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HMGA1 AND FOXM1 SYNERGISTICALLY REGULATE A COMMON GENE NETWORK MODULATING THE ANGIOGENESIS IN BREAST CANCER

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Summary

My research project was focused on the study of triple negative breast cancer (TNBC), which is highly aggressive and unresponsive to common treatments. One of the factors responsible for the aggressive features of TNBC is the High mobility group (HMG) A1 protein, a master regulator of the gene transcription, whose high expression level has been correlated with a higher grade in breast cancer. To deepen the knowledge about HMGA1 in breast cancer, we sequenced the RNA collected from a model of TNBC, the MDA-MB-231 cell line, upon HMGA1 silencing; thanks to a bioinformatic analysis, we unraveled molecular partners HMGA1 could cooperate with in regulating common downstream gene networks in breast cancer and among them we selected Forkhead box M1 (FOXM1) transcription factor, chosen for its role in breast carcinogenesis. Firstly, we validated several HMGA1/FOXM1 targets by qRT-PCR after HMGA1 and FOXM1 silencing in TNBC cell lines. Moreover, the depletion of FOXM1 in TNBC cells leads to the acquisition of a more epithelial-like phenotype, with a disruption of the vimentin network, a typical marker of the mesenchymal phenotype, and the diminution of the migratory ability of TNBC cells, results similar to those we obtained upon HMGA1 silencing, and further accentuated by the co-silencing of the two factors. In addition, upon HMGA1 depletion in TNBC cell lines and HEK-293T, FOXM1 translocates from the nucleus to the cytoplasm, suggesting that HMGA1 could modulate FOXM1 subcellular localization and activity. To validate this hypothesis, we performed a luciferase reporter assay, by transfecting the HEK293T cells with a construct containing a portion of the promoter of a known HMGA1/FOXM1 target, the Vascular Endothelial growth factor (VEGFA) A. By co-transfecting the two factors, we found that HMGA1 increases the transcriptional activity of FOXM1 on VEGFA promoter and with several deletion constructs we restricted the region responsible for this activity. Considering that the VEGFA is one of the main inducer of angiogenesis, a hallmark in breast cancer, we investigated how HMGA1 and FOXM1 are involved in this process. Thus, we treated the HUVEC endothelial cells with supernatants of MDA-MB-231 depleted of HMGA1 and/or FOXM1 and we observed that the proliferation, the migration, and the ability to form a vessel-like network of HUVEC cells were affected. We finally validated *in vivo* that HMGA1 and FOXM1 synergistically regulate the neo-angiogenesis in Zebrafish larvae. Hence, we demonstrated that HMGA1 and FOXM1 are involved in the regulation of the same gene network in TNBC and that HMGA1 influences the FOXM1 cellular localization and transcriptional activity. We showed also that breast tumor cells, on the driving force of HMGA1 and FOXM1, modulate the angiogenic process carried out by endothelial cells, both *in vitro* and *in vivo*.

Introduction

1. BREAST CANCER

1.1 Mammary gland structure

The breast is the female organ designated to the milk production and thus to the nourishment of new-borns. It is an exocrine gland located in the chest wall, atop the pectoralis muscles (Fig. 1a). It is made up with two functional units, the alveoli and the ducts. The alveoli are then grouped in lobules and are the structures in which milk is synthetized. The milk is then drained into a lactiferous duct which carries the milk from the lobule to the nipple. Both the lobules and the ducts are composed by a bi-layered cell structure, with an inner part of luminal epithelial cells and an outer layer of contractile myoepithelial cells, controlling the ejection of the milk throughout ducts (Fig. 1b). These cells are then surrounded by a stroma of adipocytes and fibroblasts infiltrated by blood and lymphatic vessels (Oakes et al., 2014).

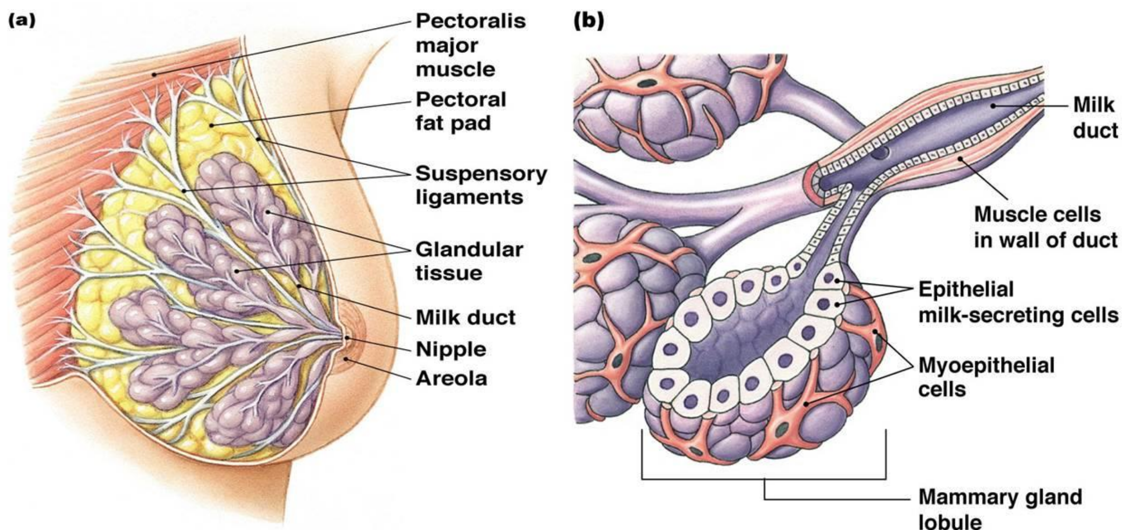


Figure 1: a) Structure of the mammary gland; b) Bilayered structure of mammary gland lobules
Adapted by
http://humanbiologylab.pbworks.com/w/page/104941359/Histology%20of%20the%20Mammary%20Gland

In vitro differentiation assays on primary epithelial cells proved the origin of these cells from common committed progenitors derived in turn from multipotent Mammary Stem cells (MaSC), present also in adult breast tissue as niches in mammary gland and characterized by self-renewal property (Prat and Perou, 2009). These cells have a key role in supporting the cellular turnover taking place during and after pregnancy (Macias and Hinck, 2012). Mammary gland development occurs in three different stages: embryonal, pubertal and reproductive. At the end of the embryonic development, a

rudimental ductal structure has formed; then, the gland grows allometrically, keeping up with overall body development, until puberty when the epithelium strongly proliferates, branching in numerous ducts with terminal endbuds or alveoli, filling the fat pad under the influence of the ovarian steroid hormone oestrogen and growth hormones (GH) (Macias and Hinck, 2012; Sternlicht, 2006). At the moment of pregnancy, the epithelial cells cleave and differentiate into distinct alveoli, becoming milk-secreting lobules during lactation. Some of these steps take place also during oestrus cycles, with a mild proliferation and differentiation of the mammary gland, followed by its involution. The hormone mainly responsible for this extensive branching and alveologenesis is progesterone, another steroid hormone released by the ovaries. Together with prolactin, progesterone brings to the alveoli differentiation. After weaning, these structures then collapse, and the mammary gland undergoes apoptosis, bringing to a pre-pregnancy state involution. In menopause, a complete involution takes place, with the fat cells replacing the mammary gland epithelial cells and the surrounding connective tissue (Macias and Hinck, 2012; Oakes et al., 2014).
The deregulation of pathways controlling hormones or other factors usually involved in the normal development of the mammary gland could bring to an uncontrollable growth of glandular epithelial cells, leading in turn to the breast cancer onset. If not treated, malignant cells can invade surrounding or distal tissues and organs, primarily lungs, brain and bones (Minn et al., 2005; Scully et al., 2012).

1.2 Breast cancer

Breast cancer is the most common malignancy in women worldwide, both in less and in more developed countries, with nearly 1,7 million estimated cases in 2012, taking into account the 25% of all tumours. It is the first and the second cause of cancer related death among women in the less and more developed countries respectively and the fifth cause of cancer related death among all cancers (Torre et al., 2015). The data presented above underline how much aggressive breast cancer is and the urgency to keep on studying the progression of this disease.
Breast cancer is an extremely heterogeneous disease, subdivided in several subtypes with different biological features and clinical outcomes, aspect that in turn deeply impinges on the choice of an adequate therapy (Vargo-Gogola and Rosen, 2007).
The breast cancer patients are classified according to the 2012 *Who Classification of tumours of the breast* (International Agency for research on cancer), which stratifies them basing on the histopathological type, tumor grading and staging, biomarkers expression and molecular profiling (Gannon et al., 2013).

At the moment of the diagnosis, one of the first evaluation made by physicians is the examination of bioptics breast specimens. The histological evaluation leads to roughly divide breast cancer in carcinoma *in situ* or infiltrating carcinoma. The *in situ* malignancy can be further classified in ductal carcinoma *in situ* (DCIS) or lobular (LCIS), less common with respect to the ductal type. The invasive carcinoma encompasses a wide variety of histological subtypes, among which the infiltrating ductal carcinoma is the most common, accounting for 70-80% of invasive lesions (Malhotra et al., 2010). In addition, the aggressiveness of breast cancer is categorized by the evaluation of the grade of the tumour basing on the differentiating status, according to the Nottighman Histologic score. Further information about the size of the primary tumour (T), the status of regional axillary lymph nodes (N) and the presence of distant metastasis (M) at diagnosis, according to the Classification of Malignant Tumours (TNM), lead to the classification of breast cancer in five stages (Webber et al., 2014). The lower the grade and the stage values are, the less aggressive the breast cancer is.

In order to better decide the patients who could benefit from a specific treatment, the evaluation of biomarkers, such as oestrogen, progesterone, epidermal growth factor receptors and Ki67 in breast cancers has been widely used in the past decades (Weigel and Dowsett, 2010). As highlighted before, the hormones oestrogen and progesterone play a crucial role in mammary gland development and differentiation, and their deregulation often leads to cancer development. For this reason, the evaluation of oestrogen and progesterone receptors (ER and PR) status is of high importance, considering that nearly 70% of invasive breast carcinoma express high levels of these receptors. The ER and PR positive breast cancers are usually treatable by hormone-interfering compounds, such as Tamoxifen, Fulvestrant and aromatase inhibitors (Gu et al., 2016; Moran, 2015). The amplification and the overexpression of the Epidermal growth factor receptor 2 (HER2) characterize approximately 15% of all primary tumours and was firstly identified as an indicator of prognosis. Indeed, patients with high levels of HER2 tend to relapse and have a shorter overall survival. Nevertheless, these patients can benefit from the use of anti-HER2 targeted therapies, such as Trastuzumab and Lapatinib (Weigel and Dowsett, 2010). Finally, Ki67 nuclear protein is used as a biomarker, being present in the active phases of the cell cycle thus indicating the proliferating cells (Scholzen and Gerdes, 2000). Ki67 evaluation is less adopted compared to the above-mentioned receptors status, due to the lack of a generally accepted system. The advent of high-throughput techniques, with microarray-based gene expression profiles, gave the physicians a further opportunity to more accurately stratify breast cancer patients in specific molecular subtypes with different overall survival rate, clinical outcome and ultimately treatment responses (Sørlie et al., 2001; Van ’t Veer et al., 2002), as represented in figure 2 in which 115 breast tumours are clustered in a dendrograms showing the different subtypes of breast

cancer, each of them with a different outcome (Vargo-Gogola and Rosen, 2007) . Indeed, breast cancer can be clustered in five molecular subtypes:

a. Luminal A characterized by the positivity to ER and PR presence and negative for HER2. They are low-grade and tend to have a good prognosis.
b. Luminal B characterized, as Luminal A, by the positivity to ER and PR presence, but it shows a more aggressive phenotype and a worse prognosis compared to the A, as it expresses high level of HER2.
c. HER2-like with an amplification and/or overexpression of epidermal growth factor receptor 2, with an unfavourable prognosis but the possibility to adopt targeted treatment
d. Normal-like genetically recalling a normal breast epithelium. There are few studies on this subtype and their clinical significance remains undetermined. Some researchers consider them technical artefacts from high contamination with normal tissue during microarrays (Weigelt et al., 2010).
e. Basal-like breast cancer which is the most aggressive subtype with a high histological grade and the worst overall survival outcome.

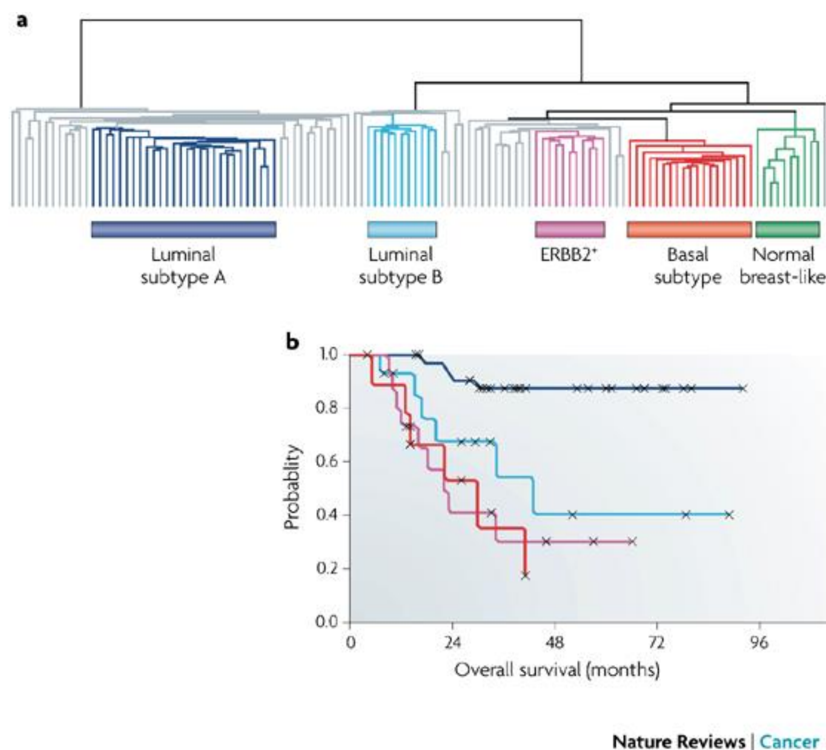


Figure 2: a) molecular classification of breast cancer subtypes basing on gene expression profile; b) prognostic outcome of different molecular subtypes of breast cancer. Adapted by Vargo-Gogola and Rosen, 2007

1.2.1 Basal-like breast cancer subtype

Basal-like breast cancer accounts for 15% of all breast cancers and encompasses a highly heterogeneous group of diseases. This molecular subtype is characterized by a gene expression profile similar to that of the basal-myoepithelial layer of the normal breast (Foulkes et al., 2010). Indeed, the basal-like breast cancer cells express high level of Cytokeratins 5/6, -14 and -17 and laminin. Basal-like breast cancer frequently shows mutations in tumor protein 53 (TP 53) gene and inactivation of the retinoblastoma (Rb) pathway (Heitz et al., 2009). The origin of basal-like breast cancer cells are still debated: indeed, it is still controversial whether basal-like breast cancer cells originate from mammary stem cells or are a product of an EMT event involving the luminal cells which lose their apicobasal polarity, acquire the ability to migrate and express basal/mesenchymal markers (Foulkes et al., 2009; Skibinski and Kuperwasser, 2015). Basal-like cancers are associated with high histological and nuclear grade and most of them are infiltrating ductal tumors with solid growth pattern, aggressive clinical behaviour, with a weak correlation between the size of primary tumour and the probability of survival, and a high rate of metastasis to the brain or lung (Heitz et al., 2009). The basal-like breast tumours lacking protein expression of oestrogen, progesterone and epidermal growth factor receptors are defined as triple negative breast cancer (TNBC). Basal-like breast cancer and TNBC are not completely synonymous, even though the majority of triple negative breast cancers are also basal-like. Indeed, TNBC comprises other subtypes of breast cancers such as the claudin-low tumours, composed by cells with stem cell-like properties (Foulkes et al., 2010). Patients affected by TNBC do not benefit from specific treatments, such as endocrine therapy or trastuzumab. Indeed, chemotherapy is the only mainstay of systemic treatment.

1.3 Cancer Hallmarks

Cancer is a heterogeneous disease that develops through the accumulation of mutations and genetic changes in normal cells. In a multistep process, neoplastic cells acquire a succession of hallmark capabilities. In 2000, Hanahan and Weinberg classified these tumour features in six hallmarks, simplifying the biology of cancer, and subsequently, in 2011, they modified their original formulation adding four new emerging hallmarks (Hanahan and Weinberg, 2000, 2011). These include: (i) unlimited proliferation, (ii) evasion from growth suppressors, (iii) escape from the surveillance of the immune system, (iv) replicative immortality of cells, (v) escape from programmed cell death, (vi) dysregulated cell metabolism, (vii) DNA mutations/instability, (viii) dysregulated angiogenesis, (ix) escape from the primary tumor site and metastasis, and (x) a tumor promoting inflammation state

(Hanahan and Weinberg, 2011) (Fig. 3). It is possible to identify dominant hallmarks driving breast cancer heterogeneity by focusing on identified biomarkers and the associated subtypes. In particular, the extent to cells having ‘sustaining proliferative signalling’ is of particular importance in breast tumor classification, especially among hormone positive tumors such as ER, PR, and HER2 versus TNBC. Whereas, in tumors lacking hormone receptors, such as basal-like, other cancer hallmarks take a dominant role and are often associated with more aggressive properties, among these, “escaping from the primary tumor site and metastasis” is the most important. Another hallmark, “DNA mutations/instability” help to take into consideration some genomic amplification that occurs frequently in breast cancer, and that have predictive value. Finally, for breast tumors is emerging the hallmark “angiogenesis”, that we will analyse in this thesis work.

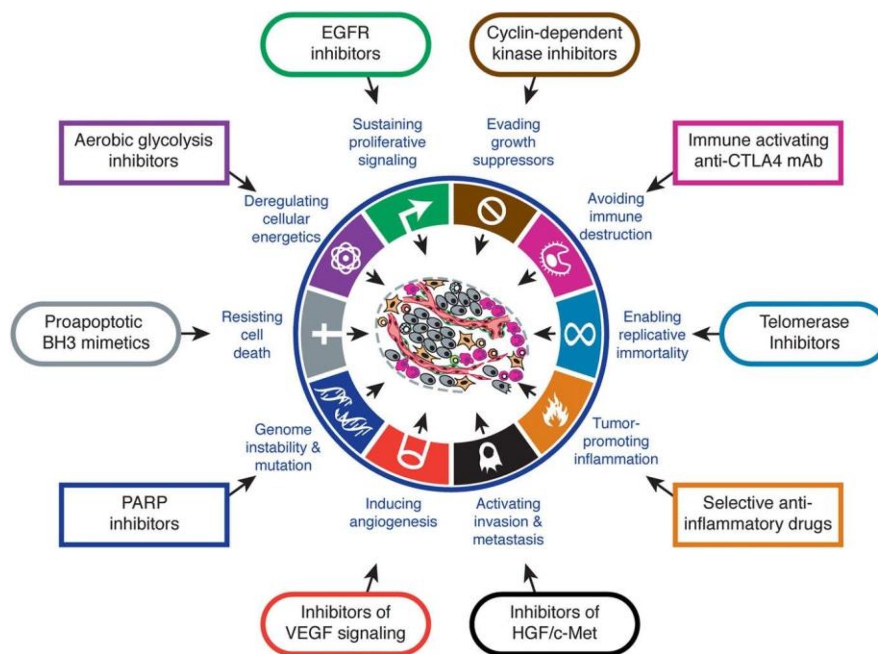


Figure 3: Cancer hallmarks, adapted by (Hanahan and Weinberg, 2000, 2011)

1.3.1 Hormone receptor status and the hallmark of “sustaining proliferative signalling”

In normal cells, the proliferation event is severely controlled. Clearly the most fundamental trait of cancer cells involves their ability to sustain chronic proliferation. They carefully regulate the production and release of growth-promoting signals, maintaining the cell number and thus the tissue homeostasis. Cancer cells find the way to escape these growth signals and start to auto-maintain (Hanahan and Weinberg, 2011). Mutations in growth factors or their receptors could lead to an uncontrollable proliferation in cancer.

In breast cancer this hallmark has a crucial role especially in some subtypes such as Luminal A and B, and HER2-positive. Indeed, oestrogen and progesterone receptors act upstream a cascade of multiple growth-promoting signals which lead to the production of growth factors and their receptors and other signalling molecules that sustain cell proliferation and survival (Nicholson et al., 1999). Therefore, in patients that are ER and PR-positive it could act on limiting the hallmarks of proliferation by treating patients with hormone-interfering compounds (like Tamoxifen, Fulvestrant and aromatase inhibitors). Moreover, another mainstay factor in promoting breast cancer proliferation is epidermal growth factor receptor 2, HER2, a transmembrane growth factor receptor, that when overexpressed in breast epithelial cells causes the deregulation of G1/S control and leads to the upregulation of cyclin D1, E and cdk6, and degradation of p27 (Timms et al., 2002). In HER2 overexpressing breast cancers, HER2-targeted therapies can be adopted, such as the monoclonal antibodies Trastuzumab and Pertuzumab. Furthermore, inhibitors of the CDK4/6 and mTOR pathways (such as Palbociclib and Everolimus, respectively) can be adopted (Manuscript under review).

1.3.2 Breast cancer genomic amplification and the hallmark of “DNA mutations/instability”

In addition to genomic amplification of HER2 that confers proliferative defects, it has been shown that breast cancer displays other amplification events, particularly in genes involved in DNA repair. The DNA repair system of normal cells is extremely efficient in detecting mutational events usually occurring. In cancer cells, the DNA mutations happens at a higher rate in comparison to normal cells, leading to genomic instability that fosters neoplastic transformation (Hanahan and Weinberg, 2011). By analysing breast tumours with fluorescence in situ hybridization and comparative genomic hybridization it has been found how breast tumours present a high number of genome copy number aberrations, such as amplification associated with adverse outcome. Commonly amplified sites include FGFR1, MYC, CCND1, MDM2, ERBB2 and ZNF217 gene loci (Chin et al., 2006). The degree of genomic instability influences the breast cancer patient’s prognosis (Auer et al., 1980, 1984), as genomically unstable tumours are indicator of a shorter disease-free survival times compared to stable genome (Kronenwett et al., 2004, 2006).

1.3.3 Basal-like breast cancers are characterized by the capacity of escaping from the primary tumor site and metastasize to other organs

In advanced cancers stages or in particularly aggressive types of tumors, malignant cells can invade surrounding tissues or spread to distal organs, thus worsening the patient conditions. Among breast

cancer, the basal-like displays the most aggressive phenotype, in which hallmark like escaping from the primary tumor site and metastatization has a crucial role. In this subtype, the related deaths are more attributable to the metastatic spread of breast cancer cells rather than to the primary tumor itself (Weigelt et al., 2005). The metastatization is characterized by a cascade of events in which neoplastic cells lose some normal features such as the capacity of cell-to-cell adhesion and acquire new abilities to degrade the extracellular matrix and escape from the primary tumor site. In breast cancer a key event in this multistep process is the epithelial to mesenchymal transition (EMT), achieved by the loss of epithelial markers, like E-cadherin, and the expression of mesenchymal markers such as N-cadherin, Vimentin and Fibronectin. Firstly, the alteration of cadherins expression, both the epithelial E- and mesenchymal N-types, causes the loss of adhesion capacity of cells to stromal cells and ultimately the stroma invasion by breast cancer cells (Cavallaro and Christofori, 2004). Beside this, in order to facilitate the tumoral cells invasion, the extracellular matrix has to be degraded: this process occurs thanks to metalloproteinases (MMPs) and the urokinase plasminogen activator system (uPA). In breast cancer context, it has been showed how uPA system has a prognostic importance in predicting the risk of distant metastases (Harbeck et al., 2004) and MMPs have been proved to mediate ECM proteolysis in invasive breast cancer cell lines (Kelly et al., 1998). Then, over-expression of mesenchymal markers such as vimentin, that has emerged as an organizer of a number of critical proteins involved in attachment and migration, confers the ability to tumor cells to move and reach distal organs through the blood stream. The preferential sites of metastasis of breast cancer cells are bone and lungs, thanks to a chemokines gradient (Minn et al., 2005). Interestingly, in breast cancer low expression of E-cadherin is associates with a poor prognosis (Gould Rothberg and Bracken, 2006), and high levels of vimentin is associated with invasion and poor prognosis (Lehtinen et al., 2013). Moreover, vimentin expression was found higher in patients with stage IV breast cancer, underlying how it could be a significant biomarker for predicting reduced disease-free survival and overall-survival in breast cancer (Patel et al., 2015). As breast cancer tissue preferentially express CXCR4 receptor, the organs with a higher expression of its ligand CXCL12, such as lymph nodes, lung, liver and bone marrow, are common sites of breast cancer metastatic invasion (Bruce et al., 1970). The cells detached from the primary or metastatic tumours which circulate in the blood vessels are called circulating tumor cells (CTCs) and they could indicate ongoing metastasis (Goodman et al., 2009). Moreover, the presence of CTCs above a certain cut-off predisposes the patients to a shorter progression-free survival and overall survival, highlighting the importance to evaluate this aspect (Consoli et al., 2011). In case of metastatic breast cancer, both standard chemotherapy and targeted therapies, such as hormonal and targeted treatments are adopted.

1.3.4 Tumor angiogenesis and breast cancer

Since the first identification of the tight dependence of the tumour growing on the vascularization process, the interest in this issue has grown rapidly in the last years. The nutrients and oxygen supply to the tumour is a crucial step for cancer growth and metastatic dissemination. Indeed, a tumor mass can grow up to 2 mm without a vasculature network (Folkman, 1971). Once a tumor lesion exceeds a few millimetres in diameter, signals such as hypoxia and nutrient deprivation trigger the "angiogenic switch" to allow the tumor progression (Folkman and Hanahan, 1991). In normal cells there is a balance between anti- and pro-angiogenic signals, balance that is then perturbed in tumoral cells. Malignant cells undergo the angiogenic switch favouring the production of pro-angiogenic molecules, event which in turn promote both primary tumor growth and a change from a quiescent to an invasive cancer phenotype (Kerbel, 2008). While in embryonic development the formation of new blood vessels initially occurs through *de novo* vasculogenesis from angioblasts, in tumor context vessels are formed from sprouting of pre-existing tubes and incorporation of endothelial progenitors into the growing vascular bed, event called angiogenesis (Rafii et al., 2002; Herbert and Stainier, 2011). This process involves the proliferation, migration and invasion of endothelial cells, their organization in functional tubular structures and moreover maturations and regression of vessels (Hicklin and Ellis, 2005). The new vasculature is characterized by abnormalities in its structure and function (Jain, 1990), the blood vessels are immature and leaky (Tong et al., 2004). An increased tumor vascularization, in terms of microvessels density (MVD), has been widely connected to an advanced tumor stage and a poor prognosis, also in breast cancer: high MVD has been correlated with a shorter relapse-free survival and overall survival in patients with lymph node-negative breast cancer (Weidner et al., 1992). In this complex scenario, one factor and its downstream pathway have a predominant role in promoting tumor angiogenesis: the vascular endothelial growth factor (VEGF). VEGF belongs to a family of angiogenic and lymphoangiogenic growth factors which includes six secreted glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor PIGF -1 and -2. Specifically, VEGFA is one of the most important factor. Several isoforms of VEGFA are produced by alternative splicing of its gene, among those the $VEGFA_{165}$ is the most abundant isoform (Giacca and Zacchigna, 2012). VEGF family members exert their role by binding several types of receptors on endothelial cells. Specifically, as represented in figure 4, VEGFA is secreted by cancer cells and, by binding VEGFR-2 on endothelial cells of the blood vessels, promotes their proliferation, migration and differentiation (Carmeliet and Jain, 2011) and thus the formation of new blood vessels from preexisting ones (Weis and Cheresh, 2011). Neurophilin (NRP) 1 functions as coreceptor for VEGF, lacking an intracellular signalling domain and enhancing the binding affinity

of VEGF ligands to their receptor (Soker et al., 1998). One of the most important process regulated by VEGFA is the vascular permeability, especially in tumor vessels. An increased permeability causes the leakage of several plasma proteins, a subsequent retardation in edema clearance from extravascular space and ultimately an activation of the stroma to a proangiogenic environment (Dvorak et al., 1995). Moreover, VEGF induces several other angiogenic properties, pivotal for the angiogenesis, such as the proliferation, invasion and migration abilities of endothelial cells, by regulating kinases ERK1/2 and uPA and tissue type plasminogen activator systems (Zachary and Gliki, 2001). Several works underline the importance of VEGF in breast cancer, in fact, high expression level of VEGFA has been indicated as an independent prognostic factor for relapse-free survival and overall survival specifically in lymph node-negative breast cancer patients (Manders et al., 2002). Moreover, a 13-gene VEGF signature has been demonstrated to predict poor outcome and distant metastases in breast cancer (Hu et al., 2009), highlighting the link between VEGF and metastasis. In addition, high levels of VEGFR2 has been shown in a subset of TNBC and correlates with shorter survival (Rydén et al., 2010). Considering the dependence of the tumor progression on the angiogenic process and the pivotal role of VEGFA in promoting the tumor angiogenesis, it has been developed a monoclonal antibody, against this pathway, which specifically targets the activation of the VEGF receptor, bevacizumab. It has been reported that the use of anti-VEGF treatment is highly recommended in TNBC cases, which show high level of VEGFA (Greenberg and Rugo, 2010). Apart from bevacizumab, other antiangiogenic treatments are used against VEGF, such as the tyrosine kinase inhibitors sorafenib and sunitinib, which target VEGF receptors (Chung et al., 2010). Nevertheless, resistance mechanisms limit the long-term benefit of VEGF-targeted therapies: for instance, anti-VEGF therapy leads to the upregulation of placenta growth factor, which in turn binds to VEGFR1 and leads to the transphosphorylation of VEGFR2 (Loges et al., 2009). Therefore, studying molecular pathways that sustain pro-angiogenic properties is of paramount importance in order to discover new target therapies.

