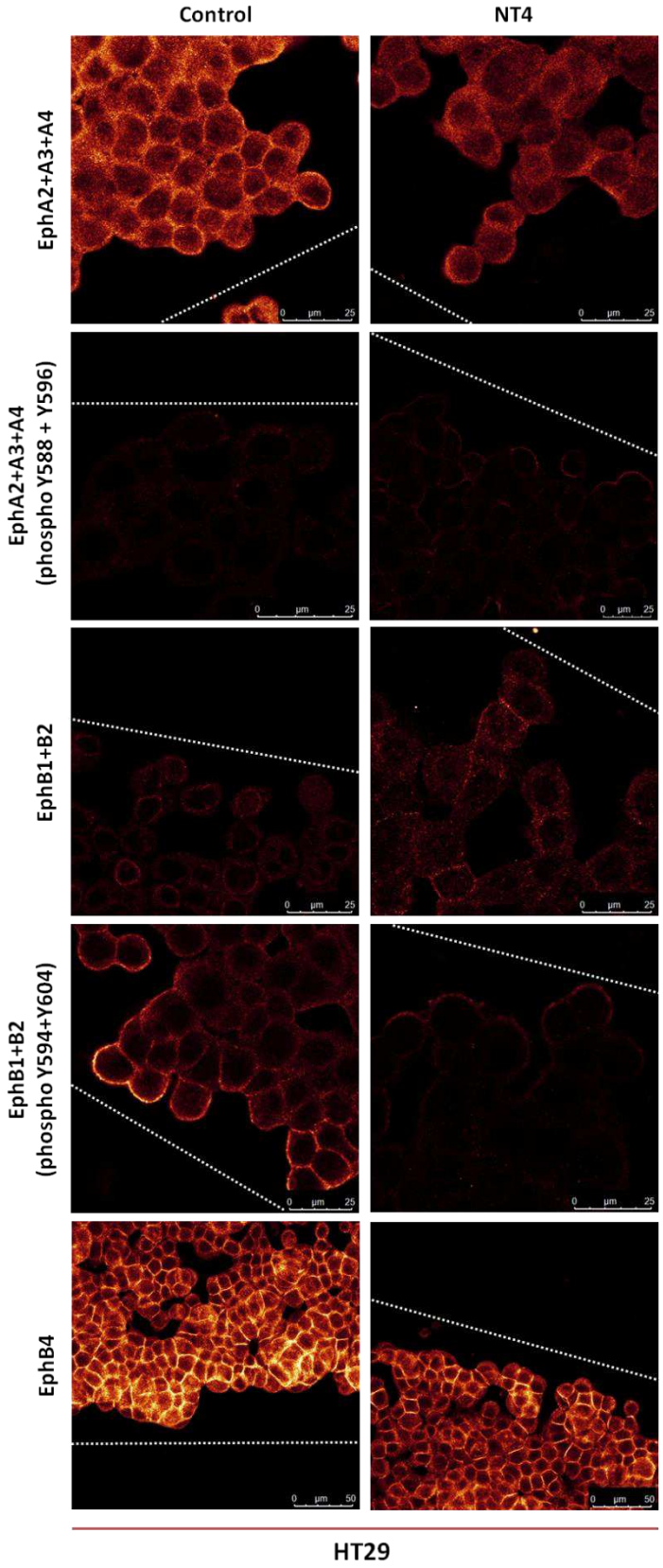


Figure 32. Effect of NT4 peptide on Eph receptors distribution. Confocal microscopy analysis of Eph receptors in HT29 cells. Each series of images shows cells under not treatment (control) or treatment with 10 μm NT4. The dotted lines indicate the cell migration front.

BINDING OF NT4 TO DIFFERENT CANCER CELL LINES IS INHIBITED BY HEPARIN

By analyzing the binding of NT4 and its inhibition in the presence of heparin by flow cytometry, it was possible to confirm previous reports [Depau et al., 2020]. NT4 turns out to be able to bind different cell lines, in this case PANC-1, TE671, HT29, MCF-7, and MDA-MB-231 cells. Heparin was tested at two concentrations: 20 µM and 10 µM. Already at the lowest concentration it inhibits binding by about 90% (Fig. 33).

