UNIVERSITAS STUDIORUM INSUBRIAE

PhD in Biotechnology – XXVIII cycle

PhD School in Biological and Medical Sciences

"In vitro studies of the novel protein Q7: role on hyaluronan regulation in breast tumour microenvironment"

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Academic year 2014-2015

L'espressione più eccitante da ascoltare nella scienza, quella che annuncia le più grandi scoperte, non è "Eureka" ma "Che strano...".
(Isaac Asimov)

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Summary

Cancer is a group of multifactorial diseases, which involves variations in multiple genes, often coupled with environmental causes. It is characterized by uncontrolled growth of cells that are able to divide continually and invade into surrounding tissues. In Italy, breast cancer is the most common cancer form among women, accounting for over 20% of the cancer cases and about 15% of the mortality (Jemal et al., 2011). Dysregulation of the composition of the extracellular matrix (ECM) is associated with cancer, by facilitating cell growth, survival and invasion. Among various ECM glycosaminoglycans, hyaluronan (HA) has a remarkable structural importance but also a role in regulating cellular processes through a binding with membrane receptors and activation of signalling pathways. The role of HA in tumour cells' functions depends on its molar mass which is regulated by the enzymes that synthesize HA, i.e. hyaluronan synthases (HAS), and hyaluronidases (HYALs). Alterations of these metabolic enzymes are correlated with breast cancer progression.

In this thesis, we aimed to explore the role of crosstalk between tumour cells and stroma, focusing our attention to the HA regulation. Specifically, we studied the mechanism by which proteins secreted by breast tumour cells alter HA metabolizing enzymes and its synthesis in the stromal cells.

Recently, in our laboratory we discovered a new protein in the conditioned medium (CM) of a breast tumour cell line, called "Uncharacterized protein of c10orf118" or "Q7z3e2". For simplicity, this protein is called "Q7". Further studies on the two well-known breast cancer cell lines MCF-7 (low invasive cells) and MDA-MB231 (high invasive cells) demonstrated a higher expression and secretion of Q7 in tumour cells than in normal cells. Information obtained from bioinformatics databanks (the Ensemble website) showed that the gene for Q7 is located on human chromosome 10 in the region q25.3. The gene is composed of 18 exons and there are six splicing variants, but only four of them code for proteins. The secreted isoform found in the CM of breast cancer cell lines is the full-length isoform that consists of 898 aminoacids and has a molecular weight of 104 kDa.

In the literature and in our data, it was shown that co-culture of breast cancer cells with fibroblasts results to an induction of HAS2 in fibroblasts and an increase of the secreted HA. Among the three HASes, HAS2 isoform was the most expressed and induced by breast tumour cells CM in fibroblasts, whereas HAS1 was not detected. When fibroblasts were treated with CM from MCF-7 cells, in the absence of Q7, the relative expression of

HAS2 was significantly decreased. This last data was further confirmed when fibroblasts were treated with a recombinant protein of Q7 and a HAS2 induction and HA increase were observed.

To sum up, the data of this thesis demonstrate that the novel protein Q7 may play a key role in the increment of pericellular HA and in the breast tumour progression.

Riassunto

Il cancro è una malattia multifattoriale, che comporta variazioni in diversi geni e, sempre più spesso, viene correlato a fattori ambientali. È una patologia caratterizzata da una crescita incontrollata di cellule che sono in grado di dividersi continuamente e invadere i tessuti circostanti. In Italia il tumore al seno è la forma più comune di cancro tra le donne e rappresenta oltre il 20% dei casi di tumore e circa il 15% della mortalità (Jemal et al., 2011). L'alterata composizione della matrice extracellulare è associata al cancro in quanto ne facilita la crescita cellulare, la sopravvivenza e l'invasione. Tra i vari glicosaminoglicani della matrice, l'acido ialuronico (HA) è noto per le sue proprietà strutturali, inoltre riveste un ruolo fondamentale nella regolazione dei processi cellulari attraverso legami con alcuni recettori di membrana, i quali permettono di attivare differenti vie di segnalazione. Il ruolo funzionale dell'HA nelle cellule tumorali dipende dalla sua massa molecolare. Quest'ultima, infatti, è regolata dagli enzimi che lo sintetizzano, ovvero le sintasi dell'acido ialuronico (HAS) e dalle ialuronidasi che invece lo degradano (HYAL). Il disequilibrio fra questi enzimi metabolici sono correlati con la progressione del tumore al seno.

In questa tesi, abbiamo voluto studiare il ruolo del crosstalk tra le cellule tumorali e lo stroma circostante, concentrando la nostra attenzione sulla regolazione dell'HA. In particolare, abbiamo valutato il meccanismo con cui le proteine secrete dalle cellule tumorali della mammella sono in grado di alterare gli enzimi responsabili del metabolismo dell'HA e la sua sintesi nelle cellule stromali.

Recentemente, nel nostro laboratorio è stata individuata, nel terreno condizionato di una linea di cellule tumorali della mammella, una nuova proteina chiamata "Uncharacterized protein of c10orf118" o "Q7z3e2". Per semplicità, questa proteina verrà chiamata "Q7". Ulteriori studi su due linee cellulari ben caratterizzate di tumore della mammella, come MCF-7 (cellule a bassa invasività) e MDA-MB231 (cellule ad alta invasività), hanno dimostrato una più alta espressione e secrezione della proteina Q7 nelle cellule tumorali rispetto a quelle normali. Informazioni ottenute da banche dati bioinformatiche (es. Ensemble), hanno mostrato che il gene che codifica per la proteina Q7 è localizzato sul cromosoma umano 10 nella regione q25.3. Nello specifico, il gene è composto da 18 esoni e 6 varianti di splicing, di cui solo quattro codificano per la proteina. L'isoforma secreta, trovata nel terreno condizionato da linee cellulari di tumore della mammella, è quella "full-length" formata da 898 aminoacidi e con un peso molecolare di 104 kDa.

Ulteriori dati ottenuti nel nostro laboratorio, e confermati da studi presenti in letteratura, hanno dimostrato che la co-coltura di cellule tumorali della mammella con fibroblasti provocano un'induzione della HAS2 nei fibroblasti ed un aumento dell'HA secreto nello stroma circostante. Fra le tre sintasi dell'HA, infatti, l'isoforma HAS2 era quella maggiormente espressa nei fibroblasti dopo trattamento con il terreno condizionato proveniente dalle cellule tumorali della mammella, mentre l'espressione di HAS1 non è stata rilevata. Quando i fibroblasti sono stati trattati con terreno condizionato proveniente dalle cellule MCF-7, privato della proteina Q7, abbiamo visto una significativa diminuzione dell'espressione relativa di HAS2. Quest'ultimo risultato è stato ulteriormente confermato quando i fibroblasti sono stati trattati con una porzione di proteina Q7 ricombinante. È stato osservato, infatti, un incremento sia della sintesi di HAS2 che dell'HA secreto. In conclusione, i risultati ottenuti dimostrano come la proteina Q7, recentemente identificata, possa avere un ruolo chiave nell'aumento dell'HA nello stroma circostante il tumore favorendone così la progressione.

Abbreviations

CAF	Cancer-associated fibroblast
CM	Conditioned media
CS	Chondroitin sulphate
DCIS	Ductal carcinoma in situ
DS	Dermatan sulphate
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
EST	Expressed sequence tag
GAG	Glycosaminoglycan
GlcNAc	N-acetyl-D-glucosamine
GlcUA	D-glucuronic acid
HA	Hyaluronan
HABP	Hyaluronan binding protein
HAS	Hyaluronan synthase
HMW-HA	High molecular weight of hyaluronan
HS	Heparan sulphate
HYAL	Hyaluronidase
IDC	Invasive ductal carcinoma
KS	Keratin sulphate
LMW-HA	Low molecular weight of hyaluronan
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor-1
MW	Molecular weight
NHDF	Normal human dermal fibroblast
oHA	Oligosaccharide of hyaluronan
ORF	Open reading frame
PDGF	Platelet-derived growth factor
Q7	Uncharacterized protein of c10orf118 or with accession number Q7z3e2
RHAMM	Receptor for hyaluronan mediated motility
SASP	Senescence activated secretory pathway
TGF-β	Transforming growth factor beta
TLR-2	Toll-like receptor 2
TLR-4	Toll-like receptor 4
TPM	Transcript per million
TSG	Tumour suppressor gene
UDP	Uridine diphosphate
VHMW-HA	Very high molecular weight of hyaluronan

Introduction

Cancer

Cancer is a group of multifactorial disease that involves variations in multiple genes, often coupled with environmental causes and is characterized by uncontrolled growth of cells that are able to divide continually and invade into surrounding tissues. In Italy, such in most industrialised countries, cancer is the second common cause of death for people aged 25-64 years, after cardiovascular diseases (Figure 1). Among gender-specific cancers, prostate cancer ranked tenth in men (7,282 deaths) and breast cancer ranked seventh in women (12,004), being the most common cause of female cancer deaths (Istat, 3 December 2014).

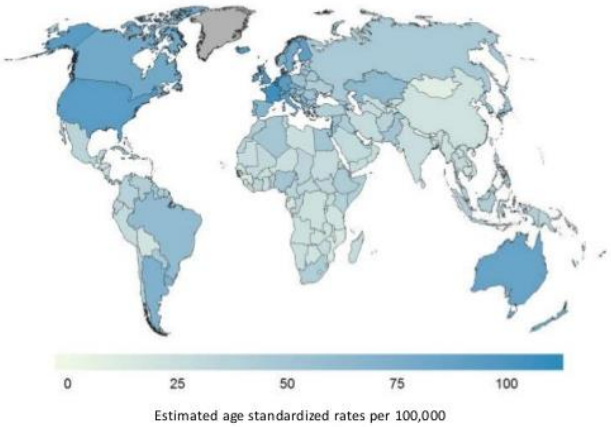


Figure 1 - Estimated Breast Cancer Incidence Worldwide WHO International Agency for Research on Cancer GLOBOCAN 2012 Estimated age standardized rates per 100,000 (from GLOBOCAN).

A striking characteristic of tumour cells is the capability to invade and damage normal tissue and consequently migrate at distant sites. These cells travel through the blood or lymph system and form secondary tumors, a procedure known as metastases (Fidler, 2002, 2003). According to the tissue origin, tumours can be classified into the three major groups of carcinomas, sarcomas and leukemias. Carcinomas, which include approximately 90% of human cancers, are solid tumors that arise of epithelial cells. Breast cancer is the most common form of carcinoma, accounting for over 20% of the cancer cases in women and about 15% of the mortality (Jemal et al., 2011). The discovery of molecular trait that leads to tumorigenesis has been defined by Hanahan and Weinberg in their review called “the Hallmarks of Cancer” (Hanahan and Weinberg,

2000). These hallmarks describe the different capabilities of tumour cells to sustain proliferative signalling, evade growth suppressor, enable replicative immortality and resist cell death (Figure 2). Moreover, solid carcinoma cells are able to induce angiogenesis and activate invasion and metastasis.

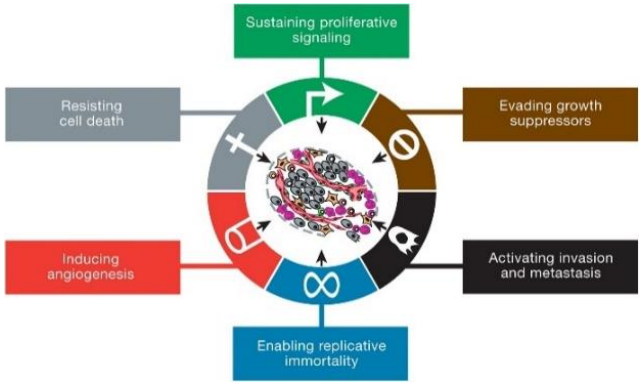


Figure 2 - The Hallmarks of Cancer
This illustration encompasses the six hallmark capabilities originally proposed (Hanahan and Weinberg, 2011).