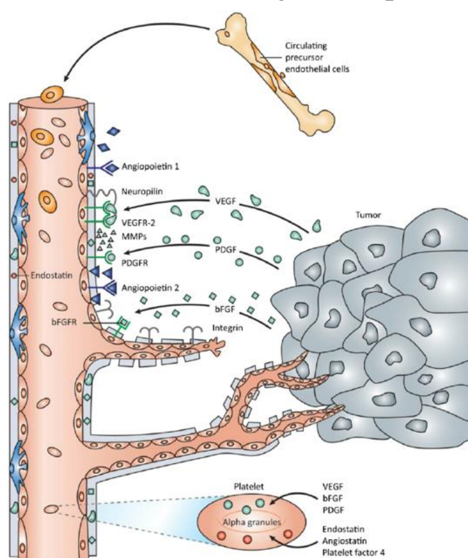


Figure 4: Representation of angiogenesis induced by tumoral cells on endothelial cells.
Adapted from Zhao and Adjei, 2015

2. HIGH MOBILITY GROUP PROTEINS

The “High mobility group” (HMG) proteins are a group of non-histone architectural factors, pivotal for several cellular processes, such as embryonic development, DNA repair control, modulation of chromatin structure and above all the regulation of gene transcription (Cleynen and Van de Ven, 2008; Reeves and Adair, 2005). They own their name to the high mobility in acidic (pH 2.4) polyacrylamide gel electrophoresis, as a consequence of their small molar mass (less than 30 KDa) in comparison with other nuclear proteins (Reeves and Wolffe, 1996). The HMG proteins can be subdivided in three different families, depending on the typical functional domain they possess:

- HMGA family, characterized by three positively charged AT-hooks involved in the binding of Adenine and Thymine stretches on the minor groove of the DNA (considered as more than 4/5 A or T consecutive nucleotides);
- HMGB family characterized by two alpha-helix HMG boxes which bind the non-B DNA form structures;
- HMGN family containing a positively charged nucleosome binding domains that associate with nucleosomes.

My thesis will be focused on HMGA proteins family.

2.1 HIGH MOBILITY GROUP A PROTEINS

2.2.1 Structure of HMGA gene and protein

As reported in figure 5, two different genes encode for the functional members of this family: HMGA1 gene, located at the chromosomal band 6p21, which produces two isoforms by alternative splicing, HMGA1a (107 aminoacid residues) and HMGA1b (96 amino-acid residues), differing for eleven aminoacid residues, and HMGA2 gene in 12q13-15 chromosome band from which the HMGA2 protein is produced (Fusco and Fedele, 2007). HMGA proteins display a natively disordered status, as they do not show any secondary or tertiary structures; nevertheless, by binding DNA or other proteins, HMGA proteins undergo several structural changes (Reeves, 2001) . This aspect gives HMGA proteins the possibility to interact with a multitude of molecular partners and thus regulate a variety of targets in a plethora of biological processes (Sgarra et al., 2010; Sumter et al., 2016). They possess three highly conserved palindromic basic amino acid motifs of proline-arginine-glycine-arginine-proline, defined AT-hooks, through which they bind the AT stretches in the minor groove

of the DNA. Furthermore, they have an acidic carboxy-terminal tail, highly modified at post-translational level, aspect that deeply impinges on HMGA activities (Sgarra et al., 2004).

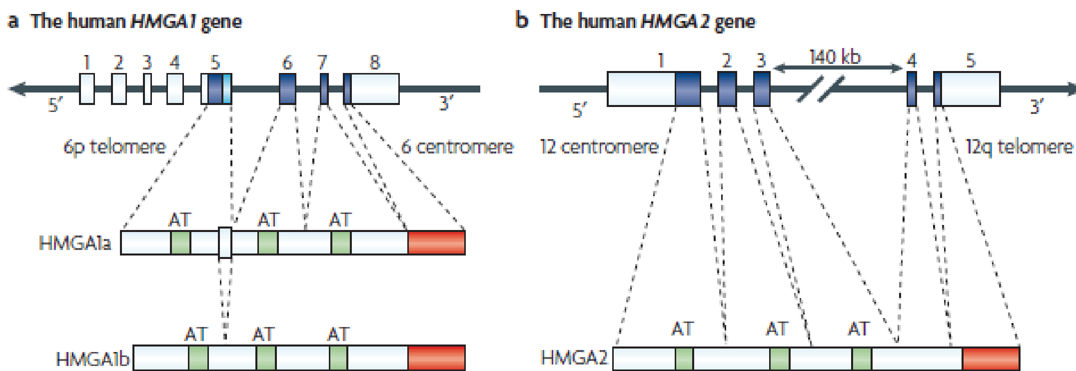


Figure 5: HMGA1 and -2 gene and protein structures. Adapted by Fusco and Fedele, 2007

2.2.2 Molecular mechanisms of action

As already described, HMGA proteins are chromatin architectural factors which do not display a transcriptionally activity *per se;* nevertheless, they are master regulator of the gene transcription, by their ability to bind both DNA and proteins, and assembling and/or modulating macromolecular complexes. Summarizing, they modulate transcription by acting through three different mechanisms. Firstly, HMGA proteins can interact with both DNA and proteins (Fig. 6a), thus forming macromolecular complexes, called enhanceosomes, on regulatory regions of their target genes, such as in the case of Interferon-Beta (IFN-β). In details, HMGA proteins bind and bend the DNA at the enhancer level of IFN- β gene promoter. HMGA1 binding enhances the binding affinity of other factors (c-JUN, ATF2 and NFκB) to the enhancer of IFN-β, thus regulating its transcription (Yie et al., 1999a). Secondly, it establishes direct protein-protein interactions (Fig. 6b), for instance with other transcription factors: among the plethora of factors bound by HMGA1, primarily it was found that NF-Y (Nuclear Transcription Factor) was directly bound by HMGA1, binding which enhances the affinity of NF-Y to its target (Currie, 1997); in addition, the SRF (Serum Response Factor) and HMGA1 collaboration positively regulate the c-FOS transcription (Chin et al., 1998). Moreover, it is noteworthy to mention the interaction between HMGA2 and pRB, which frees E2F1 from RB1 binding and activates E2F1 transcriptional program (Fedele et al., 2006a). Finally, HMGA in general alter the chromatin structure (Fig. 6c), facilitating the accessibility of the transcription machinery to the DNA. Specifically, with their AT-hooks HMGA proteins are associated to matrix and scaffold associated region (MAR/SAR), DNA genomic region with high affinity for nuclear matrix. Moreover,

HMGA proteins compete with histone H1 for DNA binding, de-repressing the histone H1-mediated inhibition of SAR transcription (Zhao et al., 1993).

Through these three different mechanisms of action, HMGA1 protein regulate a multitude of biological processes, such as cell growth, proliferation, differentiation and cell death (Cleynen and Van de Ven, 2008) and for this reason their expression level is tightly regulated during life.

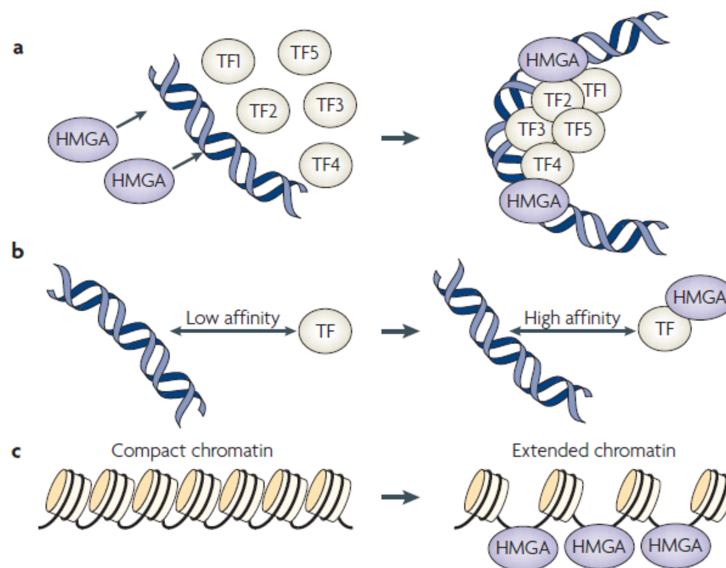


Figure 6: Mechanisms of action of HMGA proteins. a) HMGA proteins bind directly the DNA, altering the chromatin structure and facilitating the formation of the enhanceosome; b) HMGA proteins bind other transcription factors modifying their conformation and their DNA binding affinity; c) HMGA proteins alter the chromatin structure. Adapted by Fusco and Fedele, 2007

2.2.3 Expression level of HMGA1 in physiological and pathological conditions

HMGA1 proteins are highly expressed during embryonic stages when they take part in development as it has been demonstrated by *in vivo* mice through gene knockout. HMGA1 knock-out mice display myeloproliferative disorders and cardiac hypertrophy (Fedele et al., 2006b). Moreover, the double Hmga1/Hmga2 knock-out leads to the “super pygmy” feature with a reduced viability of embryos survived (Federico et al., 2014). In adult life, HMGA1 expression is very low or even absent (Chiappetta et al., 1996). Nevertheless, HMGA1 proteins are frequently re-expressed in cancer context. Overexpression of HMGA1 was first observed in HeLa S3 cells (Lund et al., 1983) and subsequently in cultured rat thyroid cells transformed by viral oncogenes (Giancotti et al., 1987). Since these discoveries the causal role of HMGA1 in cancer onset and progression has been

extensively studied during the years. Back to that observation, a high expression level of HMGA1 proteins has been confirmed in many other tumor types, where HMGA1 overexpression correlates with a high malignant phenotype and a poor prognostic index (Fedele and Fusco, 2010). In addition, Pierantoni and colleagues demonstrated also the involvement of HMGA1 in several haematological leukemias, where HMGA1 high levels are reported (Pierantoni et al., 2003). Moreover, an axis between HMGA1 and STAT3, a pivotal player in cancer progression, has been established: indeed, HMGA1 positively regulates STAT3, found highly expressed in several haematological malignancies, by directly binding its promoter (Bowman et al., 2000).

In the context of breast cancer, in 1993 Ram and colleagues found that the high expression level of HMGA1 correlates with the neoplastic transformation of mammary epithelial cells (Ram et al., 1993), induced to grow anchorage-independently and to form metastasis (Reeves et al., 2000). Moreover, the *in vivo* causal role of HMGA1 in breast cancer progression was studied in human breast epithelial cells MCF7 where the overexpression of HMGA1 transgene makes the cells able to form both primary and metastatic tumors in nude mice (Reeves, 2001). Years later, HMGA1 overexpression was also confirmed in 40% of hyperplastic lesions with cellular atypia and in 60% of ductal carcinoma, based on the analysis of breast cancer specimens (Chiappetta et al., 2004). As a confirm, a Gene expression-based outcome (GOBO) for breast cancer online analysis highlighted an enrichment of HMGA1 expression in basal and HER2 overexpressing breast cancer subtypes with respect to less aggressive subtypes, such as luminal A and B and normal-like (Pegoraro et al., 2013), thus connecting once again the HMGA1 not only to breast cancer but specifically to highly aggressive subtypes. HMGA1 has been tightly connected to several hallmarks of breast cancer. For instance, Baldassarre and colleagues demonstrated that the overexpression of HMGA1b in MCF7 cells induces a higher sensitivity to chemotherapy agents, such as cisplatin and bleomycin, enhancing the DNA damage predisposition of cells (Baldassarre et al., 2005). In addition, HMGA1 is also involved in cellular metabolism: indeed, by regulating the transcription of GLUT-3 (Ha et al., 2012) , which is a glucose carrier found at increasing doses during cancer progression (Kocdor et al., 2013) promotes the aggressive traits of invasive breast cancer. In metabolism context again, HMGA1 forms a multiprotein complex with Sp1 and C/EBP-beta proteins on the promoter of the Insulin Receptor (INSR) (Foti et al., 2003), which is overexpressed in MCF7 and human breast cancer tissues (Paonessa et al., 2006). In addition, in a triple negative breast cancer cell line model with a high proliferation and migration/invasion rates, the silencing of HMGA1 causes a reversion of the mesenchymal and less differentiated phenotype to a more polarized epithelial one: specifically, the depletion of HMGA1 brings the cancer cells to acquire an ordered monolayer sheet disposition, a reorganization of the cytoskeleton, decreases stemness and self-renewal ability. Moreover, it has a negative impact on the motility and the

invasiveness both *in vitro* and *in vivo*, and down-regulates a signature of genes related to epithelial to mesenchymal transition, stemness and poor prognosis (Pegoraro et al., 2013; Shah et al., 2013). Among the genes modulated by HMGA1, it has been found to regulate CCNE2 expression which in turn influences the nuclear localization of YAP and thus its malignant transcriptional program (Pegoraro et al., 2015). Furthermore, it has been demonstrated that HMGA1 modulates the expression level of SERPINE1 and PLAU, functional members of the Urokinase plasminogen activator system, involved in the promotion of the cellular migration and thus the metastatization process, through the remodelling of the extracellular matrix (Resmini et al., 2017).

Regarding the cancer hallmark of angiogenesis, a few evidences report the role of HMGA1 in regulating this aspect, pivotal in physiological and pathological conditions. HMGA1 factor increases the VEGF transcription by directly binding its promoter, and this effect seems to occur synergistically in cooperation with HIF1, thus in hypoxic condition, in adipose tissue (Messineo et al., 2016). Moreover, HMGA1 has been discovered to regulate VEGFA and angiopoietin 1 expression in ischemic rat brains, promoting the viability of endothelial cells and consequently having a role in brain functions repair after a stroke event (Camós et al., 2014). Furthermore, in human gliomas a correlation has been established between HMGA1 and several other factors involved in cancer aggressiveness, among them VEGFA, suggesting a role for HMGA1 in angiogenic process (Pang et al., 2012). These studies highlight that HMGA1 is strictly involved at multiple levels in breast cancer aggressive features, regulating its progression and metastatic dissemination.

Taken into consideration all these data, it appears clear that HMGA1 has a strong role in breast cancer aggressiveness acting on the majority of cancer hallmarks through the coordination of specific gene networks. Further dissecting the HMGA1–linked molecular mechanisms involved in breast cancer aggressiveness could give major chance of therapeutic intervention.

3. FOXM1

3.1 Forkhead box family

The Forkhead box M1 (FOXM1) protein belongs to the Forkhead box family of transcription factors. They are characterized by a highly conserved DNA binding domain of nearly 100 amino acids residues, the forkhead box (FKH) or "winged helix" domain at the N-terminal region, which assumes a helix-turn-helix structure, flanked by two loops called wings (Brennan, 1993). This large family of transcription factors comprises more than 100 proteins with temporally and spatially restricted biological functions, primarily involved in embryonic and adult homeostasis (Korver et al., 1997).

3.2 FOXM1 structure and function

The human forkhead box M1 gene is located on 12p13 chromosomal band and consists of 10 exons (Fig. 7). From the alternative splicing of two exons, exons Va (A1) and VIIa (A2), three different variants are produced: FOXM1a, in which both exons are present, FOXM1b with neither one the exons and FOXM1c in which only exon A1 is retained. FOXM1a is transcriptionally inactive, because of the insertion of the exon A2 in its transactivation domain, aspect which results in a dominant-negative variant without a functional transactivation domain (Ye et al., 1997), whereas both FOXM1b and FOXM1c are transcriptionally active (Korver et al., 1997; Ye et al., 1997). At the protein level FOXM1 is characterized by three domains: the FKH domain described above, which is involved in DNA binding; the transactivation domain (TAD) in its C-terminus, and the N-terminal repressor domain (NRD) involved in the autoregulatory activity of FOXM1. FOXM1 binds DNA *in vitro* preferentially to repeats of the consensus DNA motif TAAACA, highly conserved among the other Forkhead factors family members (Korver et al., 1997).

FOXM1 regulates the transcription of several targets involved in paramount cellular activities. FOXM1 is intimately involved in the regulation of a transcriptional program of cell-cycle progression, such as G1-S and G2-M transitions, mitotic progression and the maintenance of chromosome stability, by regulating Skp1-Cullin 1-F box ubiquitine ligase complex, several cyclins (A2, B and D), Cdc25B, PLK1, Auroka kinases A and B, Survivin, and Centromere protein A, B and F whose promoters are directly bound by FOXM1 (Wang et al., 2005a, 2001, 2002). Indeed, FOXM1-depleted cells do not progress beyond the prophase stage of mitosis and they show cell-cycle abnormalities, such as delay in G2-M progression, chromosome missegregation and failure of

cytokinesis (Laoukili et al., 2005; Wang et al., 2005b; Wonsey and Follettie, 2005). Besides its central role in cell-cycle regulation, as transcription factor FOXM1 promotes also several other biological processes, such as stem-cell expansion and renewal, senescence and DNA-repair, epithelial to mesenchymal transition and angiogenesis, all functions important for embryonic and adult homeostasis (Lam et al., 2013). Regarding vascular development, the importance of FOXM1 has been endorsed by *in vivo* FoxM1 knockout, in which the lungs of FOXM1 -/- mice show severe abnormalities of the lung vasculature concomitantly with reduced expression of Pecam-1 (Platelet endothelial cell adhesion molecule-1), the VEGF receptor type 1 (Flt1) and FoxF1, targets related to vasculogenesis (Kim et al., 2005).

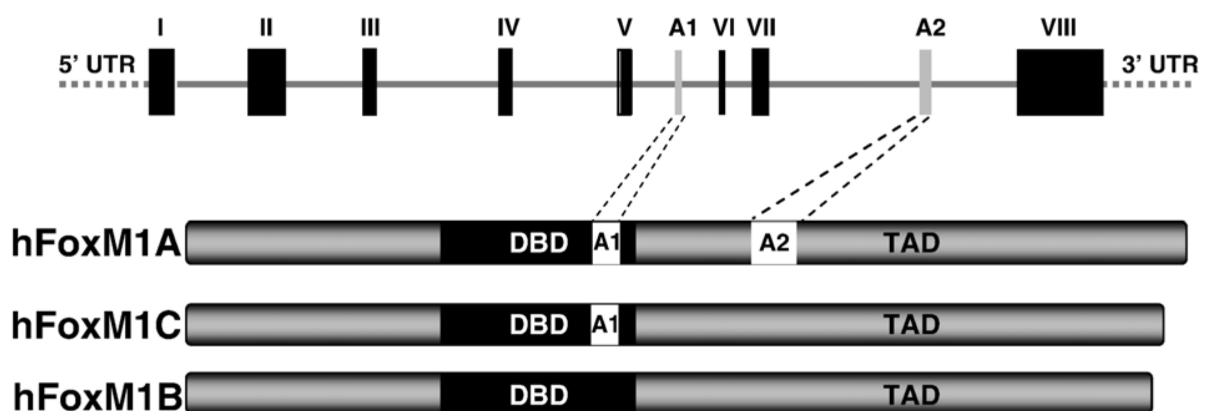


Figure 7: Structure of FOXM1 gene and proteins. Adapted by Laoukili et al., 2007

3.2.1 Expression level regulation of FOXM1

FOXM1 is defined as a proliferation associated factor, whose expression is tightly regulated during the cell cycle. Indeed, its expression level is high in proliferating cells, such as in embryonic cells, while it is negatively regulated in quiescent or terminally-differentiated cells. During adult life, FOXM1 expression is limited to highly proliferating and self-renewing cells, such as thymus, testis, small intestine and colon. Nevertheless, FOXM1 is re-expressed when cells are induced to enter the cell cycle in response to mitogenic stimuli, tissue injury or oxidative stress (Korver et al., 1997; Yao et al., 1997). The importance of FOXM1 in embryonal stages is highlighted by FOXM1 knock-out mouse models, where heterozygous mutants appear normal, whereas the homozygotes die due to embryonal lethality. Occasionally, homozygous knock-out embryos complete the development but die after birth, because of severe heart defects, suggesting the important role of FOXM1 not only in embryogenesis but also during organogenesis (Korver et al., 1998). Given the critical role of FOXM1 in cell cycle progression, its expression is tightly regulated at different levels.

It has been demonstrated that FOXM1 is subjected to a strong post-translational regulation (Fig. 8). In fact, the cellular localization and transcriptional activity of FOXM1 is dependent upon its phosphorylation state: un-phosphorylated FOXM1 is located in the cytoplasm where it is inactive at late G1 and S phases (Ma et al., 2005), then it is phosphorylated by CyclinE-Cdk2 in G1 and it translocates into the nucleus; it is subjected to further phosphorylation events in a sequential order by several Cdk-cyclin complexes and mitogenic kinases in the S- and G2-M phases of the cell cycle, leading to the hyperphosporylated and completely active form of FOXM1 by the G2-M phases, Cyclin B-Cdk2 and Plk1. Several other post-translational modifications affect FOXM1 activity by controlling its protein levels. At the late M and G1 phases of the cell cycle FOXM1 is degraded after ubiquitination by the anaphase promoting complex/cyclosome complex (APC/C), in collaboration with Cdh1, (Park et al., 2008), leading to FOXM1 degradation. FOXM1 protein stability is also affected by sumoylation, which negatively regulates FOXM1 after its cytoplasmic translocation upon APC/C-Cdh1 induction (Myatt et al., 2014). At the transcriptional level, FOXM1 is principally controlled by three pivotal tumor suppressors Rb, p53 and p19ARF (Jaiswal et al., 2014). Moreover, FOXM1 has a prominent role also on its own transcriptional activity. It has been highlighted that FOXM1 activates its own mRNA and protein expression in a positive feedback loop (Halasi and Gartel, 2009).

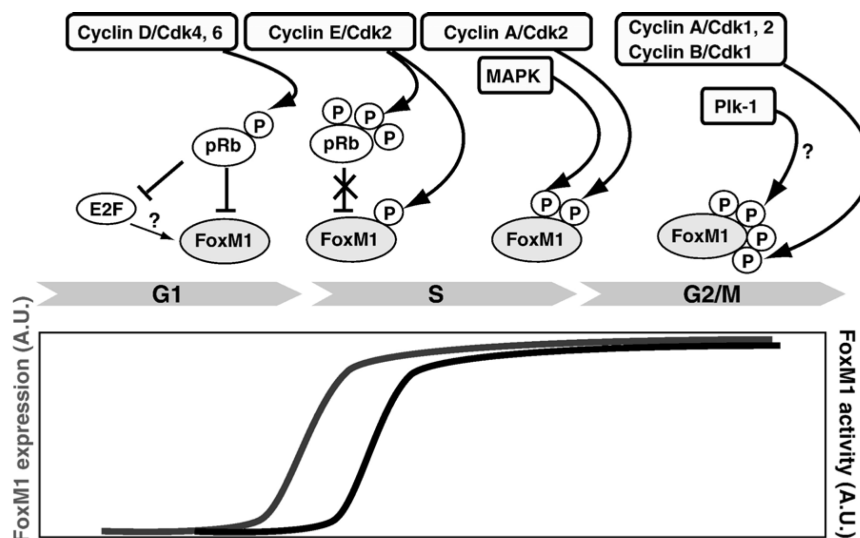


Figure 8: Cell-cycle dependent regulation of FOXM1, adapted by (Laoukili et al., 2007)

3.2.2 FOXM1 and cancer

Given a role in cell cycle, it is expected that FOXM1 plays a pivotal role in tumorigenesis. Indeed, elevated expression level of FOXM1 has been found in cancer context, implicated both at the early stages of carcinogenesis and in metastatic progression. The first identification of the causal role of FOXM1 in cancer came from a study of 2002, in which the authors demonstrated that this factor is up-regulated in basal cells carcinomas and that FOXM1 is a downstream target of Sonic Hedgehog pathway via GLI1 (Teh et al., 2002). Since that work, the awareness of the causal role of FOXM1 in cancer has grown intensively. Indeed, in 2004, a comparative microarray analysis unravelled FOXM1 as one of the most commonly overexpressed gene in solid tumours, in particular in undifferentiated cancer with respect to well-differentiated types, strongly underline the importance of FOXM1 in cancer context (Pilarsky et al., 2004). Up-regulation of FOXM1 in neoplasias correlates with advanced tumour stage, high proliferation rate and a poor prognosis, bringing to light the possibility of using this factor as prognostic biomarker (Halasi and Gartel, 2009). Different mechanisms have been proposed to induce FOXM1 overexpression in cancer: (i) amplification of FOXM1 locus, which is frequently amplified in several cancers, among which we find breast cancer; (ii) an increase in FOXM1 protein stability and expression thanks to the interaction with other molecular partners, such as nucleophosmin; (iii) an enhancement of FOXM1 transcription, for example by Rb/E2F, cMyc and HIF1 which directly bind its promoter; (iv) mutations of p53 tumor suppressor and activation of FOXM1 by multiple signalling pathways, such as PI3/Akt, EGF receptor, Raf/MEK/MAPK and Hedgehog (Halasi and Gartel, 2009). As primarily implicated in cell-cycle regulation, the dysregulation of FOXM1 leads to an increase in cancer proliferation, thus in the early stages of tumorigenesis (Laoukili et al., 2007). Moreover, it increases the resistance of cancer cells to apoptosis (Koo et al., 2012). Indeed, Foxm1-transgenic mice develop larger tumors in comparison with their wild-type counterparts (Kalin et al., 2006). Furthermore, it is found implicated in other cancer hallmarks, indeed it promotes the migration, invasion and metastatic capabilities of cancer cells and the angiogenic process, promoting in this way more aggressive tumour traits. In this context, FOXM1 is involved in the modulation of an EMT-transcriptional program. In particular, it up-regulates several EMT markers such as ZEB1 and 2, Snail 2, vimentin, fibronectin, N-cadherin and down-regulates E-cadherin (Halasi and Gartel, 2009). It promotes also the expression of VEGF, by binding its promoter (Zhang et al., 2008). In addition, it modulates the extracellular matrix degradation by regulating MMP2 and MMP9 (Lim et al., 2009; Wang et al., 2007) and transcriptionally activates Stathmin, destabilizing the microtubules (Park et al., 2011), leading thus to an increased cancer cell invasiveness

and motility. All these evidences reveal FOXM1 as a key player of cancer disease, highlighting how it is involved at multiple stages of cancer progression.

3.2.3 FOXM1 and breast cancer

Among the cancer tissues in which FOXM1 has been found up-regulated, it has been intensively connected to breast cancer. In a subset of breast cancer patients FOXM1 was associated to larger tumour size, lymphovascular invasion, lymph node metastases and higher stage of breast cancer (Ahn et al., 2015). In addition, overexpression of HER2 correlates with high level of FOXM1 mRNA (Bektas et al., 2008). Moreover, a whole genome and transcriptome sequencing of TNBC revealed a consistent overexpression of FOXM1 in this breast cancer subtype, suggesting a causal role of this factor. Altogether, these studies clearly show as FOXM1 has a prominent role in breast cancer and particularly in more aggressive subtypes. It exerts this tumor promoting role by modulating breast cancer at multiple levels. In particular, it is involved in the proliferation of malignant cells by binding the promoter and activating the transcription of Estrogen Receptor α, which in turn regulates FOXM1 in a positive feedback loop (Madureira et al., 2006). In addition, this factor has a critical role in promoting drug resistance: by keeping $p27^{kip1}$ expression low, it bypasses the Herceptin-induced G1 arrest (Carr et al., 2010). It up-regulates anti-apoptotic genes, such as XIAP and Survivin, contributing to chemoresistance (Nestal de Moraes et al., 2015). Furthermore, FOXM1 enhances DNA repair, thus leading to resistance to cisplatin and epirubicin in breast cancer cells (Kwok et al., 2010; Millour et al., 2011). FOXM1 has a role also in promoting EMT and metastatization processes: it binds the promoter of SLUG (Yang et al., 2013) and by interacting with SMAD3 and stabilizing the SMAD3/4 complex activates the TGF-beta pathway (Xue et al., 2014), modulating in this way a crucial oncogenic pathway. Given the previously described central role of angiogenesis in the growth, progression and metastasis of solid tumors such as breast tumors, it is of interest to underline the involvement of FOXM1 in the direct binding and transcription of the VEGF promoter by competing with another Forkhead member, FOXO3A, in breast cancer cell lines (Karadedou et al., 2012).
From these evidences it is obvious that a deregulation of FOXM1 has a causal role in triggering and fostering breast and other types of cancer. Indeed, it is considered a strong candidate biomarker for cancer and several anti-cancer drugs directly and indirectly targeting FOXM1 have been tried, such as proteasome inhibitors, which negatively regulate this factor. Nevertheless, much effort has to be made to better elucidate how to target FOXM1, a paramount factor for breast cancer progression.