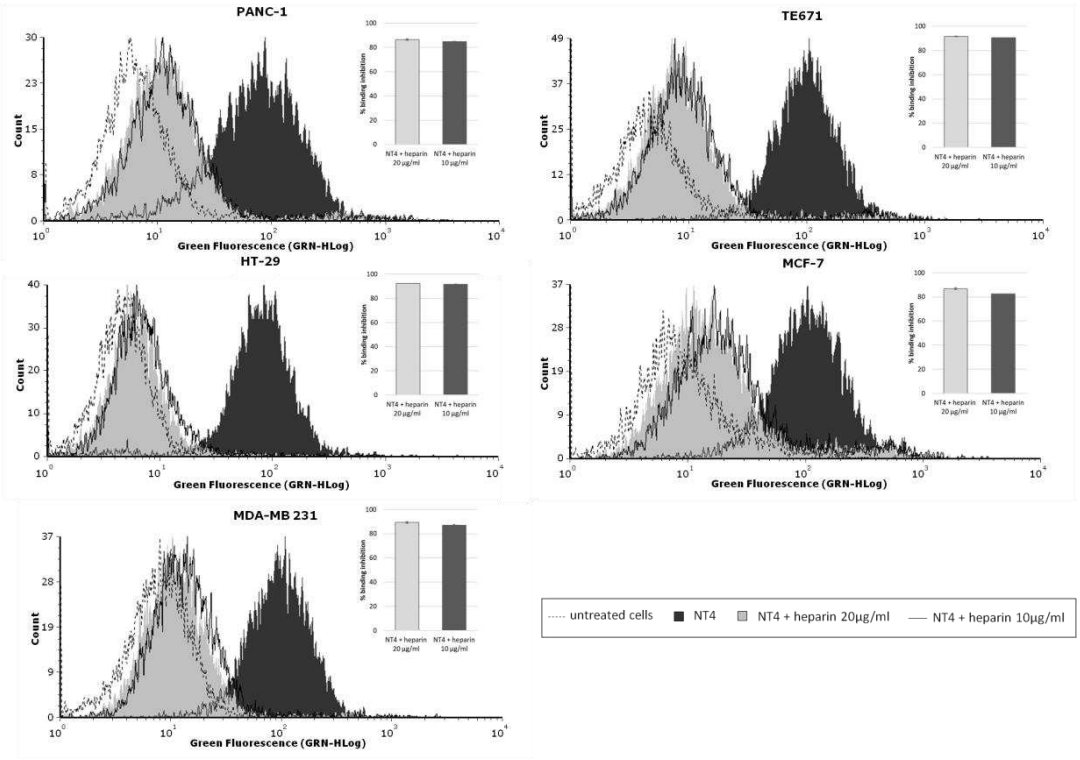


Figure 33. Binding of NT4 and inhibition of the same binding by heparin to PANC-1, TE671, HT29, MCF-7 and MDA-MB-231 cells.

EFFECT OF NT4 AND HEPARIN ON CANCER CELL COLONIES FORMATION

The ability of individual cells to give rise to colonies was analyzed by colony formation assay (Fig. 34). Highly diluted cells were seeded on collagen-coated wells

and incubated for 6 days. The results obtained showed some differences. NT4 succeeded, in fact, in inhibiting colony formation in PANC-1 cells and to a greater extent in MDA-MB-231 cells, but the process was also inhibited in HT29, although the number of colonies formed by these cells was very low. Moreover the peptide did not significantly modify colony formation in MCF-7 cells, while it increased that in TE671 cells, in agreement with NT4-induced stimulatory effect on the migration of this cell line. Heparin, on the other hand, did not significantly alter colony formation in all cells tested, with the exception of MDA-MB-231, where it seemed to inhibit colony formation, although to a lesser extent than NT4.

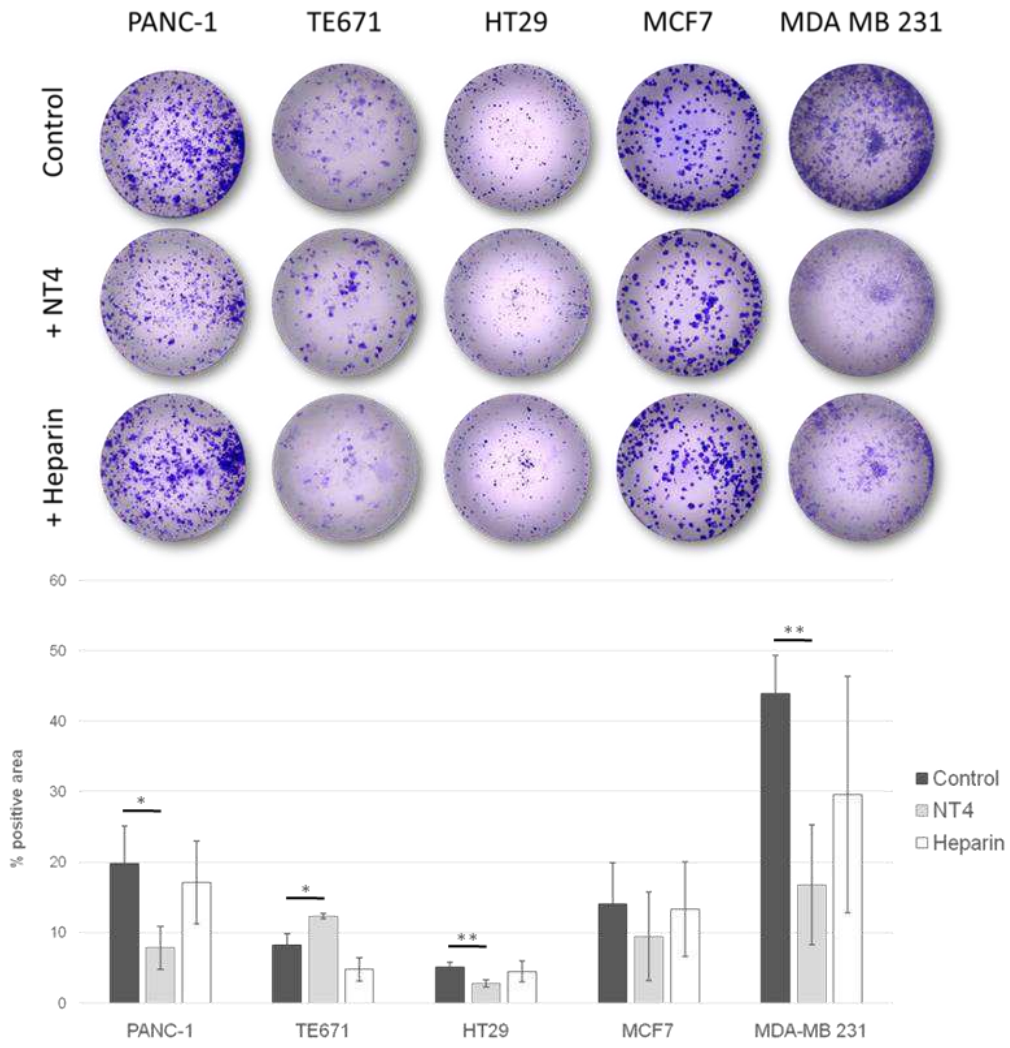


Figure 34. Colony formation assay. PANC-1, TE671, HT29, MCF-7 and MDA-MB-231 cells were plated on wells previously coated with 20 µg/mL human collagen IV, in the presence or absence of 10 µM NT4 and 70 µg/mL Heparin. The images of cell colonies, fixed and stained with crystal violet, were obtained by confocal microscopy, on the entire well surface, and were analyzed with ImageJ. Errors bars represent SD. * $p < 0.05$ and ** $p < 0.01$ by one-tailed Student's t-test.

