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The role of the long non coding RNA HAS2-AS1 in breast cancer cells

Ruolo dell' RNA non codificante HAS2-AS1 in cellule di tumore della mammella

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Abbreviations

4-MU	4-methylumbelliferone
AMPK	5' AMP-activated protein kinase
ARL6IP1	ADP Ribosylation Factor Like GTPase 6 Interacting Protein
BACE1	Beta-Secretase 1
ECM	Extra Cellular Matrix
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen Receptor
ERK1/2	Extracellular signal–Regulated Kinase
ESYT2	Extended Synaptotagmin 2
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast Grow Factor
GAGs	Glycosaminoglycans
HA	Hyaluronan
HAS1	Hyaluronan Synthase 1
HAS2	Hyaluronan Synthase 2
HAS2-AS1	Hyaluronan Synthase 2 Antisense 1
HAS3	Hyaluronan Synthase 3
HASes	Hyaluronan Synthases
HER2/neu	Human Epidermal grow factor Receptor 2
HGF	Hepatocyte Grow Factor
HMW-HA	High Molecular Weight Hyaluronan
HOTAIR	HOX Transcript Antisense RNA
HYAL	Hyaluronidase
LMW-HA	Low Molecular Weight Hyaluronan
lncRNA	Long Non Coding Rna
MALAT1	Metastasis Associated Lung Adenocarcinoma Transcript MET Mesenchymal to Epithelial transition
miRNAs	microRNAs
ncRNA	non coding RNA
NF-KB	nuclear factor kappa-light-chain-enhancer of activated B cells
NRBF2	Nuclear Receptor Binding Factor 2
OGT	O-Linked N-Acetylglucosamine (GlcNAc) Transferase

PDCD	Programmed Cell Death
PGs	proteoglycans
PKC	Protein Kinase C
PR	Progesterone receptor
RAF1	Raf-1 Proto-Oncogene, Serine/Threonine Kinase
RHAMM	ReceptorHyaluronan Mediated Motility
SLC	Solute Carrier
SLK	STE20 Like Kinase
SMAP1	Small ArfGAP 1
SUB1	SUB1 Homolog, Transcriptional Regulator
TADA2B	Transcriptional Adaptor 2B
TGFβ	Transforming Growth Factor Beta
UTR	Untranslated region
VAMP4	Vesicle Associated Membrane Protein 4

1 - SUMMARY

Extracellular matrix (ECM) is a network made by proteins and proteoglycans, whose structure is essential to maintain tissue architecture and to provide molecules diffusion and cellular communications. A deregulated synthesis of ECM components is often associated to a pathological status. Among various glycosaminoglycans, hyaluronan (HA) is a ubiquitous ECM component with a remarkable structural importance. It is able to modulate cell adhesion, motility, growth and inflammation after the binding with cellular receptors (CD44 and RHAMM) and the activation of different cellular pathways. In tumour microenvironment, the up-regulation of HAS2 and the overproduction of HA are often associated with tumour progression and metastasis. This also applies to breast cancer, where the accumulation of HA and the overexpression of hyaluronan synthases (HASes) in stromal and tumoral cells correlate with tumor malignancy and patients survival.

The study of the regulation of HAS2, the main enzyme in the production of HA, is very important to understand the development and the progression of breast cancer. Recently, it has been discovered that the lncRNA HAS2-AS1 can modulate the expression of HAS2 and the production of hyaluronan in aortic smooth muscle cells via epigenetic modifications [1]. Although the role of HA and HAS2 in breast cancer is widely described, little is known about HAS2-AS1.

Given this considerations, the aim of this project is to study the role of HAS2-AS1 in breast cancer. In particular, we compared the behaviour of MDA-MB-231 and MCF-7 cells after the modulation of HAS2-AS1 expression with functional assays evaluating cell proliferation, migration and invasion. In the same conditions, we analysed the expression of HA related genes and receptors in MDA-MB-231 cells. This analysis revealed that HAS2-AS1 knockdown stimulated the presence of a malignant phenotype, as its abrogation increased cell motility and invasion, as well as the expression of several HA related genes and the receptor CD44. These evidences suggested that HAS2-AS1 plays an important role breast tumor progression through alteration of HA metabolism.

Further analysis were conducted to understand the molecular mechanisms at the basis of the changes observed. LncRNAs can orchestrate gene expression through a variety of mechanisms, regulating transcription and translation, chromatin-remodelling and the interaction with other RNA species, i.e. miRNAs. HAS2-AS1 transcript contains a putative binding site for miRNA 186, a negative regulator of the pro-apoptotic receptor $P2X_7$ [2]. In our results we demonstrated that the

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overexpression of HAS2-AS1 decreased the abundance of miR-186, while the transcript of P2X7 and other targets of miRNA 186 (involved in cell cycle and autophagy) raised.

All together, these data suggest that the "sponge effect" of HAS2-AS1 is able to antagonise the function of miRNA 186 on its downstream targets and could explain the presence of a malignant phenotype after HAS2-AS1 silencing in MDA-MB-231.

2 - RIASSUNTO

La matrice extracellulare (ECM) è una fitta rete costituita da proteine e proteoglicani, la cui struttura è essenziale per mantenere l'architettura tissutale, per fornire la diffusione di molecole e per mediare le comunicazioni cellulari. Una sintesi deregolata dei componenti della ECM è spesso associata a uno stato patologico. Tra i vari glicosaminoglicani, l'acido ialuronico (HA) è un componente ubiquitario della ECM con una notevole importanza strutturale. Esso è in grado di modulare adesione, motilità, crescita cellulare e infiammazione dopo il legame con recettori cellulari come CD44 e RHAMM e l'attivazione di diverse vie di segnale. Nel microambiente tumorale, l'over-espressione dell'acido ialuronico sintasi 2 (HAS2) e la sovrapproduzione di HA sono spesso associate alla progressione tumorale e all'insorgenza di metastasi. Ciò vale anche per il cancro al seno, dove l'accumulo di HA e la over-espressione delle HA sintasi (HASes) da parte delle cellule stromali e tumorali, sono collegati alla malignità del tumore e alla sopravvivenza dei pazienti.

Per questo motivo, lo studio della regolazione della HAS2, principale enzima nella produzione di HA, è molto importante per comprendere lo sviluppo e la progressione del cancro al seno.

Recentemente, è stato scoperto che un *long non coding RNA* (lncRNA) chiamato *HAS2 natural antisense transcript 1* (HAS2-AS1) può modulare l'espressione di HAS2 e la produzione di acido ialuronico [1] tramite modificazioni epigenetiche. Anche se il ruolo di HA e HAS2 nel cancro al seno è ampiamente descritto, in letteratura poco è noto circa HAS2-AS1.

Tenuto conto di queste considerazioni, l'obiettivo di questo progetto è quello di studiare il ruolo di HAS2-AS1 nel cancro al seno.

In particolare, abbiamo confrontato il comportamento delle linee cellulari MDA-MB-231 (cellule ad alta invasività) e MCF-7 (cellule a bassa invasività) dopo la modulazione dell'espressione di HAS2-AS1 con test funzionali volti a valutare la proliferazione, la migrazione e l'invasione cellulare. Nelle stesse condizioni, abbiamo analizzato l'espressione di geni e recettori cellulari coinvolti nel metabolismo di HA in MDA-MB-231.

Secondo queste analisi, il silenziamento di HAS2-AS1 stimola l'insorgenza di un fenotipo maligno, in quanto la sua abrogazione è collegata ad un aumento della motilità e dell'invasione cellulare, così come l'espressione di diversi geni correlati al metabolismo di HA. Queste analisi suggeriscono che HAS2-AS1 svolge un ruolo importante nella progressione del tumore al seno attraverso l'alterazione del metabolismo di HA.

Inoltre, sono state condotte ulteriori analisi per comprendere i meccanismi molecolari alla base dei cambiamenti osservati in termini di aggressività cellulare. I LncRNA possono orchestrare l'espressione genica attraverso una varietà di meccanismi, regolando la trascrizione e la traduzione, il rimodellamento della cromatina e l'interazione con altre specie di RNA, come i miRNA. Il trascritto di HAS2-AS1 contiene, infatti, un sito di legame putativo per miRNA 186, un regolatore negativo del recettore pro-apoptotico $P2X_7$ [2]. Nei nostri risultati abbiamo dimostrato che l'over-espressione di HAS2-AS1 porta ad una diminuzione dei livelli di miRNA 186, mentre è stato osservato un incremento del trascritto di $P2X_7$ e altri target di miRNA 186 coinvolti nel ciclo cellulare e nell'autofagia.

Tutti insieme, questi dati suggeriscono che l'effetto "spugna" di HAS2-AS1 sia in grado di antagonizzare la funzione di miRNA 186 sui suoi bersagli a valle e potrebbe spiegare la presenza di un fenotipo maligno dopo il silenziamento di HAS2-AS1 in MDA-MB-231.

3 - INTRODUCTION

3.1   Breast cancer

Breast cancer is the most common malignancy and the major cause of cancer-related mortality of women in the world [3]. As it is a heterogeneous disease, the classification depends on different aspects and influences therapy response and prognosis.

Based on the anatomical origin of the tumor, breast cancer can be classified into lobular or ductal. Lobular tumors represent just a little percentage of breast cancer and originate from the lobules of the mammary gland, whereas ductal carcinomas are the most frequent (80%) and originate from the lobules. Ductal carcinomas can be further divided into luminal carcinomas (if originate from the epithelial cells of the ducts), or basal (if originate from the myoepithelial cells).

Moreover, according to the stage and TNM criteria, breast cancers can be divided into *in situ* and invasive, with a different description depending on the grade of involvement of lymph nodes and distant metastasis. There are different types of invasive carcinomas, sub classified into infiltrating ductal (80% of cases), invasive lobular (10% of cases) and other less common sub types like mucinous, medullary, tubular and inflammatory carcinomas [4].

The strategies to treat such malignancies depend on the molecular expression profile of different receptors, like ER, PR and HER2/neu.

Regarding the treatment, the endocrine therapy with compounds like tamoxifen is used in ERα positive tumours. The 17β-estradiol (E2)/ERα signalling is the most important pathways activated in human breast cancer, since about 70% of such cancers results positive to ERα. This targeted therapy cannot be used for triple negative (estrogen, progesterone and HER-2 negative) breast cancer which results in a more aggressive cellular phenotype and in a poorer outcome in terms of survival than ER positive tumours [5].

3.2   Hyaluronan (HA)

The extracellular matrix (ECM) is a molecular network made by proteins (i.e. collagen and elastin) GAGs and proteoglycans PGs whose structure and composition are critical for tissue organisation and homeostasis. This matrix, however, has not to be considered just as a scaffold which gives support to the cells; in fact ECM is able to bind secreted molecules and therefore can serve as reservoir for growth factors and cytokines allowing the diffusion of these molecules and modulating their activation status. Moreover, ECM components can have receptors on cell surface, like integrins

for collagen [6]. Modifications in the balance between the synthesis and the degradation of ECM components can modulate receptor signalling and thereby cellular behaviour, contributing to the genesis as well as to the progression of pathological processes.

Hyaluronan (HA) is a ubiquitous component of ECM found in all vertebrates and in certain bacteria. It is a polysaccharide belonging to the class of GAGs composed by repeating units of glucuronic acid and N-acetyl-glucosamine linked with β 1-3 and β 1-4 bonds, respectively (Fig. 1).

It has a simple chemical structure without typical modifications present in other GAGs as epimerisation or sulphation residues. Many of the functions of HA depend on specific HA binding proteins and PGs present on the cell surface and into the extracellular environment. HA structure and the deriving physical properties determine different functions of the polysaccharide: the anionic nature of HA along with its hydrodynamic volume create a size selective barrier for the diffusion of small molecules, while large molecules are partially or completed excluded. Moreover, such a solution create a network which regulates cell migration [7]. Its properties are also important for tissue hydration and lubrication. It is actively produced after tissue injury, tissue repair and wound healing [8]. In addition HA is an important mediator of the inflammatory process, in the modulation of the immune response and in the regulation of cell behaviour.

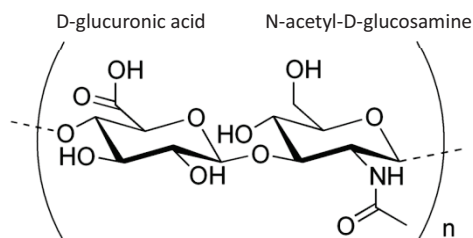**Figure 1 - Repeated disaccharide structure of HA**

3.2.1 HA biosynthesis and metabolism

Although many GAGs are synthesized and assembled in the rough endoplasmic reticulum and Golgi apparatus, HA is produced in the cytoplasm at the plasma membrane with the growing chain being extruded into the extracellular environment. HA is synthesized as a large, negatively charged and unbranched polymer with a

variable molecular weight ranging from 1,4 x 10^7 Da (HMW-HA) to 5 x 10^3 Da (LMW-HA).

In mammals three transmembrane isoenzymes are involved in HA synthesis; this family of lipid-dependent integral membrane proteins is called HAses (HA synthase 1, 2, 3 - HAS1, HAS2, HAS3). HASes have a molecular weight ranging from 42 to 64 KDa and have a double catalytic domain situated in the inner face of the membrane, which allows the interaction with the two substrates and the generation of the disaccharide units necessary for the production of HA chains. Although they have a similar structure and share a high homology in their amino acid sequence [9], the genes encoding for the three HASes are situated in different chromosomes: HAS1 is located on Chr 19q13.4, HAS2 on Chr 8q24.12 and HAS3 on Chr 16q22.1. Among HASes, HAS2 is considered the most important enzyme because of its fine regulation and its essential role for animal survival; HAS2 deficiency results in embryonic lethality and failure of the endocardial cushion formation, along with defects in yolk sac and vasculogenesis [10,11]. HAS2 mRNA expression is strictly related to HA production and it can be controlled at transcriptional and post-transcriptional levels. In particular, the availability of UDP-sugars precursors regulates the activity of the enzyme OGT (O-GlcNAc transferase), which transfer N-acetylglucosamin moieties stabilizing HAS2 protein in the membrane and increasing HA synthesis [12]; other post-translational modifications alters HAS2 protein levels, like the phosphorylation mediated by AMPK (which decrease the synthesis of HAS2 and in turn the production of HA) and the ubiquitination, which controls the dimerization and the activity of the protein [13]. Other kinases like PKC seems to have a role in the control of HAS2 [14].