Aim of the Thesis

Triple negative breast cancer (TNBC) is one of the most aggressive and less curable subtype of cancer, lacking specific treatments, aspect that motivates researchers to deepen the factors strictly responsible for the aggressive traits of this disease. Among the factors involved in TNBC progression, HMGA1 has been strongly connected to malignant features of breast cancer, for its plasticity as architecture proteins in controlling many cellular processes by associating with other factors. Thus, to better address TNBC treatments, further exploring the network HMGA1 play a role in could be of extreme interest. The purpose of this work is to unravel new molecular partners of HMGA1 and better investigate their synergistical action in controlling common pathways promoting several cancer hallmarks. To achieve this objective, we took advantage of cellular models of triple negative breast cancer overexpressing HMGA1 and we deepened the features controlled by its interaction with new molecular partners.

Materials and Methods

1. Cell Culture

The human triple negative breast cancer cell lines MDA-MB-231 and MDA-MB-157 and the human embryonic kidney cell line HEK293T were routinely grown in high glucose Dulbecco's Modified Eagle Medium (DMEM, Euroclone), with 10% tetracycline-free Foetal Bovine Serum (FBS, Euroclone), 2mM L-Glutamine (Euroclone), 100 U/ml Penicillin (Euroclone), 100 µg/ml Streptomycin (Euroclone). For routine cell culture passage, cells were dissociated with Trypsin-EDTA (0.05% trypsin, 0.02% EDTA in PBS, Euroclone). For long-term maintenance, cells were collected in FBS 10% (v/v) DMSO (AnalaR BDH) and stored in liquid nitrogen.

2. siRNA Transfection

The day before the siRNA transfection, cells were seeded in antibiotics-free DMEM at a density of $2*10^5$ cells/35-mm dish (Corning). The Lipofectamine™ RNAimax reagent (Invitrogen/Thermo Fisher Scientific) was used for transfection of 30 pmol of siRNA/35-mm dish, following the manufacturer instructions. For co-silencing experiments, 30 pmol of each specific siRNA and 60 pmol of siCTRL in the control condition were transfected. The cells were processed after 72 hours of silencing.

siRNAs used in this work are listed in the following table:

TARGET GENE	*siRNA*	*SEQUENCE (5'-3')*
	siCTRL	ACAGUCGCGUUUGCGACUG
HMGA1 a/b	siA1_3	ACUGGAGAAGGAGGAAGAG
FOXM1	siFOXM1	CCUUUCCCUGCACGACAUG

3. Plasmid transfection

Plasmid transfections were carried out using Lipofectamine 3000 (Invitrogen/ThermoFisher Scientific), following the manufacturer protocols, for MDA-MB-231 cells and the standard Calcium Phosphate transfection method for HEK293T cells. For overexpression experiments, $35*10^4$ cells were seeded in 35 mm diameter dish the day before the transfection.

The plasmids transfected are listed below:

- pEGFP-N1, pEGFP-N1 HMGA1a, pRL-CMV Renilla (Promega) and pGL4.11 (Promega) were already present in the laboratory.
- pEGFP-FOXM1 and pGL3-5BS (containing five repetitions of FOXM1 binding sites-TAAACA) were kind gifts of Dr. Muy Teck Teh from the Department of Diagnostic and Oral Sciences, Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK.
- The vectors used in this work named pGL4.10-VEGFprom (-1000-1), pGL4.10-VEGFprom (-1000-500), pGL4.10-VEGFprom (-500-1), as they contain a portion of the VEGFA promoter that goes from -1000 to -1 bp, -1000-500 bp and -500-1 bp respectively, were a gift from David Mu (Addgene plasmid # 66128, # 66129, #66130).
- The deletion mutants pGL4.10-VEGFpromWT(-388-1), pGL4.10-VEGFprom (-338-1), pGL4.10-VEGFprom (-172-1) and the pGL4.10-VEGFprom (-104-1), containing a region of the VEGFA promoter spanning from -388-1 bp, -338-1 bp, -172-1 bp, and -104-1 bp respectively, were generated in the laboratory by amplifying the pGL4.10-VEGFprom (-1000-1) with the forward primers 5'-GGGGTACCCCGGGGCGGCCGGGGGCGGGGTCC-3',5'-GGGGTACCCCCTTTTTTTTTAAAAGTCGGC-3', 5'-GGGGTACCTGGAATTTGATATTCATTGATCCG -3', 5'-GGGGTACCTGTATTGTTTCTC GTTTTAATTT- 3' for the -388, -338, -172 and -104 to -1 bp fragments respectively and the common reverse primer 5'-CCCAAGCTTAAAATCCACAGTGATTTGGGGAA - 3'. We then created a pGL4.10-VEGFpromMUT(-388-1) mutated in two SP1 binding sites (GGGCGG → GGAAGG) by amplifying the pGL4.10-VEGFprom (-1000-1) with the forward primer 5'-GGGGTACCCCGGGAAGGGCCGGGGAAGGGG TCC -3' (GC → AA) and the reverse primer used in previous experiments.

  Subsequently, the PCR products were cloned in KPNI and HINDIII (Amersham Biosciences) restriction sites of pGL4.11 vector. All the plasmids generated in the laboratory were sequenced by Eurofins Genomics sequencing service.

In particular, for immunofluorescence experiment upon HMGA1 silencing in HEK293T cells, one day after the silencing we transfected 1 µg of pEGFP-FOXM1.

For luciferase reporter assays, a total amount of 1.4 µg of plasmid DNA was transfected in HEK293T cells by Calcium Phosphate method. Specifically, we used the following amounts of plasmids:

- 200 ng of the Luciferase reporter, specific for each experiment;
- 1200 ng of pEGFP-N1 in the control condition;
- 600 ng of pEGFP-N1 HMGA1a or/and 600 ng pEGFP-FOXM1.

The luciferase assay was also carried out upon silencing condition, in which the expression of HMGA1 or FOXM1 was knocked down by transfecting 30 pmol of siRNA/35-mm dish, followed by the transfection of 1 µg of DNA, as follows:

- 200 ng of the Luciferase reporter, specific for each experiment
- 1000 ng of pEGFP-N1 in the control condition
- 600 ng of pEGFP-N1 HMGA1a or 600 ng pEGFP-FOXM1

For each condition, 10 ng of pRL-CMV Renilla was transfected. Renilla luciferase was used as normalizer.

4. Luciferase assay

The Dual-Luciferase® Reporter Assay System (Promega) was used for the luciferase reporter assay, following the manufacturer instructions. The measurements were carried out using the Berthold Lumat LB 9501 Tube Luminometer; two technical replicates were performed for each sample. As HMGA1 is insoluble in the Passive Lysis Buffer provided by the Promega Dual-Luciferase kit, we centrifuged 1/5 of the total volume of cells collected in PBS1X for 5 minutes at 200 g and the cells were lysed in 20 µl of SDS sample buffer. The amount of proteins transfected was checked by Western blot analysis.

5. Protein extraction and western blot analysis

Cells were washed in ice-cold PBS1X and then were lysed in SDS sample buffer (62.5 mM Tris, pH 6.8; 2% SDS; 10% glycerol) with 200 mM DTT, 1 mM Na3VO4, 5 mM NaF and mammalian protease inhibitor cocktail (PIC) (Sigma). Afterwards, DNA were disrupted with passing the sample through an insulin syringe and boiled for 5 minutes. Total lysates were separated by SDS-Polyacrylammide gel electrophoresis (SDS-PAGE) in SDS-PAGE gel composed by the stacking and the running gel: Stacking gel: 5%: 5% acrylamide/bisacrylamide (29:1), 0.125 M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.05% (w/v) APS and 0.05% (v/v) TEMED. Running gel: 7.5%: 7.5% acrylamide/bisacrylamide (29:1), 0.3 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) APS and 0.05% (v/v) TEMED; 10%:

10% acrylamide/bisacrylamide (29:1), 0.5 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) APS and 0.05% (v/v) TEMED; 15%: 15% acrylamide/bisacrylamide (29:1), 0.375 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) APS and 0.05% (v/v) TEMED. Gel thickness: 0.75 mm, length of stacking gel: 0.5 cm, length of running gel: 8 cm, gel width: 8 cm. The electrophoresis was carried out in running buffer composed by 25 mM Tris, 200 mM Glycine, 3.5 mM SDS (w/v) and performed at 50V for the accumulation step and then at 200V for the protein separation, till the complete separation of the molecular markers (Pre Stand, Thermo Fisher Scientific). Subsequently, the gel could be stained in solution of methanol/water/acetic acid solution (in a volume ratio of 5/4/1) containing 0.05% (w/v) Coomassie Brilliant Blue R 250, and then destained in a solution of 10% Acetic Acid in H_2O, in order to perform the protein quantification. Alternatively, the proteins were transferred to nitrocellulose membrane Ø 0.2 µm (GE Healthcare, WhatmanTM) using a wet transfer system (Transfer buffer: 25 mM Tris, 200 mM Glycine, 20% methanol) at 4°C for 16 hours at 40 V. After the transfer step, membranes were then stained with Red Ponceau solution (0.2% red ponceau S, 3% trichloroacetic acid, 3% sulfosalicylic acid). Western blot analyses were performed according to standard procedures. To revealed horseradish peroxidase conjugated to secondary antibody on autoradiography films (GE Healthcare), Liteablot Extend ECL (Euroclone) or TPro Lumilong Plus (T-Pro Biotechnology) were used.

The antibodies used are listed in the following table:

Primary Antibody	*Antibody diluition*
αHMGA1 (made in the laboratory)	1:500
αFOXM1 (A301-533A-M, Bethyl Laboratories)	1:700
αGFP (kind gift of Professor Collavin, LNCIB, Trieste)	1:1000
α-β-actin (A2055, Sigma)	1:2000

Secondary Antibody	*Antibody diluition*
α-rabbit IgG peroxidase conjugate (A0545, Sigma)	1:5000 (1:2500 for αFOXM1)
α-mouse peroxidase conjugate (A9044, Sigma)	1:5000

6. Migration Assay

For wound healing assay, MDA-MB-231 cells were seeded in antibiotics-free DMEM at a density of $2*10^5$ cells/well in a 6-multiwell dish (Corning) in biological triplicates and were silenced for HMGA1 and/or FOXM1 as previously described. The cells were cultured to 90% confluence and then scraped with a 200-µl tip, and wound closure was followed for 7 hours. Two images for the same area were taken for each well.

7. Immunofluorescence

Cells were grown on glass coverslips and treated for each experiment; they were then washed in PBS1X and fixed in a solution of PBS 1X 4% Paraformaldehyde (pH 7.2). Cells were incubated with a solution of PBS1X with 0,1 M Glycine (Sigma-Aldrich/Merck) in order to reduce autofluorescence background and then incubated with PBS1X 0.3% Triton 100X (Sigma-Aldrich/Merck) to permeabilize cells. Between each passage, the cells were washed three times in PBS1X. We then proceeded with a 45 minutes blocking step with a blocking solution of PBS1X 5% Bovine Serum Albumin (BSA-Sigma-Aldrich/Merck). Subsequently, we incubated with the appropriate primary antibody diluted in blocking solution added with 0.01% Triton for 90 minutes in a wet environment; we washed the cells in PBS1X and we incubated the cells with the secondary antibody in PBS1X for 45 minutes. We washed the cells in PBS1X, incubated them in a solution of PBS1X 1:1000 Hoescht to detect nuclei and finally we mounted the coverslips on glass supports with the Vectashield mounting medium (Vector Laboratories).

The primary and secondary antibodies used in this project are enumerated in the following table:

Primary antibody	*Dilution*
αHMGA1 (made in the laboratory)	1:50
αFOXM1 (rabbit-Bethyl)	1:100
αVIMENTIN (mouse-Dako)	1:200

Secondary antibody	*Dilution*
α-rabbit IgG Alexa 594 (Thermo Fisher Scientific)	1:500
α-mouse IgG Alexa 488 (Thermo Fisher Scientific)	1:500

The images were visualized using a Nikon Eclipse e800 microscope and acquired using Nikon ACT-1 software, then analysed by the ImageJ software analyser.

8. RNA extraction

Cells were washed in ice-cold PBS1X and lysed in 1 ml of TRIzol (Thermo Fisher Scientific). Total RNA was isolated following the manufacturer's instructions of the TRIzol reagent and resuspended in UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific). The isolated RNA was subjected to DNase-I (Deoxyribonuclease I Amplification Grade, Invitrogen) treatment as indicated in the datasheet and to a subsequent phenol/chloroform purification. The RNA quality was checked by running an aliquot of the RNA on a denaturing agarose gel MOPS 1% (w/v) agarose (Sigma), 6.67% (v/v) formaldehyde (Sigma) in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7). In addition, RNA was quantified at NanoDrop 2000 (ThermoFisher Scientific).

9. Gene expression analysis

1 μg of total RNA was reverse transcribed with Random primer by the Superscript III (Invitrogen), according to the manufacturer's instructions. The quantitative RT-PCR (qRT-PCR) was carried out using diluted cDNA combined with iQ™ SYBR Green Supermix (BIO-RAD) and specific primers listed below. The reaction was performed in technical duplicates. The PCR reaction was performed in BIO-RAD CFX instrument and the protocol used was the following: an activation step at 95°C for 3 minutes followed by 40 cycles of a denaturation step at 95°C for 5 seconds and an amplification and elongation step at 60°C for 30 seconds. The data obtained were analysed with BIO-RAD CFX Manager software and the relative gene expression was calculated by ΔΔCt method, using the GAPDH as a normalizer. The genes evaluated in qRT-PCR are enumerated below with the primer sequence used:

Target gene/primer name	Forward primer (5'- 3')	Reverse Primer (5'- 3')
hGAPDH	TCTCTGCTCCTCCTGTTC	GCCCAATACGACCAAATCC
hHMGA1/HMGA-3'UTR	ACCAGCGCCAAATGTTCATCCTCA	AGCCCCTCTTCCCCACAAAGAT
hFOXM1	AGAAACGACCGAATCCAGAGC	CTGACCCGTGGTAGCAGTG
hVEGFA	AGGAGGGCAGAATCATCACG	ACCAGGGTCTCGATTGGATG
hCCNE2	TGAGCCGAGCGGTAGCTGGT	GGGCTGGGGCTGCTGCTTAG

hLEF1	CGAATGTCGTTGCTGAGTGT	GCTGTCTTTCTTTCCGTGCT
hSNAI2/SLUG	TTCGGACCCACACATTACCTTG	AGGGCAAGAAAAAGGCTTCTCC

10. Preparation of MDA-MB-231 samples for RNA-Sequencing

MDA-MB-231 cells were silenced for HMGA1 and the RNA were collected at 24 and 72 hours after the silencing. Three biological replicates were made for each condition. The total RNA was then extracted and checked as described above. Then, an aliquot of RNA was reverse transcribed and the silencing of HMGA1 was confirmed by qRT-PCR. The RNA-Sequencing was performed in San Raffaele Institute, Milano.

11. RNA-Sequencing analysis

Demultiplexed raw reads (fastq) generated from the Illumina HiSeq were check using FASTQC tool (Version 0.11.3). All samples passed the quality standards. Then we aligned to the reference genome (UCSC-hg19) using STAR (Dobin et al., 2013), version 2.0.1a using recommended options and thresholds. HTSeq-count (version 0.6.1) was used to generate gene counts. Starting from read counts, differential gene expression analysis was performed using EdgeR (version 1.10.1, R version: 3.2.3,(Robinson et al., 2010), comparing the different time points using a quantile-adjusted conditional maximum likelihood (qCML) method. In order to identify the relationship between each sample and every other sample, the Euclidean distance between each pair of samples was calculated using the log-transformed values of the complete data set. Average linkage clustering was then used to generate a sample-to-sample distance heatmap, via the cluster3 package (Cluster3: http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm#ctv). For statistical analyses the adjusted p-values were generated via the Benjamini-Hochberg procedure. Finally, genes were selected as differentials with a cutoff of 0.5 for the log Fold change and 0.05 for the False Discovery Rate.

Differentially expressed genes were analysed using Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com). The prediction of the transcription factors and regulative molecules was obtained using the Upstream regulators function (IPA suite). For every upstream regulator an overlap p-value and a z-score are calculated: the p-value indicates the significance based on the overlap between dataset genes and known targets regulated by the molecule, while the z-score is used to infer the possible activation (z-score ≥ 1,8) or inhibition (z-score ≤ -1,8) of the molecule based on prior knowledge stored in the proprietary Ingenuity Knowledge Base.

All statistical test and calculation have been performed in R (Dean and Nielsen, 2007) environment.

12. Preparation of the Condition medium (CM) for Angiogenic assays

MDA-MB-231 were seeded and silenced for HMGA1 and/or FOXM1 as described. The day before proceeding to the collection of the cellular supernatant, the cells were washed and cultured with serum-free DMEM. After 72 hours of silencing, the condition medium was collected, centrifuged at 240Xg at 4°C for 5 minutes to deposit cells debris and stored at -80°C. The cells were then washed in PBS 1X and lysed in SDS sample buffer as described. The HMGA1 and FOXM1 protein levels were checked by Western blot analysis.

13. Endothelial cells Proliferation analysis

Endothelial cells HUVEC were seeded in 96 well plate, 5000 cells/well. The cells were incubated with HMGA1, FOXM1 and HMGA1/FOXM1 condition medium and the control medium for 18 hours. Normal Horse serum 10% was used as positive control. After a washing step, the cells were detached from the wells with trypsin/EDTA (Sigma-Aldrich), seeded on a slide, and fixed first with 4% paraformaldehyde (Sigma-Aldrich) for 1 hour and then with cold methanol for 15 minutes. After two runs in microwaves in Tris/EDTA solution and washing with PBS, the cells were incubated with the anti-Ki-67 antibody (Dako) 1:75 in permeabilization solution, 30 minutes at room temperature. After two washing steps, we incubated with the secondary antibody anti-mouse FITC (Dako) 1:50 in permeabilization solution 50 minutes at room temperature. The cells were washed twice and lysed. Measurements were performed in TECAN 480-535nm (gain 70).

14. Transwell migration assay of endothelial cells

150000 cell/well of endothelial cells HUVEC CA3 (passage 2) were seeded in 200 µl of DMEM serum free medium (Invitrogen) in the upper compartment of 8 µm pore 24 transwell plate, pre-coated with human Fibronectin (Sacco) in the lower face. We proceeded by incubating the cells with 500 µl of HMGA1 and/or FOXM1 CMs, and with serum free medium as a negative control or Normal Horse Serum 10% (NHS) as a positive control in the lower compartment of the transwell. After 18 hours of incubation, the cells were lysed with lyses buffer for Coulter and the number of migrated cells were counted with Coulter Counter BD.

15. *In vitro* tube formation of endothelial cells

5.5*10^4 of HUVEC endothelial cells were plated on wells precoated with Matrigel (12µg/ml) (Becton Dickinson) and incubated for 18 hours with CMs diluted 1:2, with 20 ng/ml VEGF as a positive control or with serum-free medium as a negative control. After 4% paraformaldehyde fixation step and staining with Phalloidin-Alexa Fluor 546 (Invitrogen), the number of tubules was counted under a Leica AF6500 microscope using LAS software (Leica).

16. Preparation of cells for Zebrafish injection

1.2*10^6 of MDA-MB-231 cells were cultured and co-silenced for HMGA1 and FOXM1 as described before. After 40 hours of silencing the cells were injected in the yolk sack of Zebrafish embryos.

17. Zebrafish Xenograft

Zebrafish were raised and maintained as previously described (Tonon et al., 2016). Embryos were generated by natural pair-wise mating and were kept and handled for all experiments in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33mM CaCl2, 0.33nM MgSO4) and PTU (1-phenyl 2-thiourea, 0.03 mg/ml) to reduce zebrafish skin pigmentation for microscope analysis. All experimental procedures were conformed to the ITA guidelines (Dgl 26/2014) in accordance with EU legislation (2010/63/UE); this protocol was approved by the Ethics Committee for Animal Experimentation (OPBA) of the University of Trieste (Protocol number PO1937ZEN16). In this study, the Tg(fli1:EGFP) (y1) Zebrafish larvae were used. This transgenic fluorescent strain expresses in the entire vasculature EGFP under the control of the fli1 promoter (Delov et al., 2014). Before all experimental procedures, animals were properly anesthetized by using 1:100 dilution of 4 mg/ml Tricaine (Sigma-Aldrich Co., St Louis, MO, USA). Two days post fertilization (2dpf), Zebrafish embryos were dechorionated and microinjected with MDA-MB-231 siCTRL and siHMGA1/FOXM1 cell lines or cellular medium alone as control. Each cells suspension was stained by 2 µg/ml DiI (Sigma Aldrich) for 10 min at 37°C, re-suspended in DMEM medium and kept on ice before injection. Microinjections were performed with the electronic FemtoJet microinjector (Eppendorf) using borosilicate glass micro-capillaries (20 mm O.D. 15 mm I.D.; Eppendorf). Approximately 500 cells were microinjected into the yolk of each embryos, which were then maintained in E3 medium/PTU for 1 hour at 28°C. Afterwards embryos were kept at 34°C to allow tumor cells survival and growth as previously described (Jung et al., 2012). 24 hours after microinjection, the larvae were observed under fluorescence microscope (Leica DM 2000). During all the procedures live animals, properly anesthetized, were positioned in 1.5% methylcellulose (Sigma Aldrich Co). The images of the tumor

masses were acquired in red signal (DiI staining) and then merged with the respective bright field image by using the Leica Application Suite X (LAS X) software. Instead, to evaluate the host angiogenic response to the injected tumor cells, vessels images were acquired in green (GFP) signal and then analyzed using the ImageJ software. For both analysis, 22 animals for each cell line, divided into two independent experiments, were considered.

For gene expression, total RNA was extracted from at least 15 larvae for each experiment using Trizol Reagent (Invitrogen, Life Technologies, Milan, Italy). RNA concentration was determined by Nanoquant (Tecan). One µg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Life Technologies). Gene expression was analyzed by real-time quantitative RT-PCR (Step One Plus, Applied Biosystems) using the SYBR Green system (Life Technologies). The primer sequences are listed below. The gene analysis was repeated twice and the values were normalized respect to medium treated animals.

Target gene/primer name	Forward primer (5’-3’)	Reverse Primer (5’-3’)
zVEGFA (Zebrafish VEGFA)	GATGTGATTCCCTTCATGGATGTGT	GGATACTCCTGGATGATGTCTACCA
zFLT-1 (zebrafish VEGF receptor 1)	AACTCACAGACCAGTGAACAAGATC	GCCCTGTAACGTGTGCACTAAA
zFLK-1 (zebrafish VEGF receptor 2	GACCATAAAACAAGTGAGGCAGAAG	CTCCTGGTTTGACAGAGCGATA
zACTIN (zebrafish actin)	CAGCAAGCAGGAGTACGATGAGT	TTGAATCTCATTGCTAGGCCATT
hKi67 (human Ki67)	GCCTGCTCGACCCTACAGA	GCTTGTCAACTGCGGTTGC
hGAPDH (human GAPDH)	CCCATCACCATCTTCCAGGAG	CTTCTCCATGGTGGTGAAGACG

18. Statistical Analysis

Data were analysed by a two-tailed Student’s t test, and results were considered significant at a p-value <0.05. The results are presented as the mean and standard deviation (±SD). Specifically, a p-value<0.05 is indicated with *, a p-value<0.01 with ** and a p-value<0.001 with ***.

Results

1. A bioinformatic analysis reveals FOXM1 as a putative molecular partner of HMGA1

HMGA1 has been widely proved to be involved in breast cancer progression and, given its mechanisms of action as architectural transcription factor, it is clearly engaged in the regulation of several gene networks where it exerts its activity by cooperating with a broad spectrum of transcription factors. In order to better unravel the yet unexplored pathways controlled by HMGA1 in regulating several malignant aspects of the highly aggressive triple negative breast cancer subtype, we performed a deep RNA-Sequencing analysis of our model of TNBC, the MDA-MB-231 cells, at 24 and 72 hours following HMGA1 silencing. From the sequencing analysis, we were interested in discovering new transcriptional molecular partners of HMGA1, therefore, we proceeded with three steps: 1. identification of differentially expressed genes (DEG) upon HMGA1 silencing; 2. analysis of gene networks DEG are involved in; 3. prediction of transcription factors regulating these gene networks (Fig. 1).

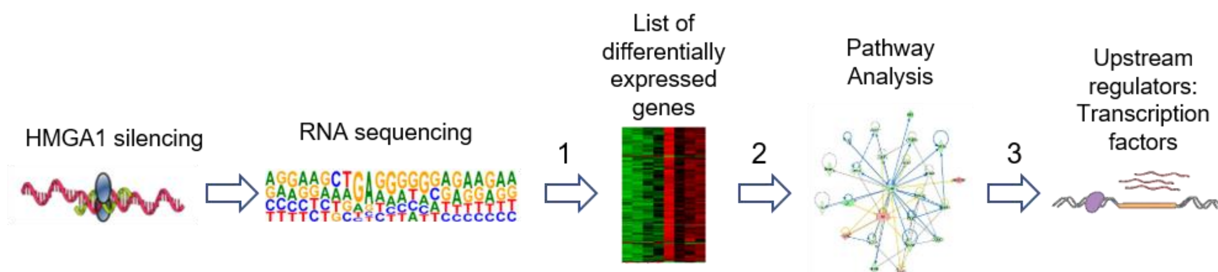


Figure 1: *A bioinformatic analysis on HMGA1-silenced MDA-MB-231 cells reveals new molecular partners of HMGA1 in regulating downstream gene networks.* At 24 and 72 hours of silencing, the RNA was collected and subjected to deep RNA sequencing. From the data, differentially expressed genes (DEG) were identified, and their pathways were rebuilt by Ingenuity Software analysis. Among the different upstream factors controlling these pathways, we focused on transcription factors, putative HMGA1 molecular partners in regulating the identified gene networks in triple negative breast cancer.

In collaboration with the Bioinformatic unit of LNCIB (Trieste), we found out, as first thing, the transcripts whose expression changed upon HMGA1 depletion on the different time points. Then, we proceeded by analysing these DEG with the Ingenuity Pathway software (IPA). This tool crosses the gene expression changes observed with known datasets collected from literature data to build up the gene networks these DEG are included in. We then focused on the hub of these networks, and we found that they could be governed by different upstream regulators, such as transmembrane receptors, transcription factors and co-activators/repressors, and compounds.

Among these identified upstream regulators, we focused our attention on transcription factors, as they could be putative molecular partners of HMGA1 in regulating the networks these DEG are involved

in. The upstream factors were then sorted by p-value which is a measure of how much the overlap between their known dataset and our HMGA1-modulated gene list is significant. Moreover, they were ranked according to a Z-score which is an indicator of the state of activation/inhibition of gene networks in the presence of activity of the transcription factors we identified as hub. A positive Z-score means an activation state of the downstream network in common between a transcription factor and HMGA1, network which in turn was negatively regulated after HMGA1 silencing. The first five upstream regulators with a Z-score of ≤ -18 or ≥ 1.8, that we chose as a cut off, in at least one-time point, are listed in table 1.

UPSTREAM REGULATORS	Z.score24	pvalue24	Z.score72	pvalue72
E2F1	0.435	1.35E-06	4.62	1.74E-22
FOXM1	1.461	0.0245	2.724	1.67E-09
RB1	-1.043	0.0289	-4.261	2.61E-17
SMAD3	1.933	0.04	1.526	1.39E-06
STAT3	0.861	0.0467	1.78	2.43E-09

Table 1: *Several known and unknown HMGA1 partner were obtained by the bioinformatic analysis on TNBC cells*. The transcription factors listed, putative molecular partners of HMGA1, were ranked by their pvalue24h.

Interestingly, with this bioinformatics analysis we found well known transcriptional partners of HMGA1, reinforcing the robustness of our bioinformatic method. In fact, the role of HMGA1 together with STAT3, E2F and RB1 in regulating gene expression has been already explored (Hillion et al., 2008; Ueda et al., 2007). We decided to focus on FOXM1, both for its Z-score values, which increases between the two time-points, and for the well-known role of this factor in breast cancer (Saba et al., 2016). The IPA outcome of FOXM1 as hub upstream regulator is represented in figure 2 and target genes of the downstream network in common between FOXM1 and HMGA1 at 24 and 72 hours after HMGA1 silencing are listed in tables 2a and 2b.