Recently, other two new hallmarks were included. One of these regards the reprogramming energy metabolism whereas the other involves the role that the immune system plays in resisting or eradicating formation and progression of tumours (Hanahan and Weinberg, 2011) (Figure 3).

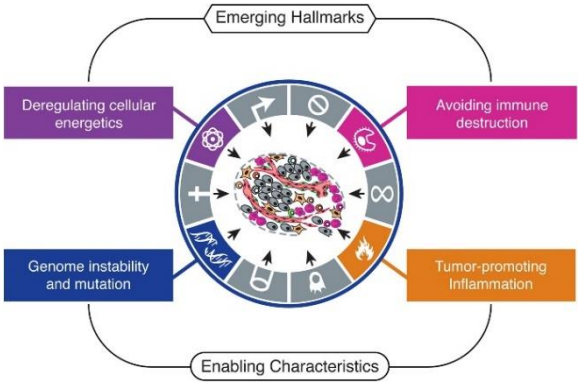


Figure 3 - **Emerging Hallmarks and Enabling Characteristics**
An increasing body of research suggests that two additional hallmarks of cancer are involved in the pathogenesis of some and perhaps all cancers. One involves the capability to modify, or reprogram, cellular metabolism in order to most effectively support neoplastic proliferation. The second allows cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells (Hanahan and Weinberg, 2011).

The extracellular matrix

The construction of multicellular organism from a single cell requires vast amounts of information to be transferred between different cells and organ systems. Peptide and non-peptide hormones carry signals between different organ systems, whereas polypeptide growth factors are molecules that are specialized for the intercellular communication. Originally, these growth factors were described as soluble molecules, although recent evidence suggest that growth factors are able to ligand receptor and regulate different mechanisms into the extracellular matrix (ECM)**.** The ECM provides a structural basis for multicellularity, whereas growth factors make it possible to transfer the information required for the construction of complex cellular structures (Taipale and Keski-Oja, 1997). In the past, ECM was classified as a cellular material visible in the electron microscope (Figure 4).

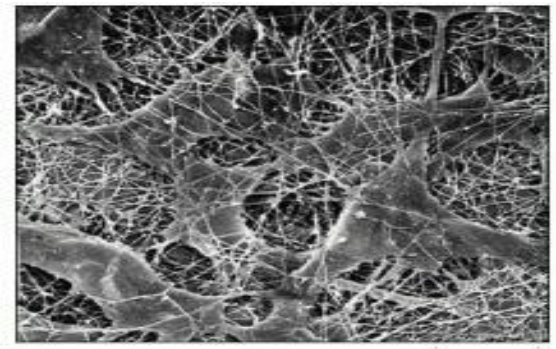


Figure 4 - Scanning electron micrograph of cells embedded in a fibrous ECM (From T. Nishida et al., Invest. Ophthalmol. Vis. Sci. 29:1887–1890, 1988. © Association for Research in Vision and Opthalmology).

Now it is defined more widely because it is well known that ECM includes secreted molecules that are immobilized outside cells, even if they lack organization that is detectable in the microscope. Major constituents identified initially in the ECM included collagens, noncollagenous glycoproteins and proteoglycans (Reichardt and Tomaselli, 1991).

In many organs, the principal components of the ECM are collagens. These are secreted mostly by fibroblasts, but also by a variety of stromal cells. The presence of collagens provides much of the scaffold necessary for the organization of cells that constitute the tissue. Another important class of molecules that play an essential role in the

composition of the ECM is the proteoglycans whose protein core is covalently bound to glycosaminoglycans (GAGs).

ECM plays a key role in tissue organization and homeostasis and is by definition nature's ideal biologic scaffold material (Badylak, 2007) for adhesion and migration (Aumailley and Gayraud, 1998). This matrix, however, is not only a scaffold for the cells of a given tissue. As the ability to bind secreted molecules, ECM serves also as a reservoir for growth factors and cytokines and modulates their activation status and turnover (Kresse and Schonherr, 2001). Dysfunctions and altered composition of ECM are correlated with different pathologies, such as inflammation and cancer (Lu et al., 2012).
Among matrix molecules, an important group with a variety of important biological functions is the GAGs. These are linear heteropolysaccharides composed of repeating disaccharide units, generally of an acetylated amino sugar alternating with a uronic acid. The high negative charge (linear anionic polysaccharide) is important for the molecular function because they are able to attract and bind a lot of water molecules (Perrimon and Bernfieldb, 2001), rendering the tissue hydrated and lubricated. GAGs can be divided into different categories: chondroitin sulphate (Cs), dermatan sulphate (Ds), heparan sulphate (Hs) and heparin, keratin sulphate (Ks) and hyaluronan (HA) (Rozario and DeSimone, 2010). HA is a typically not sulphated or covalently bound to a protein core (Stamenkovic, 2003) member of the GAG family and do not form proteoglycans, although several proteins can interact with it forming the hyaladherins.

Hyaluronan

HA was first isolated from the viscous vitreous humor of the eye in 1934 by Meyer and Palmer. They reported that it was composed of "a uronic acid, an amino sugar, and possibly a pentose (the last is incorrect) and proposed "for convenience, the name hyaluronic acid, from hyaloids (vitreous) + uronic acid" (Meyer and Palmer, 1934).

Twenty years after the initial discovery of HA, Meyer's laboratory determined the exact chemical structure of HA, a nonsulfated, high molecular-weight glycosaminoglycan composed of repeating polymeric disaccharides D-glucuronic acid and *N*-acetyl-D-glucosamine linked by a glucuronidic β (1→3) bond. The disaccharide units are then linearly polymerized by hexosaminidic β (1→4) linkages (Figure 6Figure 5). The number of repeat disaccharides in a completed HA molecule can reach 10,000 or more, with a molecular weight ranges from 10^4 - 10^7 kDa (Fraser et al., 1997; Jiang et al., 2007). HA is present in all vertebrates but has also been identified in some of the lower marine organism and in certain bacteria. It is a ubiquitous component of all connective tissues

and skin in mammals (Fraser et al., 1997) and is more abundant in remodeling tissues in processes like embryonic development. HA is also found in locations which are not connective tissues, such as in the neural nerve system (Delpech et al., 1997).

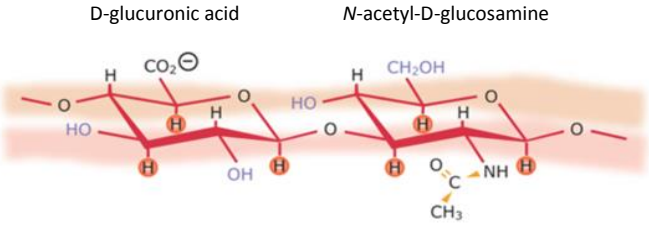


Figure 5 - Structure
Repeating disaccharide units of HA.

Hyaluronan biosynthesis & regulation synthases

Three different, but related enzymes are involved in HA synthesis. The synthesis of HA does not take place in the Golgi, as do all other GAGs, but occurs instead on the cytoplasmic surface of a complex within the plasma membrane. This family of enzymes are called HA synthases (HAS). HAS are glycosyl transferases that occur in vertebrates, bacteria, and algal viruses (Stern et al., 2006). HAS isoforms (HAS1, HAS2 and HAS3) are predicted plasma-membrane proteins with molecular masses from 42 to 64 kDa. Each protein is expected to span the plasma membrane several times, with the sequences of catalytic activity on the inner face of the membrane. The vertebrate HAS proteins share

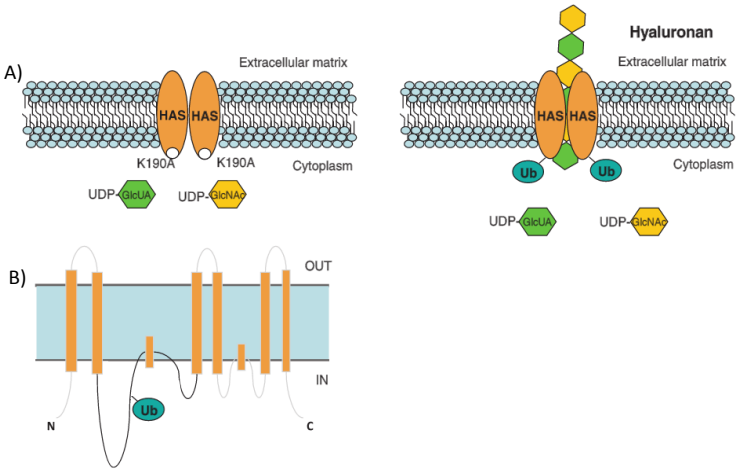


Figure 6 - A predicted structure of mammalian HAS. Schematic representation of hyaluronan synthase (HAS) orientation in the plasma membrane and its ubiquitin (Ub) modification. Dimerization occurs with or without ubiquitination (A), but the synthesis of hyaluronan requires monoubiquitination of K190. This lysine residue is located in the predicted glycosyltransferase activity domain of HAS (B). (Modified from Tammi et al., 2011).

55±71 % sequence identity whereas homologous isoforms of human and mouse share about 96±99% sequence identity (Jacobson et al., 2000). In mammals, the expression of the HAS genes also appears to be tissue and cell-specific. Even though all HAS isoforms catalyze the same reaction, they differ in the size of their reaction products. They have distinct expression patterns under the control of a wide variety of cytokines and growth factors. The changes in HA synthesis can be related to HAS mRNA expression, to the availability of the UDP-sugar precursors or to modulation by phosphorylation of HAS in response to cytokines and growth factors (Vigetti et al., 2009) (Tammi et al., 2011). The HA polymer is transported out of the cell using a pore-like formed by HAS2 dimer during its synthesize (Karousou et al., 2010) (Weigel, 2015) (Figure 6).

Hyaluronidases

The enzymes responsible for HA catabolism are the hyaluronidases (HYAL) (Stern, 2003). The HYAL enzymes hydrolyze mainly the hexosaminidic β(1→4) linkages between N-acetyl-D-glucosamine and D-glucuronic acid residues in HA. These enzymes also hydrolyze β(1→4) glycosidic linkages between N-acetyl-galactosamine or N-acetylgalactosamine sulfate and glucuronic acid in other GAGs, such as CS, CS-4, CS-6 and DS. (Jiang et al., 2007).

From the expressed sequence tag (EST) database, it was established that there are six sequences in the human genome. In the human, three genes (HYAL1, HYAL2, and HYAL3) are found tightly clustered on chromosome 3p21.3, coding for hyaluronidase 1, 2 and 3. Another three genes HYAL4, PHYAL1 (a pseudogene) and SPAM1 (Sperm Adhesion Molecule 1) are clustered in a similar fashion on chromosome 7q31.3. They code, respectively, for hyaluronidase 4, transcribed but not translated in the human, and PH-20. The HA is degraded when they reach their destination at the various sites, thus presumably depriving such cells of continued motility. HYALs are obviously critically important in embryology for the removal of the HA in the conversion from the morphogenetic and proliferative stages to the differentiating stages during development. The identities of the HYALs in embryology are unknown. However, it has been established that Hyal-2 is expressed in early development, whereas Hyal-1 is not (Stern, 2003). Intriguingly, both of these loci occur at sites of putative tumour suppressor genes (TSGs) (Stern, 2008).

Turnover

HA is the predominant component in the ECM of skin, which contains about 50% of total body of HA. Elevated levels of HA are synthesized during tissue repair (Papakonstantinou et al., 2012). HA has a rapid turnover in the body that may reach a degradation up to 30%/day. A major part of the circulating HA is taken up by the liver and a minor part by the kidneys (Laurent and Fraser, 1992). In joints 20-30% of HA is catabolized by local degradation. The lymphatic tissue carries HA to the blood stream where 80-90% in degraded by receptor mediated catabolism (Fraser et al., 1997).