HA is degraded by hyaluronidases (HYALs), which are responsible for the hydrolysis of the β 1-4 bonds between N-acetyl-D-glucosamine and D-glucuronic acid residues. In the human genome 6 HYAL-encoding genes have been identified, of which HYAL1, HYAL2 and PH-20 exhibit hyaluronidase activity. HYAL1 can be found in serum and urine as a lysosomal protein active a low pH, while HYAL2 and PH-20 are situated on the cell surface associated with cholesterol-rich lipid rafts [15]. Thanks to its localisation, HYAL2 binds HA and degrades it into fragments of about 20 KDa, which in turn are bound by CD44 to be internalized in the cells and further digested by HYAL1 in lysosomes [16].

According to the size, HA can exist as a high molecular weight polymer (HMW-HA), with a molecular weight reaching up to $2x10^4$ KDa and a prevalent extracellular localisation. This form of HA plays important hygroscopic properties and it is important to organize the ECM. Moreover, it has been demonstrated that HMW HA

has anti-inflammatory, anti-angiogenic and immune-suppressive functions, playing an important protective action in cell microenvironment. On the contrary, low molecular weight HA (LMW-HA) is able to stimulate angiogenesis, inflammation and the immune response. These fragments can derive from the action of hyaluronidases, reactive oxygen species or a dysfunction in HASes activity and can reach 10kDa [17]. Therefore, HMW-HA in general, correlates with tissue integrity and quiescence, whereas fragmented HA products are produced in presence of stress signals.
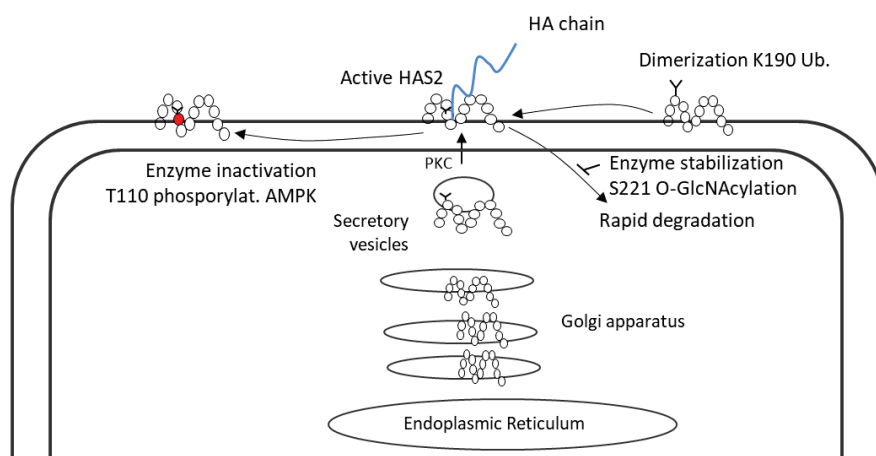
Figure 2 – Model of HAS2 post-translational modifications

3.2.2   *HA receptors and signalling*

The plethora of effects modulated by HA in physiological and pathological processes is mediated by different cell membrane receptors, which can trigger several signalling pathways.

The most common receptor involved in HA signalling is CD44, a type I transmembrane glycoprotein responsible for the communication and the adhesion between adjacent cells and between cells and ECM. The receptor is encoded by a single gene and it is expressed by cells mostly as the non-variant standard isoform (85 KDa). It is constituted by 20 exons which encode about 20 different CD44 isoforms [18] and possesses different levels of glycosylation. The intracellular domain is highly conserved and could be phosphorylated after the bound of the ligand to the extracellular domain. CD44 has no intrinsic kinase activity, thus it requires kinases and

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adaptor proteins able to link its cytoplasmic tail to the actin cytoskeleton and induce signalling cascades. Alternatively, CD44 can interact with other receptor acting like a co-receptor.

The interaction between HA and CD44 induces the phosphorylation of the cytoplasmic domain activating pathways like PI3K/AKT, RAS/RAF1, ERK1/2, WNT/β catenin, which all have effects on cell cytoskeleton and stimulate cell migration, proliferation and development [19].

Another HA biding molecule is RHAMM, which takes its name from the acronym Receptor for Hyaluronan Mediated Motility. The receptor can be produced in different isoforms through an alternative splicing mechanism and its cellular localization is ubiquitous, from the cell surface to the cytoplasm, from the nucleus to the extracellular matrix. Intracellular RHAMM can interact with the cell cytoskeleton modulating cell motility, proliferation, tissue repair and inflammation. HA-RHAMM interaction can trigger several pathways like the ERK1/2, PKC (protein kinase C9), FAK, NF-KB and PI3K [20,21].
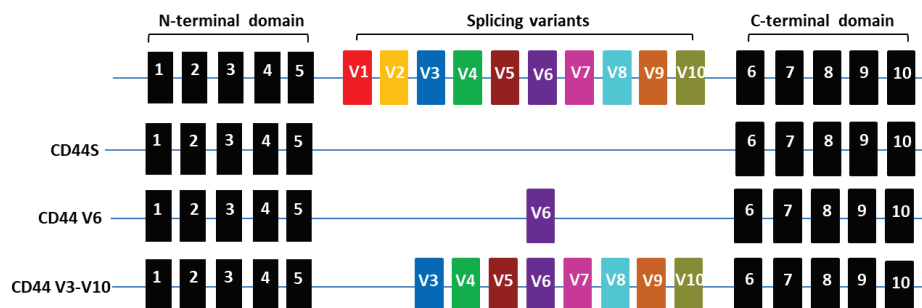

Figure 3 - CD44 gene structure and schematic representation of some of its variants
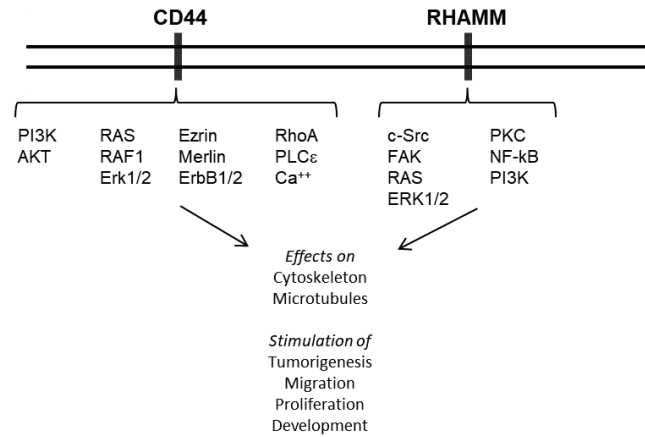
CD44   RHAMM

PI3K   RAS   Ezrin   RhoA   c-Src   PKC
AKT   RAF1   Merlin   PLCε   FAK   NF-kB
        Erk1/2 ErbB1/2 Ca++   RAS   PI3K
                                       ERK1/2

Effects on
Cytoskeleton
Microtubules

Stimulation of
Tumorigenesis
Migration
Proliferation
Development

Figure 4 - Schematic representation of HA receptors signaling and their involvement in cell function

3.2.3   HA metabolism and breast cancer

The remodelling of ECM during carcinogenesis plays a key role in tumor progression, favouring an environment with increased inflammation and angiogenesis that sustains tumor growth. Together, tumor cells and fibroblasts release grow factors and produce pro-inflammatory cytokines including FGF, TGFβ and HGF, which stimulates the synthesis of HASes mRNA and the production of HA.

A wide number of evidences have demonstrated that HA regulates breast tumor progression and cell aggressiveness *in vivo* and *in vitro* [22–24]. Cell culture studies showed that invasive breast cancer cells like the triple negative MDA-MB-231 and HS578T expressed higher levels of HASes mRNA and synthesised higher amounts of HA than breast cancer cell lines with a less aggressive phenotype like MCF-7 [25]. Moreover, HAS2 is powerfully increased in bone metastatic cells (MDA-MB-231-BM) compared to the parental MDA-MB-231 cells. Furthermore, it has been demonstrated that HAS2 expression promotes cells invasion [26,27], whereas its suppression by RNA antisense or by 4-MU inhibits tumorigenesis and progression of breast cancer cells [28–30]. Koyama et al. demonstrated that the overexpression of HAS2 in mammary epithelial cells of MMTV-Neu transgenic mice increased the amount of HA and enhanced the growth of mammary tumors [31].

Other studies conducted on mammary tumor biopsies reported that HAS2-overexpressing tumors showed increased angiogenesis and inflammatory cells recruitment, supporting the progression of the malignancy [32].

In breast tumors HA can be directly produced by tumoral cells or by stromal cells, which are a rich source of this biopolymer. As shown in figure 5, HA staining patterns within breast tumors showed a high deposition in the stroma and this accumulation was related to poor patient survival [23]. Interestingly HA levels were related to lymph node positivity and poor differentiation [23]. HA presence in tumoral stroma is also related to the stage of breast tumors: a comparison between *in situ* and invasive ductal carcinomas demonstrated that HA levels associated with the invasive phenotype [33]. Increased levels of HA in tumor stroma are often associated to high levels of HASes mRNA and proteins. Indeed, the expression of the three HASes in the stroma corresponded with reduced overall survival [34].

However, HAS2 expression is particularly linked to basal-like and triple negative tumors, where its expression is often associated with reduced survival of patients [35].

Other HA related molecules have a crucial role in breast cancer progression: the receptor of HA CD44 is considered a marker of stamness in breast cancer. The subpopulation CD44$^+$/CD24$^-$ of breast tumor is associated to invasive properties and a poorer prognosis. In fact, the interaction between HA and its receptor CD44 promotes cytoskeletal remodelling favouring growth, survival, adhesion and invasion. This behaviours would be influenced by the interaction with LMW-HA or HMW-HA, triggering different (and sometimes opposite) downstream signalling [25,36]. It has also been demonstrated that CD44 plays an important role in the formation of metastasis, promoting the invasion and the adhesion of tumoral cells to bone marrow. Moreover, as shown for HAS2 and HA, CD44 levels are enhanced in triple negative breast cancer [25]. Several studies reported that there is a peculiar expression of CD44 variants among different breast cancer subtypes and that this heterogeneity is associated to different clinical marker like HER2, ER and PR, suggesting the involvement of CD44 splice variants in specific oncogenic signalling pathways [37].

CD44 and HAS2 are also involved in the epithelial to mesenchymal transition (EMT), a physiological process typical of the developmental stage which can occur in cancer. EMT is characterized by the loss of adhesion molecules which allow cell-cell and cell-matrix connections, leading to a mesenchymal phenotype characterised by migration and invasion of the surrounding areas [38–40].

Also HA degrading enzymes are described to play a key role in this pathology. For example HYAL1 and HYAL2 displayed an aberrant expression in different type of

tumors including breast cancer [41–44], and the upregulation of HYAL1 promoted tumor cell proliferation, migration, invasion and angiogenesis [42].

Concluding, all of these evidences describe HA and its metabolizing genes as important regulators in breast cancer initiation progression.
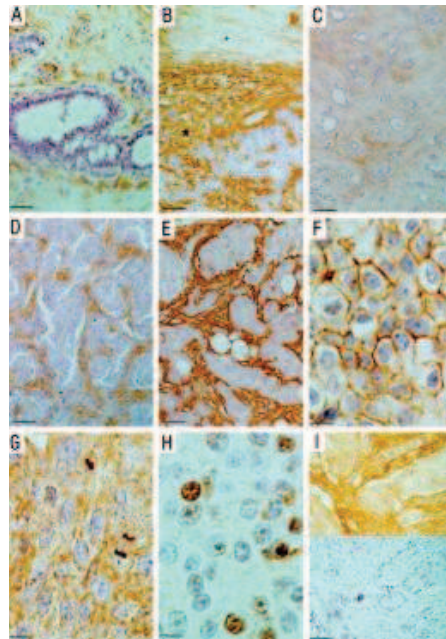
Figure 5 - The expression patterns of HA in breast carcinoma lesions. (A) Normal breast tissue. (B) Typical HA signal intensity difference between normal (+) and peritumoral (*) stroma. Views of breast cancer cases in which the intensity of HA signal in stroma is weak (C), moderate (D), or strong (E). Examples of tumor cell-associated HA from areas with HA signal on plasma membranes (F), cytoplasm (G), and some of the nuclei (H). A breast cancer case with cytoplasmic HA signal and its negative control treated with *Streptomyces hyaluronidase* before the staining (I).

*Image from "Hyaluronan in Peritumoral Stroma and Malignant Cells Associates with Breast Cancer Spreading and Predicts Survival", Auvinen at al; Am. J. Pathol. 2000;156:529–36.
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3.3 Non-coding RNAs (ncRNAs)

Emerging studies from the human genome sequencing project revealed that more than 80% of the human genome is actively transcribed into RNA, but just a little amount (3%) of the total RNA codifies for translated proteins. Although non-coding RNA was believed to be "transcriptional noise" or a sequencing artefact, recent studies demonstrated that this portion of the transcriptome could play a key role in the regulation of gene expression. Interestingly, in eukaryotes, the proportion of ncRNAs increases with the complexity of the organism, suggesting that this ncRNAs provide the extra layer of developmental complexity required for the evolution of eukaryotes [45].