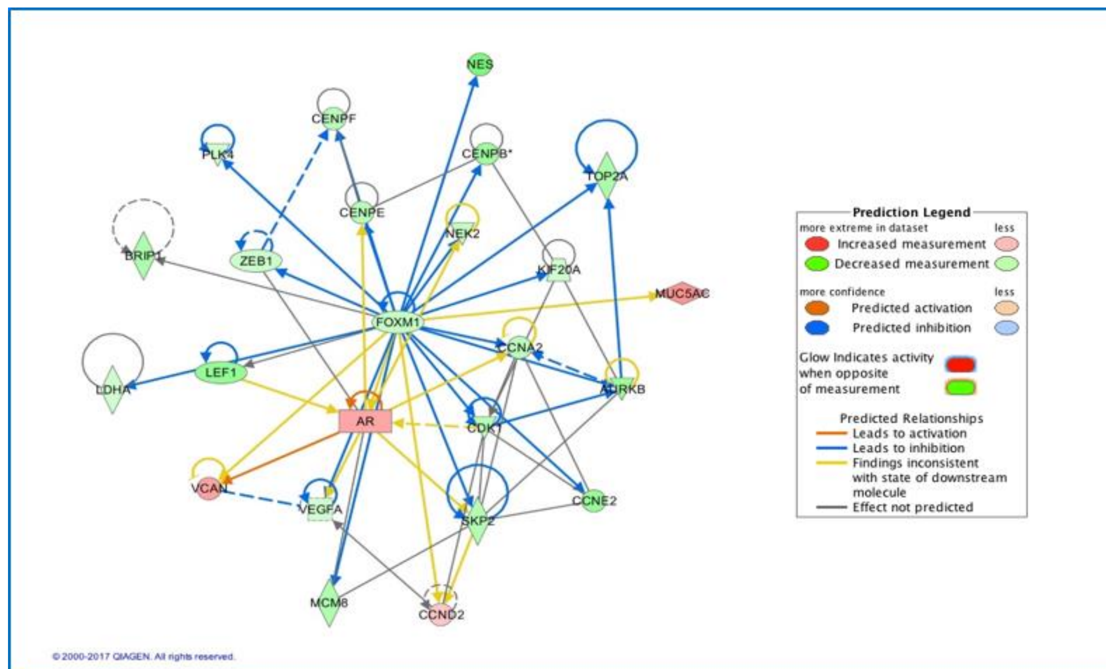


Figure 2: *Ingenuity pathway analysis outcome of FOXM1*. At the centre, we find the upstream regulator (FOXM1) of the pathways (connected-factors) found activated or inhibited by HMGA1, predicted to be in common with FOXM1, hub of this network.

a) 24 hours HMGA1 silencing

ID	Exp Log Ratio
CAV1	1.158
CENPE	0.801
LEF1	0.758
SNAI2	0.614
CCNE2	0.587
BRIP1	0.548
CCNE1	-0.661

b) 72 hours HMGA1silencing

ID	Exp Log Ratio
NES	1.457
LEF1	1.183
CCNE2	1.132
AURKB	1.061
CENPB	1.059
TOP2A	0.909
BRIP1	0.9
MCM8	0.86
SKP2	0.834
CDK1	0.799
CENPE	0.759
CENPF	0.741
CCNA2	0.74
NEK2	0.627
FOXM1	0.626
ZEB1	0.547
PLK4	0.525
KIF20A	0.513
VEGFA	0.507
LDHA	0.502
CCND2	-0.726
AR	-1.134
VCAN	-1.187
MUC5AC	-1.35

Table 2: *Several HMGA1 differentially expressed genes (DEG) promoting breast cancer aggressive traits belong also to the FOXM1 network*. The 24 hours targets are listed in panel a, while the data referred to the 72 hours HMGA1 silencing are listed on the table in panel b.

From these data, we hypothesized that HMGA1 could cooperate with FOXM1 in regulating specific gene networks that modulate crucial cancer hallmarks in conferring breast cancer aggressiveness. In support of the bioinformatic analysis, we took advantage of The Cancer Genome Atlas (TCGA) data, a public database collecting information from a high number of cancer patients obtained from high-throughput approaches, searching for a connection between HMGA1 and FOXM1. From this catalogue, we selected a dataset of 818 breast cancer patients and we firstly evaluated HMGA1 expression, thanks to the cBioportal tool, which is an open-access resource comprising large-scale genomic data about several types of cancer (Cerami et al., 2012). HMGA1 was found altered in 4% of TCGA dataset patients, particularly its expression level is up-regulated. Subsequently, we looked for FOXM1 expression level: interestingly we found that FOXM1 was enriched in HMGA1 overexpressing patients, both at RNA and protein levels, as reported in figure 3a. We then narrowed our analysis on a particular subset of 174 breast cancer patients lacking the expression of the Oestrogen receptor, subset in which we know that HMGA1 has a major role, evaluating the expression of HMGA1. Among these cases, in 16% of the patients HMGA1 was found up-regulated. Then, we interrogated the dataset for FOXM1 expression and both RNA and protein levels were found enriched in HMGA1 up-regulated patients (Fig. 3b). These results suggest a tight relation between HMGA1 and FOXM1 expression both in breast cancer and particularly in a more specific subset, such as the ER- subtype, more similar to the triple negative subtype we study, suggesting that these two factors could work together.

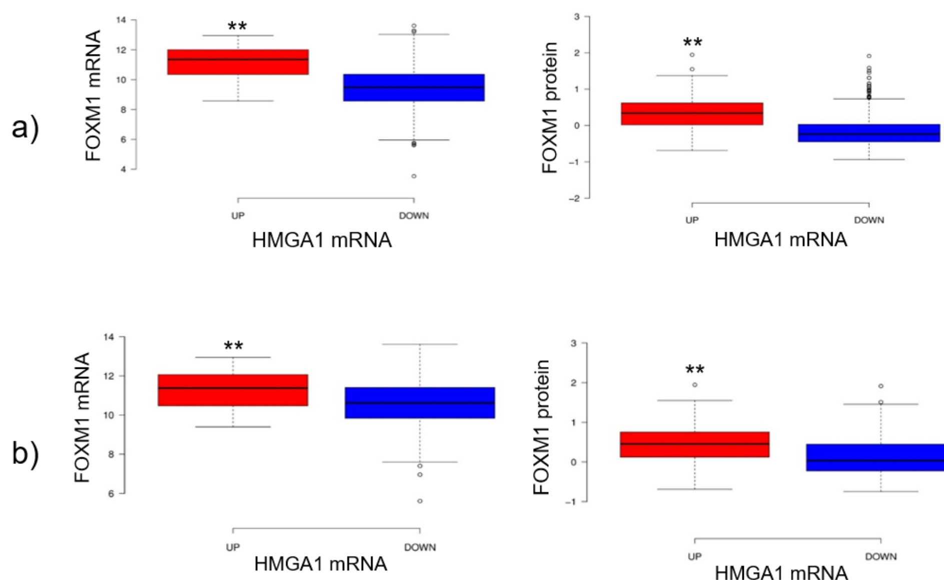


Figure 3: *FOXM1 mRNA and protein levels are enriched in HMGA1-overexpressing breast cancer patients*. a) Boxplots of the enrichment of HMGA1 mRNA levels with FOXM1 mRNA and protein levels in a dataset of 818 breast cancer patients and b) in a dataset of 174 ER-breast cancer patients. **p< 0.01.

2. HMGA1 and FOXM1 regulate a common gene network and similar cellular characteristics in TNBC

2.1 HMGA1 and FOXM1 regulate a common gene network

On the basis of the bioinformatic analysis and of DEG list belonging to the HMGA1/FOXM1 network, we proceeded by validating the data we obtained using qRT-PCR, in our cellular model of triple negative breast cancer cell line, the MDA-MB-231. We knocked down the expression of HMGA1 and checked the efficacy of the silencing (Fig. 4a). Hereafter, from the DEG list, we chose Cyclin E2 (CCNE2), Lymphoid enhancer binding factor 1(LEF1), Snail family transcriptional repressor 2 (SNAI2) and Vascular endothelial growth factor (VEGFA), that are known for their role in malignant aspects of breast cancer, such as epithelial to mesenchymal transition, cellular migration and angiogenesis (Pegoraro et al., 2015; Gebeshuber et al., 2007; Chen et al., 2009; Tanaka et al., 1993). We validated their differential expression at the two time-points chosen for the RNA-Seq analysis, 24 and 72 hours: as a matter of fact, as predicted by the bioinformatic analysis, we observed a decrease in their expression level after HMGA1 depletion (Fig. 4b). Furthermore, to generalize the role of HMGA1 in regulating this gene network, we confirmed these data in another cellular model of TNBC, the MDA-MB-157, observing, upon HMGA1 silencing, even a higher decrease in expression of some target genes, as in the case of SNAI2 and VEGFA (Fig. 5 a and b).

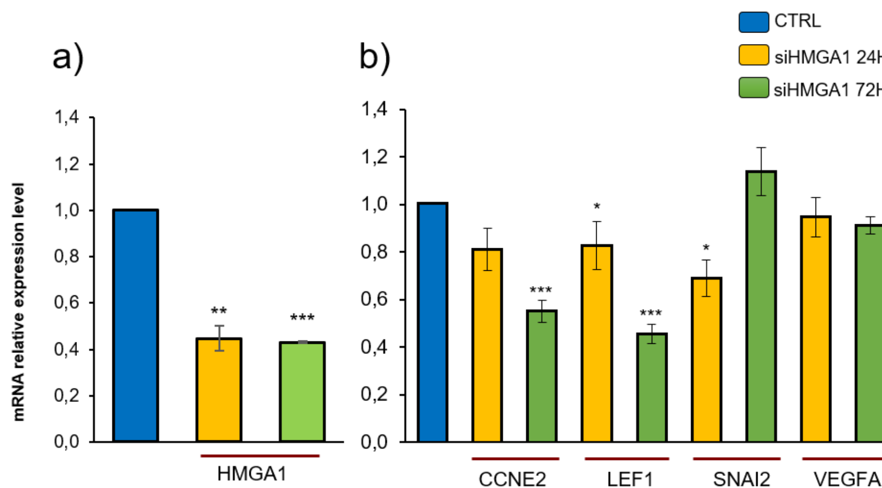


Figure 4: *CCNE2, LEF1, SNAI2 and VEGFA are regulated by HMGA1 in MDA-MB-231 as predicted by bioinformatic analysis*. qRT-PCR of HMGA1 (a) and HMGA1/FOXM1 target genes (CCNE2, LEF1, SNAI2, VEGFA) (b) in MDA-MB-231 cells after 24- and 72 hours HMGA1 silencing (yellow and green bars respectively). GAPDH was used for normalization. The data are compared to control condition and are presented as the mean±SD (n = 3) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; two-tailed Student's *t*-test.

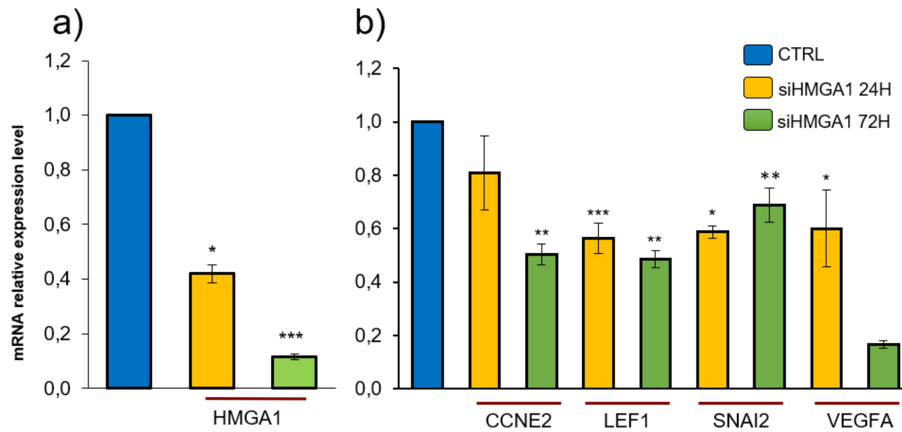


Figure 5: *The differential expression of CCNE2, LEF1, SNAI2 and VEGFA upon HMGA1 silencing were confirmed in MDA-MB-157*. qRT-PCR of HMGA1 (a) and HMGA1/FOXM1 target genes (CCNE2, LEF1, SNAI2, VEGFA) (b) in MDA-MB-157 cells after 24- and 72 hours HMGA1 silencing (yellow and green bars respectively). GAPDH was used for normalization. The data are compared to control condition and are presented as the mean±SD (n = 3) *p< 0.05, **p < 0.01, ***p< 0.001; two-tailed Student's *t*-test.

Then, to confirm the functional relation between the DEG selected and FOXM1 as predicted by the bioinformatic analysis, we silenced FOXM1 in MDA-MB-231 cells and after 72 hours we checked their expression levels (Fig. 6 a and b). We observed a down-regulation of CCNE2, LEF1 and VEGFA, confirming FOXM1 involvement in the regulation of this gene network as was previously demonstrated for HMGA1.

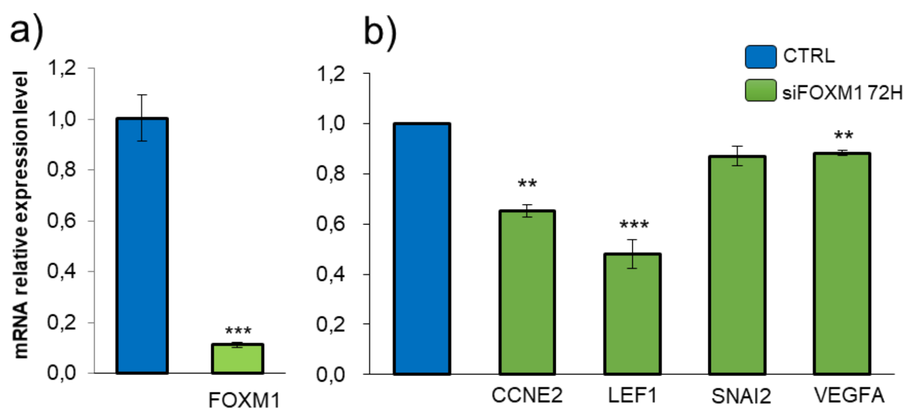


Figure 6: *CCNE2, LEF1, SNAI2 and VEGFA are regulated by FOXM1 in MDA-MB-231 as predicted by bioinformatic analysis*; qRT-PCR of FOXM1 (a) and HMGA1/FOXM1 target genes (CCNE2, LEF1, SNAI2, VEGFA) (b) in MDA-MB-231 cells after 72 hours FOXM1 silencing. GAPDH was used for normalization. The data are compared to control condition and are presented as the mean±SD (n = 3); **p < 0,01,***p < 0.001; two-tailed Student's *t*-test.

Finally, with the aim of evaluating if the knocking down of both HMGA1 and FOXM1 has a higher impact on the expression of the DEG taken into consideration, we silenced the expression of both factors in MDA-MB-231 cells at the same time and, after checking the expression level of HMGA1 and FOXM1, we evaluated the targets already mentioned (Fig. 7 a and b). Indeed, the silencing of both factors decreases the expression level of CCNE2, LEF1 and VEGFA more efficiently, with respect to the single silencing conditions. In particular, as we can observe if we compare figures 4, 6 and 7, among the DEG evaluated, the additive effect of the co-silencing on the VEGFA expression is stronger with respect to the other DEG suggesting a possible synergistic action of HMGA1 and FOXM1 on this target.

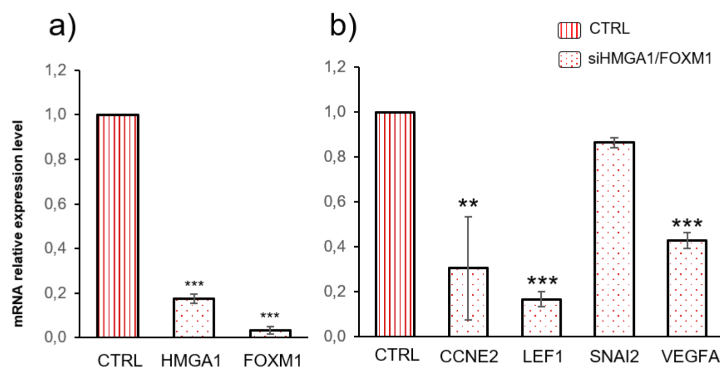


Figure 7: *CCNE2, LEF1, SNAI2 and VEGFA are regulated by HMGA1 and FOXM1 in MDA-MB-231*. qRT-PCR of HMGA1 and FOXM1 (a) and HMGA1/FOXM1 target genes (CCNE2, LEF1, SNAI2, VEGFA) (b) in MDA-MB-231 cells after 72 hours HMGA1 and FOXM1 silencing. GAPDH was used for normalization. The data are compared to control condition and are presented as the mean±SD (n = 3), $**p < 0.01$, $***p < 0.001$; two-tailed Student's *t*-test.

2.2 HMGA1 and FOXM1 modulate similar features of TNBC

Based on the evidence that HMGA1 and FOXM1 are involved in the regulation of the same gene network and that we knew from previous data that HMGA1 push cancer cells to undergo an epithelial to mesenchymal transition (Pegoraro et al., 2013), we wondered whether FOXM1 resembles the action of HMGA1 in promoting malignant aspects in TNBC. With this aim, we silenced the expression of HMGA1 and FOXM1 in the TNBC cell lines MDA-MB-231 and -157 and evaluated the morphology of cells (Fig. 8 a and b). Similarly to what happens for HMGA1 silencing, after FOXM1 depletion, cells acquire a more flattened and polygonal structure, typical of an epithelial phenotype, in comparison with the control condition, where the cells show the spindle-like shape of mesenchymal cells. Moreover, by cosilencing the two factors, this morphological change is even more emphasized, suggesting, once again, a strong molecular relation between HMGA1 and FOXM1.

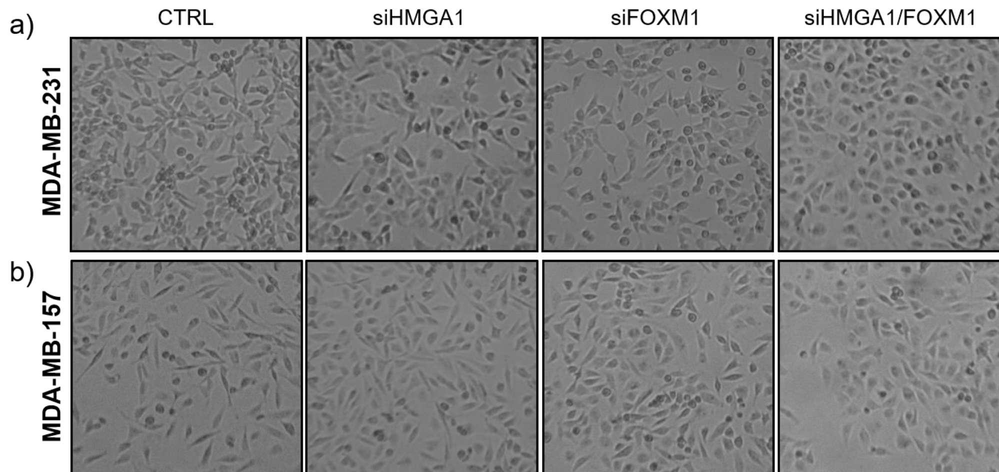


Figure 8: *The silencing of HMGA1 and/or FOXM1 induces morphological changes typical of a MET in TNBC cells*. Representative pictures of HMGA1, FOXM1 and HMGA1/FOXM1 depletion (siHMGA1, siFOXM1 and siHMGA1/FOXM1) in MDA-MB-231 (a) and MDA-MB-157 (b) cells, taken at X 4 magnification.

Considering that the morphological changes observed recall a MET (mesenchymal to epithelial transition), we explored this scenario by evaluating a molecular marker of the MET event, such as Vimentin, an intermediate filament protein of the cytoskeleton, specific of the mesenchymal cells, which is associated with an increased cellular motility (Ivaska et al., 2007). We silenced the expression of HMGA1 and FOXM1 in MDA-MB-231 and we evaluated by immunofluorescence the cellular distribution of Vimentin. As it is possible to see in figure 9, after the silencing of HMGA1 and FOXM1, the Vimentin network appears disrupted, forming aggregates in the cytoplasm, probably due to the disassembly of the protein. In addition, the expression of Vimentin seems affected, as we observed a lower intensity in the fluorescence signal with respect to the control condition. Moreover, the co-depletion of HMGA1 and FOXM1 worsened this feature, suggesting an implication of both proteins in the regulation of this MET marker.

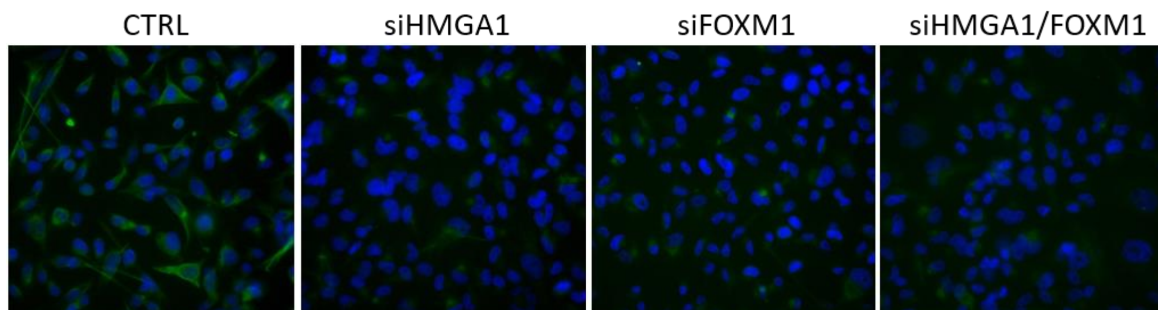


Figure 9: *The silencing of HMGA1 and/or FOXM1 disrupts the Vimentin molecular network*. Representative immunofluorescence images of Vimentin (green) in MDA-MB-231 cells after HMGA1, FOXM1 and HMGA1/FOXM1 (siHMGA1, siFOXM1 and siHMGA1/FOXM1). Nuclei are stained with Hoechst (blue). Images were taken at X 40 magnification.

As proved by the previous evidences, considering that HMGA1 and FOXM1 are involved in modulating same malignant features of TNBC, we evaluated if they synergistically promote the cellular migration. Thus, we knock-down the expression of HMGA1 and FOXM1 alone or in combination in MDA-MB-231 cells and we evaluated the migration ability of cells by wound healing assay. As reported in the representative images in figure 10a and in the graph in 10b, either the depletion of HMGA1 or FOXM1 dampened the migration capacity of the cells, but the co-silencing of the factors further diminishes the migratory ability of TNBC cells, in comparison to single silencing conditions. These results confirmed the paramount involvement of these factor in promoting together the motility of the cells and in turn the metastatization process in TNBC.

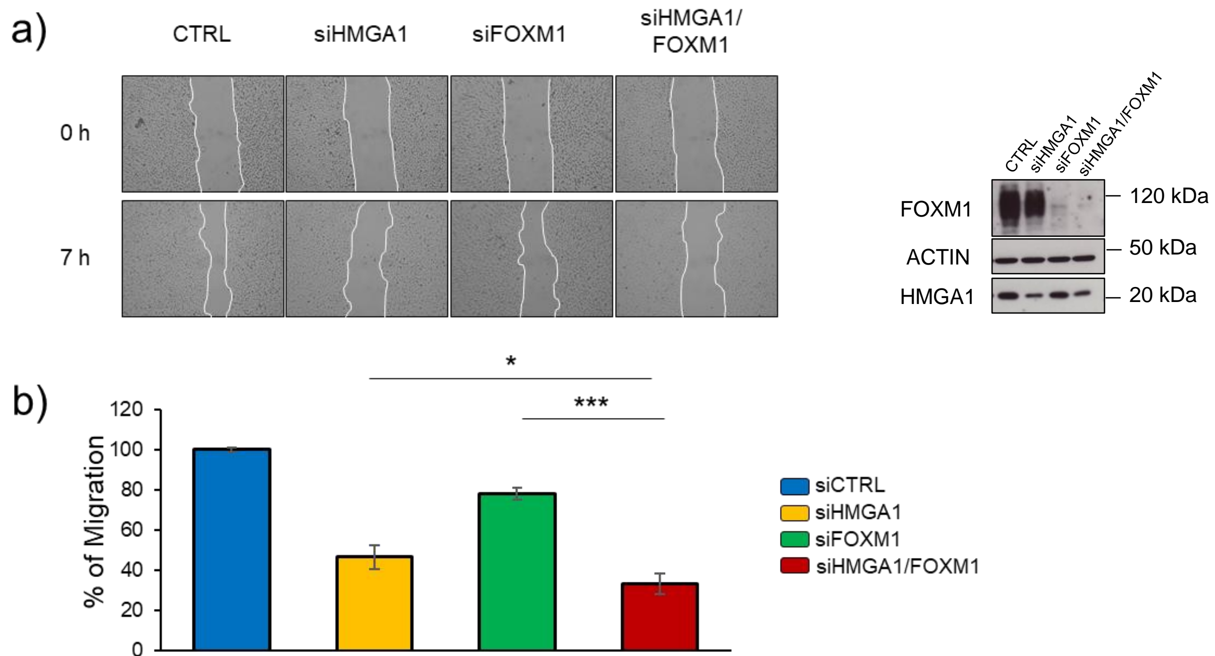


Figure 10: *HMGA1 and FOXM1 separately affect the MDA-MB-231 migration rate, but their cooperative action impinges on this aggressive feature at a higher extent.* a) Representative images of wound closure. Images were taken at X 4 magnification. b) The wound areas were measured with ImageJ software and plotted on the graph. The data are compared to control condition and are presented as the mean±SD (n = 3), *p< 0.05, ***p< 0.001; two-tailed Student's *t*-test. Western blot validation of HMGA1 and/or FOXM1 silencing in MDA-MB-231 cells is reported. ß-actin was used as a loading control.

3. HMGA1 regulates FOXM1 at a post-transcriptional level by retaining FOXM1 in the nucleus and enhancing its transcriptional activity

As highlighted by the experiments done so far, HMGA1 and FOXM1 control a common gene network responsible for mediating several malignant features in TNBC and, interestingly, the contemporary presence of both factors worsened these characteristics in cancer cells, as shown by the co-silencing experiments. Given these premises and that FOXM1 had been indicated by the bioinformatic analysis as molecular partner of HMGA1, we wondered which type of relation exists between these two factors. HMGA1 could regulate FOXM1 gene networks working on two levels: HMGA1 could modulate the transcriptional activity of FOXM1 and/or it could control the expression level of FOXM1. Therefore, we firstly asked if HMGA1 has an influence on FOXM1 transcriptional activity: with this aim, we transfected the HEK293T cells with a vector containing a synthetic FOXM1-responsive element (pGL3-5BS), represented by five repetitions of FOXM1 binding sequences (TAAACA) upstream of the luciferase gene (Fig. 11a), and tested its activity after HMGA1, FOXM1 or FOXM1 plus HMGA1 over-expression. We found that HMGA1 did not have any action on this synthetic reporter, while, as expected, FOXM1, by binding its consensus sequence, transactivates this synthetic promoter of about 3-fold with respect to the control condition (Fig. 11b). More importantly, we found that when co-transfected with FOXM1, HMGA1 was able to cause a significant increase (about 35%) in the transcriptional activity of FOXM1, potentiating its action (Fig.11b).

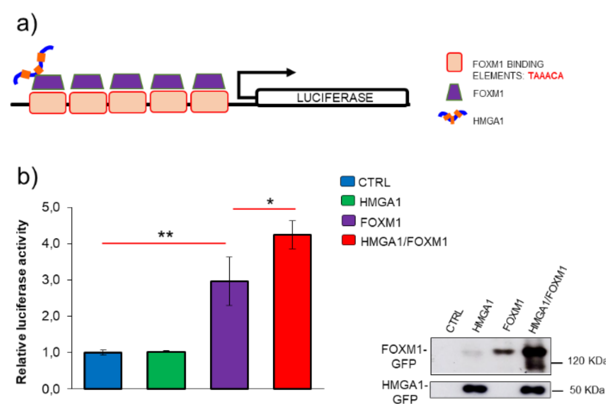


Figure 11: *HMGA1 reinforces the FOXM1 transcriptional activity in an artificial reporter vector*. (a) Schematic representation of the pGL3-5BS reporter vector with 5 binding elements of FOXM1 upstream the luciferase sequence. (b) HEK293T cells were transiently co-transfected with the luciferase reporter plasmid pGL3-5BS with the expression plasmid pEGFP-HMGA1 (green bar), pEGFP-FOXM1 (purple bar) or the combination of the two (red bar). pRL-CMV Renilla luciferase expression vector was included to normalize for transfection efficiencies and the measurements were performed with the Promega Dual-Luciferase kit. Values are reported as relative luciferase activity comparing to cells transfected with the reporter vector pGL3-5BS. The data are represented as the mean±SD ($n > 3$); *$p < 0.05$, **$p < 0.01$; two-tailed Student's *t*-test. On the right, Western blot of GFP-HMGA1, GFP-FOXM1 or GFP-HMGA1/FOXM1, using an anti-GFP as primary antibody.

Subsequently, we asked whether HMGA1 influences the expression level of FOXM1. With this aim and on the basis also on the bioinformatic analysis which highlighted a slight decrease in FOXM1 RNA level upon HMGA1 depletion (table 2b, chapter 1 of results section), we evaluated whether FOXM1 mRNA and protein levels were affected by HMGA1 knock-down. Thus, we silenced the expression of HMGA1 in MDA-MB-231 cells and evaluated the level of endogenous FOXM1 mRNA and protein by qRT-PCR and immunoblot analysis. As reported in figure 12 a and b, upon HMGA1 depletion we did not detect any FOXM1 mRNA variation after 24 hours, whereas at 72 hours mRNA level has a little decrease and the protein is strongly reduced. Furthermore, we transfected HEK293T cells with FOXM1 and increasing amounts of HMGA1 and as represented in figure 12c we noticed that exogenous FOXM1 protein was increased in a HMGA1-dose dependent manner suggesting that HMGA1 stabilizes FOXM1 protein. As it is known from the literature that FOXM1 regulates its own mRNA and protein levels by an autoregulatory loop (Halasi and Gartel, 2009) and considering all the data about the expression of both endogenous and exogenous FOXM1 after HMGA1 modulation, we hypothesized that HMGA1 could regulate FOXM1 mainly at a post-transcriptional level, and that FOXM1 regulates itself at the transcriptional level on its own promoter.