Hyaluronan receptors

HA interacts with a variety of hyaladherins also known as HA binding proteins (HABPs). These include receptors such as CD44, RHAMM (receptor for hyaluronan mediated motility expressed protein), TLR4-2 (toll-like receptors) and LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1). Some hyaladherins are associated with cell membranes, whereas others are found in the ECM.CD44 is the major cell-surface HABP. It is a polymorphic type I transmembrane glycoprotein (Figure 7). It is expressed by cells mostly as a standard isoform, which is an 85 kDa, protein that undergoes posttranslational modification. Most cells, including stromal cells such as fibroblasts and smooth muscle cells, epithelial cells, and immune cells such as neutrophils, macrophages, and lymphocytes, express CD44 (Sherman et al., 1994). HA-CD44 interactions may play an important role in development, inflammation and tumour progression (Jiang et al., 2007)

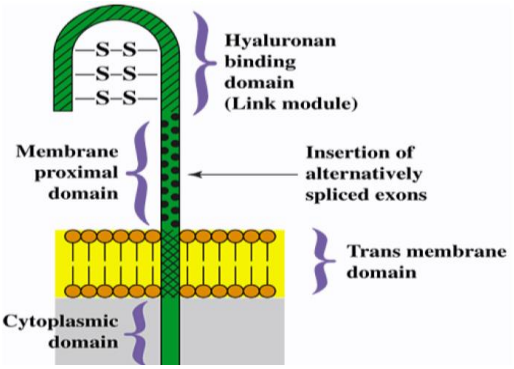


Figure 7 - Model for the structure of CD44, showing the protein domains for the standard isoform of CD44. The products of various combinations of ~10 alternatively spliced variant exons are inserted at the position indicated by the arrow, giving rise to numerous variant isoforms of CD44. The extracellular domains of CD44 are highly, but variably, glycosylated, and several serine residues in the cytoplasmic domain can be phosphorylated (From Glycoforum. All Rights Reserved).

Hyaluronan function

HA exist in a variety of sizes that have different properties. Among matrix molecules, the largest is the very-high-molecular-weight HA (VHMW-HA). The high-molecular-weight HA (HMW-HA) has an extracellular localization, is distributed in free space and has various regulatory and structural functions. It can reach a length up to 10^5 saccharides ($2x10^4$ kDa), depending on tissue (Figure 8) and the physiological conditions (Monslow et al., 2015). The polymers are able to incorporate a very large volume of water and for this reason, they have peculiar physical and mechanical properties.

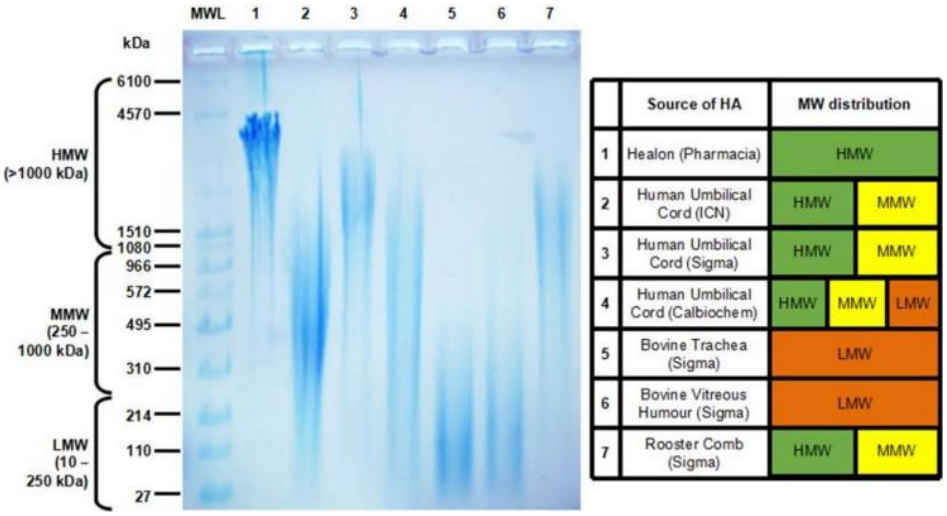
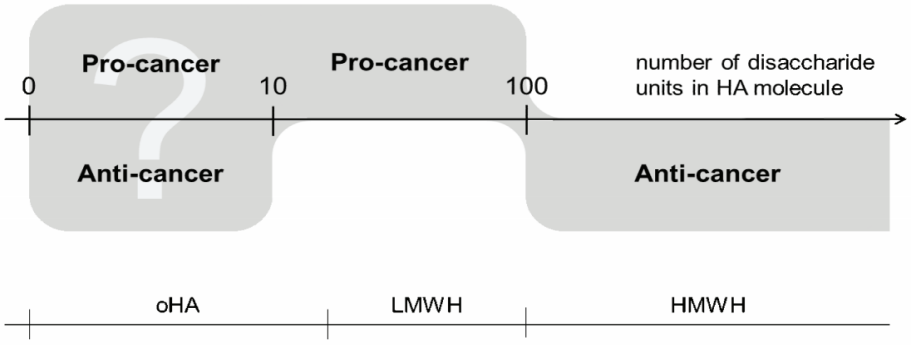


Figure 8 - Agarose gel electrophoresis showing the molecular weight (MW) distribution of commercially available HA (left) and charted for comparison (right). Molecular weight HA ladder (MWL) was purchased from Hyalose (combined mega, high, and low ladders). HA MW is divided into high (HMW >1000 kDa), medium (MMW, 250-1000 kDa), and low (10-250 kDa). (From Monslow et al., 2015 - Copyright © 2015 Monslow, Govindaraju and Puré.)

Because of its hygroscopic nature, HMW-HA is very important to organize the ECM, including tissue remodelling, hydration and molecular sieving. Moreover, it is a very important biopolymer in many pathologic situations, such as in shock and blood loss, because HMW-HA polymers increase in circulatory system and extend the intravascular volume. HA with low molecular weight (LMW-HA) is able to stimulate angiogenic, inflammatory, and immunostimulatory response.

A)



B)

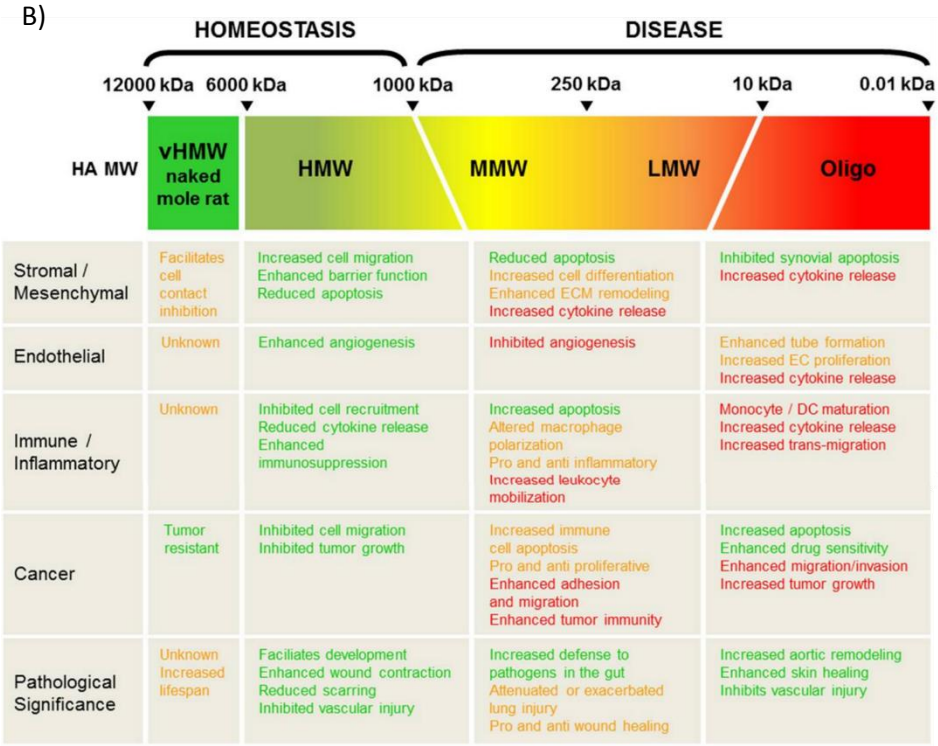


Figure 9 - A)The overall hypothesis for pro- and anti-cancer activity of various length hyaluronan molecules (from Karbownik and Nowak, 2013. **B)** Summary and pathological significance of HA size *in vivo*. HA molecular weight (MW) is divided into very high (vHMW 6000-12000 kDa; naked mole rat HA), high (HMW > 1000 kDa), medium (MMW 250-1000 kDa), low (10-250 kDa), and Oligo-HA (< 10 kDa). Green text highlights positive roles for each HA MW in tissue function and recovery, whereas red text favors pathological decline. Opposing and/or unclear tissue responses are designated in orange-colored text. (From Monslow et al., 2015 - Copyright © 2015 Monslow, Govindaraju and Puré).

These short polymers are usually involved in alarm system, generating different ‘‘danger signals’’. Another important class, which has different biological functions, is the oligosaccharides of HA (oHA) with MW up to a few kDa. The function of HA is strictly related to polymer length and mass. Therefore, HMW-HA, in general, correlates with tissue integrity and quiescence, while fragmented HA products are in presence of stress signal. These fragments may be truncated products of the synthetic reaction, but may also be the result of HYAL activities (Stern, 2008). In particular, oHA represent a very interesting class of macromolecules that may show equivocal properties. They can be involved in different pathological processes that can promote tumour cells adhesion, angiogenesis and metastatic potential or inhibit cell growth evoking apoptosis, as shown in Figure 9 (Karbownik and Nowak, 2013).

Hyaluronan in crosstalk tumours-stroma interaction

It is well known that cancer progression is not determined only by tumour cells, but also by the tumour microenvironment. This compartment includes ECM components, stromal cells as fibroblast, endothelial, mesothelial and pro-inflammatory cells. Therefore, cancer cells create a “cross-talk” with surrounding cells and ECM through cell-to-cell contacts and paracrine/endocrine signals using soluble factors affecting cancer cell behaviour. In fact, HA in the pericellular matrix interacts with membrane receptors of the surrounding cells and activates intracellular signallings that alternate different cellular functions such as cell migration, growth and differentiation. During tumour formation, the stroma becomes “activated” and induces an increase of number of fibroblasts, and a production of new blood capillary (Kalluri and Zeisberg, 2006). Additionally, it was demonstrated that the signal generated by stromal cells are used by tumour cells to invade the surrounding tissue (Hanahan and Weinberg, 2011). To date, several studies have demonstrated that ECM and fibroblasts play a key role in tumour progression.

In the last decade, research efforts have focused on the importance of crosstalk between cancer cells and their surrounding stroma. In clinical and experimental studies, it was demonstrated that the interaction of two different compartments, epithelial tumour cells and stroma, are able to support tumour progression by permitting angiogenesis and simplifying the invasion and metastasis process of tumour cells. In particular, several studies have reported a relation between invasiveness and an increased amount of HA in the stroma that surrounds tumours then in parenchymal regions. Studies on patients with cancer have demonstrated that HA concentration in ECM is usually higher in malignant tumours than in corresponding benign or normal

tissues. For example, in patients with breast and ovarian cancer, the aberrant amount of HA in the stroma is predictive of malignancy and aggressiveness and often it is associated with low survival rates (Toole, 2004).

The communication among cells occurs by secretion of soluble factors, such as growth factors (Mantovani et al., 2006). Different studies have demonstrated that overexpression of PDGF and TGF-β in tumour cells was often correlated with an overexpression of HAS2, rendering these growth factors as good candidates for the increased amount of HA in stromal cells (Porsch et al., 2013); (Mueller and Fusenig, 2004). Importantly, these growth factors released by tumour cells are able to activate stromal cells that in turn are stimulated to produce specific soluble factors used to modulate the ECM. In addition, by interaction with its specific receptor CD44, HA is capable of intracellular signal transduction that can promote the malignant phenotype (Udabage et al., 2005). CD44 seems to be essential in the initial phase of malignant transformation. It has been identified at the surface of cancer stem cells that are a small population of cancer cells responsible for maintaining the tumour and the progression of tumours in metastatic areas. This finding suggests that CD44 may be a potential diagnostic target for early cancer detection, contributing to early initiation of anti-cancer therapy and its better outcomes (Karbownik and Nowak, 2013).