In general, these ncRNAs included some of the classical housekeeping RNAs like ribosomal RNA (rRNA) and transfer RNA (tRNA); however, relying on transcript size, new classes of ncRNAs with a regulative function have been described: short ncRNAs with less than 200 nt and long ncRNA (<200 nt). Short ncRNA can be further classified into microRNAs (miRNAs), short interfering RNA (siRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA) and PIWI-interacting RNA (piRNA).
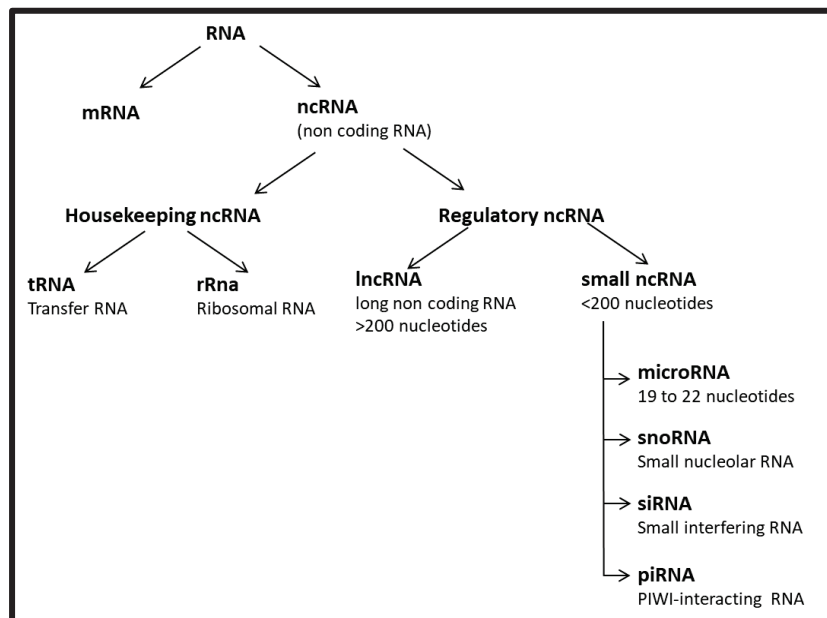

Figure 6 - Schematic classification of RNA

3.3.1 *MicroRNAs biogenesis*

Micro RNAs (miRNAs) are a class of small ncRNA, which post transcriptionally regulates the expression of target genes through the complementarity with the mRNA sequence (generally in the 3' UTR). The generation of miRNAs is a multi-step process, which starts with synthesis of a primary capped and polyadenylated transcript by RNA polymerase II (pri-miRNA). This long transcript is generally longer than 1 Kb in length, contains more than one hairpin structure and can generate different mature miRNAs. Following the transcription, pri-miRNAs are recognised in the nucleus and cleaved by the Microprocessor complex, which contains the RNAse III enzyme Drosha. The cleaved portion of the hairpin (pre-miRNA) is exported via Exportin-5 to the cytoplasm, where it forms the classical 60-90 nucleotides double-stranded structure consisting in the 5p arm and the 3p arm. This structure is further processed by the RNAse III enzyme Dicer into a ~ 22 nt duplex [46]. According to the canonical maturation process, the guide strand (5p arm) is the one to bind the mRNA target sequence, while the 3p strand is discarded. Nevertheless, new studies demonstrated that the 3p arm would be not degraded and would be selectively recognised by the RNA-induced silencing complex (RISC) to bind the predicted targets [47]. The selection of mature miRNA (5p or 3p arm) is determined by the Argonaute (Ago) protein on the basis of the hydrogen bonding selection mechanism. Generally, the targets occurs at the mRNA 3'-untranslated region (UTR), forming a partial hybrid with the miRNA seed region (nucleotides 2-7). However, some recent reports have shown that the targeting can occur also at the 5' UTR and coding regions of genes.

The final result of this process consists in the inhibition of protein expression through the suppression of the transcription or degradation of the target mRNA.

MiRNAs are highly conserved across different species. Recent analysis about the grade of conservation of miRNAs revealed 34 miRNAs families common to protostomes and deuterostomes [48]. Interestingly, the number of miRNAs seems to be related to the complexity of a species: for example, within vertebrates, there is a further increase in the miRNA numbers in the lineage leading to placental mammals [49], underlying their role in the evolution of lineages.

Finally, their expression can be tissue specific and they can perform important functions in cell proliferation, cell differentiation, senescence, apoptosis, cell division, migration, morphogenesis, tissue development, tumor growth, angiogenesis and metastasis.
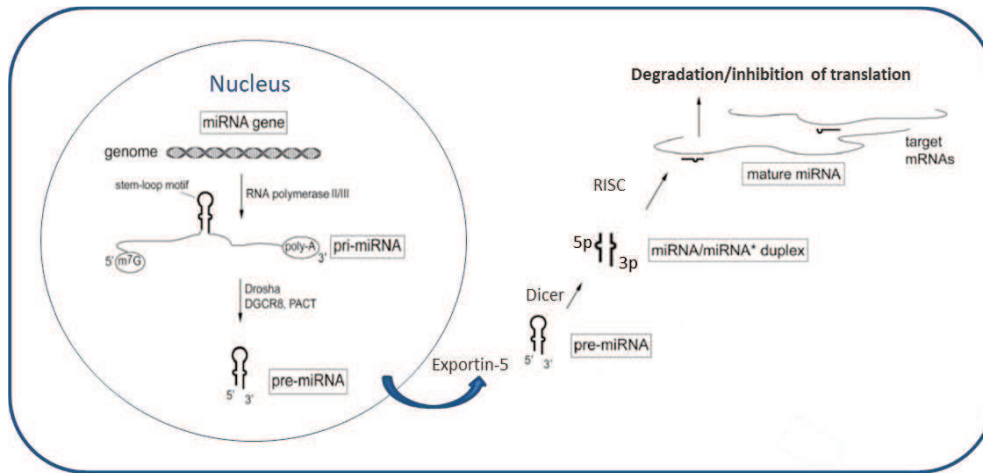
Figure 7 - MiRNAs synthesis pathway. Production and maturation of miRNAs starts with the transcription of a pri-miRNA and the action of DROSHA, Exportin-5 and Dicer to create a mature RNA, which in turn can bind the RISC complex. The final effect consist in the degradation of the mRNA target or in the inhibition of translation.

3.3.2   MicroRNAs and breast cancer

Recent studies have found that miRNAs are closely related to tumorigenesis and can act as oncogenes or tumor suppressor genes to influence the occurrence and development of tumor. Approximately the 50% of miRNAs are situated close to fragile regions and malignancy-related genomic sites, underlying the associations with cancer progression.

The axis HA-HAS2-CD44 is involved in the regulation of different miRNAs and can actively influence breast cancer cells malignancy. MiRNA 21 is often upregulated in different cancer types and it is currently considered an oncogene. The interaction between HA and its receptor CD44 stimulates the expression of miRNA 21 [50], which in turn can modulate cell invasion, metastasis, drug resistance and proliferation through the stimulation of survival proteins and inhibition of apoptosis [50]. Another study demonstrated that the activation of CD44 after HA binding, promoted the activation of JNK and c-JUN, a signalling pathway involved in the onset of breast cancer [51]. Moreover, HA-CD44 interaction promotes miRNA 302 expression, with repercussion on the chemoresistance of breast cancer stem cells [52]. A similar stimulation is obtained with the oncogene miRNA 10b, which has been reported to

promote invasion and metastasis of breast cancer cells [53]. Furthermore, a recent study demonstrated that 200 KDa HA fragments can stimulates a panel of miRNAs (including miRNA 10b) in cancer stem cells [54].

On the other hand, HAS2 expression can be actively regulated by miRNAs; a study of Liu et al., demonstrated that HAS2 is targeted and negatively regulated by miRNA 26b, resulting in the stimulation of apoptosis mediated by caspase 3 and CD44 [55].

HAS2 mRNA has been demonstrated to be the target of miRNAs from the tumor suppressor family let7 and a study of Yang et al. showed that the abrogation of HAS2 expression via let7 influenced cell survival, adhesion and invasiveness [56].

3.3.3 Long non coding RNAs biogenesis

LncRNAs have gained widespread attention in recent years as key regulators of a variety of cellular functions and diseases. The human transcriptome contains up to 16000 lncRNAs, which are transcribed by RNA polymerase II, capped, polyadenylated and spliced like common mRNA.

Although several ncRNAs , such as miRNAs, show a high conservation rate across species genome, most of lncRNAs are not highly conserved in their sequence [57], suggesting that they can be under different selection pressures. Differently from mRNAs, which have to preserve the codon sequence to prevent mutations, selection may only conserve little portions of lncRNAs sequences with a particular structure or sequence specific interactions but exhibit a tissue specific expression [58].

LncRNAs are mainly situated into the nucleus but a substantial proportion reside within, or is shuttled, to the cytoplasm where they regulate mRNA translation and protein localisation [59]. They are ubiquitous molecules and exhibit specific expression. The expression patterns are often associated to a single cell line, but recent researches showed that there could be specific cohorts of lncRNAs in a given tissue [60].

Depending on their orientation with reference to protein-coding genes, lncRNAs can be classified into sense, antisense, intronic and intergenic [61]. Although the functions of lncRNAs are widespread and still to be elucidated, they may be defined into four archetypes of molecular mechanism [62]:

- Archetype I – signals: the lncRNAs function as molecular signals or indicators of transcriptional activity;
- Archetype II – decoy: as decoys, lncRNAs can titrate away other molecules, like RNAs, transcription factors or proteins acting like negative regulators. In

particular, they can bind and compete for the binding of miRNAs, preventing their interaction with mRNA targets. These lncRNAs are known as ceRNAs (Competive Endogenous RNAs) and their effect on miRNAs is also known as "sponge effect";

- Archetype III – guide: as guides, lncRNAs are able to direct the localization of ribonucleoprotein complexes to specific targets:
- Archetype IV – scaffolds: lncRNAs can act like a structural supports upon which molecular components are assembled. This would help to stabilise nuclear structures and signalling complexes affecting chromatin structure and histone modifications.
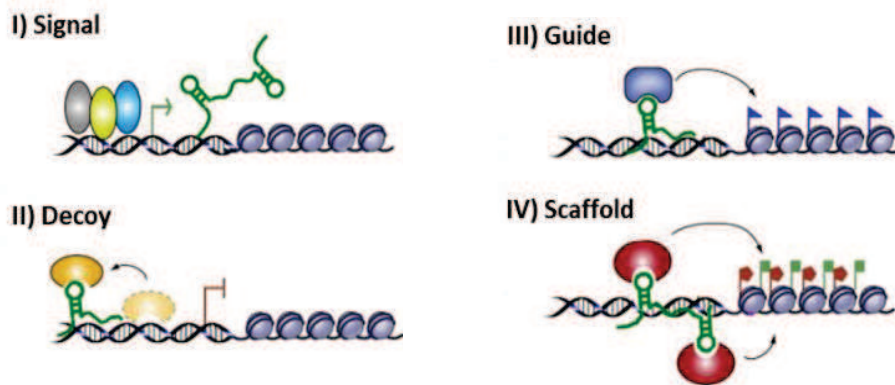

Figure 8 – Schematic representation of of lncRNAs molecular mechanisms. I) As signals lncRNAs expression can reflect the combinatorial actions of transcription factors or signaling pathways to indicate gene regulation in space and time**.** II) As decoys, lncRNAs can titrate transcription factors away from chromatin. A further example is lncRNAs decoy for miRNA target sites (not shown in the figure). III) As guides, lncRNAs can recruit chromatin modifying enzymes to target genes, either in cis or in trans to distant target genes. IV) As scaffold, lncRNAs can bring together multiple proteins to form ribonucleoprotein complexes.

3.3.4   Long non coding RNAs and breast cancer

Although the function of lncRNAs remains largely unknown, recent studies have demonstrated the functional importance of lncRNAs in embryonic development, cell differentiation, and various human diseases, including cancer.

Like proteins, lncRNAs may mediate oncogenic and tumor-suppressive functions; for instance, the lncRNA HOTAIR (HOX Antisense transcript RNA) is suggested to silence tumor suppressor genes favouring metastasis formation and malignancy in breast

cancer [63], while lincRNAp21 mediates global gene repression in the p53 pathway [64]. Genome wide association studies on cancer revealed that more than 80% of cancer associated-polymorphisms occur in non-coding regions, suggesting that a significant fraction of genetic etiology of cancer is related to lncRNAs [45]. Several studies demonstrated that lncRNAs are frequently deregulated cancer. Interestingly, numerous lncRNAs show different expression patterns in breast cancer tissues compared to normal breast tissues [58] and display a dissimilar expression also within different breast cancer subtypes; for example, lncRNAs expression can be associated to ER signalling [58] or triple negative status [57,65,66]. These differences in lncRNAs expression could serve as diagnostic biomarker tools and may be potential targets for individual therapy.

3.3.5   Functional interactions among miRNAs and lncRNAs

Several studies in the last years began to demonstrate post transcriptional interactions among lncRNAs and miRNAs in mammals.

Some miRNAs can degrade lncRNAs, controlling their amount and functions in different cellular processes, as described for lincRNA-p21 (degraded by let 7), HOTAIR (by let-7), MALAT1 (by miRNA 9) and LOC28594 (by miRNA 211) [67].

As mentioned above, other lncRNAs can serve as sponges/decoys for miRNAs as described for linC-MDI (sequestering miRNA 133 and miRNA 135), MALAT 1 (miRNA 206) [68] and PVT 1 (miRNA 186).

Moreover, several lncRNAs can compete with miRNAs for mRNA binding, like the antisense BACE1, which competes with miRNA 485-5p for binding to BACE 1 mRNA [69].

Lastly, there are some examples of lncRNAs which can generate miRNAs and other small RNAs, as shown for linc-MD1, which generates miRNA 206 and miRNA 133b [67].

3.3.6   HAS2 natural antisense transcript (HAS2-AS1)

The production of HA mainly depends on the activity and regulation of the enzyme HAS2. Recently, it has been described that HAS2 expression is regulated by the lncRNA HAS2-AS1 [1]. HAS2-AS1 is a tetra exonic natural antisense transcript synthesized from the opposite genomic DNA strand of the HAS2 locus on chromosome 8. HAS2-AS1 was first identified as a lncRNA by Chao et al. [29] who described two variants of different length generated by alternative splicing named

HAS2-AS1 short (174 nucleotides) and HAS2-AS1 long (257 nucleotides). HAS2 exon 1 and HAS2-AS1 exon 2 share sequence complementary starting ~70 bp from the presumed transcription start site of human HAS2 [1].