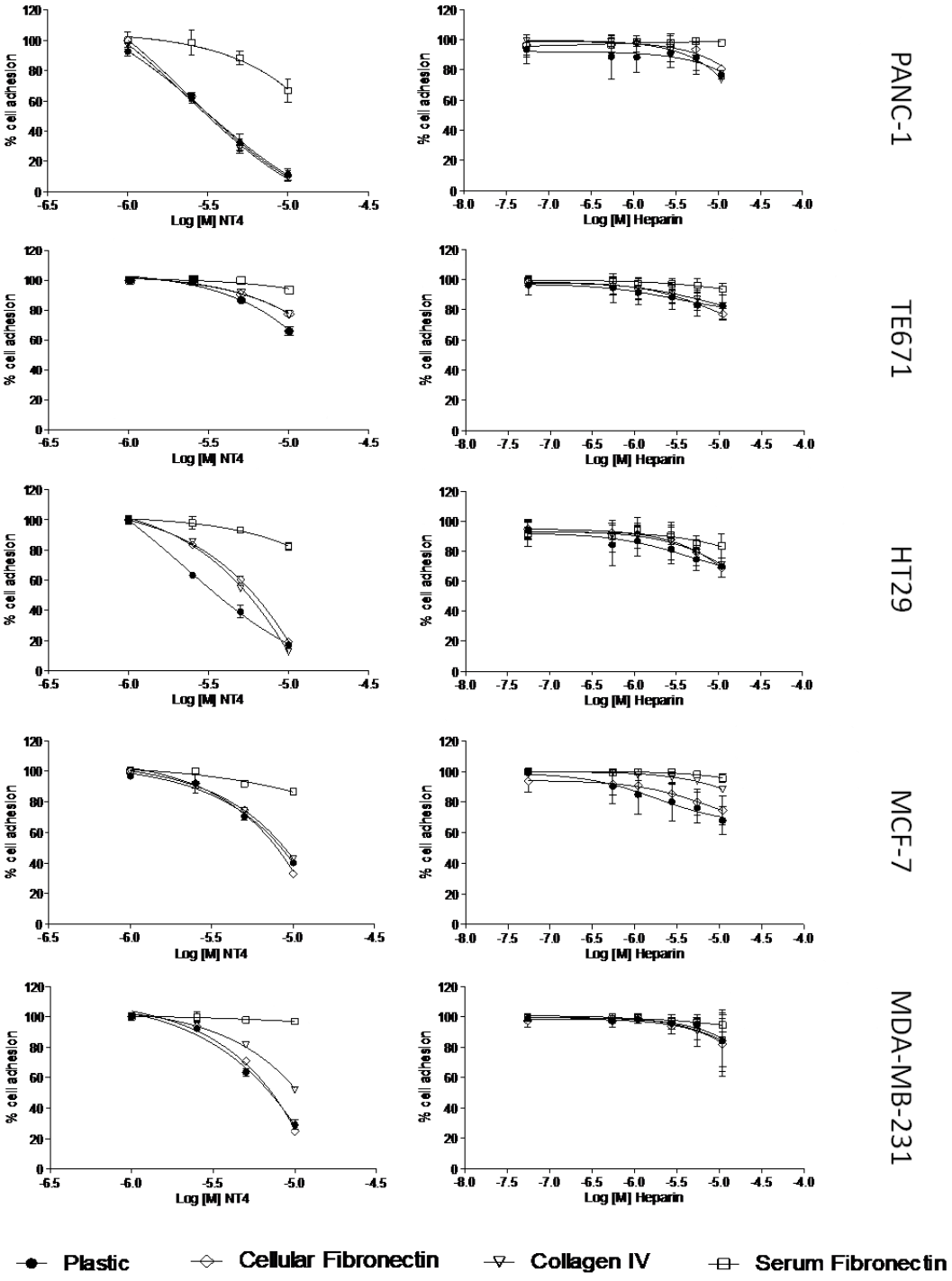


Figure 35. Adhesion of different human cancer cell lines on plastic, cellular fibronectin, plasma fibronectin and collagen IV, in the presence of different molar concentrations of NT4 and heparin.

Adhesion of PANC-1, TE671, HT29, MCF-7 and MDA-MB-231 cells was tested on culture plates coated with different supports including cellular fibronectin, plasma fibronectin and collagen IV or on uncoated plastic wells (Fig. 35). In each of these

cases, the effect induced by the peptide was compared with that produced by heparin. As reported in previous papers, NT4 inhibited adhesion on all media except plasma fibronectin, but the peptide-induced effect appears to be highly variable depending on the cell line under consideration [Depau et al., 2020]. It can inhibit adhesion from as high as 90% in PANC-1 and HT29 cells, down to about 30% in TE671 cells. On the other hand, heparin did not inhibit adhesion of any cancer cell line to all the tested supports.

EFFECT OF NT4 AND HEPARIN ON CANCER CELL MIGRATION

The effect of NT4 and heparin on cancer cell oriented migration were tested in wound healing experiments on collagen-coated well (Fig. 36). Also in this case, different cancer cell lines were analyzed: PANC-1, TE671, MCF-7 and MDA-MB-231 cells. As demonstrated by the results, heparin did not modify oriented migration in any of the tested cells, whereas NT4 induced either inhibition or enhancement of directional migration in different cancer cell lines. Indeed, in PANC-1, MDA-MB-231 and MCF-7 cells, migration was inhibited upon treatment with NT4. On the contrary, in TE671 cells migration resulted increased by the peptide.