Currently, there are different mechanisms suggested about the stimulatory effects of HA in crosstalk between tumour-stroma.
HA seems to affect tumour cell behaviour and cancer progression by regulating the hydration and osmotic balance in the tumour environment. This molecule is extruded in the ECM and it is involved in cell motility, even if in normal cells, decrease the cell-cell interaction and intracellular communication (Toole and Hascall, 2002). *In vitro* studies have demonstrated that HA is an important molecule for anchorage-independent growth because high levels of HA are crucial for tissue organization and promote cell anchorage-independent cell proliferation (Laurent et al., 1996). During tumour proliferation, cancer cells prefer to use anaerobic glycolysis than an oxidative phosphorylation. This mechanism is described as Warburg effect. The lactate produced by tumour cells during their anaerobic glycolysis is used by themselves to stimulate stromal cells to increase the amount of HA in the surrounding area. In turn, the increased amount of HA facilitates the formation and proliferation of new cells. (Stern et al., 2002).

The recruitment of fibroblast by neoplastic cells occurs through various growth factors and cytokines. Several findings underline the role of CAF in tumour growth. For instance, the correlation of “cancer-associated fibroblasts” (CAFs) cells with the metastatic

process of cancer cells is mainly due to the induction of epithelial-mesenchymal transition (EMT) (Yu et al., 2014). In EMT process, epithelial cells acquire fibroblast-like properties, exhibit reduced cell-cell adhesion and increased motility as mesenchymal cells. Also, in this case, TGF-β induces an overproduction of HA in mammary carcinoma that in turn results in the suppression of E-cadherin (a hallmark of EMT) expression and the nuclear translocation of β-catenin. All of this suggests that increase amount of HA is sufficient to promote EMT, which facilitates the escape of tumour cells from primary tumours (Kalluri and Zeisberg, 2006; Zeisberg et al., 2007).

Aim of PhD project

Breast cancer is one of the leading causes of cancer related deaths among women and alterations of ECM have a central importance in basic cancer biology and therapy. HA, a component of cell microenvironment, has critical functions in modulating tumorigenesis and metastasis.

In this thesis, we focused our attention on the crosstalk interactions between tumour cells and stroma compartment and we found a new uncharacterized protein, called Q7, secreted in medium of breast cancer cell lines and in breast tumour samples. Specifically, there were two subjects to explore:

- firstly, characterization of Q7 protein;
- secondly, investigation of the role of Q7 protein in the crosstalk between tumour and surrounding stroma favouring HA synthesis.

Materials & Methods

Cells and culture conditions

For the experiments, we used the neoplastic cell lines as MCF-7 and MDA-MB231 kindly provided by Dr. Martin Götte (University of Münster, Germany). As nontumoral cells control we used the Normal Human Dermal Fibroblast (NHDF) obtained by Lonza. All cells were grown in RPMI1640 with stable L-Glutamine culture medium (ECM2001L; Euroclone) supplemented with 10% fetal bovine serum (FBS) EU Approved (ECS0180D; Euroclone) and 100U/ml penicillin + 100µg/ml streptomycin (ECB3001D; Euroclone) in an atmosphere of humidified 95% air, 5% CO_2 at 37 °C in tissue culture T 75-cm^2 flasks.

Transwell system

A Transwell system with a porous (0.4 µm pore size) polycarbonate membrane filter (Costar, Corning Incorporated) and 12-well plastic tissue culture plates were used for the NHDF-Tumour cell co-cultures. NHDF cells were first seeded into 12-well culture plates at a subconfluent density of 4×10^4 cells/well. Then, different types of tumour cells (1×10^4/well) were added to the upper chambers. The resultant three groups were as follows: 1) NHDF - NHDF control group, 2) NHDF - MCF-7 group, and 3) NHDF - MDA-MB231 group.

Cell treatment with CM of MCF-7 cells

MCF-7 cells were plated in T25 flask at a density of $2x10^6$ cells/flask. At about 70% confluence in a T-25, the culture medium was removed and incubated with fresh complete medium for 48h. For abrogation the effect of Q7 protein, CM from MCF-7 cells was harvested, centrifuged to remove cell debris and incubated with gentle agitation for 1h at 37°C with 4 µg/ml (final concentration) of anti-c10orf118 rabbit polyclonal antibody (HPA018019; Sigma). The same concentrations of α-actin (sc-1616; Santa Cruz) were used as control. NHDF cells were plated in 6-well at a density of $5x10^5$ cells/well. At 70-80% confluence, 800 µl of pre-incubated conditioned media with blocking antibodies were added to NHDF cells. After 48h, NHDF cells were harvested for RNA in TRI Reagent® to study HASes gene expression and their modulation in fibroblasts.

Cell treatment with Q7 recombinant protein

The recombinant protein Human C10orf118 full-length ORF (AH30557.1) obtained by Abnova was produced in *in vitro* wheat germ expression system and consisting of the C-terminal 221 amino acids of Human C10orf118, fused with GST-tag at N-terminal (MW = 50kDa).
NHDF cells were plated in 6-well at a density of $5x10^5$ cells/well. At 70-80% confluence, different concentrations of recombinant protein (4 pM-40 nM final concentration) were added in each well. The same concentrations of BSA were used as control. After 24 h, NHDF cells were harvested for RNA in TRI Reagent® to study the relative expression of HAS2 in NHDF cells after treatment.

RNA extraction and cDNA synthesis

Total RNAs were obtained from different cell lines cultures. At confluence, the cells were washed twice with PBS (Euroclone), and the total RNAs were extracted by using TRIzol reagent (Invitrogen). Each of the total RNAs sample was treated with 0.5µl of RNAse Inhibitor 20U/µl (Roche).
The purity of the RNAs was verified by measurement of A_{260}/A_{280} value using spectrophotometer.
Total RNA was retro-transcribed using the High Capacity cDNA synthesis kit (Applied Biosystems) in a total reaction volume of 50 µl. The reaction mix contained 4 µg of purified total RNA, 3 µl of RT-Buffer (10X), 1.2 µl of DNTPs (25X), 3 µl of random primers (10X), 1.5 µl of Multiscribe (50U/µl) and DEPC water. The cDNA synthesis program consisted of an initial step at 25°C for 10 min, followed by 37 °C for 2 h.

qRT-PCR (quantitative Reverse Transcription PCR)

qRT-PCR was performed by means of TaqMan technology and a Real-Time ABI Prism 7000 apparatus (Applied Biosystems, CA, USA). All mammalian HASs (HAS1 Hs00155410_m1; HAS2 Hs00193435_m1 and HAS3 Hs00193436_m1), the more common mammalian HYALs (HYAL 1 Hs00537920_g1 or Hs00738390_m1; Hyal2 Hs00186841_m1) and C10orf118 Hs00215984_m1 gene expression were studied, using β-actin Hs99999903_m1 as a housekeeping gene (endogenous control). For these experiments, oligonucleotide primers and TaqMan® probes were Assays-on-Demand of

Applied Biosystems. PCR reaction mix contained 2.5 µl of cDNA, 12.5 µl of Universal PCR Master Mix (Applied Biosystems), 1.25 µl of Assay-on-Demand primer and probe and 8.75 µl of nuclease-free water.
The PCR program consisted of an initial hot start at 50 -C for 2 min, followed by 95 °C for 10 min and 45 amplification cycles (95 °C for 15 s and 60 °C for 60 s). For each sample, ABI PRISM 7000 Sequence Detection System (SDS) software plotted an amplification curve by relating the fluorescence signal intensity (ΔRn) to the cycle number. A relative quantitative analysis was performed, using the $2^{-(\Delta\Delta t)}$ value, where ΔCt=Ct (target) – Ct (endogenous control) and ΔΔCt=ΔCt (sample - ΔCt (calibrator) (being Ct=number of cycle of exponential fluorescence beginning) (Livak and Schmittgen, 2001).

Immunoprecipitation

At 70-80% confluence in a T-75 flask, the CM was harvested from all the cell lines and centrifuged at 14000 g for 15 min at 4°C, in order to remove insoluble debris. A 4-ml aliquot was concentrated to 1 ml volume into GyroVap and desalted using PD-10 Desalting Columns (GE Healthcare). The CM were poured into 15-ml tubes, frozen and lyophilized to dryness. The lyophilized samples were resuspended with 50µl of RIPA buffer (150mM NaCl, 50mM Tris-HCl, 0.1% NP-40, 0.25% sodium deoxycholate) containing 1X Sigma FAST Protease Inhibitor. Samples were pre-cleared with 50% G-Sepharose beads in RIPA buffer + protease inhibitor for 2 h at 4°C on an orbital shaker. Beads were removed by centrifugation at 10000rpm for 1 min. The Q7-antibody (2.5µg) was added to the sample and incubated at 4°C overnight on an orbital shaker. To collect the immunocomplex, G-Sepharose beads were added to the sample and incubated at 4°C overnight. All immunoprecipitates were washed five times with RIPA buffer. Beads were boiled in 3X sample buffer for 5 min at 95°C and centrifuged. Eluates were analysed with SDS-PAGE and Western Blot.

SDS-PAGE and Western Blot

Cells were grown to confluence in appropriate medium. The CM were collected, spin down to remove cellular debris, and frozen at -20°C until further use. The cells were rinsed twice with ice-cold PBS followed by lysis in 300 µl of ice-cold RIPA buffer + Protease Inhibitor and mixing. Cell lysates were scraped into microfuge tube on ice and incubated O.N in agitation at 4°C.

The protein content in the samples (cell lysates and CM) was measured using Coomassie Protein Assay kit (Thermo Scientific). Aliquots containing equal protein concentration were mixed with 1 volume of reducing sample buffer, denatured at 95°C for 5 min and separated by SDS-PAGE, followed by transfer to nitrocellulose. Membranes were blocked O.N. at 4°C with 5% BSA in Tris-buffered saline (TBS; 20 mM Tris-HCl, 137 mM NaCl, pH7.6) supplemented with 0.1% Tween-20. After blocking and washing with TBS and 0.1% Tween-20 (T-TBS), the nitrocellulose membranes were probed with anti-c10orf118 rabbit polyclonal antibody (HPA018019; dilution 1:500)(Sigma) in T-TBS containing 5% BSA O.N. at 4°C. After five washes with T-TBS, the membranes were incubated with HRP-conjugated anti-rabbit IgG diluted 1:400 (Santa Cruz Biotechnology) in 5% BSA in T-TBS for 1h at RT and immunocomplexes were detected by enhanced chemiluminescence (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare) according to the manufacturer's instruction. As a control, we also used a primary polyclonal antibody against α-tubulina (goat; dilution 1:300) and donkey anti-goat IgG-HRP (dilution 1:200) (Santa Cruz Biotechnology). Signal was detected by standard X-ray films. Densitometry analysis was performed using the ImageJ Gel Analysis tool, where gel background was also removed individually for each band.

Immunostaining

Cells seeded on coverslips in six-well plates were rinsed with PBS and fixed in 4% paraformaldehyde for 15 min and permeabilized with 0,1%Triton X-100 for 15 min. The coverslips were preincubated with PBS containing 5% BSA O.N. at 4°C and then incubated in the same solution containing antibody against Q7 (Sigma; 1:225 dilution) O.N. at 4°C. The coverslips were washed with PBS, and then incubated with secondary antibody Alexa Fluor 488-labeled goat anti-rabbit (dilution 1:400) in PBS 1% BSA. After washing in PBS, the coverslips were mounted using mounting medium Vectashield with DAPI (Vector Laboratories). Photographs were taken with an Olympus IX51 immunofluorescence microscope.