The study of Chao et al. demonstrated that the overexpression of the variants HAS2-AS1 short and long reduced HAS2 expression and HA production and inhibited the proliferation of human osteosarcoma cells [29]. On the contrary, other studies demonstrated that HAS2-AS1 showed a coordinated expression with HAS2 in the renal proximal tubular epithelial cell [70] and that HAS2-AS1 stabilised HAS2 mRNA via chromatin remodelling in human aortic smooth muscle cells [1]. Recently, the coordinated expression between HAS2-AS1 and HAS2 was also described in oral squamous cell carcinoma, where HAS2-AS1 mediated hypoxia-induced cell invasiveness and epithelial to mesenchymal transition stabilizing HAS2 [71]. Considering such a different regulation of HAS2-AS1 on HAS2 expression and cell behaviour, it could be possible that the function of HAS2-AS1 might vary in different types of cell.
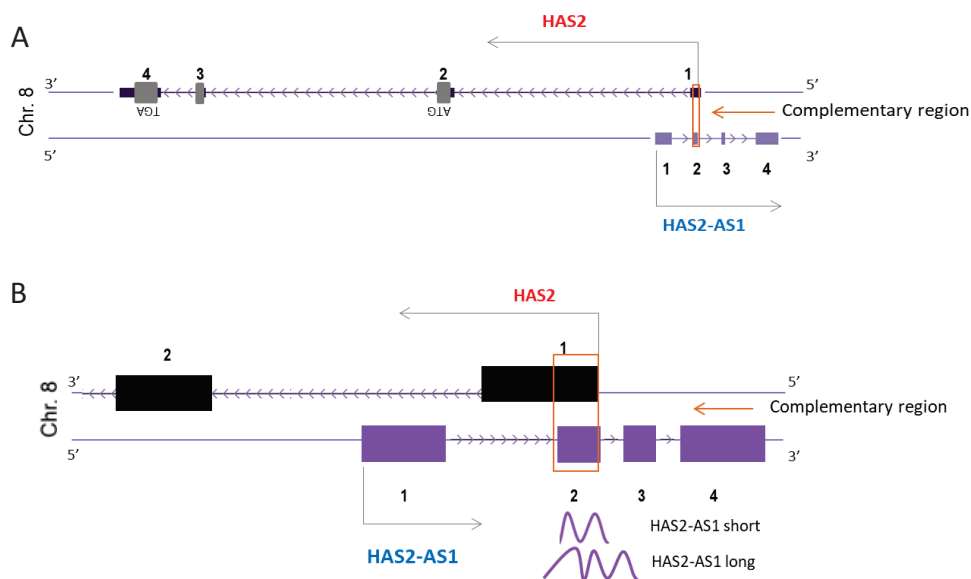

Figure 9 - HAS2-AS1 gene structure. (A) Schematic representation of HAS2 and HAS2-AS1 locus on chromosome 8. (B) Representation of the complementary region between HAS2 and HAS2-AS1 and relative isoforms of HAS2-AS1.

4 - AIM OF THE WORK

Breast cancer is one of the leading causes of death among women in western countries and alterations of ECM have a key role in the onset and progression of the disease.

Considering that HA and HAS2 are active modulators of breast cancer aggressiveness and metastasis and that HAS2-AS1 can regulate HAS2 expression and HA synthesis, the aim of this study was to investigate the role of HAS2-AS1 in breast cancer cells.

Since little is known about HAS2-AS1 function, in particular in breast cancer, the first step in this project was to modulate HAS2-AS1 expression (silencing and overexpression) and perform functional assays in order to evaluate cell viability, invasion and migration of the breast cancer cell lines MDA-MB-231 and MCF-7. Subsequently, further analysis about gene expression and microRNA expressions were conducted to understand the possible molecular mechanisms that mediated the changes observed in cell behaviour.

5 - MATERIAL AND METHODS

5.1 Cell cultures

MDA-MB-231 (triple negative breast cancer cell line) was purchased by the American Type Culture Collection (ATCC), grown and maintained in complete DMEM medium supplemented with 10% of FBS at 37°C in the presence of 5% CO_2. MCF-7 (low metastatic, ERα-positive) breast cancer cell line was obtained from ATCCand routinely harvested in a humidified 95% air/5% CO_2 incubator at 37 °C in RPMI with 10% FCS at 37°C. MCF-7 Csh and MCF-7 sp10 (ERα-negative) were a kind gift from N. Karamanos Lab, University of Patras, Greece [72]. They were harvested in complete medium DMEM 10% FBS with 0.8 µg/mL puromycin dihydrochloride (sc-108071; Santa Cruz Biotechnology, Inc.) and maintained at 37°C in the presence of 5% CO_2

5.2 Cell transfection

MDA-MB-231 and MCF-7 cells were plated in a six-well plate one day before the transfection to reach 70-80% confluency. The cells were transiently transfected using Dharmafect (Dharmacon) in OPTI-MEM medium (Gibco) and the siRNA n265529 targeting a coding region of the lncRNA HAS2-AS1 (20 nM, Thermo Fisher Scientific). Twenty-four hours after transfection, OPTI-MEM was replaced by Dulbecco's modified Eagle medium (MDA-MB-231) or RPMI (MCF-7) with 10% FBS. The two variants of HAS2-AS1 (long and short) exon 2 were transfected with lipofectamine 2000 (500ng, Invitrogen) in OPTI-mem medium.

In other set of experiments HAS2-AS1 siRNA and HAS2-AS1 were transfected in MDA-MB-231 using a nucleofector apparatus (Amaxa) and the Kit V (Lonza) following manufacturer's instructions.

In both the cases control samples were transfected with a scrambled sequence (silencer negative control #1, Ambion) or a pcDNA3.1 empty vector. Target downregulation/upregulation was confirmed by qPCR and the transfection of a GFP encoding plasmid was used as positive control.

5.3 Cell viability (MTT assay)

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Forty-eight hours after siRNA transfection, 100 cells were seeded in a 96-well plate and cultured for 72 hours. Afterward, 4 hours of incubation in the presence of MTT followed. The reaction was stopped with 10% SDS and read at 595nm.

5.4 Cell invasion (Matrigel invasion chamber assay)

The matrigel invasion assay was performed in MDA-MB-231 cells. Forty-eight hours after the transfection, $2,5 \times 10^5$ cells were seeded in each invasion filter (BD Biosciences) with complete medium. The day after, the medium was replaced with a free medium serum in the upper part of the chamber. The bottom of the well was filled with DMEM 10% FBS as a chemoattractant. After 18 hours, the cells in the upper chamber were removed with a cotton swab and the ones on the lower surface were fixed and stained with Diff-Quik dye (Medion). Excised and mounted filter membranes were photographed using a Zeiss Axiovert microscope equipped with Axiovision software (Zeiss) at 100 magnification. Five fields per each image were counted. Relative invasiveness was expressed as percentage of the number of cells vs the control.

5.5 Gene expression determinations by quantitative RT-PCR

Total RNAs were extracted with the innuPREP RNA Mini Kit (Analytik Jena), retro transcribed using the High Capacity cDNA synthesis kit (Applied Biosystems) and amplified on an ABI Prism 7000 instrument (Applied Biosystems). Taqman probes and primers and are reported in the tables below. The relative gene expression was determined by comparing ΔCt [73].

Table 1 – Taqman assays used for quantitative RT-PCR

SPECIES	ASSAY	ASSAY ID
Homo Sapiens	HAS2	Hs00193435_m1
Homo Sapiens	HAS3	Hs00193436_m1
Homo Sapiens	HAS2-AS1	Hs03309447_m1
Homo Sapiens	HYAL2	Hs00186841_m1
Homo Sapiens	TOTAL CD44	Hs01075861_m1
Homo Sapiens	CD44 V8-V10	Hs01081475_m1
Homo Sapiens	CD44 V3-V10	Hs01081480_m1
Homo Sapiens	CD44S	Hs01081473_m1
Homo Sapiens	P2RX7	Hs00175721_m1
Homo Sapiens	P21	Hs00355782_m1
Homo Sapiens	ACTIN	Hs99999903_m1

Table 2 – Sybr green assays used for quantitative RT-PCR

GENE	FARWARD PRIMER	REVERSE PRIMER
LC3b	5' AGCAGCATCCAACCAAAATC 3'	5' CTGTGTCCGTTCACCAACAG 3'
VIMENTIN	5' TCAGCATCACGATGACCTTGAA 3'	5' CTGCAGAAAGGCACTTGAAAGC 3'
TWIST	5' GCGGCCAGGTACATCGACTT 3'	5' TGCAGCTTGCCATCTTGGAG 3'
SNAIL	5' CGAGCCCAGGCAGCTATTTC 3'	5' CCCGACAAGTGACAGCCATT 3'
OCLUDIN	5' CCACTGGCTGAGCTGATTGTG 3'	5' TCTGTTCTTTGCCCATGAGTGG 3'
ZO1	5' GTGTTGTGGATACCTTGT 3'	5' GATGATGCCTCGTTCTAC 3'
E-CADHERIN	5' CACCTGGAGAGAGGCCATGT 3'	5' TGGGAAACATGAGCAGCTCT 3'
HAS2-AS1 VECTOR	5' AAGCCTGTGGAAGACTCAGC 3'	5' CTAGAAGGCACAGTCGAGGC 3'
ACTIN	5' TCAAGATCATTGCTCCTCCTGAG 3'	5' ACATCTGCTGGAAGGTGGACA 3'

5.6   MiRNAs expression determinations by quantitative RT-PCR

Total RNA and miRNAs from MDA-MB-231 lysates were isolated using the mirVana miRNA isolation kit (Thermofisher Scientific) according to the manufacturer's instruction. Concentration and purity of miRNAs were determined by spectrophotometry at 260 nm. The High Capacity cDNA synthesis kit (Applied Biosystems) and the Taq Man MicroRNA Assay (PN 4427975, Applied Biosystem) were used to perform quantitative RT-PCR and to determine the levels of miRNA 186-5p, miRNA 186-3p and U6 snRNA (endogenous control) using a 7000 real time PCR system instrument (Applied Byosistem). The following Taq Man assays from Appliyed biosystem were used: hsa-miR-186-3p (002105), hsa-miR-186-5p (002285) and U6 snRNA (001973).

Relative quantification was calculated as described above by comparing ΔCt.

5.7   Migration assay

A wound healing assay was performed to determine MDA-MB-231 migration. Eight hours before the assay the complete medium was replaced with a free serum medium. Three scratches per well were done with a 20 µl pipette tip. Cells were washed once with PBS to remove the detached cells and fresh new medium (without serum) was added to each well. Pictures were taken at 0, 3, 6 and 16 hours. Results are presented as migration index.

To evaluate the role of tumor microenvironment, untreated MDA-MB-231 were incubated with the medium of transfected MDA-MB-231 with HAS2-AS1 long, HAS2-AS1 short, and HAS2-AS1 siRNA. After the creation of 3 scratches/well, cells were washed in 1X PBS and incubated with the conditioned medium diluted 1:1 in DMEM

10% FBS. Pictures were taken at 0, 3, 6 and 24 hours. Results are presented as migration index.

5.8 HA quantification and pericellular coat of MDA-MB-231 evaluation

To detect the amount of HA in MDA-MB-231 after HAS2-AS1 silencing and overexpression, cell culture media were collected 48 hours after the transfection and diluted 1:100. The quantification of HA was performed with the Hyaluronan quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions.
To evaluate the pericellular coat of HA a particle exclusion assay was performed [74,75]. Briefly, 48 hours after the transfection $1x10^6$ fixed human red blood cells were washed in PBS and added to each well. After an incubation time of 30 minutes, cells were examined by contrast microscopy and 10 pictures per well were taken. As a control, cells were treated with 2U/ml of bovine testis hyaluronidase (SIGMA). The analysis of the images and the relative quantification was performed using the image analysis software Imagej.

5.9 Western blot analysis

Protein samples were separated on 8 % SDS-PAGE at 30 mA for 2 hours in 1X running buffer and transferred to nitrocellulose membrane 16 V for 45' in 1X transfer buffer. The membrane was blocked in 5% non-fat dry milk/PBS-Tween 20 0,1% for 1 hour and incubated with primary antibody overnight at 4°C. After 3 washes in PBS-T for 30 minutes, the membrane was incubated with secondary antibody for 1 hour at room temperature. The membrane was washed three times in PBS-T and then incubated with the reagent ECL Prime® (Amersham) to detect chemioluminescence. Used Antibodies: p44/42 (#4695, Cell signaling, diluted 1:1000), pp44/42 (#9101, Cell signaling, diluted 1:1000), α-tubulin (#2125, Cell signaling, diluted 1:1000).

5.10 Flow cytometry analysis

To detect cell surface breast cancer stem cell markers, control and HAS2-AS1 siRNA transfected cells were incubated with 10 µl of anti-CD44-FITC, anti-CD24-PE and the FITC and PE isotype control antibodies for 30 min at room temperature in the dark. Stained cells were analysed by a cube-8 flow cytometer (Sysmex/Partec, Muenster, Germany). Afterwards, the cells were incubated for 1 h at 37 °C in water bath in dark with agitation at 10 min interval. Finally, the cells were centrifuged at 400xg for 5 min and were resuspended in 1 mL assay buffer and analysed by flow cytometry.

5.11 Affymetrix screening

The transcriptome MDA-MB-231 cells after HAS2-AS1 transient silencing was compared against negative control siRNA-transfected cells using Affymetrix U133 v2 Gene Arrays and GeneSpring GX 11.0. After Robust Multiarray Average (RMA) normalization, the list of candidate genes regulated by HAS2-AS1 was generated using the filtering criteria of $p < 0.05$ and fold change of 2 [76]. Three independent experiments were performed.

5.12 Bioinformatical analysis

The informations on the expression of HAS2-AS1 in different tumours were obtained using available genomic data on Mitranscriptome site (www.mitranscriptome.org/) [77]. Data on HAS2-AS1 mRNA expression in different tumoral cell lines were obtained on GEO2R (www.ncbi.nlm.nih.gov/geo/geo2r/ GEO2 Accession number GSE58643 and GSE58844). MDA-MB-231 and MCF7 cell lines data were confirmed by quantitative RT-PCR.

To determine predicted miRNA 186 binding sites on HAS2-AS1 we used the bioinformatic tools RNA hybrid (https://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid) [78], miRanda (www.microrna.org) [79,80] and DIANA-microT-CDS (http://diana.imis.athena-innovation.gr) [81].

The informations about miRNA 186 regulated pathways were found at http://www.microrna.gr/miRPathv3 [82].

The analysis on the conservation of miRNA 186 binding site on HAS2-AS1 were performed at www.ensembl.org.

5.13 Statistics

All experiments were repeated at least three times in duplicates. Data are shown as the mean values ± s.e.m. The data were tested for significance employing the one-way ANOVA test followed by Tukey's post hoc test to identify differences between the means. Statistical comparison between two groups was made using an unpaired Student's t-test. The level of significance was set at p<0.5.