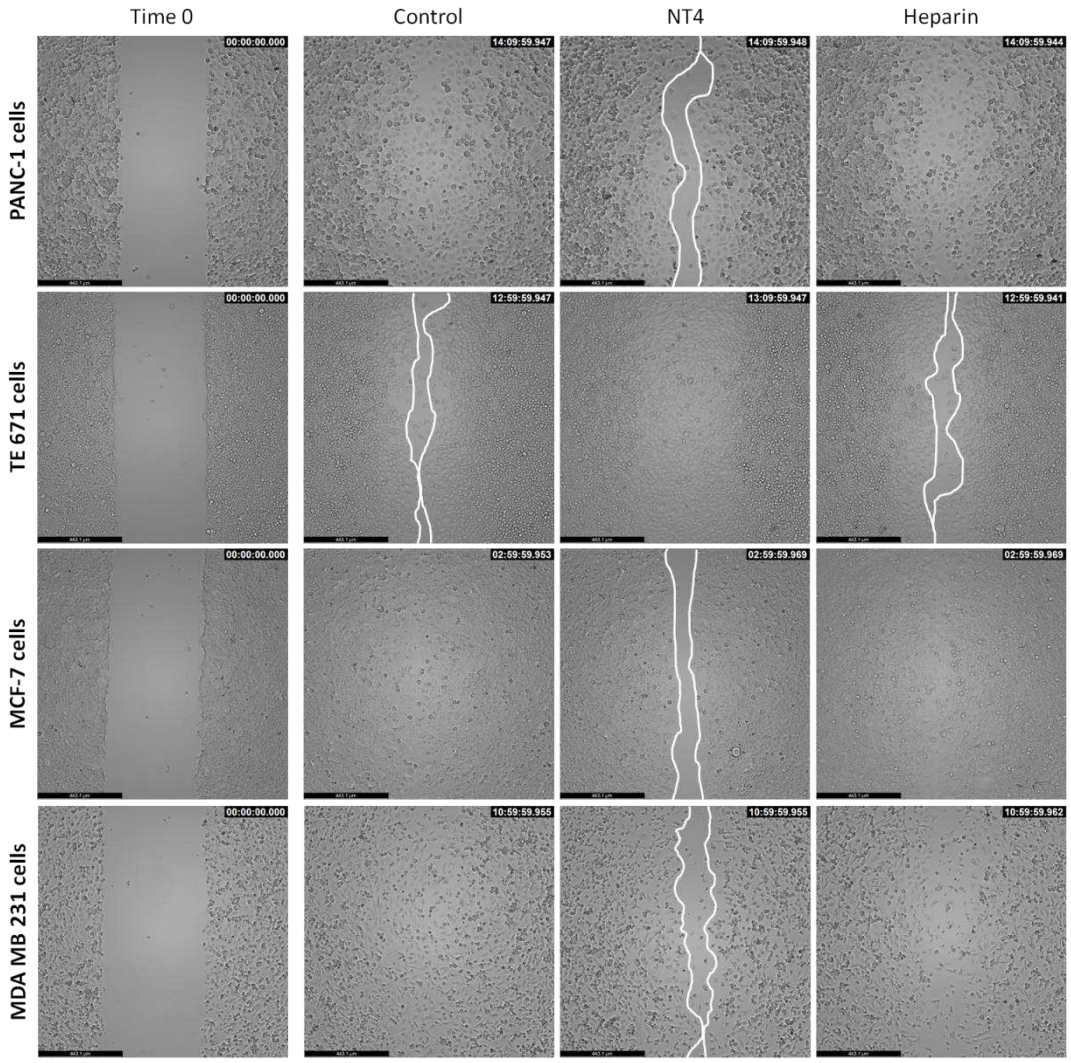


Figure 36. Wound healing assay. PANC-1, TE671, MCF-7 and MDA-MB-231 cancer cells were plated on wells coated with collagen where a silicon spacer had been placed immediately before cell plating. Once cells had reached confluence, the silicon spacer was removed and cells were treated with 10 μM NT4 and 70 μg/ml heparin until the gap is completely closed.

STABLE TRANSFECTION OF PANC-1 AND TE671 CELLS

Previous experiments on PANC-1 and TE671 cells have shown that NT4 can influence, more or less obviously, the organization of actin filaments. In order to visualize the actin cytoskeleton in live cell imaging, both of these cell lines were transfected with a plasmid able to mark actin filaments, named mApple LifeAct7 (Fig. 37).