Generation and establishment of stable transfectant

To study the function of Q7 in crosstalking tumour and stromal fibroblast cell interaction, the cDNA sequence corresponding to human c10orf118 open reading frame (ORF) was cloned into pCMV6-Entry vector to overexpress the protein (Origene). To

stably knock-down the protein Q7, we used four siRNA target sequences obtained from ABM Good. Cells transfected with an empty vector or a GFP-vector (Mock), were used as a control.
Transfection of MDA MB-231 and MCF-7 human breast cancer cells were performed using ExGene transfection reagent according to manufacturer's recommendations (Fermentas). Stable transfectant cells were selected using G418 (neomycin) at 500 µg/ml and Puromycin at 0.4 µg/ml. Individual colonies were established by cloning antibiotic-resistant cells. Overexpression or knock-down of Q7z32e were confirmed using western blot analysis and/or qRT-PCR.

HPLC analysis

Medium from cell cultures was frozen at -80°C and lyophilized. Each pellet was dissolved in 300 µL water followed by an addition of 96% ethanol (or absolute ethanol) in a ratio of 1:4. The mixtures were precipitated overnight at -20°C. Following centrifugation at 12000 rpm at 4°C for 40 min, the pellets were left to dry and then digested with 10 µl protease K. In particular, the pellet was dissolved in 300 µl 0,1 M ammonium acetate buffer, pH 7.0, containing 20 U/ml proteinase K (Finnzymes), and digestion was done at 500 rpm, 50°C for 3 h in a thermomixer. The enzymic treatment was terminated by boiling for 5 min. Thereafter, 4 volumes of 96% ethanol per sample volume were added, and the GAGs in the mixture were precipitated at -20°C overnight. Ethanol-precipitated GAGs were centrifuged at 12000 rpm at 4°C for 40 min. The obtained pellets were dried and dissolved in 100 µL 0,1 M ammonium acetate, pH 7.0, containing 10 mU/ml hyaluronidase SD and digested at 37°C for 1 h. A 10 mU/ml chondroitinase ABC (Seikagaku Corp.) was added, and the mixture was incubated at 37°C for 3 h. The samples were then frozen at -80°C and then lyophilized. Derivatization of standard HA and CS D-disaccharides was done as described by Calabro (Calabro et al., 2000). In particular, 10 nmol of each standard D-disaccharide in water was completely evaporated in a microcentrifuge tube at 12000 rpm at room temperature. A 40 µL volume of 12.5 mM AMAC solution in glacial acetic acid/DMSO (3:17 v/v) was added and samples were incubated for 10 –15 min at room temperature. A 40 µL volume of a freshly prepared solution of 1.25 M $NaBH_3CN$ in water was added to each sample followed by an overnight incubation at 37°C and 500 rpm. Separation and analysis of derivatized products were performed with a Jasco–Borwin chromatograph system with a fluorophore detector (Jasco FP-920, λex=442 nm and λem=520 nm). Chromatography was carried out using a reversed phase column (C-18, 4.6150 mm, Bischoff, Germany) at room temperature, equilibrated with 0.1 M ammonium acetate, pH 7.0, filtered through

a 0.45 µm membrane filter. A gradient elution was performed using a binary solvent system composed of 0.1 M ammonium acetate pH 7.0 (eluent A), and acetonitrile (eluent B). The flow rate was 1 ml/min and the following program was used: pre-run of column with 100% eluent A for 20 min, isocratic elution with 100% eluent A for 10 min and gradient elution to 30% eluent B for 30 min. Sample peaks were identified and quantified comparing the fluorescence spectra with standard unsaturated disaccharides, using a Jasco–Borwin software.

Results

Immunolocalization of HA in cancer biopsies

During my PhD project, I worked on the study of the ECM with particular attention to one of its main components, hyaluronic acid (HA). This glycosaminoglycan has been proved to be very in many inflammatory diseases but also in cancer. It has been known that the amount of HA in the cancer cells and in the surrounding stroma correlates with tumour progression and metastatic behaviour in different cancer cell lines (Delpech et al., 1997; Itano et al., 1999). To better understand the role of HA in human tumours, in our laboratory, it was performed analyses on human breast carcinoma biopsies with immunostaining using biotinylated hyaluronan binding protein (bHABP). As shown in Figure 10, in ductal carcinoma in situ (DCIS) at G3 stage (panel A) and in invasive ductal carcinoma (IDC) at G2 stage (panel B), HA staining is present in the stroma while the signal is faint in the tumour epithelial cells. Moreover, in panel C, it is shown in detail the differential HA staining between cancer cells (low signal) and stroma (high signal).

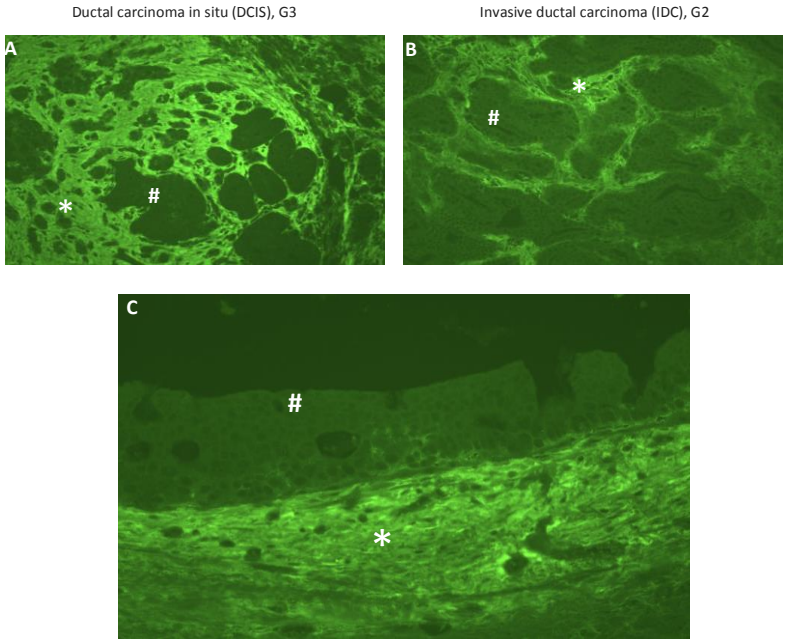


Figure 10 - Immunofluorescence microphotographs of human breast carcinoma biopsies with immunostaining using biotinylated hyaluronan binding protein (bHABP). The hashtag points the (tumour) epithelial cells and the asterisc the stromal compartment.

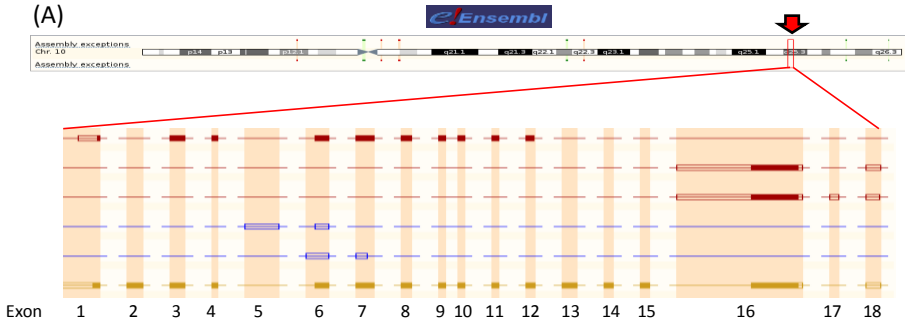
In silico characterization of Q7

It is well-known that the communication between tumour cells and stroma occurs by growth factors and cytokines produced by tumour cells. These soluble factors induce the stroma and peritumoral cells to produce some macromolecules of the ECM which in turn support tumour progression, such as HA. Thus, to increase the knowledge about cross-talk between tumour and stroma, in our laboratory it was analysed the conditioned media (CM) of a breast cancer cell line (BC8701 from Palermo) using a MALDI-TOF mass spectrometer and a "bottom-up proteomics" approach. The result obtained by Mascot search showed a new "Uncharacterized protein of c10orf118 or Q7z3e2". From now on, for simplicity, the protein will be called Q7.

To date, there is no data in public databases and in the literature about Q7. Therefore, to better characterize the protein we used bioinformatics tools using different public databanks and the Ensemble website. The information that was obtained demonstrated that the gene for Q7 is located on human chromosome 10 in the region q25.3.

Moreover, the gene is composed of 18 exons and exists in 6 splicing variants (Figure 11A), which are shown together with the relative protein products in the panel B (Figure 11B). Notably, two of these variants do not produce any protein. The last one with 898 aminoacids represents the "full-length" isoform (FL), while the isoform with 436 aminoacids is the "short" one (S).

(A)



(B)

Show/hide columns (3 hidden) Filter

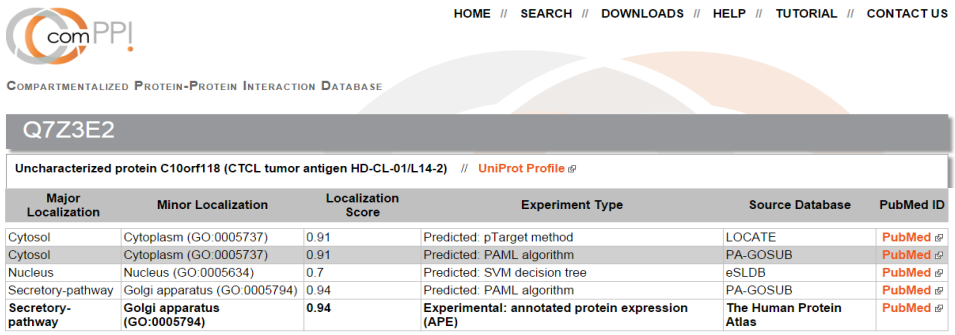
Name	bp	Protein	Biotype	CCDS	UniProt	RefSeq
CCDC186-001	1576	436aa	Protein coding	-	H0Y7V5	-
CCDC186-002	1928	211aa	Protein coding	-	A0A0C4DFU7	-
CCDC186-003	2035	211aa	Protein coding	-	A0A0C4DFU7	-
CCDC186-004	661	No protein	Processed transcript	-	-	-
CCDC186-005	477	No protein	Retained intron	-	-	-
CCDC186-006	7245	898aa	Protein coding	CCDS7587	Q7Z3E2	NM_018017 NP_060487

Figure 11 - (A) The scheme shows chromosomal location and the alternative splice variants. In panel (B) is shown a portion of the NCBI Gene report for Q7 gene. The table includes information about the different isoforms of protein. The last one represents the "full-length" isoform (FL), while the isoform with 436aa is the "short" one (S).

Immunolocalization of Q7

After examining *in silico* Q7 consensus sequences, we decided to explore the localization of the protein. Using compartmentalized protein-protein interaction database (Figure 12A), Q7 protein was predicted to be localized in the secretory pathway with a localization score of 0.94. Analysis of immunofluorescence using two breast cancer cell lines (MCF-7 and MDA-MB231) demonstrated that protein Q7 is localized in the perinuclear region but also appeared as distinct cytosol dots dispersed in the cytoplasm of cells (Figure 12B). This *in vitro* result is in accordance with the previous *in silico* prediction.