6 - RESULTS

6.1   HAS2-AS1 is highly expressed in tumors and correlates with cell aggressiveness

The expression of HAS2-AS1 in human healthy and tumoral tissues was determined through bioinformatic data available online on www.mitranscriptome.org, a catolog containing analysis of high-throughput RNA sequencing (RNA-Seq) data from over 6,500 cancer and tissue samples [77]. As reported in figure 10A, the expression levels of HAS2-AS1 was generally higher in tumoral than in normal tissues, with particular attention to prostate, thyroid, bladder and breast. Regarding breast cancer, further analysis on GEO2R (www.ncbi.nlm.nih.gov/geo/geo2r/) revealed that the levels of HAS2-AS1 were increased in more aggressive cancer cell lines, like the triple negative MDA-MB-231 and Hs578T. On the contrary the expression of HAS2-AS1 was lower in non-invasive breast cancer cells, such as MCF-7 and Zr-75-1 (Fig. 10B.). To confirm such bioinformatical analysis, qPCR experiments were conducted in two different breast cancer lines: MDA-MB-231 (high invasive and triple negative for ER, PR and HER2) and MCF-7 (low invasiveness and ER positive). In line with bioinformatical data, our results showed that the expression of HAS2-AS1 was higher in MDA-MB-231 than MCF-7 (Fig. 10C).

To better investigate the role of HAS2-AS1 in breast cancer cells, we also tested its expression in the cell lines MCF-7 sp10+ (a peculiar cell line that derives from MCF-7 but has been transformed in a highly aggressive phenotype via stably silencing of ERα), a kind gift from N. Karamanos, University of Patras, Greece [72]. Interestingly, when MCF-7 acquired a more aggressive phenotype after ERα knockdown, HAS2-AS1 and HAS2 mRNA increased (Fig. 10C).

Our results suggest that HAS2-AS1 could be important in the switch from healthy to tumoral tissue and that there could be a possible relationship between its expression and the severity/aggressiveness of breast tumours and cell lines.
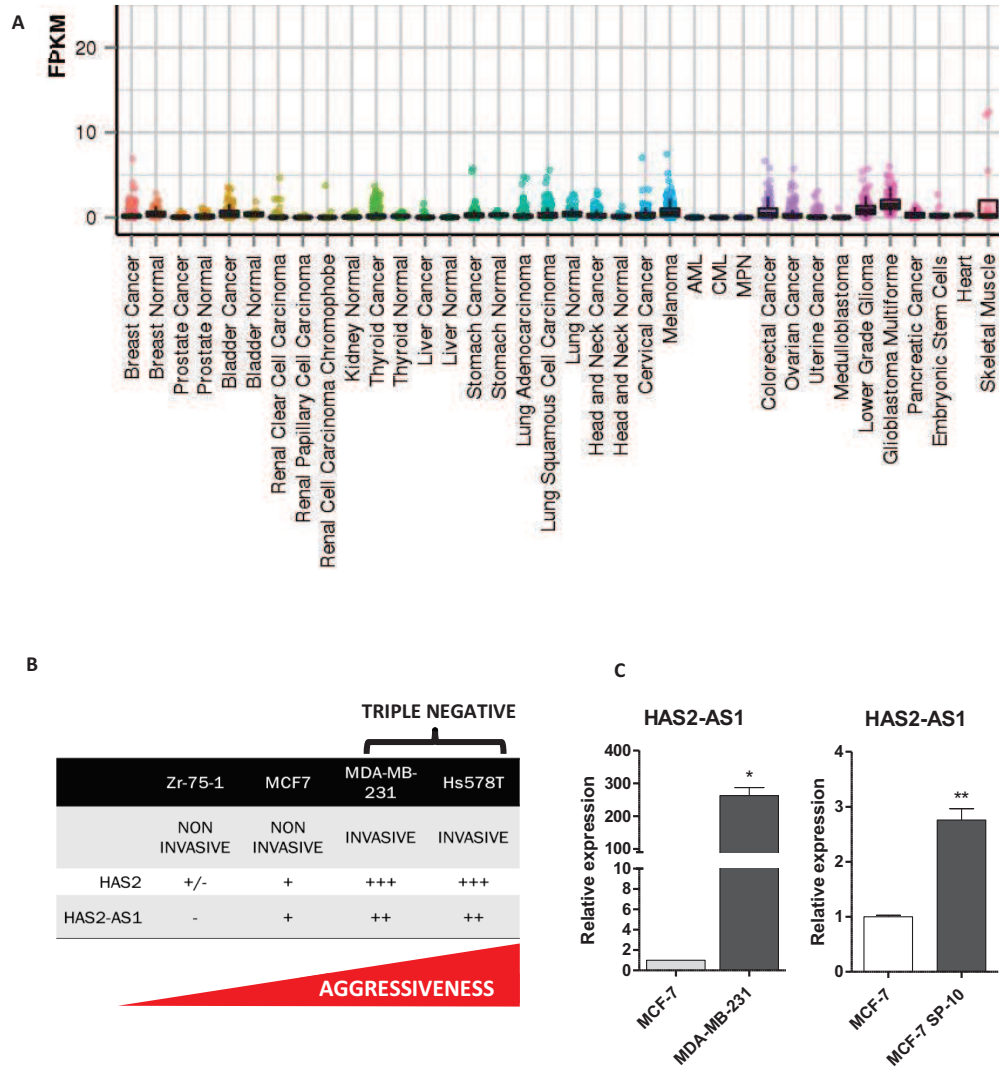
Figure 10 - **HAS2-AS1 expression in cancer**. (A) HAS2-AS1 levels in tumoral and healthy tissues. Ref-seq results are displayed as Fragments Per Kilobase of exon per Million of fragments mapped. (B) In silico analysis of HAS2 and HAS2-AS1 expression on GEO2R. Accession number GSE58643 and GSE5884. (C) Relative gene expression of HAS2-AS1 in breast cancer cell lines of different aggressiveness. Results are presented as mean ± s.e.m. The experiments were repeated three times in duplicates. $p < 0.05$.

6.2 HAS2-AS1 modulation affected functional properties related with breast cancer aggressiveness

The aggressiveness of a cancer is usually determined considering its ability to proliferate and to migrate in the surrounding areas as well as to invade the basement membrane to originate metastasis. To investigate the biological effects of HAS2-AS1 in breast cancer cells, we performed functional assays evaluating cellular proliferation, invasion and migration. In the triple negative cell line MDA-MB-231 the transient silencing of HAS2-AS1 induced a higher capability to invade a 3D matrigel support (Fig. 11A) and to migrate in a confluent monolayer after 16 hours (Fig. 11B). As a control, the abrogation of HAS2 expression by siRNA inhibited MDA-MB-231 invasion as previously reported [30]. Furthermore, the MTT analysis showed a 2.5 fold increase of cell viability (Fig.11c).

On the other hand, the overexpression of the two exon 2 isoforms of HAS2-AS1 (HAS2-AS1 short and HAS2-AS1 long) decreased cell viability and their ability to penetrate into matrigel. Interestingly, the biological effects after HAS2-AS1 overexpression were related to the length of the two isoforms, as HAS2-AS1 long caused a stronger effect in the both processes (Fig. 11A, 11C). No significant differences were detected in the viability of the low aggressive cell line MCF-7 (Fig. 11D).

All together, these data suggest that the abrogation of HAS2-AS1 could have an impact in the regulation of MDA-MB-231 aggressiveness without altering the behaviour of MCF-7 cell line.
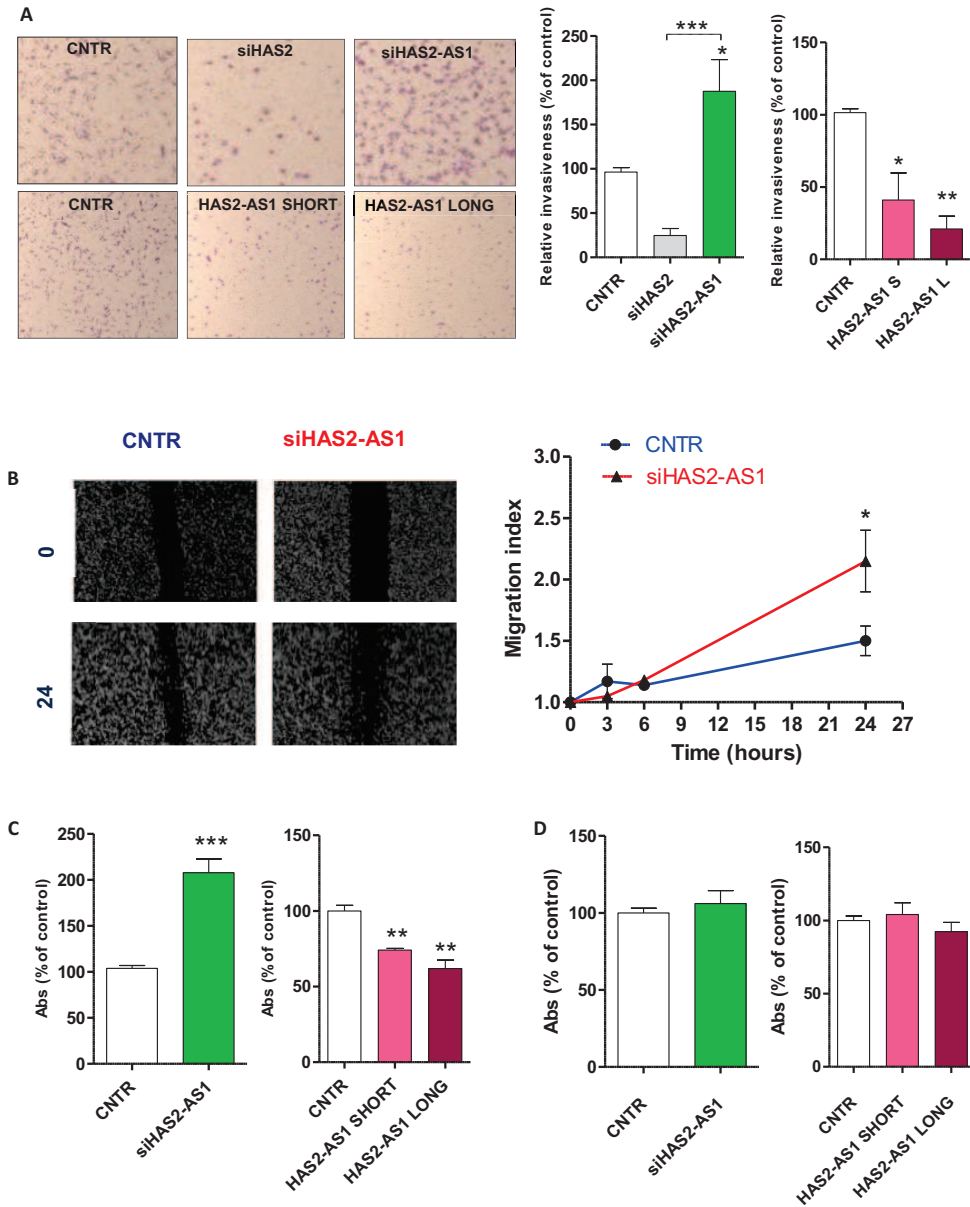
Figure 11 – Effects of HAS2-AS1 modulation on cell behaviour. (A) Representative pictures of HAS2-AS1 overexpression/silencing on cell invasion and relative quantification. Results are represented as the mean ± s.e.m. based on 4 independent experiments conducted in duplicate. (B) Evaluation of MDA-MB-231 migration after a wound healing assay and relative quantification. Results are expressed as mean of migration index ± s.e.m. of 3 independent experiments. (C) Viability of MDA-MB-231 and (D) MCF-7 after HAS2-AS1 overexpression/silencing. Data are showed as % of absorbance of the control. Experiments were conducted 4 times in duplicate.

6.3 HAS2-AS1 abrogation influenced HA metabolizing genes expression

The functional changes observed in MDA-MB-231 after the silencing of HAS2-AS1 could be associated to modifications of ECM composition. Since HAS2-AS1 can modulate HAS2 expression and HA production [1,29,70], we evaluated the expression levels of HA and its metabolizing genes. Interestingly, the abrogation of HAS2-AS1 stimulated HAS2 and HAS3 mRNA expression (HAS1 mRNA was not detected), as well as HYAL2 transcript levels.

Our analysis about the increment of HAS2 after HAS2-AS1 knockdown was also confirmed using on line data (ATCG-BRCA) available on Tanric (http://ibl.mdanderson.org/tanric/_design/basic/index.html) on biopsies of breast tumors. Moreover, HAS2-AS1 silencing stimulated the expression of total CD44 mRNA and some of the variants that are important for tumor progression like the V3-V10, V8-V10 and CD44s. However no significant differences were detected by FACS analysis in the changes of the breast cancer stem cells marker CD24$^-$/CD44$^+$.

To better understand the mechanism which mediated MDA-MB-231 malignancy after HAS2-AS1 knockdown, the CD44-dependent pathway p44/42 was evaluated by western blot analysis. The results indicated a lack of the activation of the phosphorylated form of P44/42, suggesting that other signalling pathways could mediate the aggressive behaviour of MDA-MB-231 cells.