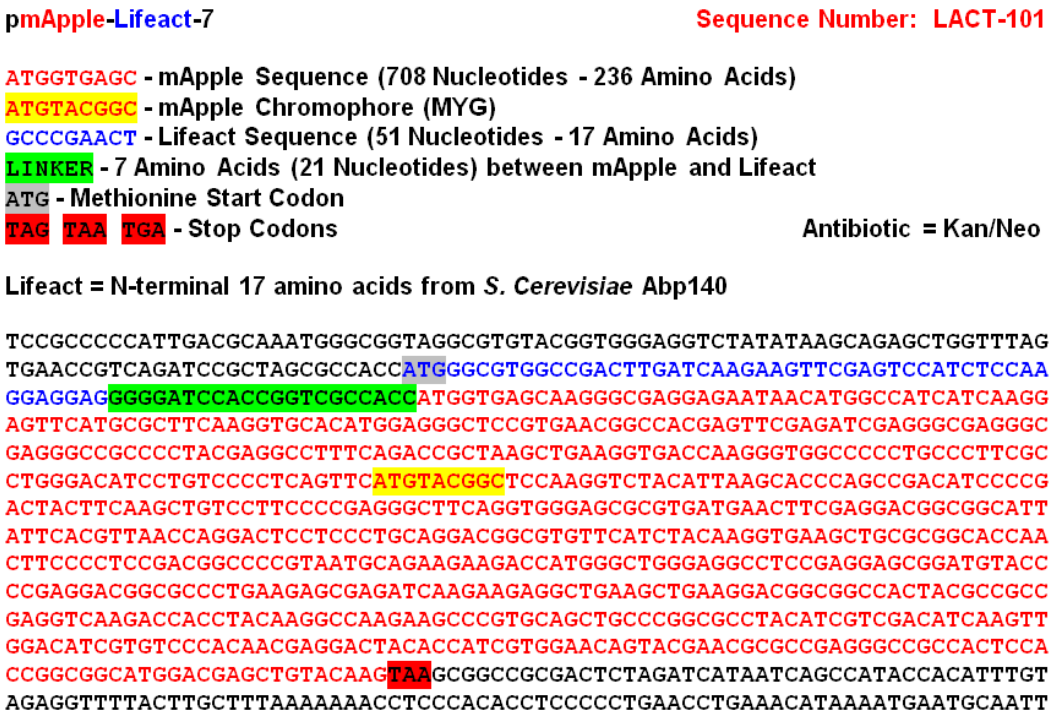


Figure 37. Plasmid mApple Life Act7 sequence [Addgene plasmid # 54747; http://n2t.net/addgene:54747 ; RRID:Addgene_54747].

To obtain a stable transfection, 24 hours after the addition of the plasmid, the antibiotic neomycin was added to the cell culture medium, as it was fundamental for the selection of transformed cells only. The plasmid consists of the mApple and LifeAct sequences interspersed with a 7-amino acid linker sequence. It also contains a chromophore, responsible for fluorescence, and a gene that in bacteria confers resistance to the antibiotic kanamycin, while in eukaryotic cells to neomycin. To proceed with wound healing experiments in fluorescence, it was essential to have cells with at least 70% transfection efficiency and therefore sub-cloning was necessary. Few cells/well (2/3) were grown in 96-well bottom plates U for fluorescence in culture medium supplemented with neomycin until 30-50%

confluence was reached. The individual clones were then observed by confocal microscopy, both in brightfield and in fluorescence, to verify and select clones with higher transfection efficiency, resulting in clone C4 for PANC-1 cells (Fig. 38) and clone F3 for TE671 cells with a transfection efficiency of almost 100% (Fig. 39).