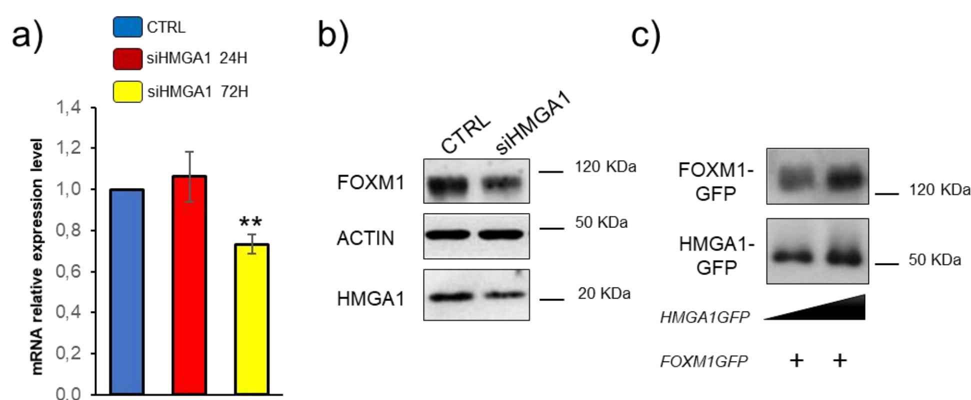


Figure 12: *HMGA1 regulates FOXM1 expression levels mainly at post-transcriptional level*. a) qRT-PCR of FOXM1 in MDA-MB-231 cells after 24- and 72 hours HMGA1 silencing (red and yellow bars respectively). GAPDH was used for normalization. The data are compared to the control condition and are presented as the mean±SD ($n = 3$), **$p < 0.01$; two-tailed Student's *t*-test. b) FOXM1 Western blot analysis of MDA-MB-231 cells upon 72 hours HMGA1 silencing. ß-actin was used as a loading control. c) Western blot analysis of HEK293T transfected with pEGFP-FOXM1 and increasing amounts of pEGFP-HMGA1, using an anti-GFP as primary antibody. pRL-CMV Renilla luciferase expression vector was used to normalize for transfection efficiencies and the measurements were performed with the Promega Dual-Luciferase kit.

Moreover, taking into account that one of the level of FOXM1 protein regulation is its nucleo-cytoplasmic shuttling (Kelleher and O'Sullivan, 2016), we investigated if HMGA1 potentiates

FOXM1 activity by influencing also its nuclear retention. For this purpose, we evaluated, by immunofluorescence analysis, the cellular localization of transfected FOXM1-GFP upon HMGA1 silencing in MDA-MB-231: indeed, in control cells FOXM1-GFP localizes in the nucleus, whereas, after HMGA1 depletion, FOXM1-GFP shows a more cytoplasmic localization, indicating that HMGA1-depletion induced the translocation of FOXM1-GFP from the nucleus to the cytoplasm (Fig. 13). This result was even more evident when we repeated the same experiment in HEK293T cells that have a higher efficiency of transfection (Fig. 14).

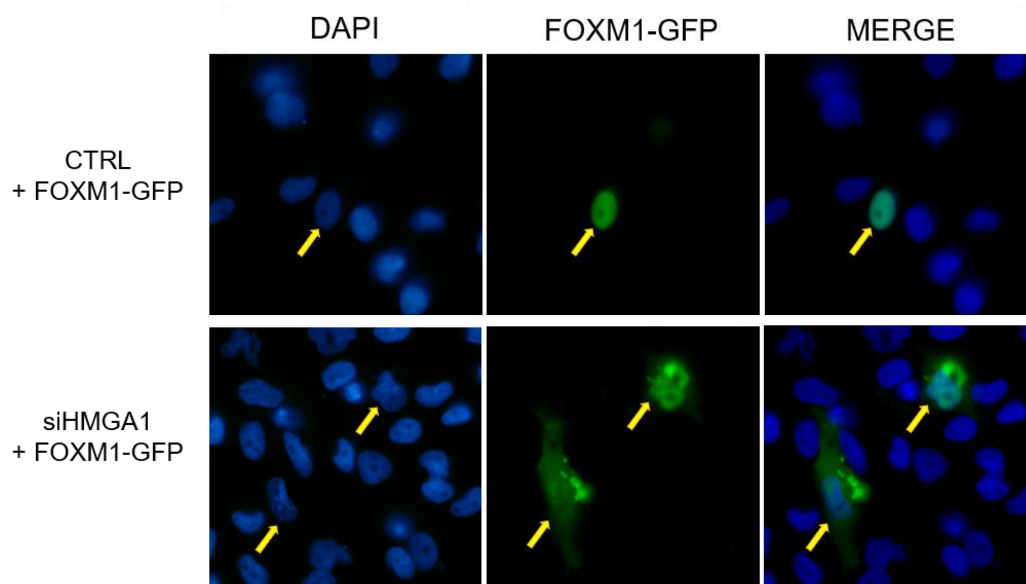


Figure 13: *HMGA1 induces a relocalization of FOXM1 in MDA-MB-231 cells*. Representative images of FOXM1-GFP after HMGA1 silencing and pEGFP-FOXM1 transfection.

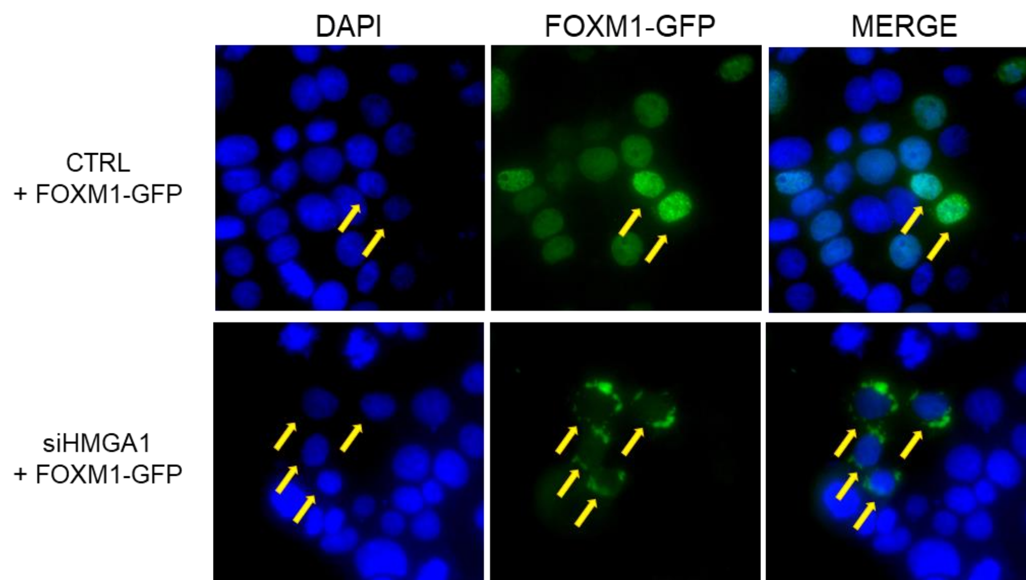


Figure 14: *HMGA1 induces a relocalization of FOXM1 in HEK293T cells*. Representative images of FOXM1-GFP after HMGA1 silencing and pEGFP-FOXM1 transfection.

Furthermore, we confirmed these results by exploring the localization of endogenous FOXM1 upon HMGA1 knock-down in MDA-MB-231 and -157 cell lines. We observed that the silencing of HMGA1 causes a more nucleo-cytoplasmic localization of FOXM1 with respect to control condition in which FOXM1 is more nuclear (Fig. 15 and 16). The data obtained led us to hypothesize that HMGA1 stabilizes FOXM1 in the nucleus and potentiates FOXM1 transcriptional activity on its target genes, among those FOXM1 itself.

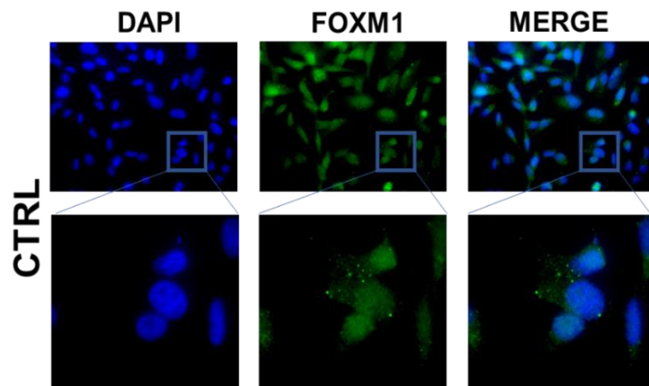


Figure 15: *HMGA1 induces a cellular re-distribution of endogenous FOXM1 in MDA-MB-231*. Representative immunofluorescence images of FOXM1 in after HMGA1 silencing are shown. Images were taken at X 60 magnification.

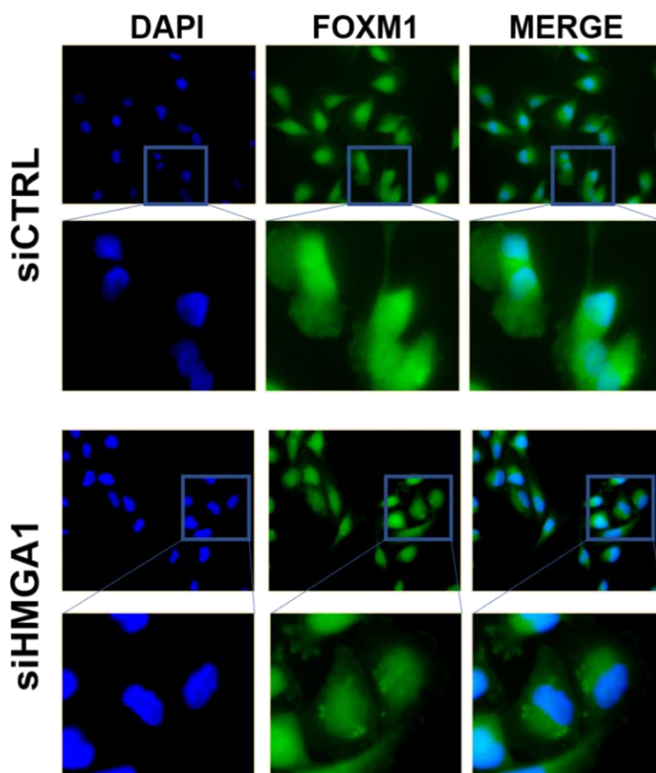


Figure 16: *HMGA1 induces a cellular re-distribution of endogenous FOXM1 in MDA-MB-157*. Representative immunofluorescence images of FOXM1 after HMGA1 silencing are shown. Images were taken at X 60 magnification.

4. HMGA1 and FOXM1 synergistically mediate the transcription of the VEGFA

Our data indicate that with high level of HMGA1, FOXM1 shows a more nuclear distribution; moreover, HMGA1 increases FOXM1 transcriptional activity and guides the control of a common gene network in TNBC. In literature, it is reported the involvement of FOXM1 in the angiogenic process, particularly in regulating VEGFA transcription by directly binding its promoter (Zhang et al., 2008; Karadedou et al., 2012), while very little is known about the role of HMGA1 in this process. Although some reports demonstrated that HMGA1 transcriptionally regulates VEFGA (Messineo et al., 2016).

By analysing the TCGA dataset, we evaluated the expression of VEGFA mRNA in HMGA1 over-expressing breast cancer patients. As shown in figure 17, we found a strong enrichment of VEGFA in this subset of patients, supporting the hypothesis of a role of HMGA1 in the angiogenic context and an involvement in VEGFA expression. In addition, as shown before, we demonstrated that HMGA1 and FOXM1 have a slight effect on VEGFA expression, whereas the co-silencing of the two factors induced a stronger reduction in VEGFA expression (Fig. 18 a and b).

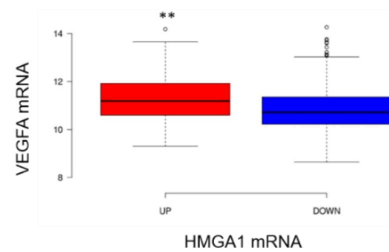


Figure 17: *VEGFA mRNA level is enriched in HMGA1-overexpressing patients.* Boxplots of the enrichment of HMGA1 mRNA levels with VEGFA mRNA in a dataset of 818 HMGA1-overexpressing breast cancer patients. **p< 0.01.

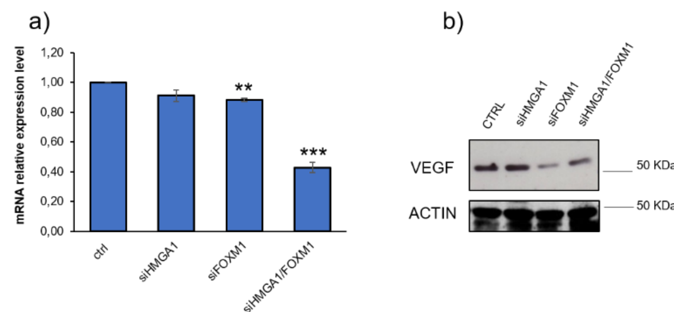


Figure 18: *HMGA1 and FOXM1 regulate VEGFA expression.* a) qRT-PCR of VEGFA in MDA-MB-231 cells after 72 hours HMGA1, FOXM1 and HMGA1/FOXM1 silencing. GAPDH was used for normalization. The data are compared to control and are presented as the mean±SD (n = 3); **P < 0.01, ***P<0.001; two-tailed Student's t-test. b) VEGFA Western blot analysis of MDA-MB-231 cells upon 72 hours HMGA1, FOXM1 and HMGA1/FOXM1 silencing. ß-actin was used as a loading control.

On the basis of these evidences and considering the key role of the angiogenic process in promoting the progression of breast cancer, we investigated if HMGA1 controls VEGFA transcription and whether it has an impact on the transcriptional activity of FOXM1 on the VEGFA promoter.

To study the action of HMGA1 and FOXM1 on the transcriptional regulation of VEGFA we used a luciferase reporter vector containing a region of the VEGFA promoter spanning from -1000 to -1 bp from the transcriptional start site (TSS). Firstly, we transfected the HEK293T cells with the luciferase promoter and increasing amounts of FOXM1 and HMGA1 expressing vectors. As reported in figure 19 a and b, we observed that not only FOXM1 but also HMGA1 increases VEGFA-luciferase activity. Thus, we co-transfected FOXM1 and increasing quantities of HMGA1, in order to see if HMGA1 has an effect on FOXM1 activity. Indeed, HMGA1 enhances FOXM1 transcriptional activity on VEGFA promoter, in a dose dependent manner (Fig. 19 c).

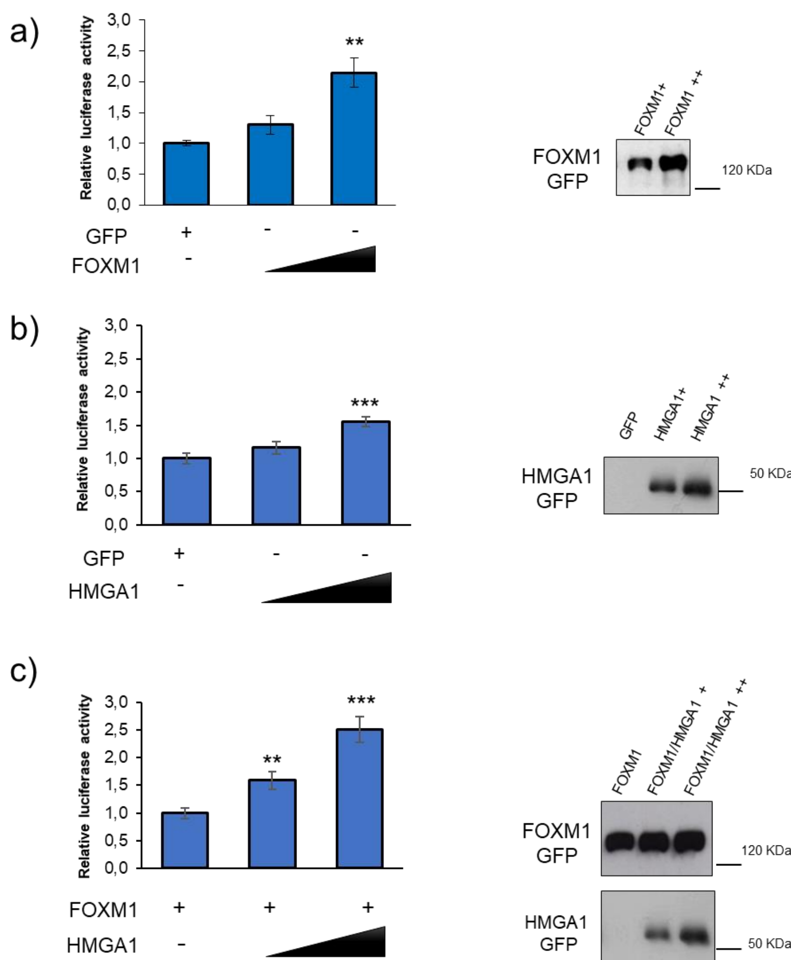


Figure 19: *HMGA1 enhances FOXM1 transcriptional activity in a dose-dependent manner on a VEGFA promoter*. HEK293T cells were transiently co-transfected with the luciferase reporter plasmid pGL4.10-VEGFprom (-1000-1) with the expression plasmid pEGFP-FOXM1 at increasing concentrations (a), pEGFP-HMGA1 at increasing concentrations (b) and pEGFP-FOXM1 and increasing quantities of pEGFP-HMGA1 (c). pRL-CMV Renilla luciferase expression vector was included to normalize for transfection efficiencies. Values are reported as relative luciferase activity comparing to cells transfected with the reporter vector pGL4.10-VEGFprom (-1000-1). The data are compared to control condition and are represented as the mean±SD ($n > 3$). **$P < 0.01$, ***$P<0.001$; two-tailed Student's *t*-test. On the right, are reported the correspondent Western blot validation.

In addition, to further explore the tight dependence of FOXM1 transcriptional activity on HMGA1, we silenced the expression of HMGA1 and transfected FOXM1 in HEK293T cells.
We observed (Fig. 20) that the transfection of FOXM1 strongly increases the activity of the luciferase, while the silencing of endogenous HMGA1 does not have any significant effect on the transactivation of this promoter. When we overexpressed FOXM1 and at the same time we silence the expression of HMGA1 we observed a significant reduction of FOXM1 ability in inducing the activity of the reporter gene. These results confirmed that HMGA1 boosts FOXM1 activity on VEGFA promoter.

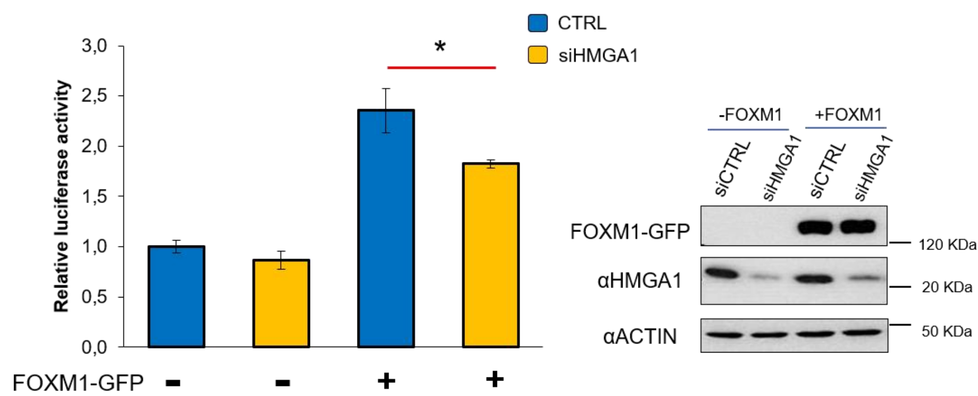


Figure 20: *FOXM1 transcriptional action on VEGFA promoter is dependent on HMGA1*. HEK293T cells silenced for HMGA1 by siRNA and transiently cotransfected with the luciferase reporter plasmid pGL4.10-VEGFprom (-1000-1) with the expression plasmid pEGFP-FOXM1. pRL-CMV Renilla luciferase expression vector was included to normalize for transfection efficiencies. Values are reported as relative luciferase activity comparing to cells transfected with the reporter vector pGL4.10-VEGFprom (-1000-1). The data are represented as the mean±SD ($n > 3$), *$p < 0.05$; two-tailed Student's *t*-test. On the right, the correspondent western blot validation. ß-actin was used as a loading control for endogenous HMGA1.

5. Identification of FOXM1 putative binding sites relevant for HMGA1 activity on the VEGFA promoter

Subsequently, we asked where HMGA1 and FOXM1 exert their transcriptional action along VEGFA promoter and for this reason we analysed the -1000 to -1 bp sequence of VEGFA promoter used by searching sequence elements important for the two factors. Specifically, for HMGA1 we looked for AT-rich regions searching for at least three consecutive A or T (Watanabe et al., 2013), and for FOXM1 we looked for its consensus sequence, which is the common binding element of Forkhead family proteins: in particular, we considered the sequence RYAAAYA (R can be A or G and Y can be substituted with C or T) (Littler et al., 2010). The schematic representation of the analysis performed is illustrated in figure 21. We found several AT-rich sequences (indicated with a red arrow

in the figure) particularly present in the region from – 979 to 907 bp, from -641 to -521 bp, from -355 to -322 bp, from -169 to -75 bp, where the AT stretches are particularly long, and from -17 to -13 bp from the TSS of the VEGFA promoter. In addition, we found 9 putative FOXM1 binding sites (represented as yellow boxes in the figure) from -993 to -922 bp, from -643 to - 638 bp, from -326 to -322 bp, from -124 to -104 bp, where it is located the FOXM1 preferential binding sequence TAAACA, and from -17 to -13 bp from the TSS of the VEGFA promoter.

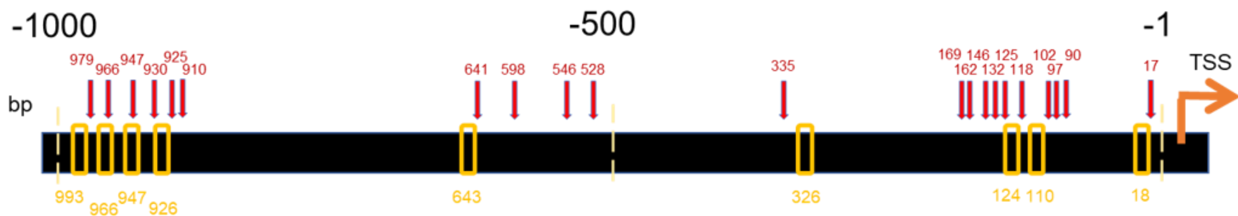


Figure 21: *VEGFA promoter vector contains several putative HMGA1 and FOXM1 binding regions.* Schematic representation of the bioinformatic analysis of the 1000 bp of VEGFA promoter used in reporter experiments. FOXM1 binding sites are represented with yellow boxes, whereas the AT-enriched sequences bound by HMGA1 are figured as red arrows.

On the basis of this analysis, we decided to explore whether the cooperative action of HMGA1 and FOXM1 occurs in the region spanning from -1000 to -500 or from -500 to -1 (Fig. 22a). We thus transfected the HEK293T cells with the reporter vectors pVEGFprom (-1000-1), pVEGFprom (-1000-500) and pVEGFprom (-500-1) and with the same amount of plasmids for over-expressing HMGA1 and FOXM1. The activation of the luciferase by HMGA1 and FOXM1 in the deletion mutants was compared to the full length VEGFA promoter (-1000 to -1 bp), in order to compare the action of the two factors in these three promoter constructs. As shown in figure 22b, the synergic action of HMGA1 and FOXM1 drops in the mutant promoter -1000 to -500 bp while it remains constant in the -500 to -1 bp VEGFA promoter, with respect to the full-length promoter condition. From this experiment, we inferred that the region of the VEGFA promoter where the action and the binding of HMGA1 and FOXM1 are mainly localized goes from -500 to -1 bp.

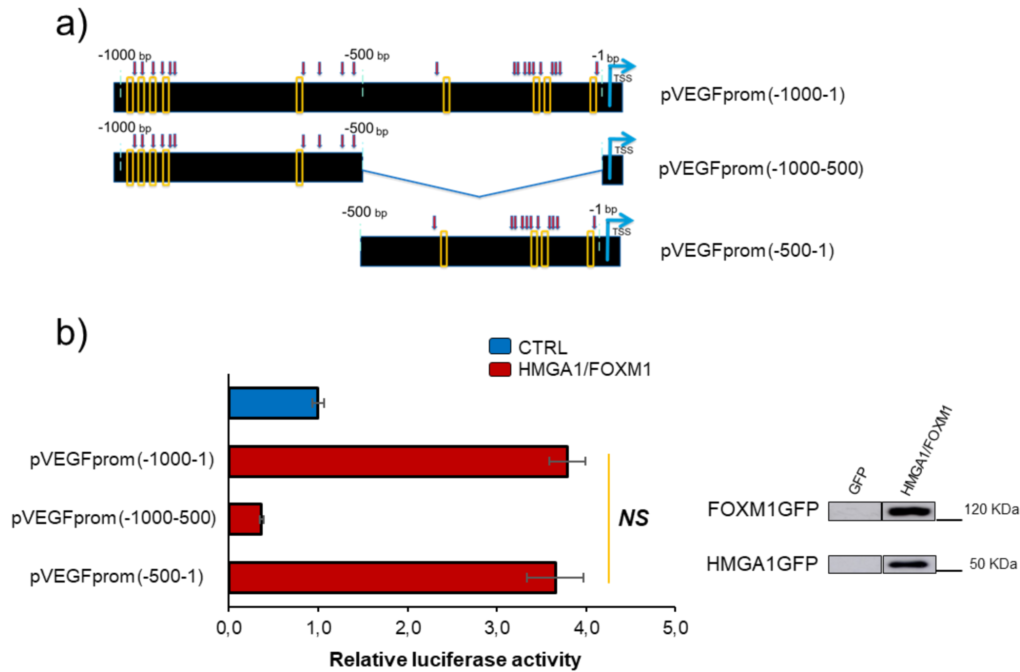


Figure 22: *The cooperative action of HMGA1 and FOXM1 resides mainly in (-500-1 bp) VEGFA promoter region*. a) Schematic representation of deletion reporter vectors transfected; b) HEK293T cells were transiently cotransfected with the luciferase reporter plasmid pVEGFprom (-1000-1), the deletion mutants pVEGFprom (-1000-500) or pVEGFprom (-500-1) with the expression plasmids pEGFP-HMGA1 and pEGFP-FOXM1. pRL-CMV Renilla luciferase expression vector was included to normalize for transfection efficiencies. Values are reported as relative luciferase activity comparing to cells transfected with the reporter vector pVEGFprom (-1000-1). The data are represented as the mean±SD ($n > 3$). *NS*: not significant; two-tailed Student's *t*-test. An example of western blot validations is reported.

Furthermore, in order to narrow down the regulatory region involved in the transcriptional control by HMGA1 in cooperation with FOXM1 we introduced progressive deletion of the -500-1 bp VEGFA promoter (Fig. 23a), on the basis of the presence of AT-rich regions and the FOXM1 putative binding sites and we generated the following constructs:

- pVEGFprom (-338 to -1) bp from the TSS comprising all the AT rich sequences and the FOXM1 binding elements and excluding the region between -500 -338 bp, where no putative binding sites for both factors were found;
- pVEGFprom (-172 to -1) bp from the TSS excluding one putative binding site for HMGA1 and FOXM1, but comprising all the long AT stretches and the preferential FOXM1 putative binding site described above.
- pVEGFprom (-104 to -1) bp from the TSS which excluded the specific putative binding sites for FOXM1 and all the long AT sequences of HMGA1, including only a region containing one putative binding site for FOXM1 (-17 to -13 bp).

Thus, we transfected these VEGFA deletion mutant reporter vectors together with expression vectors for HMGA1 and FOXM1 and evaluated the luciferase activity of these constructs with respect to the pVEGF prom (-500 to -1) bp reporter, (Fig. 23b). Specifically, we observed that FOXM1 maintains its transcriptional activity in all the reporters, except in the construct pVEGF prom (-104 to -1) bp promoter, where there is a decrease probably due to the loss of its preferential binding site which is located at -124 -118 bp of VEGFA promoter (Fig. 23b see purple bars). Overexpression of HMGA1 does not increase the activity of all three mutant reporters, in comparison with the pVEGF prom (-500 to -1) bp promoter (Fig. 23b see green bars), revealing the importance of the region spanning from -500 to -338 bp for its activity, even though from the analysis we made this region does not contain any AT stretch. Finally, we found that even if HMGA1 loses its ability to transcriptionally regulate the deletion mutants, it maintains its capacity to potentiate the activity of FOXM1 both on pVEGF prom (-338-1) bp and pVEGF prom (-172-1) bp luciferase vectors, whereas, on the pVEGF prom (-104 to -1) bp promoter, it fails in increasing FOXM1 activity (Fig. 23b see red bars).