These data indicated that HAS2-AS1 could globally control HA metabolism, not only through the expression of its synthases but also through its receptor and degrading enzyme.
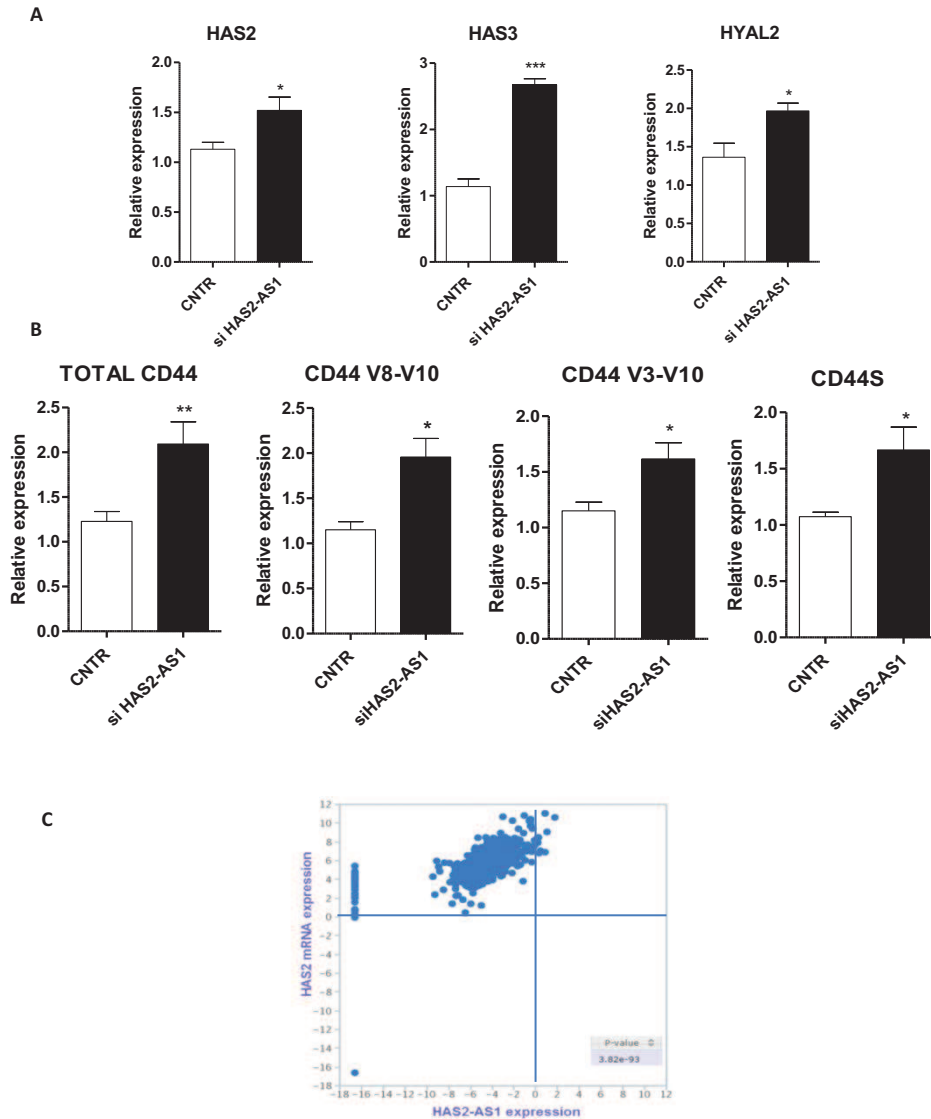
Figure 12 - HA related genes expression after HAS2-AS1 transient silencing. (A) qRT-PCR of HAS2, HAS3 and HYAL 2. Results are represented as the mean ± s.e.m of three independent experiments conducted in duplicate. p<0.05 (B) mRNA expression levels of HA receptor CD44 and CD44 variants. Results displayed the mean ± s.e.m of three independent experiments conducted in duplicate. p<0.05 (C) Correlation between HAS2 mRNA and HAS2-AS1 transcript levels. Analysis made in silico using the ATGC study on breast cancer available at http://ibl.mdanderson.org/tanric/_design/basic/index.html)

A

B
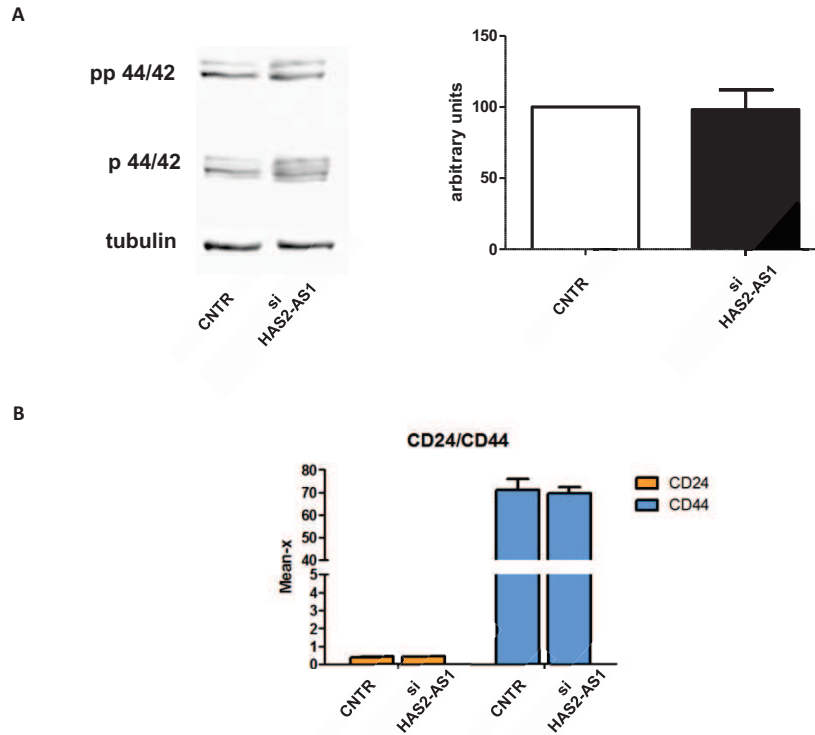
Figure 13 - Evaluation of CD44 signaling after HAS2-AS1 abrogation. (A) Western blot analysis and relative quantification of p44/42 and the phosphorylated form pp44/42. Quantification results are reported as phosphorylated/total p44/42 amount. Tubulin was used to normalize protein levels. Data are displayed as the mean ± s.e.m. of 3 independent experiments. $p < 0.05$. (B) Flow cytometry analysis quantification of CD24⁻/CD44⁺ breast cancer stem cell marker. Results are reported as the mean ± s.e.m. of 3 independent experiments.

6.4 HAS2-AS1 modulation did not alter HA production in the medium or in the pericellular matrix

Considered the results shown above about HASes expression and the importance of HA in cancer progression, we measured the levels of HA in MDA-MB-231 culture medium by ELISA assay. Although the silencing of HAS2-AS1 increased HAS2 and HAS3 mRNA levels, no differences in the amount of secreted HA were detected, in fact the levels of HA in control medium was 9.1 ng/μg of proteins, while after HAS2-AS1 silencing 8.6 ng/μg of proteins (Fig. 14A). Furthermore no significant differences were found between control medium and HAS2-AS1 transfected samples (5.3 vs 4.9 ng/μg of secreted proteins). In Figure 14A results are represented as % of the control.

As HA can also remain associated to the plasma membrane via HASes or receptors, we evaluated the pericellular amount of HA by particle exclusion assay using fixed red blood cells. The overexpression of HAS2-AS1 did not alter the production of HA in the pericellular space. As a control, the knockdown of HAS2 inhibited the production of secreted HA (3,7 ng/μg of secreted proteins) and its presence around cell membrane, as well as the digestion with 2U/ml of bovine testis hyaluronidase. These results indicate that the modulation of HAS2-AS1 expression in MDA-MB-231 did not affect the secretion of HA and its presence around the cell membrane, suggesting that other mechanisms mediate the regulation of MDA-MB-231 malignancy upon alteration of HAS1-AS1 levels.
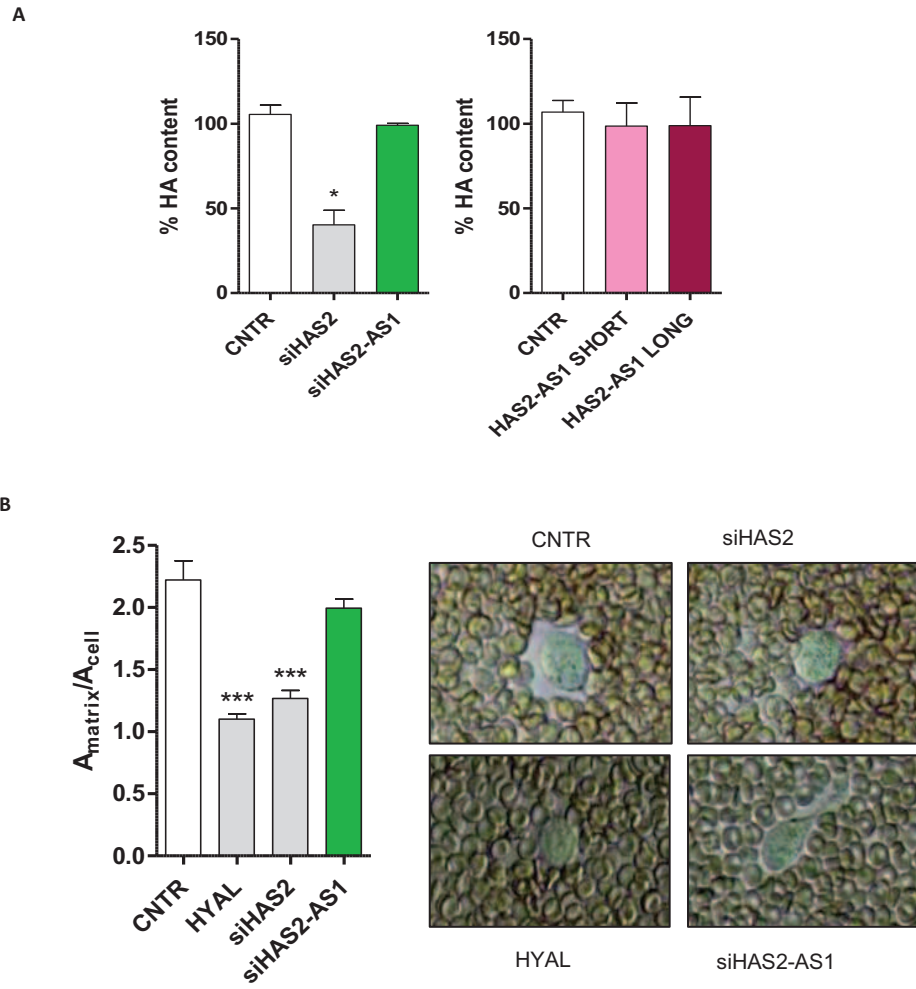
Figure 14 – Effects of HAS2-AS1 modulation on HA production. (A) HA quantification by ELISA of MDA-MB-231 culture media. Results are expressed as the mean of 3 independent experiments ± s.e.m., normalized to the µg of extracted proteins and reported as % of the control. (B) Particle exclusion assay and relative quantification indicating the pericellular amount of HA. Data are presented as the mean ± s.e.m. of 3 independent experiments. Results are expressed as the ratio between the area of ECM and the area of the cell.

6.5   HAS2-AS1 did not regulate MDA-MB-231 malignancy via secreted HA

The presence of HA in cancer microenvironment is often considered as a negative prognostic factor for the progression of the disease [23]. To mimic the effects of tumor microenvironment, MDA-MB-231 were transfected with plasmids encoding for HAS2-AS1 short or long and with HAS2-AS1 siRNA. After 48 hours cell culture media were collected and added to untreated MDA-MB-231. A wound healing assay was performed to evaluate the migration of the cells. The results show that the modulation of HAS2-AS1 did not significantly change cell motility neither after the knockdown, nor after the overexpression of the two isoforms of HAS2-AS1. These results suggest that HAS2-AS1 was able to modulate cell aggressiveness without altering HA levels or other soluble ECM components.
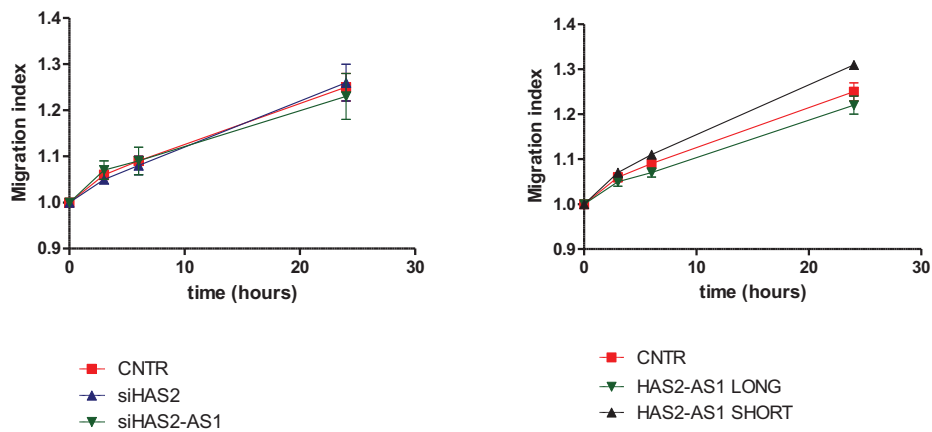

Figure 15 – Wound healing assay of MDA-MB-231. The scratch assay was performed using the medium of transfected MDA-MB-231 mixed with complete medium (ratio 1:1) to treat MDA-MB-231 for 24 hours. A scratch with a p200 tip was made at time 0. Pictures were taken at 3, 6 and 24 hours. Results are expressed as mean of migration index ± s.e.m. Experiments were conducted 3 times.

6.6   HAS2-AS1 silencing in breast cancer cells: a microarray analysis

To screen for HAS2-AS1-regulated candidate genes in our system, we compared the transcriptome of MDA-MB-231 transiently silenced with a siRNA against HAS2-AS1 and a control group using Affimetryx U133v2 Gene Arrays (in collaboration with the Department of Anatomy, National University of Singapore, Singapore). Thirty-two upregulated and 8 downregulated genes satisfied the filtering criteria of fold change≥1.5 and p<0.05.

Several genes were involved in vesicular trafficking (Syntaxin 12, VAMP4, NSF, ESYT2, ARL6IP1, SMAP1), transport (SLC transporters) and cell signalling. Among the genes involved in cell signalling we confirmed the array results measuring by quantitative RT-PCR the expression of the hepatocyte growth factor receptor c-MET which is a critical factor in breast carcinogenesis [83,84].

Further alterations were also found in genes controlling apoptosis (C6orf120, PDCD and SLK) and autophagy (NRBF2). Lastly, we found variations in the expression of genes involved in DNA and protein binding, in particular some regulators of transcription (SUB1, TADA2B) and cellular trafficking (ARL6IP1, LZTFL1).

These results suggest that HAS2-AS1 can regulate the expression of several genes not related to HAS2 as previously reported in vascular smooth muscle cells or tumor [1,29].
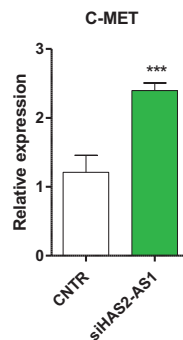

Figure 16 –C-MET upregulation after HAS2-AS1 transient silencing in MDA-MB-231. Confirmation by quantitative RT-PCR of c-MET expression levels in MDA-MB-231. Data are reported as the mean ± s.e.m of three independent experiments conducted in duplicate. p<0.5

Table 3 – Affymetrix microarray analysis of differentially regulated genes in MDA-MB-231 upon HAS2-AS1 transient silencing.