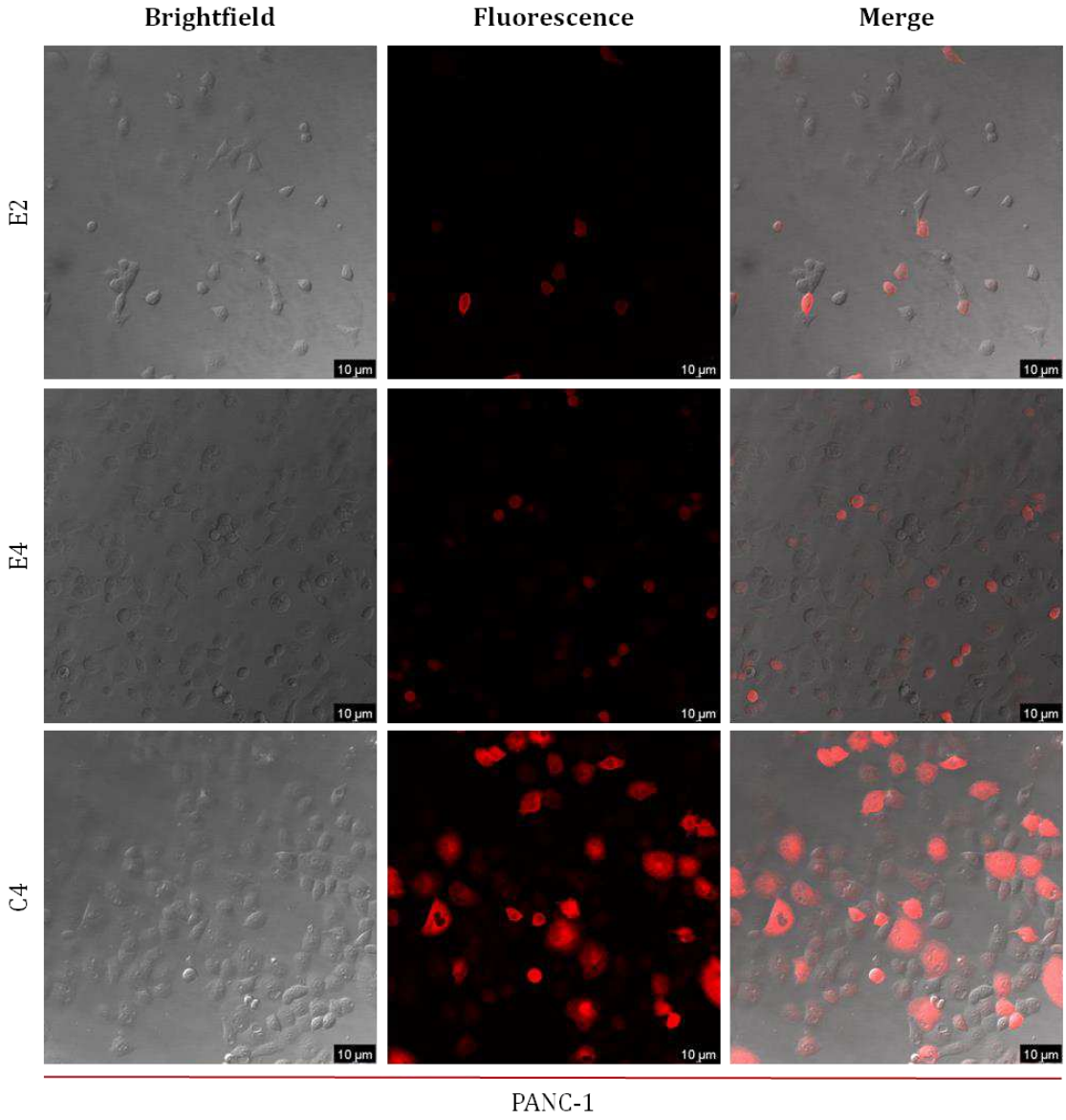


Figure 38. Visualization by fluorescence of successful transfection in 96-well plate into PANC-1 cells. On the left are visible the clones in Brightfield, in the center in fluorescence, on the right a merge of the two images.

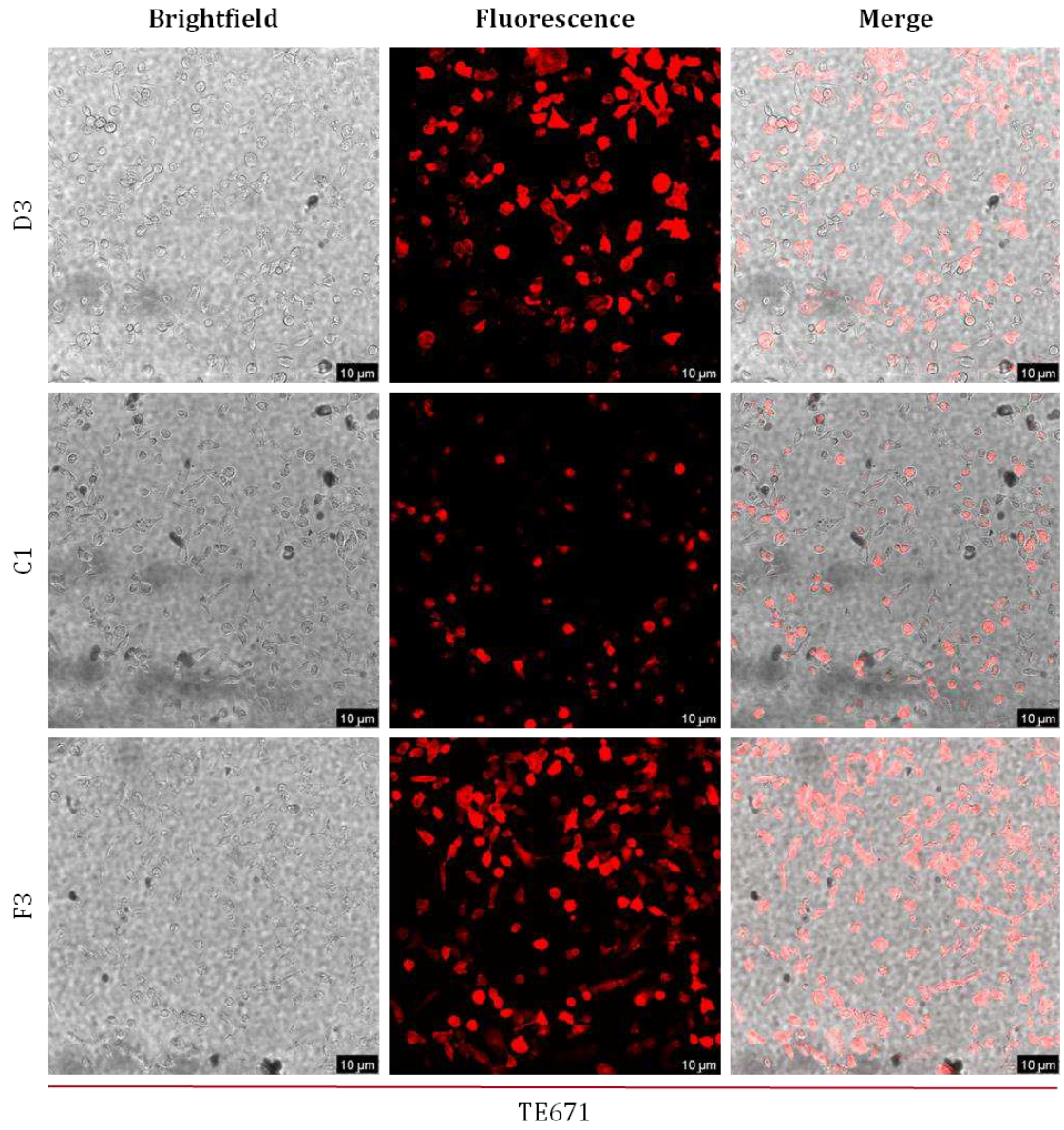


Figure 39. Visualization by fluorescence of successful transfection in 96-well plate into TE671 cells. On the left are visible the clones in Brightfield, in the center in fluorescence, on the right a merge of the two images.

LIVE-CELL IMAGING OF MIGRATING TRANSFECTED PANC-1 CELLS

Using the selected clone of transfected PANC-1 cells, wound healing experiments were performed to observe in live cell imaging the effects produced by NT4 on actin filaments organization and cell migration (Fig. 40).

Initially, observation of live migration was performed using a fluorescence microscope, but in this case the images had low definition, which did not allow to see

in detail actin filaments. Therefore, in order to obtain higher definition images, a confocal microscope was used. The obtained movie showed how the untreated cells had an oriented migration and evident lamellipodia and stress fibers. On the other hand, treatment with NT4 induced a loss of directionality and polarity of cell movement, actin appeared to be organized into filopodia rather than lamellipodia and the cells assumed a "sea urchin" conformation, with protrusions all along the membrane.