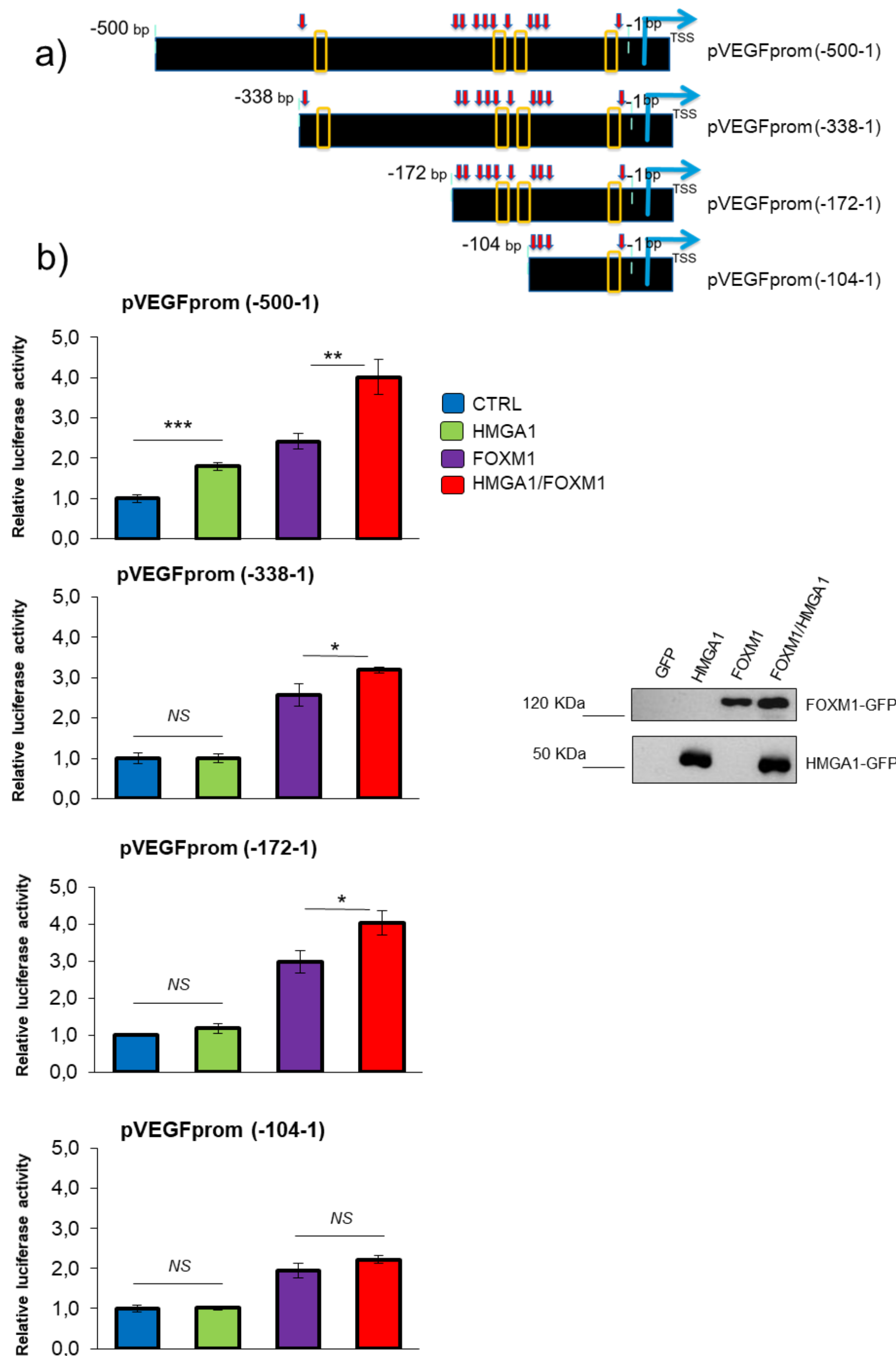


Figure 23: *HMGA1 enhances the luciferase activity on VEGFA promoter in two independent fashions*. a) Schematic representation of deletion reporter vectors transfected. b) HEK293T cells were transiently co-transfected with the luciferase reporter plasmid pGL4.10-VEGFprom (-500-1), the deletion mutants pGL4.10-VEGFprom (-338-1), pGL4.10-VEGFprom (-172-1) or pGL4.10-VEGFprom (-104-1) with the expression plasmid pEGFP-HMGA1(green bar), pEGFP-FOXM1 (purple bar) and pEGFP-HMGA1/pEGFP-FOXM1 (red bar). pRL-CMV Renilla luciferase expression vector was included to normalize for transfection efficiencies. Values are reported as relative luciferase activity comparing to cells transfected with the reporter vectors used. The data are represented as the mean±SD ($n > 3$). *$p<0.05$, **$p<0.01$, ***$p<0.001$, *NS*: not significant; two-tailed Student's *t*-test. An example of western blot validations is reported.

These results led us to conclude that HMGA1 acts on VEFGA promoter through two independent ways:

- a FOXM1-independent mechanism that resides in the region from -500 to -338 bp of the promoter;
- a FOXM1-dependent mechanism since it is able to potentiate FOXM1 transcriptional activity, mainly in the region from -338 to -104 bp of VEGF promoter, where we detect most of the cooperative action between HMGA1 and FOXM1.

6. HMGA1 acts on VEGFA promoter through two transcriptional factors: Sp1 and FOXM1

Given the data obtained and since the analysis we carried out did not reveal any AT stretch in the region from -500 to -338 bp of VEGF promoter, we hypothesized that the FOXM1-independent action of HMGA1 on the VEGFA promoter is mediated through other factors, possible molecular partners of HMGA1. We thus searched in the literature other known important factors involved in the transcription of VEGFA in the region -500 to -338 bp and we found that the binding of a well-known transcription factor, Sp1, occurs in the region from -385 to -352 bp of VEGFA promoter. Specifically, in this region three Sp1 binding sites (Sp1 consensus sequence: 5’-GGGCGG-3’) are described and it has been demonstrated that when the first and the second binding sites are mutated, the activity of Sp1 in VEGFA promoter decreases significantly (Pore et al., 2004). Intriguingly, it has been reported that HMGA1 cooperates with Sp1 in regulating gene expression. For example, HMGA1 physically interacts with Sp1 and supports its binding to the Insulin receptor promoter (Foti et al., 2003). Therefore, we decided to further explore whether HMGA1 cooperates with Sp1 on the VEGFA promoter, by introducing a further deletion in -500 -388 bp and mutating the two Sp1 binding sites described by Pore N. and colleagues (Fig. 24a), in this new deletion mutant pVEGFprom(-388-1). We transfected the HEK293T cells with the pVEGFprom(-500-1), pVEGFpromWT(-388-1) and pVEGFpromMUT(-388-1) reporters and evaluated by luciferase assay the activity of HMGA1 and FOXM1 alone or in combination on the activation of these promoters. As shown in figure 24b, the transcriptional activity of HMGA1 persists in the pVEGFpromWT(-388-1) with respect to the pVEGFprom(-500-1), highlighting that the action of HMGA1 does not occur in the region from -500 to -388 bp of the promoter (Fig. 24b, see green bars). Very interestingly, when we mutate Sp1 binding elements, HMGA1 is not able to transactivate the pVEGFpromMUT(-388 to -1), drawing the attention on the dependence of HMGA1 upon Sp1 factor on this promoter region (Fig. 24b, see green bars). In addition, when we transfected FOXM1, in all the reporters used in this experimental setting the activity is comparable to the pVEGFprom(-500-1) (Fig. 24b, see purple bars), consistently with

the analysis presented above. Finally, if we co-transfected HMGA1 and FOXM1, the synergic activity of the factors is not affected by the mutation of Sp1 elements (Fig. 24b, see red bars). This evidence confirmed our previous hypothesis speculating two independent mechanisms of action of HMGA1 on VEGFA promoter: the first one that we demonstrated goes by SP1 binding to the region -385 to -352 bp of the VEGFA promoter and the second one which involves the cooperation with FOXM1.

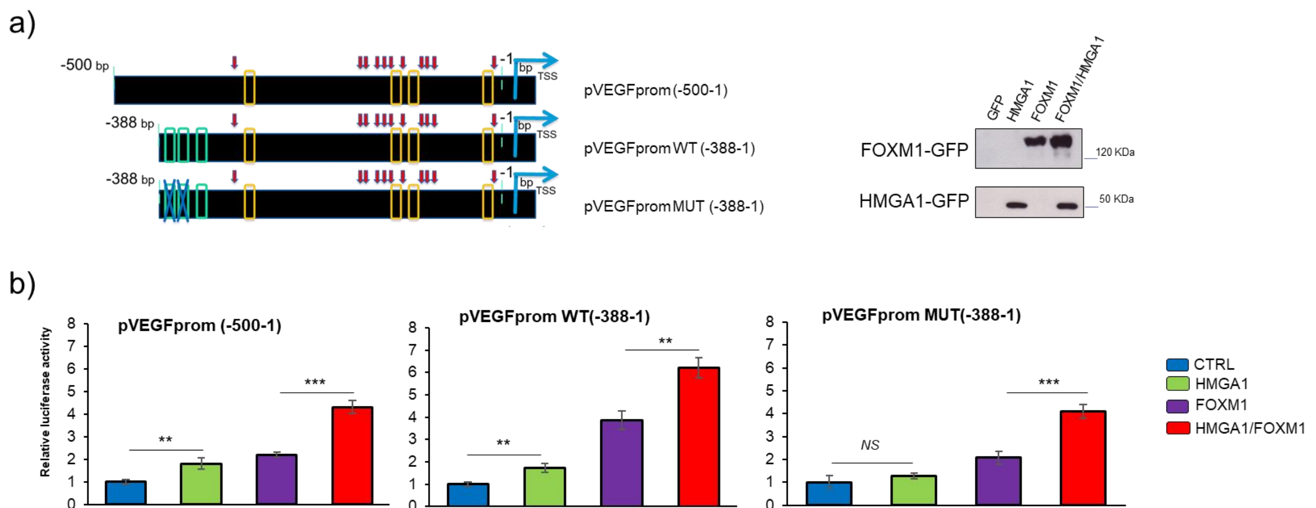


Figure 24*: HMGA1 transcriptional activity on VEGFA promoter is dependent upon SP1 and FOXM1 in different promoter regions*. a) Schematic representation of deletion reporter vectors transfected b) HEK293T cells were transiently cotransfected with the luciferase reporter plasmid pGL4.10-VEGFprom (-500-1), the deletion mutants pGL4.10-VEGFprom (-388-1) wild-type or mutated in SP1-binding sites with the expression plasmisd pEGFP-HMGA1(green bar), pEGFP-FOXM1 (purple bar) or pEGFP-HMGA1/ pEGFP-FOXM1 (red bar). pRL-CMV Renilla luciferase expression vector was included to normalize for transfection efficiencies. Values are reported as relative luciferase activity comparing to cells transfected with the reporter vectors used. The data are normalized to control condition and represented as the mean±SD ($n > 3$). **p<0.01, ***p<0.001, *NS*: not significant; two-tailed Student's *t*-test. An example of western blot validations is reported.

Subsequently, in order to deeply investigate the FOXM1-dependent activity of HMGA1, we carried out a reporter assay in HEK293T cells with the pVEGFpromWT(-388 to -1) or pVEGFpromMUT(-388 to -1) and the pVEGFprom(-338 to -1) and we evaluated the luciferase activity upon FOXM1 knock-down and HMGA1 overexpression. As reported in figure 25, the silencing of endogenous FOXM1 diminishes the luciferase activity in all the reporters used, regardless of the presence of Sp1 binding elements, underlying once again the importance of this factor on the VEGFA transcription. Moreover, the HMGA1 overexpression increases the pVEGFpromWT(-388 to -1) promoter activity,

as expected, while this increment is lower in pVEGFpromMUT(-388 to -1) or in the pVEGFprom(-338 to -1), confirming the dependence of HMGA1 upon Sp1. When we silenced the expression of FOXM1, HMGA1 over-expression is not able to induce the same level of luciferase activation of all the three reporters taken into consideration. Moreover, by comparing the silencing conditions (yellow bars), the overexpression of HMGA1 increases the VEGFA transcription to some extent only in the pVEGFpromWT(-388 to -1), while the mutation or the deletion of Sp1 binding elements diminishes HMGA1 ability to increase luciferase, confirming the existence of two different ways this factor works through, both pivotal for driving VEGFA transcription by HMGA1 (Fig. 25).

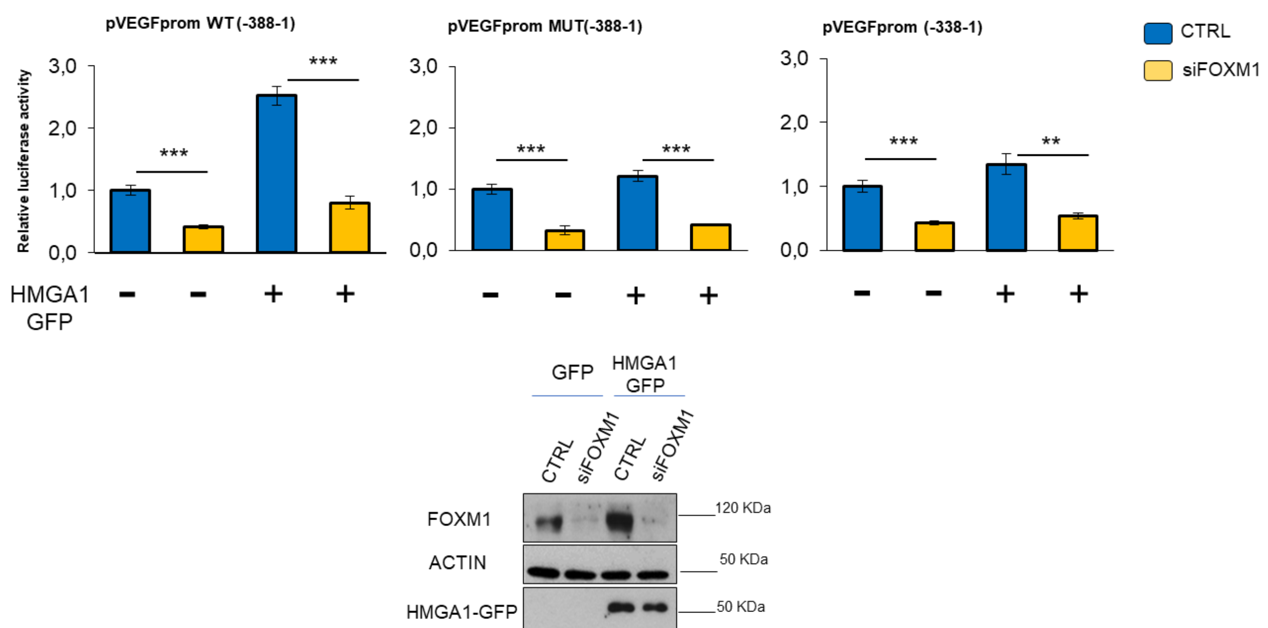


Figure 25: *The deletion of SP1 binding sites is sufficient to decrease the HMGA1 transcriptional activity on VEGFA promoter but is not necessary for its FOXM1-dependent action.* HEK293T cells were silenced for FOXM1 expression and transiently co-transfected with the luciferase reporter plasmid pGL4.10-VEGFprom (wild type -388-1), pGL4.10-VEGFprom (SP1 mutated -388-1) or pGL4.10-VEGFprom (-338-1) with the expression plasmids pEGFP-HMGA1. pRL-CMV Renilla luciferase expression vector was included to normalize for transfection efficiencies. Values are reported as relative luciferase activity comparing to cells transfected with the reporter vectors used. The data are normalized to control condition and are represented as the mean±SD ($n > 3$). **p<0.01, ***p<0.001; two-tailed Student's *t*-test. An example of western blot validations is reported. ß-actin was used as a loading control for endogenous FOXM1.

On the other hand, the ability of FOXM1 to induce VEGFA-luciferase reporter is dependent only on the presence of HMGA1 and not on that of Sp1. In fact, the overexpression of FOXM1 increases the VEGFA promoter transactivation regardless the presence of Sp1 binding elements as described before (Fig. 26). In addition, the presence of endogenous HMGA1 is crucial for FOXM1 activity, because

when we depleted the cells of this factor, FOXM1 decreases its transcriptional action on VEGFA promoter at the same extent both in the wild-type and in the Sp1-mutated vector.

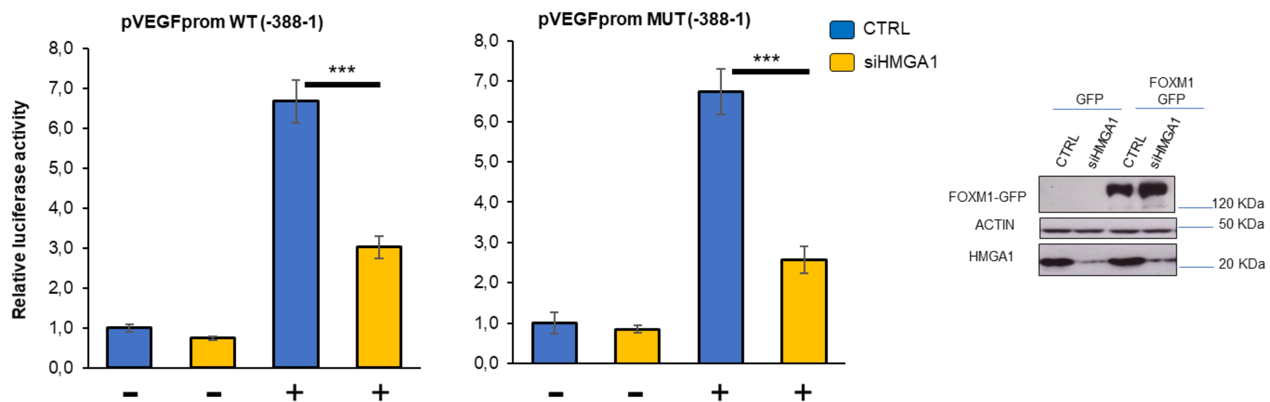


Figure 26: *FOXM1 action on VEGFA promoter does not need SP1 factor but is strictly dependent upon HMGA1 action*. HEK293T cells were silenced for HMGA1 expression and transiently co-transfected with the luciferase reporter plasmid pGL4.10-VEGFprom (wild type -388-1) or pGL4.10-VEGFprom (SP1 mutated -388-1) with the expression plasmid pEGFP-FOXM1. pRL-CMV Renilla luciferase expression vector was included to normalize for transfection efficiencies. Values are reported as relative luciferase activity comparing to cells transfected with the reporter vectors used. The data are represented as the mean±SD ($n > 3$). ***p<0.001; two-tailed Student's *t*-test. An example of western blot validation is reported. ß-actin was used as a loading control for endogenous HMGA1.

All these experiments underline the importance of HMGA1 and FOXM1 in regulating VEGFA transcription. Specifically, they pointed out that HMGA1 acts in at least two different ways, one dependent on Sp1 transcription factor and the other one on FOXM1 factor. Moreover, the results underline the tight cooperation between HMGA1 and FOXM1 in enhancing the transcription of the VEGFA factor, pivotal for the angiogenic process.

7. HMGA1 and FOXM1 induce TNBC cells to influence pivotal angiogenic processes of endothelial cells

We showed that HMGA1 and FOXM1 regulate the transcription of VEGFA, one of the most crucial factor in inducing the angiogenic process which is an important cancer hallmark. For this reason, in collaboration with the laboratory of Professor Roberta Bulla at the Department of Life Sciences of the University of Trieste, we decided to explore if HMGA1 and FOXM1 drive TNBC cells to influence the surrounding endothelial cells, enhancing their angiogenic properties and leading to the formation of new vessels important for tumor survival and spreading.

We then performed different *in vitro* experiments to test the ability of MDA-MB-231 supernatant in modulating angiogenesis. To this end, we proceeded by analysing if breast cancer cells influence some pivotal properties of endothelial cells, such as cellular proliferation and migration. Thus, we silenced the expression of HMGA1 and FOXM1 alone or in combination in MDA-MB-231 cells and treated the HUVEC endothelial cells with the supernatant collected by the cancer cells. In order to assess the proliferation rate, we measured the Ki67 marker, factor present in the active phases of the cell cycle (Scholzen and Gerdes, 2000). We observed that the endothelial cells grow to a lower rate if treated with the supernatants of cells silenced for HMGA1 and FOXM1 expression; nevertheless, the cosilencing of the two factors does not potentiate the effect of the single silencing conditions (Fig. 27a). In addition, we investigated the migration rate of endothelial cells, pivotal aspect for the angiogenic process. We treated the HUVEC cells with the supernatants of MDA-MB-231 depleted for HMGA1 and FOXM1; the single silencing conditions decrease the ability of the endothelial cells to migrate, while the co-silencing of HMGA1 and FOXM1 does not further boost this effect (Fig. 27b). From these experiments, we concluded that HMGA1 and FOXM1 individually control proliferation and migration rates of endothelial cells, considering that the cosilencing does not potentiates the action of the breast cancer cells on the aspects analysed.

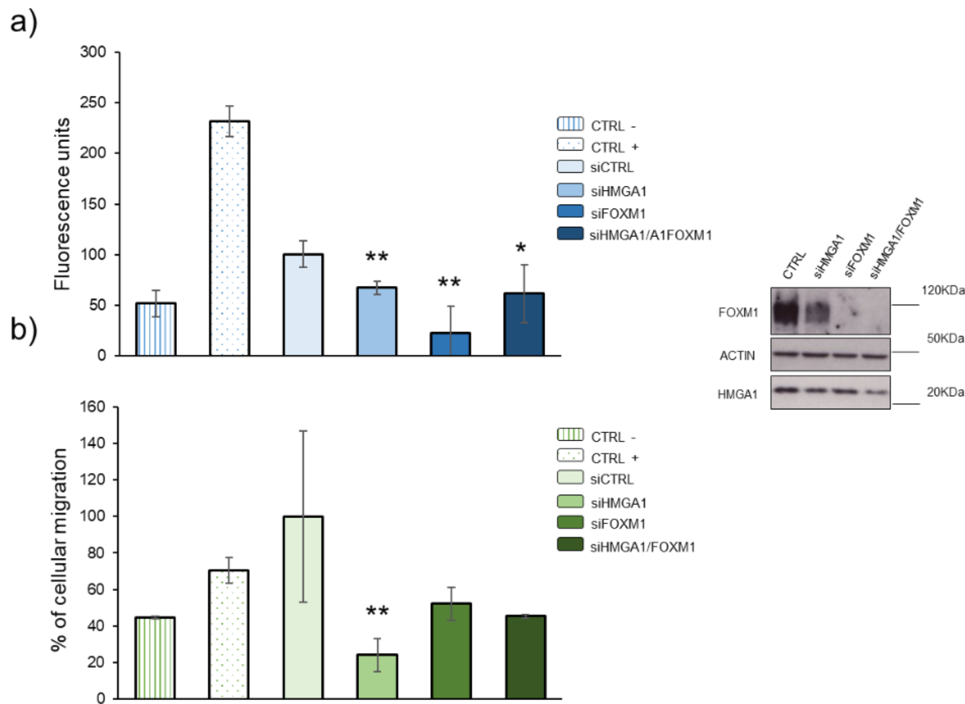


Figure 27: *HMGA1 and FOXM1 induce the TNBC cells to enhance in turn the proliferation and migration abilities of endothelial cells*. HUVEC endothelial cells were treated with MDA-MB-231 supernatants, who had been previously silenced for HMGA1, FOXM1 or cosilenced for HMGA1 and FOXM1. Serum-free medium and normal horse serum were used as negative and positive controls respectively. a) The cells proliferation was investigated by the positivity to the Ki67 marker and expressed in terms of fluorescence units. The comparison has been done with respect to the siCTRL condition. The data are represented as the mean±SD ($n > 3$). b) The migration of endothelial cells was assessed by Transwell assay, adding the supernatants of MDA-MB-231 in the lower chambers of the multiwell. The number of cells migrated were counted and the results are expressed as % of migration, respect to the siCTRL condition. The data are represented as the mean±SD ($n > 3$). *p<0.05, **p<0.01, ***p<0.001; two-tailed Student's *t*-test. Western blot validations of HMGA1/FOXM1 silencing in MDA-MB-231 cells is reported. ß-actin was used as a loading control.

Finally, we evaluated the ability of endothelial cells to form capillary-like structure, a crucial aspect of the angiogenesis, in response to breast cancer supernatants induction. To this aim we plated HUVEC cells on a Matrigel support and incubated them with the supernatant of MDA-MB-231 cells depleted for HMGA1, FOXM1, or a combination of both. As represented in figure 28a, the supernatants of control breast cancer cells induce the formation of capillary-like structures of endothelial cells at a level comparable to the one obtained by HUVEC cells stimulated by VEGFA, as a positive control. This result indicates the pro-angiogenic potential of the TNBC cells we used. Furthermore, if we give the supernatant of MDA-MB-231 cells silenced for HMGA1 and FOXM1 to endothelial cells, they lose their ability to organize themselves in a vessels network. Very interestingly, when we knocked down the expression of HMGA1 and FOXM1 together, the ability of HUVEC cells to form capillary structures is severely compromised. The number of vessels formed by endothelial cells were then counted and plotted in the graph in figure 28b. The induction of the

TNBC cells in promoting vessels formation is comparable to the positive control condition in which VEGFA was added, in terms of number of structures formed. The depletion of HMGA1 and FOXM1 in breast cancer cells decreased by 50% the tube formation capacity of HUVEC cells while the co-silencing of these factors further decreases the formation of capillaries in a significant manner.

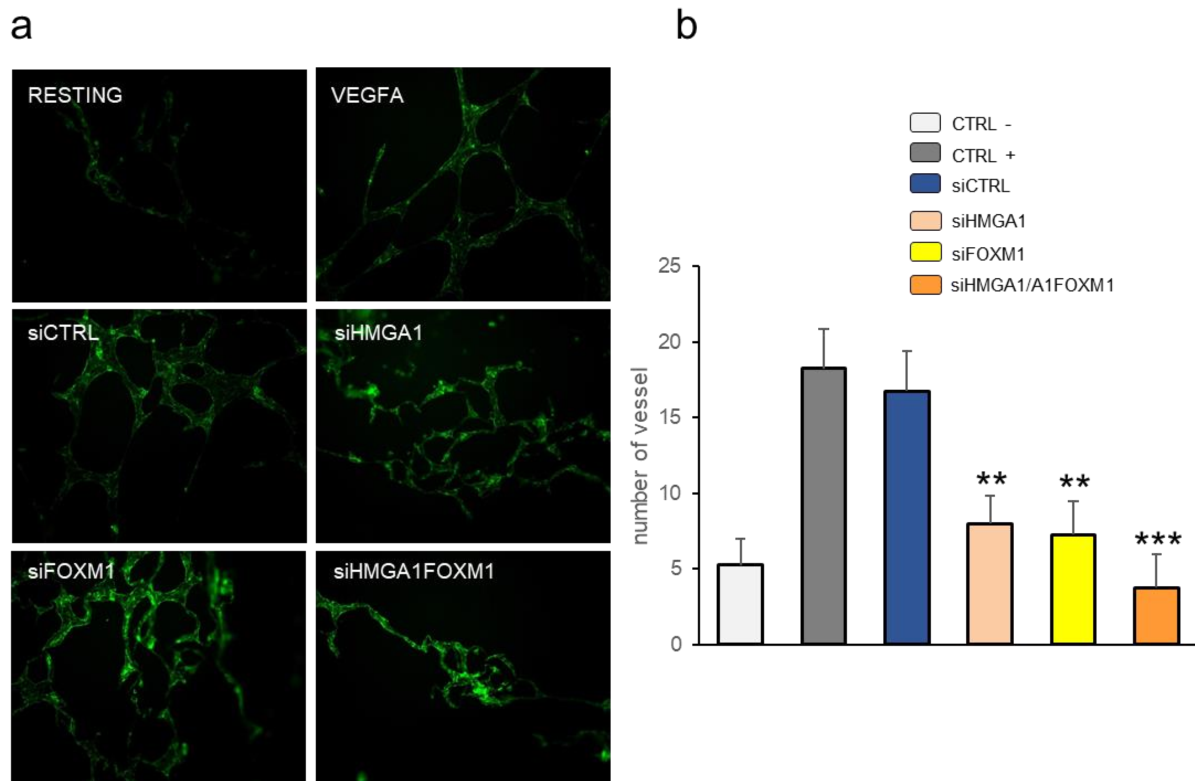


Figure 28: *HMGA1 and FOXM1 in TNBC cells strongly induce the endothelial cells to organize themselves in a vessel-like network*. a) Representative immunofluorescence images of the vessels formed. Capillary-like structures were allowed to form from endothelial cells exposed to VEGFA as a positive control or to supernatants of MDA-MB-231 cells silenced for HMGA1, FOXM1 or HMGA1/FOXM1 on Matrigel. b) The number of vessels formed by HUVEC cells were counted and plotted on the graph. The data are expressed respect to siCTRL condition and are represented as the mean±SD (*n = 4*). **P<0.01, ***P<0.001*;* two-tailed Student's *t*-test is calculated respect to siCTRL.

This experiment, which mimics better the angiogenic process with respect to the measure of proliferation and migration rates, confirmed the positive stimulus of the TNBC cells in enhancing the angiogenesis carried out by the surrounding endothelial cells. Moreover, it confirms the key role of HMGA1 and FOXM1 in breast cancer cells in promoting several angiogenic aspects of endothelial cells, by acting in a synergistical manner.