(A)



Q7Z3E2

Uncharacterized protein C10orf118 (CTCL tumor antigen HD-CL-01/L14-2) // UniProt Profile

Major Localization	Minor Localization	Localization Score	Experiment Type	Source Database	PubMed ID
Cytosol	Cytoplasm (GO:0005737)	0.91	Predicted: pTarget method	LOCATE	PubMed
Cytosol	Cytoplasm (GO:0005737)	0.91	Predicted: PAML algorithm	PA-GOSUB	PubMed
Nucleus	Nucleus (GO:0005634)	0.7	Predicted: SVM decision tree	eSLDB	PubMed
Secretory-pathway	Golgi apparatus (GO:0005794)	0.94	Predicted: PAML algorithm	PA-GOSUB	PubMed
Secretory-pathway	**Golgi apparatus (GO:0005794)**	**0.94**	**Experimental: annotated protein expression (APE)**	**The Human Protein Atlas**	PubMed

(B)

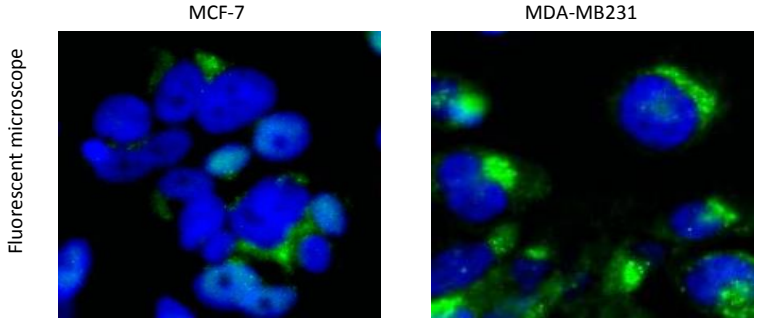


Figure 12 - (A) The table shows the localization predicted by different software for *in silico* analysis. (B) IF images show the localization of Q7 protein as determined by the use of the polyclonal antibody for Q7. Cells are counterstained with the nuclear probe DAPI (blue).

Analysis of gene expression of Q7 in different breast cancer cell lines

Preliminary studies regarding the expression profile of Q7 were performed *in silico* taking the advantage of bioinformatics data. Using NCBI UniGene database the human gene C10orf118, that encoding for Q7 protein, is represented by 139 ESTs from 87 cDNA libraries and corresponds to reference sequence NM_018017.2. [UniGene 155543 - Hs.159066]. Analyzing data reported in the database, it is possible to note that the number of transcript per million (TPM) coding for Q7 protein is different in normal and pathological tissue. In fact, taking in consideration the same body sites, the pathological tissues present more TPM respect to normal tissues (Figure 13A). Next, we decided to explore in NHDF and in two different breast cancer cell lines the relative gene expression of Q7 *in vitro* using qRT-PCR. Results show that MCF-7 and MDA-MB231 have from 4- to 2-fold increased levels of Q7 mRNA compared to NHDF cells, respectively (Figure 13B).

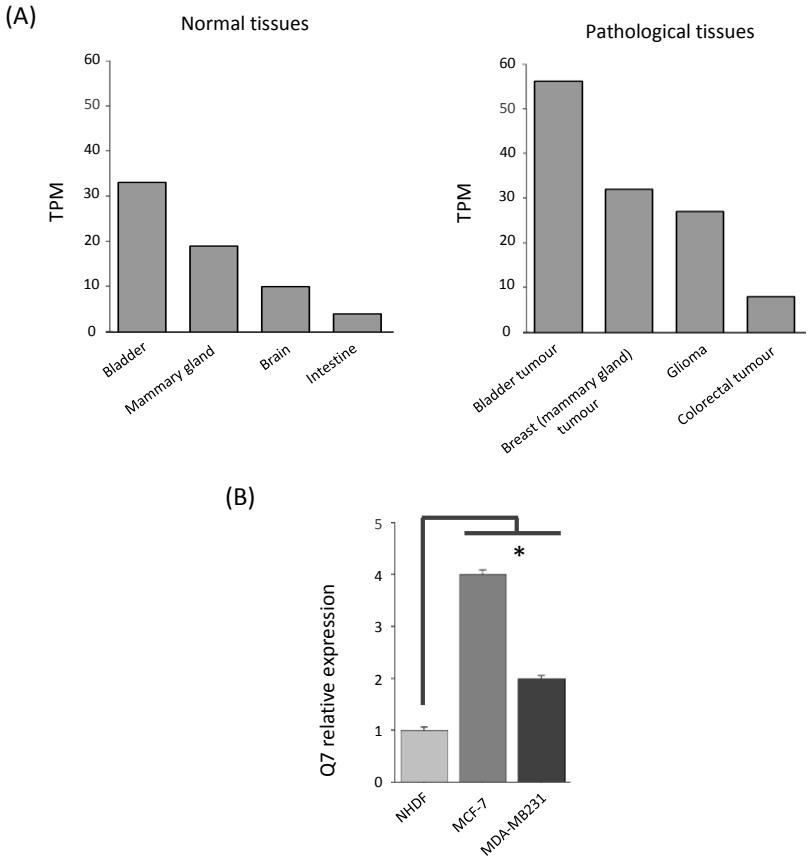


Figure 13 - (A) Different expression level of Q7 expressed as 'transcripts per million' (TPM) between normal and pathological tissues. (B) Relative gene expression of Q7 in MCF-7 and MDA-MB231 compared to NHDF cells.

Evaluation of Q7 protein in cell lysates, CM and biopsies

After evaluating the presence of Q7 transcript in both normal and cancer cells, we decided to perform Western Blots for analysis of Q7 protein obtained from cell lysates and CM. The scheme in Figure 14A represents the different isoforms of protein produced by cells. In particular, antibody HPA018019 (Sigma) was able to recognize only two isoforms. The first one is the "full-length" form composed by 898 amino acids (Q7-$_{FL\ 1-898}$), whereas the second one is the "short" isoform with 436 amino acids (Q7-$_{S\ 256-692}$). These proteins derive from alternative splicing as shown in Figure 11.

The analyzed cell lines express several intracellular forms corresponding to Q7-$_{FL\ 1-898}$ isoform with a MW of 104 kDa, whereas the 80 kDa form could be identified as Q7-$_{S\ 256-692}$. As expected from the *in silico* data, MCF-7 and MDA-MB231 cells express more Q7 bands than NHDF. Immunoprecipitation with antibody α-Q7 was performed in the CM in order to enrich the samples. In particular, we were able to detect Q7 in MCF-7 CM indicating that Q7 could be secreted in the ECM. Notably, cells lysates showed different

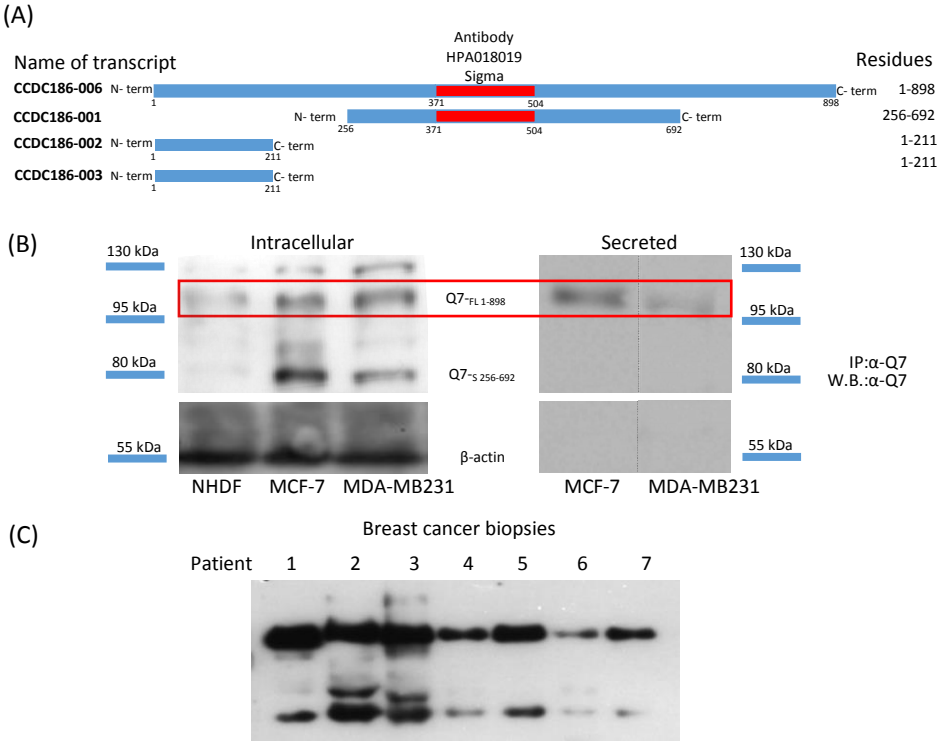


Figure 14 - (A) Schematic representation of the different isoforms of Q7 protein. In red highlights the region recognized by the antibody in each isoform. (B) Q7protein level was determined by W.B. analysis in total cell lysate (intracellular) and in CM after IP with α-Q7 (secreted). (C) W.B. analysis using antibody against Q7 on different breast cancer biopsies.

isoforms of the Q7 protein whereas in the CM it was found only Q7-$_{FL\ 1\text{-}898}$ isoform (Figure 14B).
After evaluating the pattern of expression in normal and breast cancer cell lines, we focused our attention on breast cancer biopsies. In collaboration with Dott. Patrizia Cancemi of laboratory of biochemistry, University of Palermo, we analysed seven different breast cancer biopsies from seven different patients. Western blot analyses showed that protein Q7 is expressed in all patients with a similar pattern of bands obtained using tumour cells (Figure 14C).

Analysis of HA enzymes expression and modulation of HA amount in NHDF in transwell with breast tumour cells

As Q7 is expressed in breast cancer biopsies and in tumour cell line MCF-7 and MDA-MB231, we studied its role in tumour biology focusing our attention of cancer-stromal cross-talk. Since HA is an important player in tumour microenvironment and favors cancer progression, we investigated whether Q7 released from MCF-7 and MDA-MB231 could affect HA synthesis in normal fibroblasts. We co-cultured MCF-7 and NHDF in transwell (Figure 15A) in order to permit soluble factors (and Q7 protein) diffusion. After 24 hours of incubation, HA was quantified in the CM in the lower chamber and RNA was extracted from NHDF for HA metabolizing enzymes expression analysis. As shown in Figure 15B, it is possible to note that HA production was significantly increased in NHDF co-cultured with tumour cells.
We decided to evaluate the relative expression of HA enzymes (HAS2 and HYAL-2) to verify whether the increasing amount of HA was due to an overexpression of HAS2 or a downregulation of HYAL-2. The relative expression of HAS2 and HYAL-2 was performed using qRT-PCR. Transcript levels of HAS2 were increased in NHDF in the presence of MCF-7 and MDA-MB231 in the upper chamber of the transwell, whereas the HYAL-2 transcript levels were increased exclusively in NHDF co-cultured with MCF-7. Therefore, HA increase in CM of NHDF co-cultured with cancer cells is correlated with the increased activity of HAS2.
In previous experiments, we evaluated also the relative expression of all the three HASes. HAS2 was the most expressed and HAS1 was not detected. HAS3 was found to be expressed at lower amount than HAS2 and HAS3 levels were not affected by cancer cells co-culture (data not shown).
These results show that CM of tumour cells is able to modify the expression of HA enzymes in NHDF and the amount of HA secreted. These data are in accordance to several studies performed using different cell lines, as well as dermal fibroblast (Li et al., 2007).