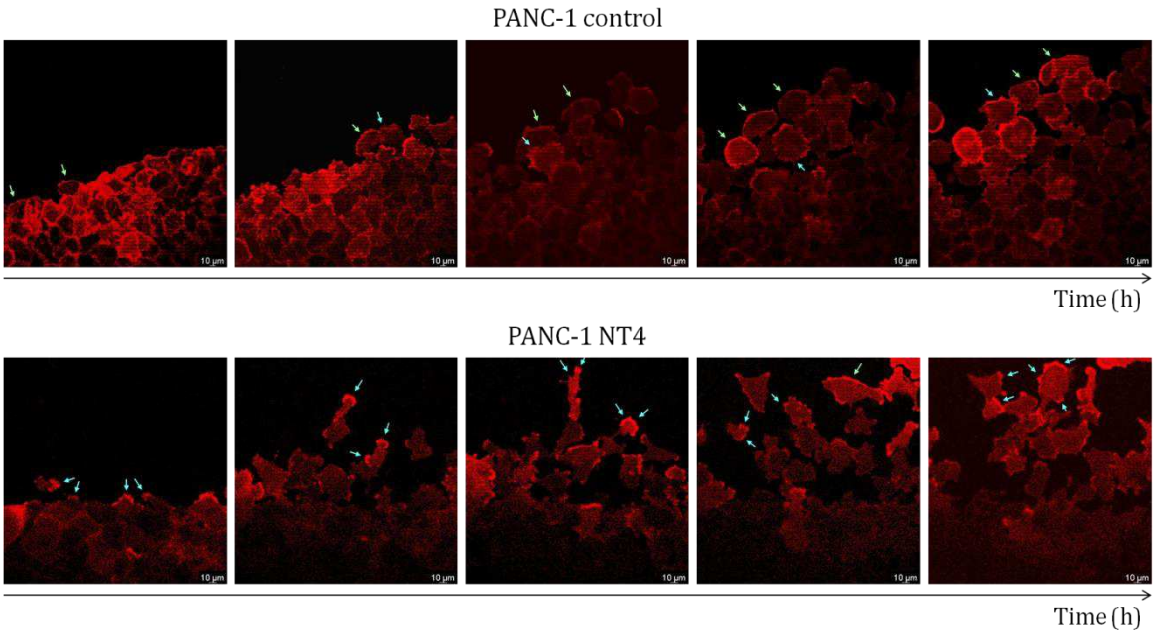


Figure 40. Live-cell imaging of migrating transfected PANC-1 cells in the absence and presence of NT4 (10 µm). Green arrows indicate lamelipodia and blue arrows indicate filopodia.

The procedure was fine-tuned with the transfected PANC-1 cells, arriving at the complete data analysis. Cells from the TE671 line have also been transfected and the analysis of their movies is still in progress.

DISCUSSION

Heparan sulfate proteoglycans are glycoconjugates consisting in a core protein to which one or more GAG chains, mainly represented by heparan sulfate, are covalently attached. The structure of these chains and especially the high variability regarding the amount and position of their sulfated groups give HSPGs the capacity to interact with different heparin binding proteins and consequently allow them to play distinct biological functions. Membrane HSPGs can be divided into two main subfamilies: syndecans, characterized by a transmembrane core with intracellular domains, and glypicans, that interact with the cell membrane via glycosyl–phosphatidyl–inositol (GPI) anchor.

NT4 is a tetra-branched peptide containing four neurotensin sequences bound to a lysine core. This conformation confers some advantages as compared to the monomeric form:

- an increased metabolic stability by preventing proteolytic enzymes from degrading it;
- the possibility to modulate the peptide in order to satisfy several purposes by means of the same biological sequence, without affecting the target binding but simply by coupling different functional units such as tracking molecules or chemotherapeutical drugs;
- a higher selectivity toward cancer cells and tissues due to its multimeric binding to sulfated GAGs of cell surface HSPGs.

To all these features can also be added another one, namely, the ability to interfere, through its binding to HSPGs, with adhesion, migration and invasion of different cancer cells. Indeed, by evaluating the effects produced by the peptide in cell migration, NT4 was found to inhibit migration of PANC-1 cells, which show a lamellipodia-based migration phenotype, and increase migration of TE671 cells, wich show a filopodia-based migration phenotype, suggesting a crucial, but different role of HSPGs [Depau et al., 2020].

To better decode their role, further analyses have been carried out with the aim of adding more information to the gradually emerging picture. In addition to PANC-1, TE671 and HT29 cells, two human breast adenocarcinoma cell lines, MDA-MB-231

and MCF-7 cells, characterized by two distinct migration modes, were also used in this project. The former, as well as PANC-1 and TE671 cells, are able to move as single cells, while the latter migrate collectively.

First, cadherins, specifically N-cadherin and E-cadherin, were examined. These proteins, creating specialized adhesions between cells, are involved in many cellular processes, proving essential for embryonic development and tissue organization in adults. For example, they are involved in EMT, actin reorganization, and intracellular signaling, all functions similar to those exerted by HSPGs. Based on this consideration and given the possibility that cadherins are also implicated in several diseases, the relationship between these two classes of proteins was analyzed using NT4. The results showed in untreated cells important differences related to the expression and distribution of cadherins in all five cell lines. In particular, N-cadherin was expressed in PANC-1 and TE671 cells, where it was predominantly distributed at the points of contact between cells while it appeared to be less expressed in cells at the migration front. In contrast, E-cadherin was found in PANC-1 cells, with a distribution similar to N-cadherin, and in HT29 cells, where it was present at contact points and along the entire membrane in both inner and gap-facing cells. In MCF-7 cells, E-cadherin was expressed in large amounts, a finding consistent with the fact that these cells, as they migrate as a group, keep the bonds between them firm. Finally, MDA-MB-231 cells did not express either N-cadherin or E cadherin. No change was induced by NT4 treatment, suggesting that the regulation of these proteins is independent of HSPGs, which therefore appear to use alternative signaling pathways especially in relation to the regulation of actin dynamics. In addition, to further confirm the linkage that is created between NT4 and HSPGs, a common thread throughout this thesis project, the distribution of HSPGs in migrating cells was evaluated by using both the peptide NT4 and the anti-heparan sulfate 10E4 monoclonal antibody. Both showed a similar distribution in all cell lines analyzed, thus providing an indication of where HSPGs were found. They were present along the membrane of singly moving cells, on the leading cell front in collectively migrating cells, and on the edge exposed on empty space in HT29 cells.