8. HMGA1 and FOXM1 regulate a specific angiogenic-transcriptional programme

In support of these experiments, in collaboration with the Bioinformatic unit of LNCIB in Trieste, we performed a bioinformatic analysis aimed to unravel other possible factors, regulated by HMGA1 and FOXM1, with a role in the angiogenic process. We thus performed a gene ontology analysis on the differentially expressed genes upon HMGA1 silencing and found more than 100 angiogenic factors whose expression is modulated by the depletion of HMGA1 in MDA-MB-231 TNBC cells (table 3a). Furthermore, a considerable amount of genes related to properties associated with angiogenesis were found significantly affected by HMGA1 knock-down (table 3a). The number of genes per function and the high significance of the data suggest an important involvement of HMGA1 in regulating these angiogenic-related processes. In addition, we crossed the DEG found with the FOXM1 network obtained by Ingenuity pathway analysis software, and we discovered several angiogenic factors, other than VEGFA, regulated by the two factors, both in angiogenic and in other angiogenic-related processes, such as the morphology of the cardiovascular system and the vasculogenesis (table 3b). Very interestingly, we found factors, such as CCNE2 and LEF1, whose differential regulation upon HMGA1 and FOXM1 expression had already been validated (see Fig. 4-7 section Results). Indeed, the silencing of HMGA1 and FOXM1 decreased the level of expression of these targets, whereas the co-depletion of HMGA1 and FOXM1 increased the effect of single factors. These data strongly support the involvement of HMGA1 and FOXM1 in the regulation of the angiogenic process, not only by modulating VEGFA, but also controlling other factors, and in other cellular processes related to it.

a)

Function Annotation	p-value	Number of Genes
morphology of cardiovascular system	2.36E-08	89
angiogenesis	7.64E-07	118
abnormal morphology of cardiovascular system	2.59E-06	76
vasculogenesis	4.70E-06	96
hemangioma	6.11E-06	51
vascular tumor	1.80E-05	52
vascular lesion	9.96E-05	33
function of cardiovascular system	1.22E-04	48

b)

	morphology of cardiovascular system	angiogenesis	vasculogenesis
	CCND2	CCNE2	CCNE2
	CCNE2	FOXM1	FOXM1
	FOXM1	LDHA	LDHA
	LEF1	LEF1	LEF1
	VEGFA	SKP2	SKP2
	ZEB1	VEGFA	VEGFA
		ZEB1	ZEB1

Table 3*: Several factors belonging to angiogenic-related functions are induced by HMGA1 and FOXM1's cooperative action*. a) A Gene ontology analysis was carried out on DEG upon HMGA1 silencing in MDA-MB-231 cells to cluster these genes in functions related to angiogenesis. The functions are ranked by p-value and the number of genes found to be regulated by HMGA1/function was annotated. b) The DEG of angiogenesis-related functions were crossed with the HMGA1/FOXM1 network and the factors involved in angiogenesis and its related functions were listed.

9. TNBC cells promote the angiogenic process in an *in vivo* model of Zebrafish

Considering the role of TNBC cells in promoting angiogenesis under the guidance of HMGA1 and FOXM1, we investigated whether cancer cells are able to influence the formation of new blood vessels also in an *in vivo* animal model. In order to evaluate the potential action of TNBC cells *in vivo,* we took advantage of *Danio rerio* (Zebrafish) embryos, which is a well-established animal model to study angiogenesis. As a matter of fact, Zebrafish embryos develop a circulatory system within one day after fertilization and it is possible to easily visualize the vessels due to the optical transparency of embryos. Moreover, the larvae manipulation and injection are simple, and no xenograft rejection occurs since the immune system is not mature enough at this stage (Tobia et al., 2011). Thus, in collaboration with the laboratory of Cristina Zennaro at the Department of Medical, Surgery and Health Sciences of the University of Trieste, we co-silenced the expression of HMGA1 and FOXM1 via siRNA in MDA-MB-231 cells and injected the cells in the yolk sack of Zebrafish embryos, two days after the fertilization. Firstly, we monitored the dimension of the tumor mass, which had colonized the site of injection, at one and three days after the injection in embryos. As reported in figure 29a, the dimension of the tumoral mass of MDA-MB-231 knocked-down for HMGA1 and FOXM1 decreases in comparison with the control cells within three days after injection. Moreover, we observed that the tumor mass in the cosilencing condition is less compact with respect to the control condition at three days post injection (representative images in figure 29 b and c). We assessed the proliferation index by measuring the Ki67 marker and noticed that the TNBC cells depleted of HMGA1 and FOXM1 proliferate less compared to control cells, specifically at day one post injection. This result was then reverted at three days post injection, probably due to the loss of the silencing effect in TNBC cells (Fig. 29d). These observations brought to light the evidence that the co-silencing of HMGA1 and FOXM1 has a strong impact on the growth of the primary tumor, underlying the importance of these factors in promoting cancer.

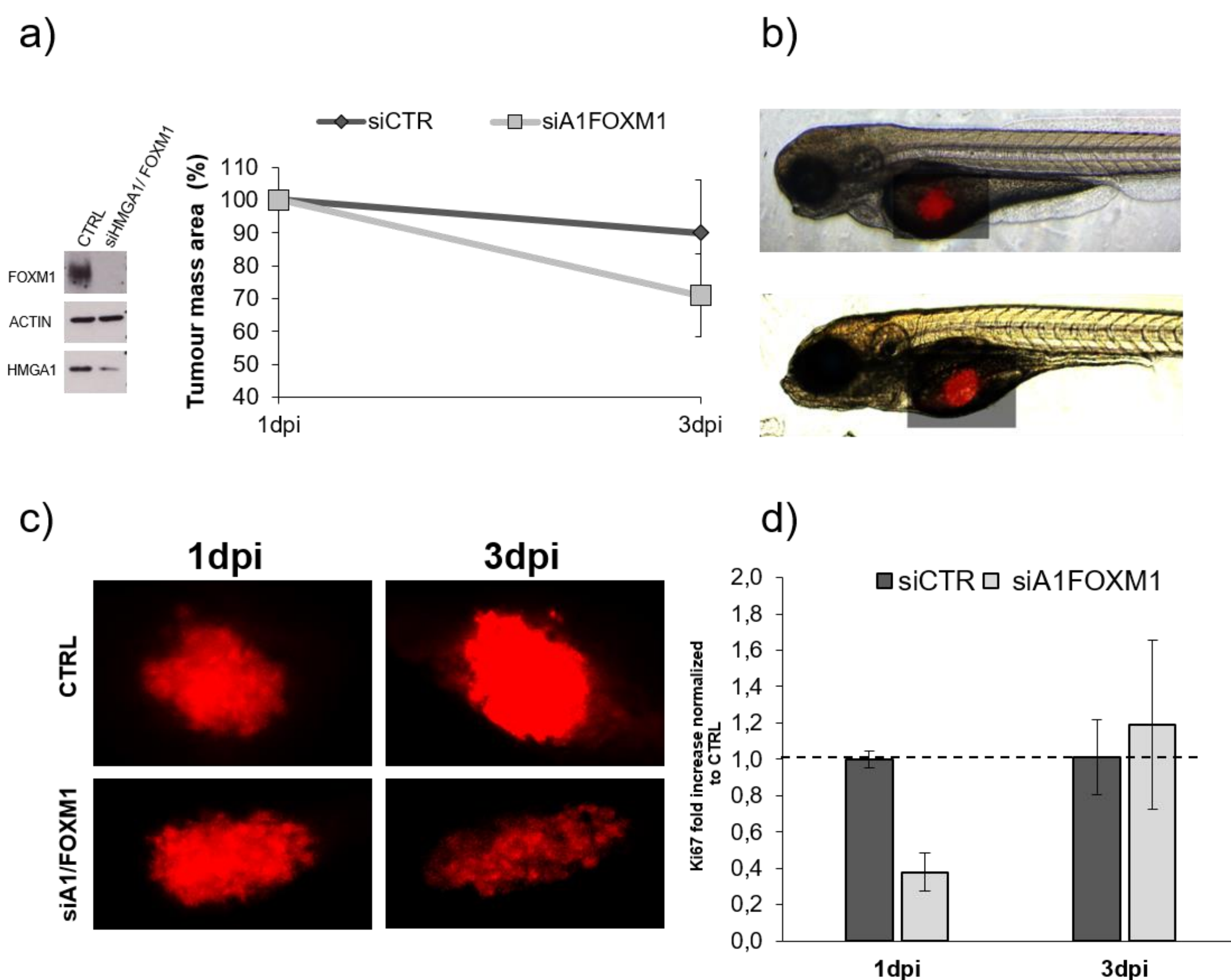


Figure 29: *HMGA1 and FOXM1 increase the tumor mass in vivo*. Evaluation of tumor mass growth in Zebrafish larvae after 1 and 3 days post injection of MDA-MB-231 cosilenced for HMGA1 and FOXM1 (1 dpi and 3 dpi). a) Western blot validations of HMGA1/FOXM1 silencing is reported. ß-actin was used as a loading control. Tumor mass evaluation was expressed as percentage of siHMGA1/FOXM1 with respect to siCTRL condition, normalized to 1dpi. The data are represented as the mean±SD (*n = 10);* b) Representative live images of tumor mass in larvae at 1 dpi and 3 dpi. Overlapping immunofluorescence images of the tumor mass are reported. c) Control and siHMGA1/FOXM1 MDA-MB-231 tumor mass evaluation in immunofluorescence at 1 and 3 days post injection. d) qRT-PCR analysis of hKi67 at 1 and 3 dpi. Data were normalized to the human GAPDH mRNA amount. The data are represented as the mean±SD (*n = 10).*

From these preliminary experiments, we decided to perform the evaluation of the angiogenic process at day one after TNBC cells injection. Therefore, the co-silenced cells were injected in Zebrafish embryos and the proliferation rate of the tumor mass was assessed by Ki67 measurement, (Fig. 30a). Subsequently, we evaluated the vessels of the sub-intestinal venous plexus (SIV), since it is an easily observable vessels network at only 2 days post fertilization (Goi and Childs, 2016), (Fig. 30b). Specifically, we investigated whether the co-depletion of HMGA1 and FOXM1 affects the normal

development of this circulatory system; we noticed that HMGA1 and FOXM1 silencing reverts the alteration observed in control cells, almost to the normal condition (Fig. 30b). Then, we counted the number of larvae whose SIV was branched and plotted the results on a graph (Fig. 30c): the knock-down of the HMGA1 and FOXM1 decreases the number of embryos altered in their sub-intestinal vessels with respect to the control condition, where more than half of the larvae taken into consideration was altered.

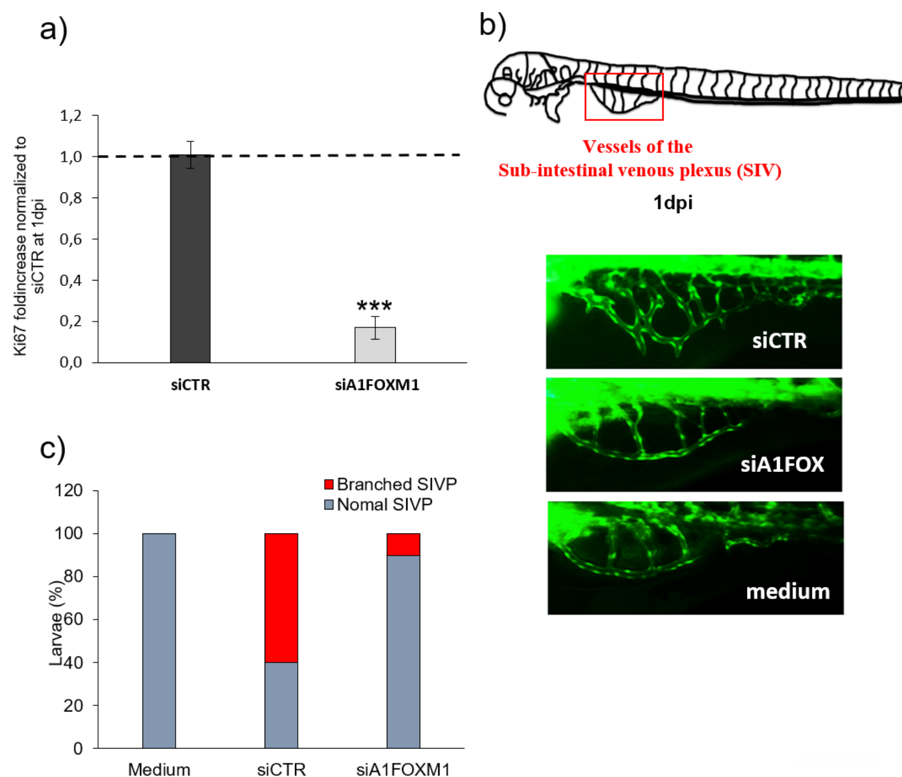


Figure 30: *The synergic action of HMGA1 and FOXM1 of MDA-MB-231 cells induce the angiogenic process in vivo*. a) Validation of proliferation of control and HMGA1/FOXM1 TNBC cells by qRT-PCR analysis of hKi67 at 1 dpi. Data were normalized to the human GAPDH mRNA amount. The data are represented as the mean±SD (*n = 10).* ***p<0.001. b) Schematic representation of the sub-intestinal venous plexus (SIVP) evaluated for the assessment of the Zebrafish angiogenic process. Representative images of vessels of the SIVP. The medium was used as a negative control. c) The larvae with a branched SIVP (red) or unaltered SIVP were counted and plotted on the graph, expressed in percentage (*n = 22*).

Furthermore, in order to better investigate the level of alteration of the vessels network, several specific parameters of the SIV were considered. The length, area and diameter of the SIV were measured and it was assessed that the knock down of HMGA1 and FOXM1 decreases the alteration on these parameters to the level of an unstimulated situation, while control cells significantly modify the structure of the SIV plexus (Fig. 31 a, b and c).

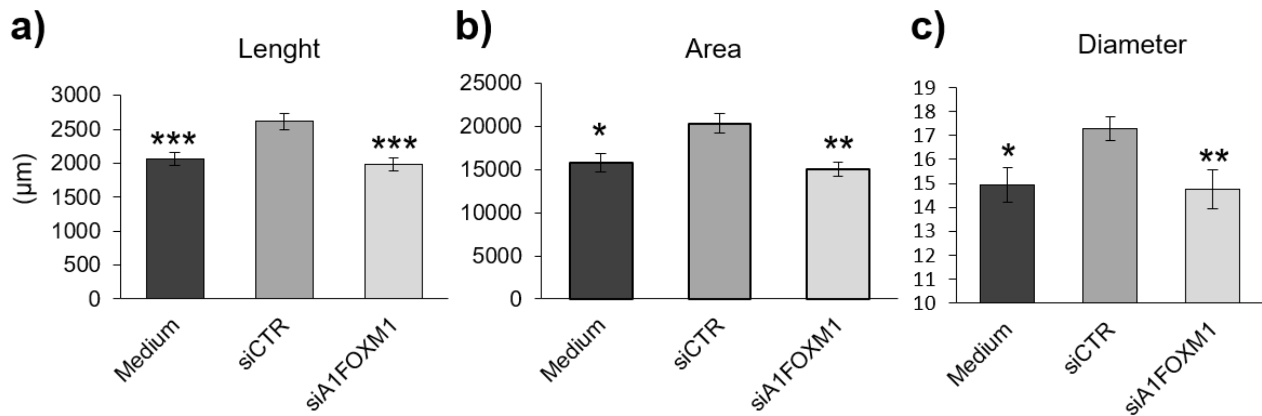


Figure 31: *TNBC cells under the driving forces of HMGA1 and FOXM1 affect the length, area and diameter of the sub-intestinal venous plexus of Zebrafish larvae*. The SIVP vessels parameters taken into account were measured in Zebrafish larvae at 1 dpi of MDA-MB-231 cells silenced for HMGA1 and FOXM1 (a, b and c respectively). The data are normalized to control and are represented as the mean±SD (*n = 22).* *p<0.05, **p<0.01, ***p<0.001.

Finally, the level of expression of several Zebrafish angiogenic markers were evaluated in qRT-PCR. Specifically, the co-silencing of HMGA1 and FOXM1 reduces significantly the level of expression of Zebrafish Vegfa2 and its receptors Ftl-1 and Flk-1, confirming the action of MDA-MB-231 cells on Zebrafish endothelial cells (Fig. 32).

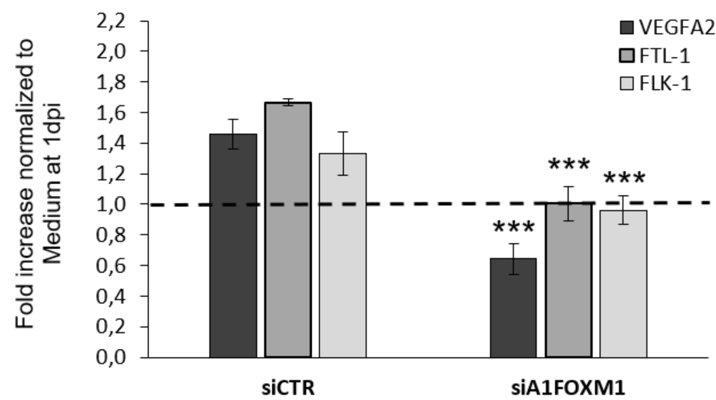


Figure 32: *HMGA1 and FOXM1 in TNBC cells induce the expression of Zebrafish angiogenic molecular targets*. qRT-PCR analysis of zebrafish VEGFA, FLT1 and FLK1 at 1dpi. Data were normalized to the Zebrafish actin mRNA amount. The data are compared to the respective controls and are represented as the mean±SD (*n = 22*). ***p<0.001.

The results obtained in this animal model sustained the observations made in the *in vitro* experiments about the vital importance of HMGA1 and FOXM1 in leading the breast cancer cells to modulate the angiogenic potential of Zebrafish endothelial cells.

Discussion and Conclusions

HMGA1 has been widely proved to have a causal role in breast cancer, both at early stages, bringing the mammary epithelial cells to acquire a malignant phenotype (Ram et al., 1993, Reeves et al., 2001), and during breast cancer progression, by promoting the cellular migration and invasion capacities, thus leading to the metastatization event (Pegoraro et al., 2013; Shah et al., 2013). In addition, HMGA1 expression is particularly enriched in the basal-like breast cancer subtype in comparison to other subtypes, demonstrating its correlation with an advanced tumor stage (Pegoraro et al., 2013). All these evidences demonstrated how HMGA1 is a crucial factor for breast cancer and how deepening the knowledge about the features it controls will be paramount for the discovery of new-more targeted and efficacious therapies. Given these premises, the aim of this work was to find new molecular partners HMGA1 could synergistically work with in regulating common gene networks, implicated in breast cancer hallmarks. HMGA1 is an architectural transcription factor binding general AT-enriched sequences on the DNA rather than specific regulatory elements; moreover, by interacting with a plethora of transcription factors, guides their transcriptional action on a high number of targets involved in many cellular processes, such as cell growth, proliferation, differentiation and cell death (Cleynen and Van de Ven, 2008), aspect which characterizes HMGA1 as a molecular instigator in normal and tumoral cells. Thus, by bioinformatic analysis on differentially expressed genes upon HMGA1 silencing in a model of TNBC cells, we identified several possible molecular partners of HMGA1 in regulating common gene networks in breast cancer. Among the factors obtained, we found RB1, E2F and STAT3, whose connection with HMGA family members has been described. Specifically, HMGA2 enhances E2F transcriptional activity by directly binding RB1, inducing the development of pituitary adenomas (Fedele et al., 2006a); furthermore, HMGA1 regulates STAT3 by directly binding its promoter, causing haematological malignancies (Bowman et al., 2000). Our data suggest that HMGA1 could work with these factors also in breast cancer progression, indicating common crucial HMGA1-oncogenic pathways in different cancer types. Basing on the fact that the role of HMGA factors has been already explored in association with these factors, we focused on FOXM1, a transcription factor primarily involved in cell cycle regulation (Wierstra and Alves, 2007), with a well-known role in breast cancer onset and progression (Saba et al., 2016). Considering all these aspects and that very little is known about the relation between HMGA1 and FOXM1, we decided to explore the molecular connection occurring between HMGA1 and FOXM1, new putative molecular partner of HMGA1. We thus explored the downstream gene network in common between HMGA1 and FOXM1 and we focused in particular on targets with a role in cancer epithelial to mesenchymal transition, migration and angiogenesis, processes in which both factors are known to be involved. In particular, CCNE2, which has been connected to migratory ability of tumoral cells (Caldon and Musgrove, 2010), has been proved to be under the transcriptional

control of HMGA1, thus promoting the migratory and invasive abilities of breast cancer cells (Pegoraro et al., 2015). Furthermore, HMGA1 has been also found to regulate the EMT, a process necessary to cell migration, by impacting on the WNT-beta catenin pathway, known to contribute to metastatization (DiMeo et al., 2009); indeed, HMGA1 regulates LEF1, one of the actor of this pathway (Pegoraro et al., 2013). Moreover, a few evidences highlighted a connection between HMGA1 and VEGFA, one of the main inducer of angiogenesis (Carmeliet and Jain, 2011) found to be under the transcriptional control of HMGA1, thus suggesting an involvement of HMGA1 in the angiogenic process, pivotal for tumor growth and dissemination. Taken into account these data, we validated the expression level of CCNE2, LEF1, SNAI2-another important factor involved in EMT- and VEGFA upon HMGA1 and FOXM1 silencing in triple negative breast cancer cell lines models. We were then able to validate the expression changes predicted by the bioinformatic analysis, confirming the involvement of HMGA1 and FOXM1 in the same gene network. Furthermore, by co-silencing HMGA1 and FOXM1 in our model of TNBC, we observed a stronger reduction of the above-mentioned targets, suggesting that HMGA1 cooperates with its molecular partner FOXM1 in regulating these factors. A considerable amount of data sustains the involvement of FOXM1 in several features of breast cancer progression, such as the EMT, the migration and invasion abilities of tumour cells and tumoral angiogenesis, in which FOXM1 controls the transcription of several metalloproteinases, the EMT inducer SNAI2 and the angiogenic factor VEGFA (Saba et al., 2016). Taken into account these evidences and considering that the co-depletion of HMGA1 and FOXM1 in our TNBC model has an additive effect on the expression level of CCNE2, LEF1, SNAI2 and VEGFA with respect to the single silencing conditions, we inferred a common mechanism of action of HMGA1 and FOXM1 in important malignant hallmarks of TNBC, such as EMT, migration and angiogenic process, highlighting a dependence of breast tumor cells on HMGA1 and FOXM1 synergic action. The cooperative action of HMGA1 and FOXM1 on breast cancer features is also confirmed by the *in vivo* injection of TNBC cells co-silenced for HMGA1 and FOXM1 in a model of Zebrafish. Indeed, it has been brought to light that the proliferation rate of breast cancer cells diminishes upon HMGA1 and FOXM1 co-depletion, proving the role of these factors in promoting in concert aggressive traits of triple negative breast cancer. Previously, a potentiating action of HMGA proteins on transcriptional activity of other transcription factors, such as NF-KB has been extensively studied (Cleynen et al., 2007; Yie et al., 1999b). Subsequently, considering the cooperative action of HMGA1 and FOXM1 on different biological aspects, we evaluated whether HMGA1 has a general impact on FOXM1 transcriptional activity. Indeed, we observed a potentiating action of HMGA1 on FOXM1 in regulating a synthetic promoter with its own binding sites, confirming the tight dependence of FOXM1 on HMGA1. Several studies support the idea that, in

order to exert its function of transcription factor, FOXM1 cellular localization has to be finely regulated and that it occurs mainly at a post-translational level (Jaiswal et al., 2014); moreover, considering that HMGA1 regulates FOXM1 transcriptional activity, we explored the possible ways of FOXM1 regulation by HMGA1. Upon HMGA1 action, we demonstrated that the endogenous and exogenous FOXM1 protein level and nuclear localization are affected by HMGA1. Thus, we speculated a new molecular mechanism of regulation of FOXM1, in which HMGA1 enhances FOXM1 transcriptional activity at a post-transcriptional level, by influencing the stability and thus the nuclear presence of FOXM1 on its targets. A profound molecular relation between HMGA1 and FOXM1 has been further highlighted by TCGA analysis on breast cancer patients, which confirmed a strong enrichment of FOXM1 presence in HMGA1 over-expressing patients, in particular in ER-cases, breast cancer subtype in which HMGA1 is highly expressed. All these results clearly confirm a fine dependence of FOXM1 on HMGA1, both for its cellular localization and subsequently for its transcriptional activity on pathways pivotal for breast cancer aggressive traits. Therefore, we considered that, among the hallmarks in which HMGA1 has a profound impact, the angiogenesis is the one in which HMGA1 role has been poorly explored. As one of the main inducer of angiogenesis, VEGFA is regulated by a plethora of factors; indeed, VEGFA promoter contains binding sites for several factors, such as Sp1/Sp3, AP-2, Egr-1, STAT3 and HIF1, which finely control VEGFA transcription depending on the patho-physiological situation, thus confirming VEGFA crucial impact on the angiogenic process (Pagès and Pouysségur, 2005). In particular, Sp1 is a potent inducer of VEGFA transcription and its down-regulation brings to a reduced tumor vascularization (Wei et al., 2004). Literature data also highlight that HMGA1 and FOXM1 regulates VEGFA transcription (Messineo et al., 2016; Zhang et al., 2008) and our results confirmed the regulation of VEGFA by these two factors both independently but above all in a synergistical manner. In fact, we further deepened the action of HMGA1 and FOXM1 on VEGFA promoter by several reporter assays and we demonstrated for the first time that HMGA1 acts through two independent pathways in regulating VEGFA transcription: on one side, HMGA1 action depends on FOXM1, confirming once again the action of this factor on VEGFA transcription and a cooperation with HMGA1 in its transcriptional activity; on the other, HMGA1 regulates VEGFA through Sp1 in a different promoter region. The fact that HMGA1 could work with Sp1 in regulating gene transcription is also reported in the control of insulin receptor (IR) gene transcription, in which HMGA1 interacting with Sp1 and C/EBP beta facilitates the binding of both factors to the IR promoter and synergistically activates IR transcription (Foti et al., 2003). These evidences suggest a similar mechanism of action on VEGFA promoter via Sp1 and FOXM1. These results highlight that HMGA1 control VEGFA with different molecular partners and suggest a possible context-dependent VEGFA activation by HMGA1. Given the action

of HMGA1 and FOXM1 on VEGFA transcription, we wondered whether they influence the angiogenic process in general by evaluating the presence of other factors involved in angiogenesis or related processes. By crossing the DEG list upon HMGA1 silencing and FOXM1 IPA network, we found several factors connected to angiogenesis and related processes like vasculogenesis. Very interestingly, among these DEG, LEF1 and CCNE2 were already been validated as transcriptional targets of HMGA1 and FOXM1, not only independently but, more importantly, in a cooperative way. A lot of studies proved the causal role of HMGA1 at multiple stages of breast cancer progression, acting on proliferation of tumoral cells, thus on tumor growth (Shah et al., 2013), but also on migration and invasion abilities of cells, leading to metastatic dissemination (Pegoraro et al., 2013). An event which represents a fundamental step in cancer growth and metastatic spread is the formation of blood vessels by tumoral angiogenesis, which sustains the tumor mass with nutrients and oxygen and promotes the cancer cells to reach distal organs (Folkman, 2002). Among the multitude of factors inducing tumoral angiogenesis, HMGA1 and FOXM1 have been found to play an important part, considering not only the literature evidences, but also the results obtained in this work, strongly relating their transcriptional action on VEGFA and other angiogenic factors. We thus evaluated whether HMGA1 and FOXM1 can lead the TNBC cells to influence the surrounding tumoral environment and promote angiogenic events carried out by endothelial cells. In accordance to this, we previously demonstrated that HMGA1 has a profound impact in breast cancer cell secretome, inducing the release of a pool of pro-migratory proteins that act on breast cancer cells itself (Resmini et al., 2017). We then performed several *in vitro* experiments on endothelial cells induced by TNBC supernatants depleted for HMGA1 and FOXM1 and we proved the leading role of these factors in guiding breast cancer cells to secrete angiogenic factors, promoting the proliferation and the migration of endothelial cells and their ability to organize in vessel-like structures. We confirmed the angiogenic potential of HMGA1 and FOXM1 in breast cancer cells by injecting HMGA1 and FOXM1 depleted-TNBC cells in Zebrafish embryos, which are highly suitable animal models for the evaluation of the blood vessels formation (Tobia et al., 2011). We thus proved that the contemporary down-regulation of HMGA1 and FOXM1 severely impairs the *in vivo* angiogenic process of Zebrafish endothelial cells, confirming that HMGA1 and FOXM1 in concert profoundly impact on triple negative breast cancer cells in promoting the angiogenic process. HMGA1 is a highly plastic factor which, with its ability to bind non specific regions of the DNA and the possibility to interact with a high number of molecular partners, regulates several cellular processes, hallmarks of cancer, in a way dependent on the molecular context. In our study, we discovered a new partner of HMGA1, the transcription factor FOXM1: the cooperation with these factors has been proved to severely influence the angiogenic induction by breast cancer cells on surrounding endothelial cells, bringing

the idea of a higher impact of HMGA1 and FOXM1 on this hallmark with respect to others. In the last years, giving the importance of FOXM1 in cancer context, the perspective of targeting FOXM1 has grown. Indeed, several anti-FOXM1 drugs have been tested, such as proteasome inhibitors like Thiostrepton and Syomicin A and the new small molecule FDI-6, which more specifically binds to FOXM1 protein and interferes with FOXM1 binding to DNA (Kalinichenko and Kalin, 2015). Considering that HMGA1 control several cellular pathways, which are intersected by FOXM1, and together they regulate common features of breast cancer aggressiveness, as we demonstrated in this work in the case of the angiogenesis process, deepening the molecular relationship occurring between HMGA1 and FOXM1 could be extremely useful to better target TNBC patients and to contribute to breast cancer defeat.