Gene Symbol	Description	Selected GO term	Fold Change	FDR p-value
ESYT2	extended synaptotagmin-like protein 2	transport	2,22	0,007
C6orf120	chromosome 6 open reading frame 120	apoptotic process	2	0,008
PDCD6	programmed cell death 6	apoptotic process	2,19	0,012
SLK	STE20-like kinase	apoptotic process	2,13	0,009
NRBF2	nuclear receptor binding factor 2	autophagy	2,2	0,010
COL8A1	collagen, type VIII, alpha 1	cell adhesion	2,06	0,012
BOD1	biorientation of chromosomes in cell division 1	cell cycle	2,16	0,008
EPDR1	ependymin related 1	cell-matrix adhesion	-2,76	0,007
PTPMT1	protein tyrosine phosphatase, mitochondrial 1	DNA binding	2,33	0,022
ERCC5	excision repair cross-complementation group 5	DNA binding	2,12	0,014
H3F3A; H3F3AP4	H3 histone, family 3A; H3 histone, family 3A, pseudogene 4	DNA binding	2,12	0,007
TADA2B	transcriptional adaptor 2B	DNA binding	2,07	0,020
TRDMT1	tRNA aspartic acid methyltransferase 1	DNA binding	2,17	0,007
RBPJ	recombination signal binding protein for immunoglobulin kappa J region	DNA binding	2,14	0,010
SUB1	SUB1 homolog, transcriptional regulator	DNA binding	-2,58	0,044
NDUFS3; PTPMT1	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase); protein tyrosine phosphatase, mitochondrial 1	oxidation-reduction process	2,65	0,012
ADH5	alcohol dehydrogenase 5 (class III), chi polypeptide	oxidation-reduction process	2,08	0,010
HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12	oxidation-reduction process	-2,46	0,007
MSRB3	methionine sulfoxide reductase B3	oxidation-reduction process	2,12	0,008
WDR1	WD repeat domain 1	protein binding	2,2	0,008
CALU	calumenin	protein binding	2,08	0,008
MSANTD3-TMEFF1;	MSANTD3-TMEFF1 readthrough; transmembrane protein with EGF-like and two follistatin-like domains 1	protein binding	3,39	0,024
LZTFL1	leucine zipper transcription factor like 1	protein binding	3,16	0,010
ARL6IP1	ADP-ribosylation factor like GTPase 6 interacting protein 1	protein binding	-2,3	0,024
BTBD1	BTB (POZ) domain containing 1	protein binding	-2,08	0,009
TAPT1	transmembrane anterior posterior transformation 1	signal transduction	2,49	0,023
MET	MET proto-oncogene, receptor tyrosine kinase	signal transduction	2,79	0,012
PTPN1	protein tyrosine phosphatase, non-receptor type 1	signal transduction	2	0,021
RASSF8	Ras association (RalGDS/AF-6) domain family (N-terminal) member 8	signal transduction	-2,78	0,039
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	transport	2,28	0,007
KIF1BP	KIF1 binding protein	transport	-2,34	0,010
NSF	N-ethylmaleimide-sensitive factor	transport	-2,56	0,007
SLC36A1	solute carrier family 36 (proton/amino acid symporter), member 1	transport	2,63	0,010
SLC4A4	solute carrier family 4 (sodium bicarbonate cotransporter), member 4	transport	-2,84	0,044
SMAP1	small ArfGAP 1	transport	2,37	0,007
STX12	syntaxin 12	transport	2,25	0,015
VAMP4	vesicle associated membrane protein 4	transport	2,1	0,041

6.7   HAS2-AS1 did not regulate Epithelial-mesenchymal transition (EMT) in MDA-MB-231

Epithelial-mesenchymal transition (EMT) is a developmental process in which epithelial cells lose their polarity and undergo changes in the expression of cell-cell and cell-matrix adhesion molecules. This lead to a gain of migratory and invasive properties, as well as to morphological changes typical of a mesenchymal phenotype. This process can be adopted by cancer cells to increase their ability to migrate and originate metastasis. As HAS2 and CD44 are described to play a key role in EMT [25,35,39], and considering the results obtained on cell behaviour (Fig.11) and gene expression (Fig. 12) after HAS2-AS1 silencing we evaluated the mRNA of the most important genes involved in EMT. A gene expression profiling of epithelial (E-cadherin, ZO-1, ocludin) and mesenchymal (vimentin, TWIST, SNAIL) markers by quantitative RT-PCR did not show relevant changes between control and HAS2-AS1 silenced samples (data not shown). Moreover, since EMT drives morphological changes, we evaluated the morphology of MDA-MB-231 by light microscopy. In accordance with the results obtained about the EMT markers, no gross changes were detected in cell morphology (data not shown).

All together these results indicate that EMT did not occur in MDA-MB-231 after HAS2-AS1 silencing suggesting that the malignancy acquired from the cells after HAS2-AS1 transient abrogation could be done to other processes.

6.8 HAS2-AS1 regulates miRNA 186 expression in MDA-MB-231 cells

Different studies have shown that lncRNAs can act as endogenous sponges or ceRNAs (Competing Endogenous RNAs) to modulate miRNAs expression [68,85–87]. To determine miRNAs that potentially bind to HAS2-AS1 transcript, we used different online tools like miRanda (www.microrna.org), DIANA-microT-CDS (http://diana.imis.athena-innovation.gr) and RNAhybrid (https://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid).

About 1100 miRNAs (prediction by miRANDA) were predicted to target the whole sequence of HAS2-AS1. To reduce the number of miRNAs to analyse, we decided to restrict the search on exon 2, as we observed a significant effect on aggressiveness using the long and short isoform of exon 2 (see Fig.11). According to the prediction results, HAS2-AS1 exon 2 contained 2 putative miRNA 186-3p binding sites (Fig. 17A). Interestingly, the short isoform of HAS2-AS1 contained only one miRNA 186-3p binding site with a medium free energy (MFE) of -14.9 kcal/mol, while an additional binding site was found to target the sequence of HAS2-AS1 long with a MFE of -23.2 kcal/mol (Fig. 17B). These sites are widely conserved in the DNA sequence of different species of eutherian mammals (Fig. 17C), highlighting its importance.

To test whether HAS2-AS1 could regulate miRNA 186 expression, we transfected MDA-MB-231 with plasmids encoding for the long and the short isoforms of HAS2-AS1 exon 2. The overexpression of both HAS2-AS1 isoforms decreased miRNA 186-3p levels, but not that of miRNA 186-5p (that we used as a control), showing a stronger inhibitory effect exerted by HAS2-AS1 long (Fig. 18). On the contrary, the knockdown of HAS2-AS1 did not affect miRNA 186-3P or -5p expression (data not shown).

To better understand the role of miRNA 186-3p in cell physiology and pathology, we investigated its possible involvement in different cellular pathways through the software miRPathv3, a program dedicated to the evaluation of miRNA regulatory roles and the identification of controlled pathways using standard, unbiased empirical distributions and/or meta-analysis statistics [82]. According to the software, miRNA 186-3p was able to target 419 genes belonging to different pathways. Interestingly, miRNA 186-3p could control the mitogen-activated protein (MAP) kinase, WNT signalling and cell cycle in colon, thyroid and pancreatic cancer (table 2). Moreover it could have a role in TGFβ signalling pathway, autophagy and apoptosis, targeting the transforming growth factor beta receptor 2 (TGFβR2) and SMAD2 (these data are from miRPathv3 analyses).

In the literature it is shown that miRNA 186 is able to regulate apoptosis via the purinergic receptor $P2X_7$ in MDA-MB-231 cells [2]. Our results reported that the

overexpression of HAS2-AS1 stimulated $P2X_7$ expression, showing a length dependent effect of HAS2-AS1 in the expression of the pro-apoptotic receptor (Fig. 19).

Furthermore, as miRNA 186 could control cell cycle in different type of tumors, we evaluated the expression of the Cyclin Dependent Kinase Inhibitor 1A (CDKN1A-p21), which is a master regulator of cell cycle progression leading to G1 arrest. The data reported in Fig. 19 showed an upregulation of p21 upon HAS2-AS1 overexpression.

Moreover, since miRNA 186 is involved in autophagy targeting Autophagy Related 7 (ATG7) and Beclin 1 [88], the expression of the autophagic marker LC3b was evaluated. In accordance with the results obtained about miRNA-186 targets, Fig.19 showed an increase of LC3B mRNA levels after HAS2-AS1 overexpression. Interestingly, the stimulation of HAS2-AS1 on miRNA 186 targets expression depended on the length of the isoform transfected.

In summary, these experiments demonstrate that HAS2-AS1 could act as an endogenous sponge inhibiting miRNA 186-3p levels and reducing its repressive effect on the expression of some of its targets, which, in turn, could act to promote apoptosis, cell cycle arrest, and autophagy. Further analysis will be aimed to demonstrate the effective binding of HAS2-AS1 miRNA 186-3p.
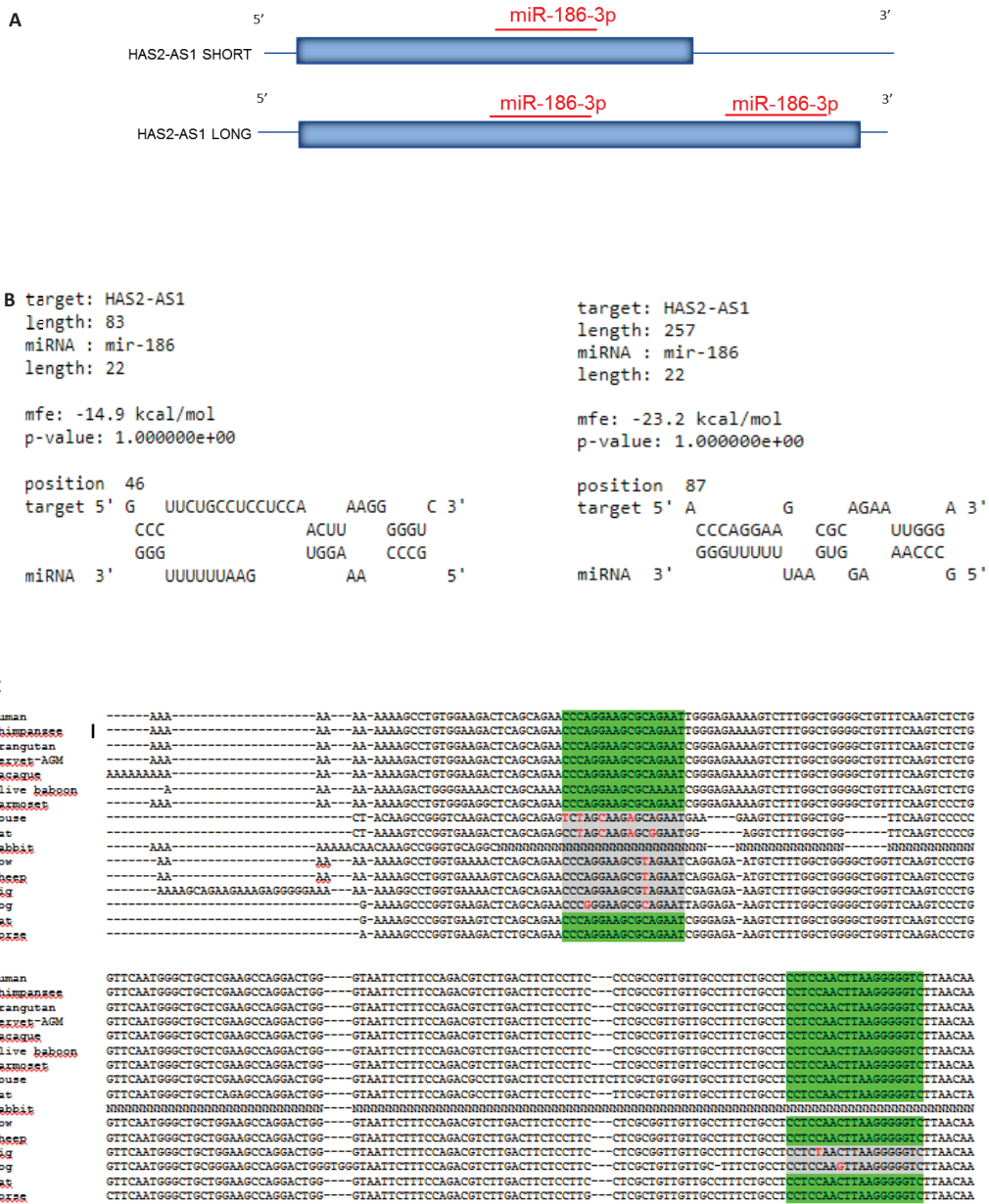
Figure 17 – miRNA 186-3p predicted binding site on HAS2-AS1. (A) Schematic representation of miRNA 186-3p binding site on has2-as1. (B) HAS2-AS1 long binding site of miRNA 186-3p predicted by RNAhybrid. (C) HAS2-AS1 short and long common binding site of miRNA 186-3p in 16 eutherian mammals (www.ensembl.org).
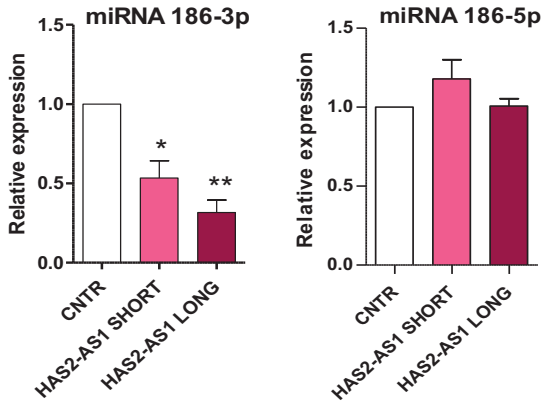
Figure 18 – HAS2-AS1 regulates miRNA 186-3p expression. Effects of HAS2-AS1 modulation on the expression of miRNA 186-3p and miRNA 186-5p. Data are displayed as gene relative expression and bars represent mean ± s.e.m. Each experiment was repeated 3 times. p<0.05

KEGG pathway	p-value	# genes
Colorectal cancer	1.45852734387 e-06	11
Pathways in cancer	0.000396459466412	21
Adherens junction	0.0008506018072	8
Proteoglycans in cancer	0.0008506018072	12
Cell cycle	0.00142549884804	15
Endometrial cancer	0.00550010335802	7
TGF-beta signaling pathway	0.0102057399918	7
Thyroid cancer	0.0110720910721	5
Prostate cancer	0.0345166951389	9
Pancreatic cancer	0.0457237575307	7

Table 4 – KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways regulated by miRNA 186-3p. The table shows the number of genes targeted by miRNA 186-3p in different pathways and cellular processes.
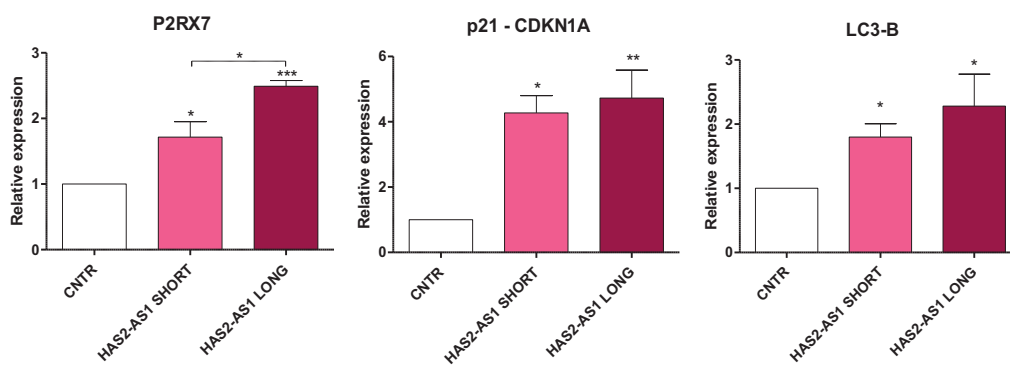

Figure 19 – HAS2-AS1 regulates miRNA 186 targets expression. Effects of HAS2-AS1 modulation on the expression of miRNA 186 targets. Data are represented as gene relative expression and bars represent mean ± s.e.m. Each experiment was performed in triplicate.