Furthermore, NT4 could interact with Eph receptors, a large family of receptor tyrosine kinases that bind ephrins and influence many cellular functions, such as cell adhesion, migration and cell-to-cell communication. In this regard, the effect the

peptide could induce on their expression and distribution was evaluated. The results obtained showed no effect following NT4 treatment, with the exception of slight changes in the expression of EphA in PANC-1 cells, Eph pA in HT29 cells and Eph pB in HT29 and MDA-MB-231 cells.

NT4 can bind heparin and heparan sulfate as a result of the introduction into the peptide, by means of its branched structure, of a positively charged "heparin binding" motif rich in positive charges that mediates interactions with the negatively charged sulfated GAGs. Therefore, in the presence of heparin, binding of NT4 to cell lines is inhibited by 90%. Starting from the consideration that heparin should compete with cell membrane HSPGs for binding to heparin binding sites on ECM proteins, we wanted to compare the efficacy of directly targeting HSPGs, using NT4, with that of using heparin as a general soluble competitor. In this regard, a series of experiments were done in which the effect of both was evaluated on the colony-forming ability, adhesion and migration of different cell lines. NT4 was shown to be more effective than heparin in inhibit cancer cell adhesion on different ECM protein support. Indeed, although to varying degrees depending on the cell line, the peptide inhibited adhesion on all media except plasma fibronectin, while heparin did not inhibit adhesion of the tested cell line on any support. NT4 was able to inhibit the migration of PANC-1, MCF-7 and MDA-MB-231 cells, while increased that of TE671 cells. Finally, with regard to colony formation, NT4 did not induce any significant change in MCF-7 cells, whereas it inhibited this process in PANC-1 and MDA-MB-231 cells, as well as in HT29 cells, although the number of colonies they manage to form was very low. On the other hand, however, heparin inhibited neither adhesion nor migration in any cell line. It also did not induce significant changes in colony formation; only in MDA-MB-231 it seemed to inhibit this process, but still to a lesser extent than NT4.

Focusing on cell migration, it is characterized by a rapid reorganization of the actin cytoskeleton, depending on the stimuli the cell receives from outside. In fact, actin filaments can give rise to different structures, located in specific areas of the cell and capable of performing essential functions for the cells to move. Previous experiments have shown that actin filaments also undergo changes related to their organization in the presence of the peptide. Specifically, in the presence of NT4, disorganization of actin filaments and stress fibers and an increase in filopodia occurs in PANC-1 cells, while in TE671 cells no particular changes occur, but an increase in the number of

filopodia can be observed. On the basis of these premises, it was decided to transfect these two cell lines with the aim of monitoring in vivo the behavior of actin during the migration process both in the presence and absence of NT4. To do this, it was necessary to transfect the cells with a plasmid capable of marking F-actin by making it fluorescent and consequently visible. Subcloning of the cells was then performed and once confluence was reached, they were observed under a microscope to verify that transfection had occurred. At this point, positive clones were harvested and those of the transfected PANC-1 cells were used for the wound healing experiment, the analysis of which was done first at a fluorescence microscope and then at confocal. There are no images from the fluorescence microscope because at first the laser power was too high and damaged the cells, thus hindering their migration. Then having found the right power to which to expose the cells, images were taken but these had low definition that did not allow the various filaments to be distinguished; so the analysis moved to the confocal microscope. That is, the results obtained confirmed what had been seen previously, namely, an oriented and organized migration, characterized by numerous lamellipodia and an obvious contraction of stress fibers, part of the untreated transfected PANC-1 cells. In contrast, in the presence of NT4 there is disorganization of actin, including that of stress fibers, and a marked increase in protrusions, particularly of filopodia, giving the cells a typical "sea urchin" appearance. The movement does not appear to have a definite direction, but appears to be rather random, and the cells seem to lack a definite "leading edge."

In conclusion NT4 can be considered a promising cancer theranostic agent and can be used to explain the role of heparan sulfate proteoglycans in biological processes such as cell adhesion, migration, EMT and invasion.

By analyzing the effect of the anti-sulfated GAG peptide NT4, on adhesion, migration, and colony formation of different cancer cell lines, we demonstrated the crucial role of HSPGs, and in particular of their sulfated GAG chains, in all these important cancer events. Moreover, by comparing the effect of NT4 with that of heparin on the same events, we could demonstrate that direct targeting of HSPGs is more efficient than using heparin as a soluble competitor for interfering with the many interactions, and the resulting functions, of HSPGs.

The involvement of HSPGs in the development of drugs may be of great interest. However, since we found that NT4, though having a general potential anti-metastatic

effect, in rhabdomyosarcoma cancer cells produced an increase in cancer cell migration, further studies are requested for decoding the role of HSPGs in cancer cells of different origin and with different migration phenotypes. On the basis of our results, any possible anti-metastatic use development of NT4 should in any case be associated with a personalized approach.

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