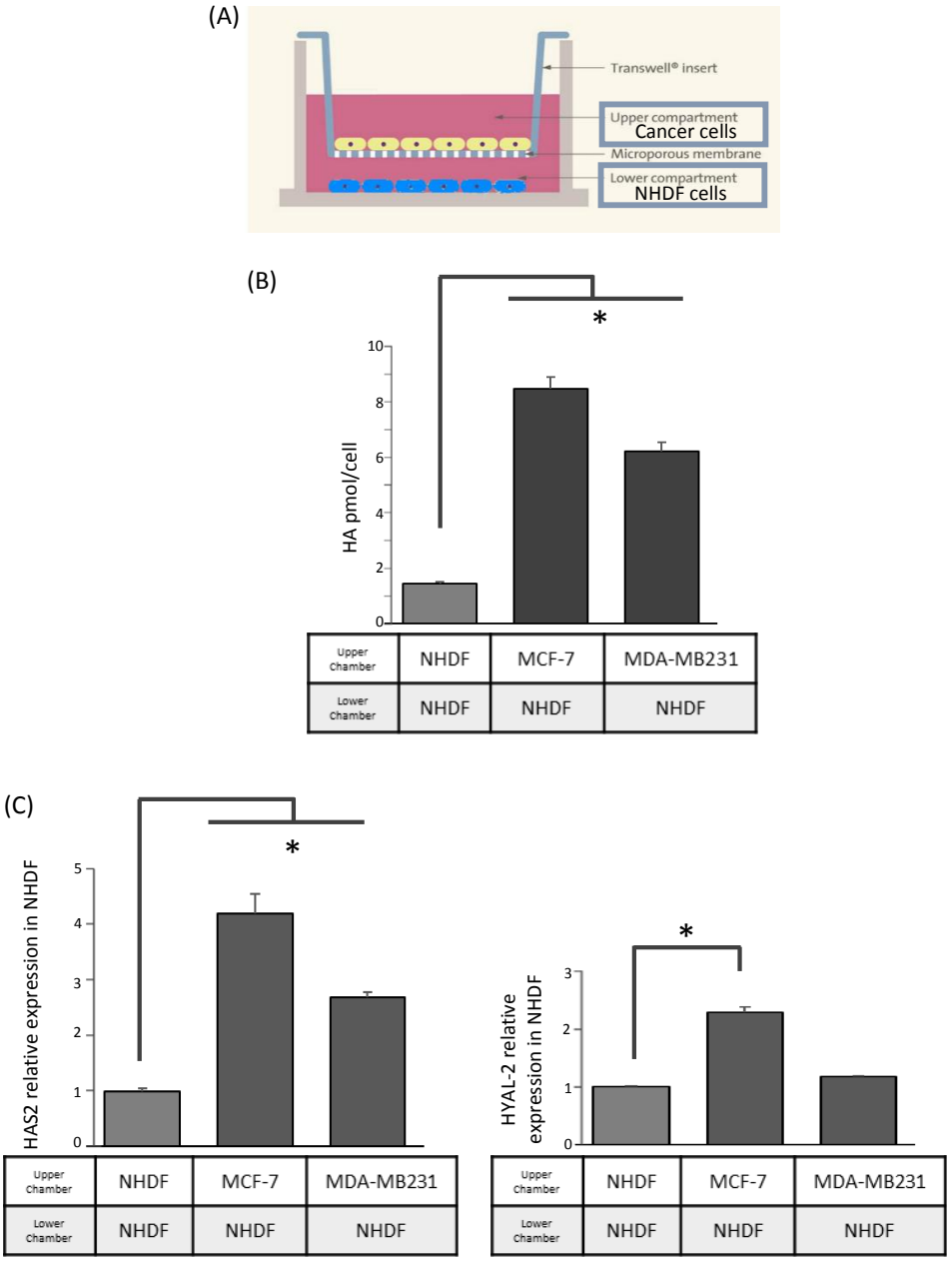


Figure 15 - **HAS metabolizing enzymes expression and HA levels in NHDF co-cultured with cancer cells.** (A) Illustration of experimental design to perform transwell assay. (B) Amount of HA secreted in the CM of NHDF in transwell with MCF-7 and MDA-MB231 cells. (C) qRT-PCR analysis was performed to examine the expression of HAS2 and HYAL-2 in NHDF.

Titration of Q7 protein in CM and its role in modulation of HAS2 in NHDF cells

To further explore the potential regulatory mechanism of the secreted Q7 protein from MCF-7 in modulation of HAS2 in NHDF cells, we removed Q7 protein from MCF-7 CM by titration assay. Preliminary experiments using several concentration of anti-Q7 antibody showed that 4µg/ml was optimal to abrogate Q7 activity. As shown in Figure 16B, when NHDF cells were incubated with MCF-7 CM and pretreated with anti-Q7 antibody, the relative expression of HAS2 in NHDF was significantly decreased. However, there was no significant change in HAS2 expression when we incubated NHDF with MCF-7 CM pretreated with anti-α-actin antibody. These results suggest that soluble Q7 secreted from cancer cells can induce the expression of HAS2 in NHDF.

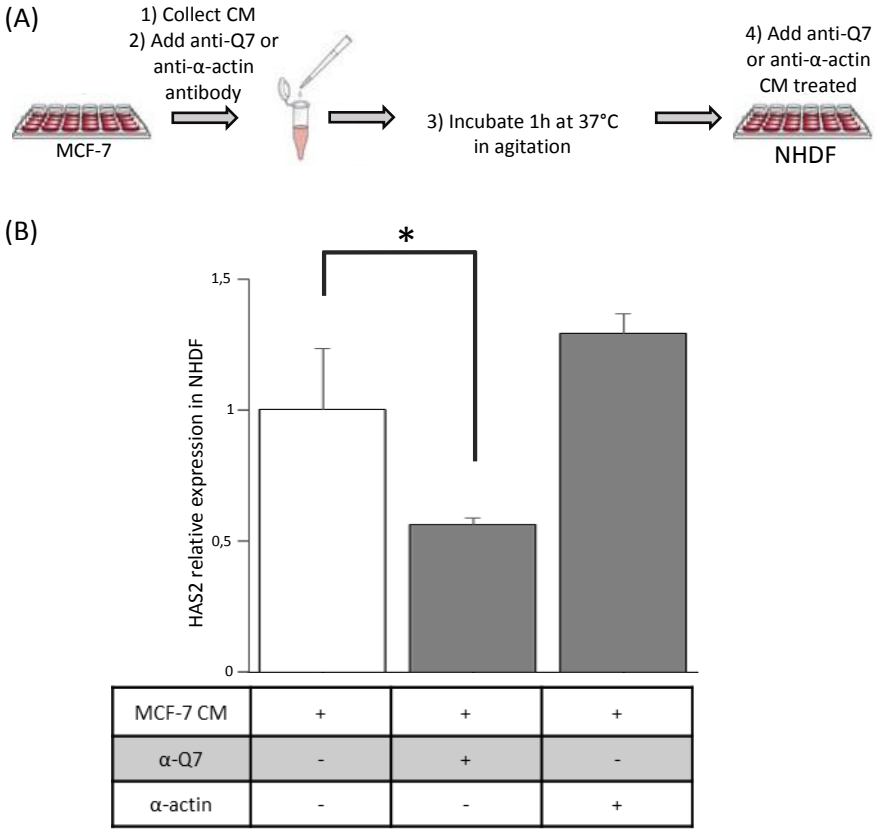


Figure 16 - Q7 titration assay. (A) Illustration of experimental design. (B) Relative expression of HAS2 in NHDF after treatment with CM and CM treated with 4µg/ml of α-Q7 or α-actin.

Treatment with Q7 recombinant protein and its role in modulation of HAS2 in NHDF cells

To confirm the role of Q7 to induce HAS2 expression in NHDF, we treated the cells with a commercial peptide spanning aminoacids 1 to 211 (Figure 17A). The quality purification procedure was checked by gel electrophoresis (Abnova). Analysis using ImageJ demonstrated that Q7 recombinant protein was purified at 85%.
It was demonstrated that there was a 2-fold increase in HAS2 relative expression in NHDF treated with different concentration, up to 40nM, of Q7 recombinant peptide for 24h respect to treatment with the same concentration of BSA used as control (Figure 17B). This result confirms that Hr_Q7$_{1\text{-}211}$ contain the functional region used to regulate the expression of HAS2.

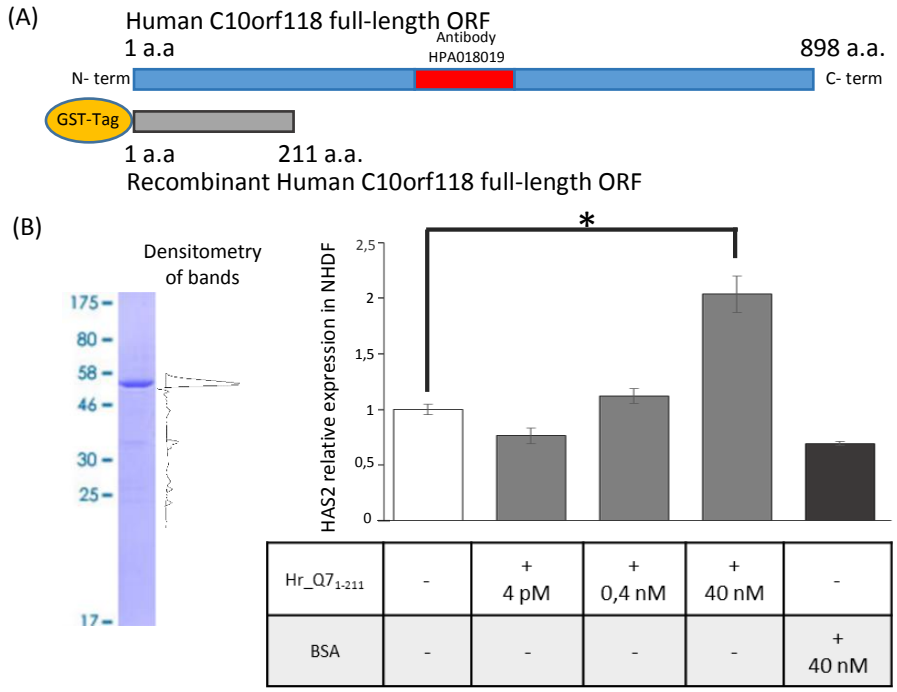


Figure 17 - Treatment with Q7 recombinant protein. (A) Schematic representation of the Human C10orf118 full-length ORF and the shorter Recombinant Human C10orf118 full-length ORF (Hr_Q7$_{1\text{-}211}$). In red highlights the region recognized by the antibody. In panel (B) is shown the quality control testing performed using 12.5% SDS-PAGE and stained with Coomassie Blue with relative densitometry of bands obtained using ImageJ. The graph on the right shows the HAS2 relative expression in NHDF untreated and treated with different concentration of Hr_Q7$_{1\text{-}211}$. As control, is used BSA at the highest concentration (40nM).

Direct co-culture

As transwell experiments highlighted that HAS2 gene expression and HA in CM of NHDF were increased when these cells were treated with MCF-7, we directly co-cultured NHDF with MCF-7 cells in the same well. As shown in Figure 18A, tumor cells were easily visible on NHDF forming a single layer or a multilayer colony. Interestingly, when we nucleofected a plasmid coding for $Q7_{FL}$, an increase of HA staining was clearly observable in NHDF as well as within the tumor cells (Figure 18B). Moreover, the overexpression of $Q7_{FL}$ coding vector in MCF-7 cells produced a 10-fold increase of HAS2 gene expression respect to MCF-7 mock cells. This result correlates with a strong staining of HA in MCF-7 cells (Figure 18B, panel VI).