7 - DISCUSSION

The objective of this study was to gain a better understanding about the role of the lncRNA HAS2-AS1 in breast cancer cells. Although the importance of HA and HASes is well described in the literature, little is known about the involvement in breast cancer of HAS2-AS1, a new regulator of HAS2 expression and HA production.

Our data reported that HAS2-AS1 expression correlated with the aggressiveness of a tumor, as its levels were higher in the aggressive cell lines MDA-MB-231 and MCF-7 sp10[+]. To investigate the function of HAS2-AS1 in breast cancer, we performed functional assays in the high aggressive and triple negative cell line MDA-MB-231 and in MCF-7, which is, on the contrary, a low aggressive cell line expressing ER and PR.

These experiments demonstrated that the transient silencing of HAS2-AS1 stimulated cell proliferation in MDA-MB-231 but not in MCF-7, suggesting that HAS2-AS1 could be important in the regulation of the viability of high aggressive breast cancer cells. In addition, HAS2-AS1 abrogation increased MDA-MB-231 ability to invade and go through a matrigel layer (3D migration) and to migrate on a bi-dimensional surface. On the other hand, we observed opposite effects in MDA-MB-231 after the overexpression of two isoforms of HAS2-AS1 called HAS2-AS1 short and HAS2-AS1 long. Interestingly, the inhibition of cell viability and invasion upon HAS2-AS1 overexpression is related to the length of the isoform transfected, as the effects observed were stronger after the transfection of HAS2-AS1 long. These results, along with the data showing high expression levels of HAS2-AS1 in aggressive breast cancer cells, indicate that this lncRNA could have a protective role and that breast cancer cells could stimulate its expression as a rescue mechanism.

The changes in cell behaviour correlated with alterations in the expression of HA related genes. Interestingly, the knockdown of HAS2-AS1 stimulated the expression of HAS2 mRNA. The relation between HAS2-AS1 and HAS2 expression has begun to be studied during the last years. The first group which described HAS2-AS1 showed that the transfection of HAS2-AS1 short and long inhibited HA biosynthesis, HAS2 expression and cell proliferation in a cell line of osteosarcoma [29]. Vice versa, Michael et. al reported that HAS2-AS1 and HAS2 exhibited a coordinated expression in the renal proximal tubular

epithelial cell [89]. Similarly, our group demonstrated that HAS2-AS1 positively regulated HAS2 expression and HA production in a model of aortic smooth muscle cells, proposing a new chromatin arrangement in the region of HAS2 promoter [1]. Such a different regulation of HAS2 among different cell lines underlies the tissue specificity of action of lncRNAs; lncRNAs expression is much more cell-, tissue-, and developmental specific than those of mRNA. For instance, numerous lncRNAs display different expression patterns in breast cancer tissue compared to normal breast tissue [90]. Moreover, they have been demonstrated to be differentially expressed within different subtypes of breast cancer. Yang and colleagues identified more than 1300 lncRNAs typically expressed by HER-2 enriched subtypes of breast cancer [91], while other 1750 have been demonstrated to specifically expressed in triple negative breast cancer [66]. Furthermore, the lncRNA DSCAM-AS1 has been described as an estrogen receptor α-dependent lncRNA with a high specificity for luminal breast cancer [57], suggesting its possible use as biomarker of this subtype. Another study reported that the modulation of the lncRNA HOTAIR by knockdown or ectopic overexpression affected the phenotype, cell differentiation and the expression of target genes in a different way among a panel of several cancer cell lines [92]. These findings indicate that the aberrant expression patterns of lncRNAs might play an important role in defining specific subtypes of breast cancer and that lncRNAs functions and target genes cannot simply be transferred from one cancer type to the other.

The silencing of HAS2-AS1 also increased the expression of the receptor CD44 and the variants V8-V10, V3-V10 and CD44S. Several lines of evidence have implicated CD44 in breast carcinogenesis and metastasis [25,36,37]. Particularly, CD44 expression has been detected on numerous breast cancer cell lines and primary tumours. It is also well described that CD44 can stimulates cell proliferation and invasion in breast cancer [93]. In addition, CD44 has been recognized as a breast cancer stem cell marker and it is hypothesized that a subpopulation of CD44-expressing tumor cells possess high metastatic potential [94,95].

The overexpression of HYAL2 also reflects the malignant phenotype acquired upon HAS2-AS1 silencing. Several papers report that HA degrading enzymes

can contribute to the process of tumorigenesis [27] favouring the degradation of HA and stimulating the inflammation of cell microenvironment. HA fragments are usually internalized by the cells through CD44 receptor, but in presence of a high hyaluronidase activity, they remain in the cell microenvironment free to interact with CD44, thus activate signaling pathways which stimulate cell proliferation, migration and tumorigenesis [19].

The microarray analysis revealed that HAS2-AS1 silencing modulated the expression of several genes in MDA-MB-231. Several genes were involved in vesicular trafficking, DNA and protein binding, cellular trafficking, apoptosis, autophagy and cell signalling. In particular, we found an up-regulation of c-MET, an important mediator of cell aggressiveness which can stimulate cell invasion and proliferation [83][84] in a CD44-dependent manner [36] and a down regulation of RASSF8, whose knockdown contributes to cell migration and invasion acting as a tumor suppressor in different cancer types [96] [97]. Moreover, we observed an upregulation of the glucose transporter SLC2A3 (GLUT3), suggesting a possible increase of glucose intake typical of aggressive cancer cells [98].

All these data suggest that HAS2-AS1 could play an active role in the regulation of MDA-MB-231 aggressiveness and in the modulation of a malignant phenotype.

Changes in the expression/function of adhesion molecules on the cell surface can alter cell-cell and cell-matrix interactions. This process, known as EMT, can be acquired by cancer cells which lose contacts and change their phenotype from epithelial to mesenchymal becoming more aggressive and malignant. Interestingly, this event can be reverted, transforming mesenchymal into epithelial cells (MET). Despite the changes observed in cell aggressiveness, no significant differences were found in the expression of EMT/MET related genes. Our hypothesis is that MDA-MB-231 cells already have a mesenchymal and very malignant phenotype, which cannot be further stimulated after HAS2-AS1 knockdown. Similarly, MDA-MB-231 did not go through MET after HAS2-AS1 overexpression, suggesting that the modulation of HAS2-AS1 alone is not sufficient to induce these processes.

LncRNAs can orchestrate gene expression through a variety of mechanisms, regulating transcription and translation, chromatin-remodelling and the interaction with other RNA species. Recently, a new regulatory mechanism has been identified in which the crosstalk between lncRNAs and mRNA occurs by competing for shared miRNAs response elements.

A huge number of studies described the function of lncRNAs as sponges for miRNAs [67,68,86,99].

In silico analysis revealed that HAS2-AS1 exon 2 transcript contains several putative binding site for different miRNAs, among them miRNA 186. The reasons for choosing miRNA 186 were different. Firstly, HAS2-AS1 long contained 2 putative miRNA 186-3p binding sites, while HAS2-AS1 short only one. This difference could reflect the stronger response in cell migration and invasion upon the overexpression of HAS2-AS1 long. Secondly, the medium free energy for HAS2-AS1 short and long showed good scores (14.9 kcal/mol and -14.9 kcal/mol, respectively). Third, the predicted binding site of miRNA-186 displayed a high evolutionary conservation in HAS2-AS1 sequence among different species of eutherian mammals.

It has been already demonstrated that miRNA 186 targeted the pro-apoptotic receptor $P2X_7$ and that the treatment with miRNA 186 inhibitors increased $P2X_7$ mRNA [2]. Interestingly, our results fit with this description, as the overexpression of HAS2-AS1 isoforms decreased the levels of miRNA 186-3p and stimulated the expression of $P2X_7$ mRNA and other miRNA 186 targets in a HAS2-AS1 isoform-dependent manner. No significant differences in the levels of miRNA 186-5p were detected.

During the past years, the regulatory function of miRNAs was mainly attributed to the -5p arm, although miRNAs can derive from both the 3' and 5' ends of the same precursor. Today, the biological function and mechanism of miRNA arm expression preference remain unclear in breast cancer, but recent studies have reported that miRNA -5p and miRNA -3p arms can be preferentially selected among different tissues, developmental stages, and species and during cancer progression [47,100–104]. Previous studies have indicated that the arm selection of some miRNAs significantly changes among human cancers, including hepatocellular carcinoma, gastric cancer, and breast

cancer [103,105,106]. Therefore, the simple thermodynamic hydrogen bonding theory is insufficient to explain the phenomenon of flexible selection known as arm switching or arm selection preference.

A consistent number of papers described that miRNA 186 can target different lncRNAs, especially in cancer [86,88,107,108]. The role of miRNA 186 in cancer is controversial and still to be elucidated. Several studies reported that it can inhibit cancer cells proliferation and tumor growth [109–111], while other papers described its active role in the stimulation of cancer cell proliferation and tumorigenesis [22,26,27]. These differences can be due to the discrepancy in the expression of lncRNAs, whose interaction with miRNAs can differ among specific tissues or cell lines, generating a wide variant of possible lncRNA-miRNA interactions and sponge effects.

The study of the pathways regulated by miRNA 186 showed that miRNA 186 is involved in a plethora of cellular processes and targets some genes involved in cell cycle and autophagy, like the cell cycle inhibitor p21 and the autophagic marker LC3. Our results demonstrated that p21 and LC3 mRNA levels increased upon HAS2-AS1 transfection, underlying the importance of these genes in breast cancer tumorigenesis and corroborating the hypothesis of a possible sponge effect.

All these data suggest that HAS2-AS1 plays a key role in the regulation of MDA-MB-231 aggressiveness and that this lncRNA could exert a protective role during breast cancer progression. In addition, our findings report for the first time that HAS2-AS1 could function as a sponge interacting with miRNA 186-3p, thus modulating its effects in breast cancer cells.
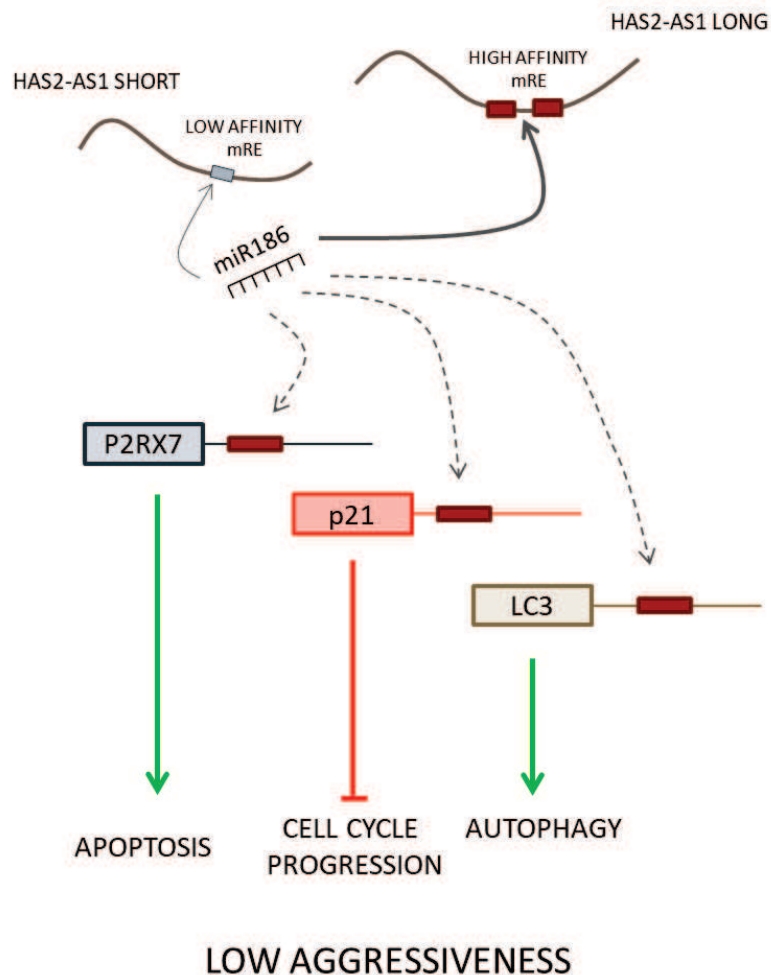
Figure 20 – Working model. The cartoon shows the proposed mechanism of action of miRNA 186 and its influence on MDA-MB-231 aggressiveness. The isoform HAS2-AS1 long contains 2 putative mRE (miRNA Responsive Element) for miRNA186, whereas HAS2-AS1 short contains just one. The titration of miRNA186 exerted by HAS2-AS1 could avoid miRNA186 repressive function on downstream targets, like the pro-apoptotic receptor P2X7 and the inhibitor of cell cycle progression p21, contributing to a low aggressive phenotype of MDA-MB-231. Moreover, the hypothesized binding of miRNA 186 to HAS2-AS1 could have repercussions on cell aggressiveness through autophagy, as described for the increase of LC3. mRNA levels.

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