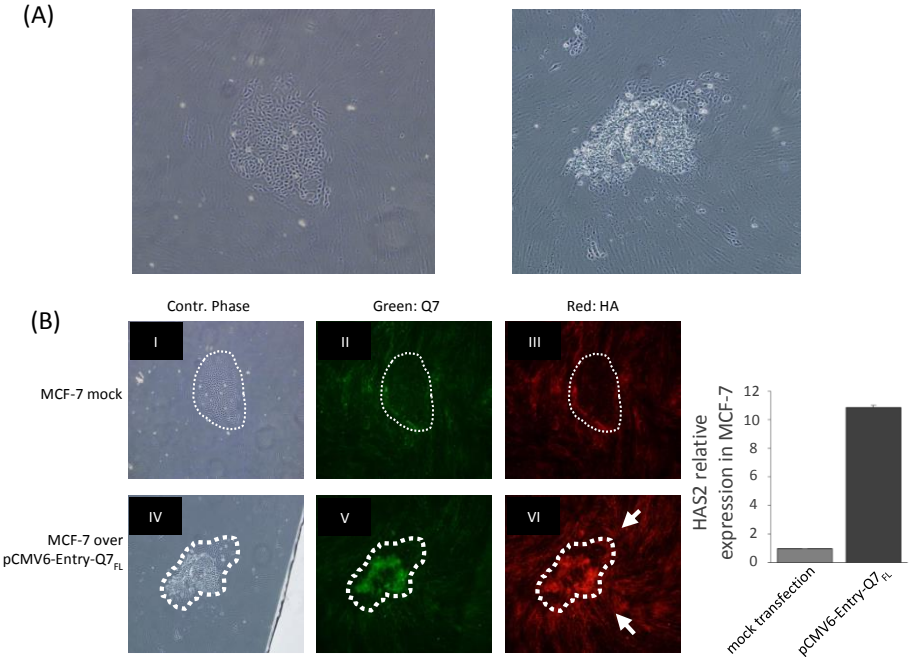


Figure 18 - (A) Representative microphotographs (phase contrast) showing morphology of direct co-culture of MCF-7 cells embedded in NHDF cells. In the panel (B), NHDF in co-culture with MCF-7 cells in the same well were stained for Q7 (green) and for HA (red). The graph shows the relative expression of HAS2 in MCF-7 cells.

Discussion & Conclusions

In this study, we demonstrated that in tissue specimens from patients with breast cancer the HA concentration is higher in the stroma of malignant tumours than in the corresponding benign or normal tissues. Additionally, *in vitro* experiments showed that the relative expression of HAS2 and the secreted HA in the stromal cells NHDF are notably increased when these cells are co-cultured with the breast cancer cell line MCF-7. Moreover, the examination of the conditioned medium (CM) of breast cancer cells revealed the presence of a novel protein called Q7. This protein was found localized in the cytoplasm and in Golgi complex. In cell lysates we observed four isoforms, whereas in CM only the full-length isoform was identified. Q7 protein is highly expressed in breast tumour cell lines and in minimal levels in fibroblasts. It is still unclear the function of this protein. However, we noticed that the expression of HAS2 in NHDF was induced after treatment with Q7 protein. This result was confirmed when NHDF where treated with MCF-7 CM in the absence of Q7 and a reduction of the induced HAS2 expression was observed. Interestingly, in a co-culture of NHDF with MCF-7 that overexpress Q7, HA decoration was increased around the tumour cells colonies where cells are in contact with NHDF.

Study on the alterations of ECM of the mammary gland and its role in tumour processes is of central importance in basic cancer biology and therapy. In particular, the HA, which is one of the principal macromolecules of ECM, is closely associated with tumorigenesis. In fact, HA influences tumour cell behaviour and cancer progression by modulating the hydration and osmotic balance in the tumour environment. These phenomena are mediated through different HA receptors. In breast cancer the serum HA level is associated with severe malignant phenotype and high metastasis (Delpech et al., 1990)

Today, several factors are involved in the regulation of cancer growth and spreading. Coller *et al.* demonstrated that stromal cells, such as fibroblasts, in the tumour microenvironment can actively support malignant transformation and metastasis. From this perspective, cancer is viewed as a parasitic disease that steals energy-rich metabolites from the host organism (Coller, 2014). In fact, tumour growth is fuelled by lactate, ketones, and glutamine provided by stromal cells that are then absorbed by cancer cells and used for its growth and proliferation. Synthesis of the two precursors of HA, UDP-GlcNAc and UDP-GlcUA, requires ATP and, for this reason, is an energy consuming process, that is less afforded by cancer cells (Vigetti et al., 2014). Thus, cancer cells secrete soluble factors that induce HA synthesis in stromal cells. As reported previously, the two representative growth factors TGF-β and PDGF upregulate

peritumoral HA, permitting by this metastasis and proliferation of tumour cells (Porsch et al., 2013).

Although breast cancer cells express HASs, they usually do not produce HA (Du et al., 2013) but they degrade it creating oHA, which in turn bind the specific membrane receptors, such as CD44 and RHAMM, and activate cell-signalling pathways that favour cell invasion, angiogenesis, inflammation (Karbownik and Nowak, 2013) and EMT (Kalluri and Weinberg, 2009). Furthermore, senescent fibroblasts are able to induce EMT in very close epithelial cells and influence tumour progression (Laberge et al., 2012). As described before, these cells secrete numerous cytokines, growth factors, degradative enzymes and proteases, called SASP (Senescence Activated Secretory Pathway), that can modify the tissue organization and, in particular, the HA composition, and may promote age-related pathologies (Laberge et al., 2012).

Here, we investigated a novel protein, called Q7, mainly produced by tumour cells. The secreted form of Q7 is the full-length isoform, whereas four different isoforms were found within the cells. The Golgi apparatus is involved in modifying, sorting, and packaging macromolecules for cell secretion or use within the cell. Thus, our hypothesis is that the intracellular soluble Q7 could be loaded into exosomes and exported via a non-classical secretion pathway from local tumour invasion site and stimulate stromal cells at distant sites. Similar findings were described by Azmi et al., showing the secretion of proteins and soluble factors *via* exosome formation (Azmi et al., 2013).

The Q7 protein is mainly expressed in breast tumour cells and in particular in the low invasive cell lines that are characterized by the presence of the hormone ER and PR receptors. Clinically, ER-positive breast cancer is less aggressive than the ER-negative and is amenable to hormone therapy by ER modulators (Karousou et al., 2014). In this work, MCF-7 showed a great expression of Q7 protein, whereas the triple negative aggressive cell line MDA-MB231 showed lower gene expression and protein secretion. Therefore, the invasiveness and/or the presence of the ER/PR receptors within the cell membrane may be correlated to the increased amounts of Q7 protein.

High levels of Q7 in breast tumour cells may have a profound biological significance. Probably, it is involved in the first step of differentiation from normal cells to tumour cells, as NHDF cells which were used here as a model of stromal cells, showed a minimal expression and secretion of this novel protein. In fact, high levels of Q7 protein produced by MCF-7 cells are able to induce HA production and HASes expression in NHDF. Therefore, MCF-7 cells may induce HA synthesis in NHDF by Q7 protein and the resulted HA-rich ECM may favour the proliferation of MCF-7 cells and explain biologically the *in*

situ growth of breast tumour. Because of a low amount of secreted protein in MDA-MB231 cells, Q7 protein does not seem to be related with advanced steps of tumorigenesis, such as migration and invasion.

Considering the above results, we suggest a possible role of Q7 protein in the breast cancer cells' functions and the mechanism by which this protein secreted by breast tumour cells induces the HAS2 expression in the stromal cells and HA accumulation in tumour microenvironment (Figure 19). Q7 protein is highly synthesized by non-metastatic MCF-7 tumour cells ① and is secreted probably using vesicular transport, such as exosomes, and/or exocytosis in the ECM as soluble protein ②. Because of the high-secreted amount of Q7, probably this protein has a role in the stroma tumour cell-cell interaction. In fact, Q7 could diffuse away from local tumour invasion site and stimulate stromal cells at distant sites. This protein is probably absorbed using endocytosis or recognised by a particular receptor present on stromal cells ③. The internalization of Q7 protein in the stromal cells induces the HAS2 gene expression ④. Therefore, Q7 produced by carcinoma cells may stimulate stromal cells to increase the production of HA ⑤. The HA secreted in ECM interacts with its receptors, in particular CD44 ⑥, and probably activate signalling pathways, such as NF-kB ⑦, which then are able to induce an overexpression of different pro-inflammatory genes, such as growth factors, MMPs and other soluble factors that are responsible for the initiation and progression of breast cancer ⑧.

All these data suggest that Q7 plays a key role in the increment of HA in tumour-stroma microenvironment and that this novel protein is an additional factor involved in the breast cancer progression. Thus, the deep knowledge of the Q7 synthesis in breast cancer cells and the definition of how this protein induces HA synthesis in stromal cells can open new approaches for the design of anticancer drugs that target the secreted Q7 in tumour microenvironment.

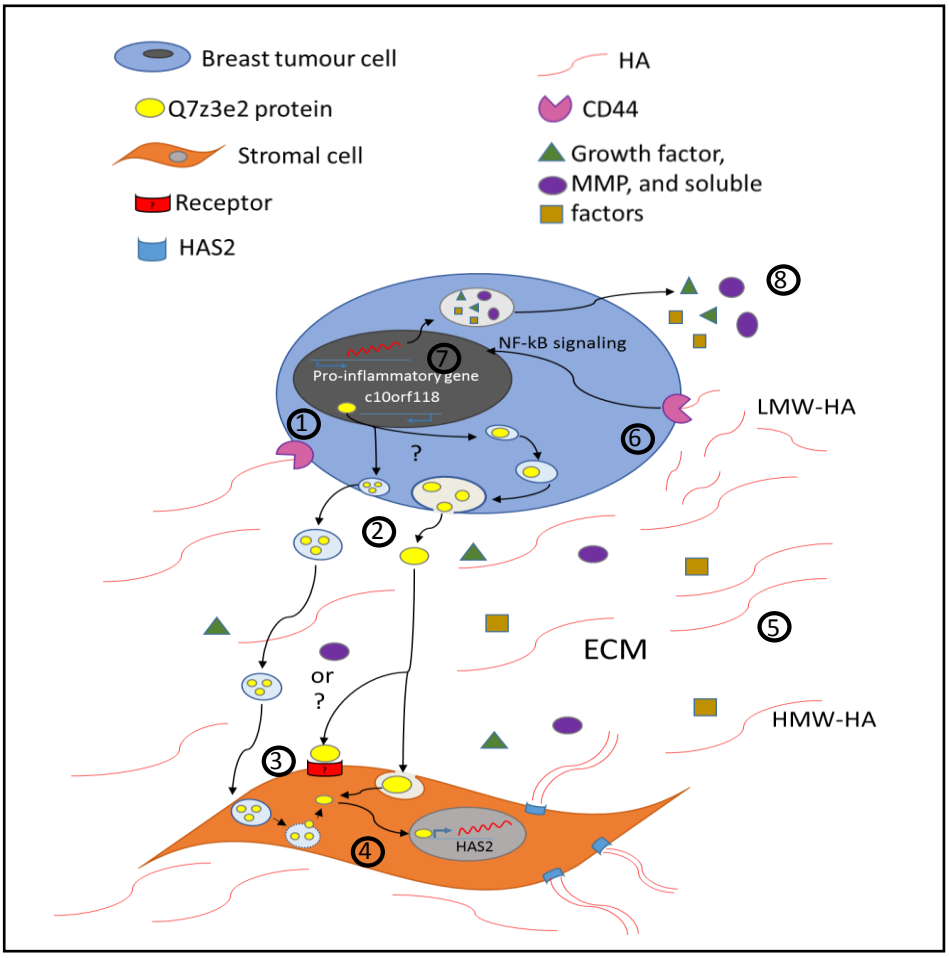


Figure 19 – Cartoon depicting the mechanism by which this protein secreted by breast tumour cells induces the HAS2 expression in the stromal cells and HA accumulation in tumour microenvironment.

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Acknowledgements

I am grateful to Prof. Albetro Passi for the continuous advice and support granted to this work.

I thank my tutor Davide Vigetti for his critical judgment and for useful suggestions and scientific discussions.

A particular acknowledgement to Jenny for being a dear friend. She gave me the enthusiasm and passion for scientific research and helped me to develop my PhD project listening to my ideas, doubts and inspirations.

A special thanks to Paola for the constant "help in the laboratory" with her precious advice and for provided me with great support and useful tips.

I would also like to thank all the guys in the lab, for these years we spent together, and, in particular, Manuela, Cristiana, Eleonora e Ilaria for all "scientific discussions" done in the morning drinking a good coffee and during the lunch.

Finally, I would like to say a VERY BIG THANK to my family and my future husband, Tonino, for always believing in me and for their love and support.