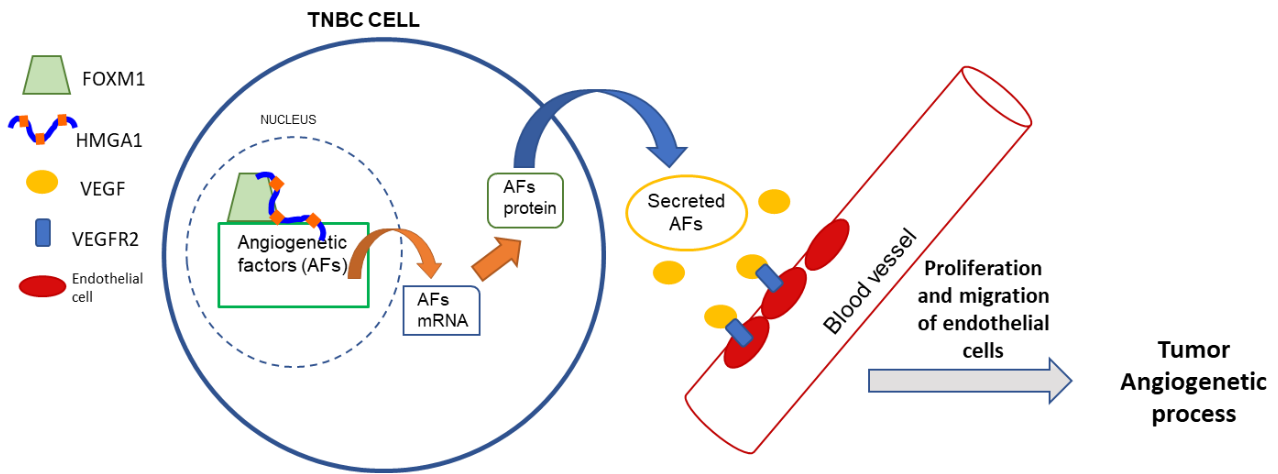
In conclusion, with this study we demonstrated several major findings:

1. HMGA1 and FOXM1 regulate a transcriptional network which control cancer hallmarks such as epithelial to mesenchymal transition, migration, invasion and angiogenesis, pivotal for triple negative breast cancer progression.

2. HMGA1 regulates the VEGFA, one of the main inducer of angiogenesis and thus tumour growth and metastatic spread, through two mechanisms, an Sp1-dependent and a FOXM1-dependent ways.

3. HMGA1 and FOXM1 control a common network of angiogenic factors secreted by TNBC cells which in turn modulate several angiogenic processes of endothelial cells.

4. HMGA1 and FOXM1 synergistically induce the *in vivo* tumoral angiogenic process, a cancer hallmark fundamental for the acquisition of aggressive traits of breast cancer



Hypothetical model proposed: HMGA1 and FOXM1 synergistically influence the production of angiogenic factors, such as VEGFA, by the TNBC cells, which in turn modulate the angiogenesis carried out by endothelial cells

References

Ahn, H., Sim, J., Abdul, R., Chung, M.S., Paik, S.S., Oh, Y.-H., Park, C.K., and Jang, K. (2015). Increased expression of forkhead box M1 is associated with aggressive phenotype and poor prognosis in estrogen receptor-positive breast cancer. J. Korean Med. Sci. *30*, 390–397.

Auer, G.U., Caspersson, T.O., and Wallgren, A.S. (1980). DNA content and survival in mammary carcinoma. Anal. Quant. Cytol. *2*, 161–165.

Auer, G.U., Fallenius, A.G., Erhardt, K.Y., and Sundelin, B.S. (1984). Progression of mammary adenocarcinomas as reflected by nuclear DNA content. Cytometry *5*, 420–425.

Baldassarre, G., Belletti, B., Battista, S., Nicoloso, M.S., Pentimalli, F., Fedele, M., Croce, C.M., and Fusco, A. (2005). HMGA1 protein expression sensitizes cells to cisplatin-induced cell death. Oncogene *24*, 6809–6819.

Bektas, N., Haaf, A. ten, Veeck, J., Wild, P.J., Lüscher-Firzlaff, J., Hartmann, A., Knüchel, R., and Dahl, E. (2008). Tight correlation between expression of the Forkhead transcription factor FOXM1 and HER2 in human breast cancer. BMC Cancer *8*, 42.

Bowman, T., Garcia, R., Turkson, J., and Jove, R. (2000). STATs in oncogenesis. Oncogene *19*, 2474–2488.

Brennan, R.G. (1993). The winged-helix DNA-binding motif: another helix-turn-helix takeoff. Cell *74*, 773–776.

Bruce, J., Carter, D.C., and Fraser, J. (1970). Patterns of recurrent disease in breast cancer. Lancet Lond. Engl. *1*, 433–435.

Caldon, C.E., and Musgrove, E.A. (2010). Distinct and redundant functions of cyclin E1 and cyclin E2 in development and cancer. Cell Div. *5*, 2.

Camós, S., Gubern, C., Sobrado, M., Rodríguez, R., Romera, V.G., Moro, M.A., Lizasoain, I., Serena, J., Mallolas, J., and Castellanos, M. (2014). The high-mobility group I-Y transcription factor is involved in cerebral ischemia and modulates the expression of angiogenic proteins. Neuroscience *269*, 112–130.

Carmeliet, P., and Jain, R.K. (2011). Molecular mechanisms and clinical applications of angiogenesis. Nature *473*, 298–307.

Carr, J.R., Park, H.J., Wang, Z., Kiefer, M.M., and Raychaudhuri, P. (2010). FoxM1 Mediates Resistance to Herceptin and Paclitaxel. Cancer Res. *70*, 5054–5063.

Cavallaro, U., and Christofori, G. (2004). Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat. Rev. Cancer *4*, 118–132.

Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., Jacobsen, A., Byrne, C.J., Heuer, M.L., Larsson, E., et al. (2012). The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data. Cancer Discov. *2*, 401–404.

Chen, H., Zhu, G., Li, Y., Padia, R.N., Dong, Z., Pan, Z.K., Liu, K., and Huang, S. (2009). Extracellular signal-regulated kinase signaling pathway regulates breast cancer cell migration by maintaining slug expression. Cancer Res. *69*, 9228–9235.

Chiappetta, G., Avantaggiato, V., Visconti, R., Fedele, M., Battista, S., Trapasso, F., Merciai, B.M., Fidanza, V., Giancotti, V., Santoro, M., et al. (1996). High level expression of the HMGI (Y) gene during embryonic development. Oncogene *13*, 2439–2446.

Chiappetta, G., Botti, G., Monaco, M., Pasquinelli, R., Pentimalli, F., Di Bonito, M., D'Aiuto, G., Fedele, M., Iuliano, R., Palmieri, E.A., et al. (2004). HMGA1 protein overexpression in human breast carcinomas: correlation with ErbB2 expression. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. *10*, 7637–7644.

Chin, K., DeVries, S., Fridlyand, J., Spellman, P.T., Roydasgupta, R., Kuo, W.-L., Lapuk, A., Neve, R.M., Qian, Z., Ryder, T., et al. (2006). Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. Cancer Cell *10*, 529–541.

Chin, M.T., Pellacani, A., Wang, H., Lin, S.S., Jain, M.K., Perrella, M.A., and Lee, M.E. (1998). Enhancement of serum-response factor-dependent transcription and DNA binding by the architectural transcription factor HMG-I(Y). J. Biol. Chem. *273*, 9755–9760.

Chung, A.S., Lee, J., and Ferrara, N. (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. Nat. Rev. Cancer *10*, 505–514.

Cleynen, I., and Van de Ven, W.J.M. (2008). The HMGA proteins: a myriad of functions (Review). Int. J. Oncol. *32*, 289–305.

Cleynen, I., Brants, J.R., Peeters, K., Deckers, R., Debiec-Rychter, M., Sciot, R., Van de Ven, W.J.M., and Petit, M.M.R. (2007). HMGA2 regulates transcription of the Imp2 gene via an intronic regulatory element in cooperation with nuclear factor-kappaB. Mol. Cancer Res. MCR *5*, 363–372.

Consoli, F., Grisanti, S., Amoroso, V., Almici, C., Verardi, R., Marini, M., and Simoncini, E. (2011). Circulating tumor cells as predictors of prognosis in metastatic breast cancer: clinical application outside a clinical trial. Tumori *97*, 737–742.

Currie, R.A. (1997). Functional interaction between the DNA binding subunit trimerization domain of NF-Y and the high mobility group protein HMG-I(Y). J. Biol. Chem. *272*, 30880–30888.

Dean, C.B., and Nielsen, J.D. (2007). Generalized linear mixed models: a review and some extensions. Lifetime Data Anal. *13*, 497–512.

Delov, V., Muth-Köhne, E., Schäfers, C., and Fenske, M. (2014). Transgenic fluorescent zebrafish Tg(fli1:EGFP)y^1 for the identification of vasotoxicity within the zFET. Aquat. Toxicol. Amst. Neth. *150*, 189–200.

DiMeo, T.A., Anderson, K., Phadke, P., Fan, C., Feng, C., Perou, C.M., Naber, S., and Kuperwasser, C. (2009). A novel lung metastasis signature links Wnt signaling with cancer cell self-renewal and epithelial-mesenchymal transition in basal-like breast cancer. Cancer Res. *69*, 5364–5373.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinforma. Oxf. Engl. *29*, 15–21.

Dvorak, H.F., Brown, L.F., Detmar, M., and Dvorak, A.M. (1995). Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am. J. Pathol. *146*, 1029–1039.

Fedele, M., and Fusco, A. (2010). HMGA and cancer. Biochim. Biophys. Acta *1799*, 48–54.

Fedele, M., Visone, R., De Martino, I., Troncone, G., Palmieri, D., Battista, S., Ciarmiello, A., Pallante, P., Arra, C., Melillo, R.M., et al. (2006a). HMGA2 induces pituitary tumorigenesis by enhancing E2F1 activity. Cancer Cell *9*, 459–471.

Fedele, M., Fidanza, V., Battista, S., Pentimalli, F., Klein-Szanto, A.J.P., Visone, R., De Martino, I., Curcio, A., Morisco, C., Del Vecchio, L., et al. (2006b). Haploinsufficiency of the Hmga1 gene causes cardiac hypertrophy and myelo-lymphoproliferative disorders in mice. Cancer Res. *66*, 2536–2543.

Federico, A., Forzati, F., Esposito, F., Arra, C., Palma, G., Barbieri, A., Palmieri, D., Fedele, M., Pierantoni, G.M., De Martino, I., et al. (2014). Hmga1/Hmga2 double knock-out mice display a “superpygmy” phenotype. Biol. Open *3*, 372–378.

Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. N. Engl. J. Med. *285*, 1182–1186.

Folkman, J. (2002). Role of angiogenesis in tumor growth and metastasis. Semin. Oncol. *29*, 15–18.

Folkman, J., and Hanahan, D. (1991). Switch to the angiogenic phenotype during tumorigenesis. Princess Takamatsu Symp. *22*, 339–347.

Foti, D., Iuliano, R., Chiefari, E., and Brunetti, A. (2003). A nucleoprotein complex containing Sp1, C/EBP beta, and HMGI-Y controls human insulin receptor gene transcription. Mol. Cell. Biol. *23*, 2720–2732.

Foulkes, W.D., Grainge, M.J., Rakha, E.A., Green, A.R., and Ellis, I.O. (2009). Tumor size is an unreliable predictor of prognosis in basal-like breast cancers and does not correlate closely with lymph node status. Breast Cancer Res. Treat. *117*, 199–204.

Foulkes, W.D., Smith, I.E., and Reis-Filho, J.S. (2010). Triple-negative breast cancer. N. Engl. J. Med. *363*, 1938–1948.

Fusco, A., and Fedele, M. (2007). Roles of HMGA proteins in cancer. Nat. Rev. Cancer *7*, 899–910.

Gannon, L.M., Cotter, M.B., and Quinn, C.M. (2013). The classification of invasive carcinoma of the breast. Expert Rev. Anticancer Ther. *13*, 941–954.

Gebeshuber, C.A., Sladecek, S., and Grunert, S. (2007). Beta-catenin/LEF-1 signalling in breast cancer--central players activated by a plethora of inputs. Cells Tissues Organs *185*, 51–60.

Giacca, M., and Zacchigna, S. (2012). VEGF gene therapy: therapeutic angiogenesis in the clinic and beyond. Gene Ther. *19*, 622–629.

Giancotti, V., Pani, B., D’Andrea, P., Berlingieri, M.T., Di Fiore, P.P., Fusco, A., Vecchio, G., Crane-Robinson, C., and Goodwin, G.H. (1987). Histone and nonhistone proteins from normal and virus-transformed rat thyroid epithelial cells. Basic Appl. Histochem. *31*, 229–238.

Goi, M., and Childs, S.J. (2016). Patterning mechanisms of the sub-intestinal venous plexus in zebrafish. Dev. Biol. *409*, 114–128.

Goodman, O.B., Fink, L.M., Symanowski, J.T., Wong, B., Grobaski, B., Pomerantz, D., Ma, Y., Ward, D.C., and Vogelzang, N.J. (2009). Circulating tumor cells in patients with castration-resistant prostate cancer baseline values and correlation with prognostic factors. Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol. *18*, 1904–1913.

Gould Rothberg, B.E., and Bracken, M.B. (2006). E-cadherin immunohistochemical expression as a prognostic factor in infiltrating ductal carcinoma of the breast: a systematic review and meta-analysis. Breast Cancer Res. Treat. *100*, 139–148.

Greenberg, S., and Rugo, H.S. (2010). Triple-negative breast cancer: role of antiangiogenic agents. Cancer J. Sudbury Mass *16*, 33–38.

Gu, G., Dustin, D., and Fuqua, S.A. (2016). Targeted therapy for breast cancer and molecular mechanisms of resistance to treatment. Curr. Opin. Pharmacol. *31*, 97–103.

Ha, T.-K., Her, N.-G., Lee, M.-G., Ryu, B.-K., Lee, J.-H., Han, J., Jeong, S.-I., Kang, M.-J., Kim, N.-H., Kim, H.-J., et al. (2012). Caveolin-1 increases aerobic glycolysis in colorectal cancers by stimulating HMGA1-mediated GLUT3 transcription. Cancer Res. *72*, 4097–4109.

Halasi, M., and Gartel, A.L. (2009). A novel mode of FoxM1 regulation: positive auto-regulatory loop. Cell Cycle Georget. Tex *8*, 1966–1967.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57–70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646–674.

Harbeck, N., Kates, R.E., Schmitt, M., Gauger, K., Kiechle, M., Janicke, F., Thomassen, C., Look, M.P., and Foekens, J.A. (2004). Urokinase-type plasminogen activator and its inhibitor type 1 predict disease outcome and therapy response in primary breast cancer. Clin. Breast Cancer *5*, 348–352.

Heitz, F., Harter, P., Lueck, H.-J., Fissler-Eckhoff, A., Lorenz-Salehi, F., Scheil-Bertram, S., Traut, A., and du Bois, A. (2009). Triple-negative and HER2-overexpressing breast cancers exhibit an elevated risk and an earlier occurrence of cerebral metastases. Eur. J. Cancer Oxf. Engl. 1990 *45*, 2792–2798.

Herbert, S.P., and Stainier, D.Y.R. (2011). Molecular control of endothelial cell behaviour during blood vessel morphogenesis. Nat. Rev. Mol. Cell Biol. *12*, 551–564.

Hicklin, D.J., and Ellis, L.M. (2005). Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. *23*, 1011–1027.

Hillion, J., Dhara, S., Sumter, T.F., Mukherjee, M., Di Cello, F., Belton, A., Turkson, J., Jaganathan, S., Cheng, L., Ye, Z., et al. (2008). The high-mobility group A1a/signal transducer and activator of transcription-3 axis: an achilles heel for hematopoietic malignancies? Cancer Res. *68*, 10121–10127.

Hu, Z., Fan, C., Livasy, C., He, X., Oh, D.S., Ewend, M.G., Carey, L.A., Subramanian, S., West, R., Ikpatt, F., et al. (2009). A compact VEGF signature associated with distant metastases and poor outcomes. BMC Med. *7*, 9.

Ivaska, J., Pallari, H.-M., Nevo, J., and Eriksson, J.E. (2007). Novel functions of vimentin in cell adhesion, migration, and signaling. Exp. Cell Res. *313*, 2050–2062.

Jain, R.K. (1990). Vascular and interstitial barriers to delivery of therapeutic agents in tumors. Cancer Metastasis Rev. *9*, 253–266.

Jaiswal, N., Chakraborty, S., and Nag, A. (2014). BIOLOGY OF FOXM1 AND ITS EMERGING ROLE IN CANCER THERAPY. J. Proteins Proteomics *5*.

Jung, D.-W., Oh, E.-S., Park, S.-H., Chang, Y.-T., Kim, C.-H., Choi, S.-Y., and Williams, D.R. (2012). A novel zebrafish human tumor xenograft model validated for anti-cancer drug screening. Mol. Biosyst. *8*, 1930–1939.

Kalin, T.V., Wang, I.-C., Ackerson, T.J., Major, M.L., Detrisac, C.J., Kalinichenko, V.V., Lyubimov, A., and Costa, R.H. (2006). Increased levels of the FoxM1 transcription factor accelerate development and progression of prostate carcinomas in both TRAMP and LADY transgenic mice. Cancer Res. *66*, 1712–1720.

Kalinichenko, V.V., and Kalin, T.V. (2015). Is there potential to target FOXM1 for “undruggable” lung cancers? Expert Opin. Ther. Targets *19*, 865–867.

Karadedou, C.T., Gomes, A.R., Chen, J., Petkovic, M., Ho, K.-K., Zwolinska, A.K., Feltes, A., Wong, S.Y., Chan, K.Y.K., Cheung, Y.-N., et al. (2012). FOXO3a represses VEGF expression through FOXM1-dependent and -independent mechanisms in breast cancer. Oncogene *31*, 1845–1858.

Kelleher, F.C., and O’Sullivan, H. (2016). FOXM1 in sarcoma: role in cell cycle, pluripotency genes and stem cell pathways. Oncotarget *7*, 42792–42804.

Kelly, T., Yan, Y., Osborne, R.L., Athota, A.B., Rozypal, T.L., Colclasure, J.C., and Chu, W.S. (1998). Proteolysis of extracellular matrix by invadopodia facilitates human breast cancer cell invasion and is mediated by matrix metalloproteinases. Clin. Exp. Metastasis *16*, 501–512.

Kerbel, R.S. (2008). Tumor angiogenesis. N. Engl. J. Med. *358*, 2039–2049.

Kim, I.-M., Ramakrishna, S., Gusarova, G.A., Yoder, H.M., Costa, R.H., and Kalinichenko, V.V. (2005). The forkhead box m1 transcription factor is essential for embryonic development of pulmonary vasculature. J. Biol. Chem. *280*, 22278–22286.

Kocdor, M.A., Kocdor, H., Pereira, J.S., Vanegas, J.E., Russo, I.H., and Russo, J. (2013). Progressive increase of glucose transporter-3 (GLUT-3) expression in estrogen-induced breast carcinogenesis. Clin. Transl. Oncol. Off. Publ. Fed. Span. Oncol. Soc. Natl. Cancer Inst. Mex. *15*, 55–64.

Koo, C.-Y., Muir, K.W., and Lam, E.W.-F. (2012). FOXM1: From cancer initiation to progression and treatment. Biochim. Biophys. Acta *1819*, 28–37.

Korver, W., Roose, J., and Clevers, H. (1997). The winged-helix transcription factor Trident is expressed in cycling cells. Nucleic Acids Res. *25*, 1715–1719.

Korver, W., Schilham, M.W., Moerer, P., van den Hoff, M.J., Dam, K., Lamers, W.H., Medema, R.H., and Clevers, H. (1998). Uncoupling of S phase and mitosis in cardiomyocytes and hepatocytes lacking the winged-helix transcription factor Trident. Curr. Biol. CB *8*, 1327–1330.

Kronenwett, U., Huwendiek, S., Ostring, C., Portwood, N., Roblick, U.J., Pawitan, Y., Alaiya, A., Sennerstam, R., Zetterberg, A., and Auer, G. (2004). Improved grading of breast adenocarcinomas based on genomic instability. Cancer Res. *64*, 904–909.

Kronenwett, U., Ploner, A., Zetterberg, A., Bergh, J., Hall, P., Auer, G., and Pawitan, Y. (2006). Genomic instability and prognosis in breast carcinomas. Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. Cancer Res. Cosponsored Am. Soc. Prev. Oncol. *15*, 1630–1635.

Kwok, J.M.-M., Peck, B., Monteiro, L.J., Schwenen, H.D.C., Millour, J., Coombes, R.C., Myatt, S.S., and Lam, E.W.-F. (2010). FOXM1 confers acquired cisplatin resistance in breast cancer cells. Mol. Cancer Res. MCR *8*, 24–34.

Lam, E.W.-F., Brosens, J.J., Gomes, A.R., and Koo, C.-Y. (2013). Forkhead box proteins: tuning forks for transcriptional harmony. Nat. Rev. Cancer *13*, 482–495.

Laoukili, J., Kooistra, M.R.H., Brás, A., Kauw, J., Kerkhoven, R.M., Morrison, A., Clevers, H., and Medema, R.H. (2005). FoxM1 is required for execution of the mitotic programme and chromosome stability. Nat. Cell Biol. *7*, 126–136.

Laoukili, J., Stahl, M., and Medema, R.H. (2007). FoxM1: at the crossroads of ageing and cancer. Biochim. Biophys. Acta *1775*, 92–102.

Lehtinen, L., Ketola, K., Mäkelä, R., Mpindi, J.-P., Viitala, M., Kallioniemi, O., and Iljin, K. (2013). High-throughput RNAi screening for novel modulators of vimentin expression identifies MTHFD2 as a regulator of breast cancer cell migration and invasion. Oncotarget *4*, 48–63.

Lim, E., Vaillant, F., Wu, D., Forrest, N.C., Pal, B., Hart, A.H., Asselin-Labat, M.-L., Gyorki, D.E., Ward, T., Partanen, A., et al. (2009). Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat. Med. *15*, 907–913.

Littler, D.R., Alvarez-Fernández, M., Stein, A., Hibbert, R.G., Heidebrecht, T., Aloy, P., Medema, R.H., and Perrakis, A. (2010). Structure of the FoxM1 DNA-recognition domain bound to a promoter sequence. Nucleic Acids Res. *38*, 4527–4538.

Loges, S., Mazzone, M., Hohensinner, P., and Carmeliet, P. (2009). Silencing or fueling metastasis with VEGF inhibitors: antiangiogenesis revisited. Cancer Cell *15*, 167–170.

Lund, T., Holtlund, J., Fredriksen, M., and Laland, S.G. (1983). On the presence of two new high mobility group-like proteins in HeLa S3 cells. FEBS Lett. *152*, 163–167.

Ma, R.Y.M., Tong, T.H.K., Cheung, A.M.S., Tsang, A.C.C., Leung, W.Y., and Yao, K.-M. (2005). Raf/MEK/MAPK signaling stimulates the nuclear translocation and transactivating activity of FOXM1c. J. Cell Sci. *118*, 795–806.

Macias, H., and Hinck, L. (2012). Mammary gland development. Wiley Interdiscip. Rev. Dev. Biol. *1*, 533–557.

Madureira, P.A., Varshochi, R., Constantinidou, D., Francis, R.E., Coombes, R.C., Yao, K.-M., and Lam, E.W.-F. (2006). The Forkhead box M1 protein regulates the transcription of the estrogen receptor alpha in breast cancer cells. J. Biol. Chem. *281*, 25167–25176.

Malhotra, G.K., Zhao, X., Band, H., and Band, V. (2010). Histological, molecular and functional subtypes of breast cancers. Cancer Biol. Ther. *10*, 955–960.

Manders, P., Beex, L.V. a. M., Tjan-Heijnen, V.C.G., Geurts-Moespot, J., Van Tienoven, T.H., Foekens, J.A., and Sweep, C.G.J. (2002). The prognostic value of vascular endothelial growth factor in 574 node-negative breast cancer patients who did not receive adjuvant systemic therapy. Br. J. Cancer *87*, 772–778.

Messineo, S., Laria, A.E., Arcidiacono, B., Chiefari, E., Luque Huertas, R.M., Foti, D.P., and Brunetti, A. (2016). Cooperation between HMGA1 and HIF-1 Contributes to Hypoxia-Induced VEGF and Visfatin Gene Expression in 3T3-L1 Adipocytes. Front. Endocrinol. *7*, 73.

Millour, J., de Olano, N., Horimoto, Y., Monteiro, L.J., Langer, J.K., Aligue, R., Hajji, N., and Lam, E.W.F. (2011). ATM and p53 regulate FOXM1 expression via E2F in breast cancer epirubicin treatment and resistance. Mol. Cancer Ther. *10*, 1046–1058.

Minn, A.J., Kang, Y., Serganova, I., Gupta, G.P., Giri, D.D., Doubrovin, M., Ponomarev, V., Gerald, W.L., Blasberg, R., and Massagué, J. (2005). Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. J. Clin. Invest. *115*, 44–55.

Moran, M.S. (2015). Radiation therapy in the locoregional treatment of triple-negative breast cancer. Lancet Oncol. *16*, e113-122.

Myatt, S.S., Kongsema, M., Man, C.W.-Y., Kelly, D.J., Gomes, A.R., Khongkow, P., Karunarathna, U., Zona, S., Langer, J.K., Dunsby, C.W., et al. (2014). SUMOylation inhibits FOXM1 activity and delays mitotic transition. Oncogene *33*, 4316–4329.

Nestal de Moraes, G., Delbue, D., Silva, K.L., Robaina, M.C., Khongkow, P., Gomes, A.R., Zona, S., Crocamo, S., Mencalha, A.L., Magalhães, L.M., et al. (2015). FOXM1 targets XIAP and Survivin to modulate breast cancer survival and chemoresistance. Cell. Signal. *27*, 2496–2505.

Nicholson, R.I., McClelland, R.A., Robertson, J.F., and Gee, J.M. (1999). Involvement of steroid hormone and growth factor cross-talk in endocrine response in breast cancer. Endocr. Relat. Cancer *6*, 373–387.

Oakes, S.R., Gallego-Ortega, D., and Ormandy, C.J. (2014). The mammary cellular hierarchy and breast cancer. Cell. Mol. Life Sci. CMLS *71*, 4301–4324.

Pagès, G., and Pouysségur, J. (2005). Transcriptional regulation of the Vascular Endothelial Growth Factor gene--a concert of activating factors. Cardiovasc. Res. *65*, 564–573.

Pang, B., Fan, H., Zhang, I.Y., Liu, B., Feng, B., Meng, L., Zhang, R., Sadeghi, S., Guo, H., and Pang, Q. (2012). HMGA1 expression in human gliomas and its correlation with tumor proliferation, invasion and angiogenesis. J. Neurooncol. *106*, 543–549.

Paonessa, F., Foti, D., Costa, V., Chiefari, E., Brunetti, G., Leone, F., Luciano, F., Wu, F., Lee, A.S., Gulletta, E., et al. (2006). Activator protein-2 overexpression accounts for increased insulin receptor expression in human breast cancer. Cancer Res. *66*, 5085–5093.

Park, H.J., Costa, R.H., Lau, L.F., Tyner, A.L., and Raychaudhuri, P. (2008). Anaphase-promoting complex/cyclosome-CDH1-mediated proteolysis of the forkhead box M1 transcription factor is critical for regulated entry into S phase. Mol. Cell. Biol. *28*, 5162–5171.

Park, H.J., Gusarova, G., Wang, Z., Carr, J.R., Li, J., Kim, K.-H., Qiu, J., Park, Y.-D., Williamson, P.R., Hay, N., et al. (2011). Deregulation of FoxM1b leads to tumour metastasis. EMBO Mol. Med. *3*, 21–34.

Patel, N.A., Patel, P.S., and Vora, H.H. (2015). Role of PRL-3, Snail, Cytokeratin and Vimentin expression in epithelial mesenchymal transition in breast carcinoma. Breast Dis. *35*, 113–127.

Pegoraro, S., Ros, G., Piazza, S., Sommaggio, R., Ciani, Y., Rosato, A., Sgarra, R., Del Sal, G., and Manfioletti, G. (2013). HMGA1 promotes metastatic processes in basal-like breast cancer regulating EMT and stemness. Oncotarget *4*, 1293–1308.

Pegoraro, S., Ros, G., Ciani, Y., Sgarra, R., Piazza, S., and Manfioletti, G. (2015). A novel HMGA1-CCNE2-YAP axis regulates breast cancer aggressiveness. Oncotarget *6*, 19087–19101.

Pierantoni, G.M., Agosti, V., Fedele, M., Bond, H., Caliendo, I., Chiappetta, G., Lo Coco, F., Pane, F., Turco, M.C., Morrone, G., et al. (2003). High-mobility group A1 proteins are overexpressed in human leukaemias. Biochem. J. *372*, 145–150.

Pilarsky, C., Wenzig, M., Specht, T., Saeger, H.D., and Grützmann, R. (2004). Identification and validation of commonly overexpressed genes in solid tumors by comparison of microarray data. Neoplasia N. Y. N *6*, 744–750.

Pore, N., Liu, S., Shu, H.-K., Li, B., Haas-Kogan, D., Stokoe, D., Milanini-Mongiat, J., Pages, G., O'Rourke, D.M., Bernhard, E., et al. (2004). Sp1 is involved in Akt-mediated induction of VEGF expression through an HIF-1-independent mechanism. Mol. Biol. Cell *15*, 4841–4853.

Prat, A., and Perou, C.M. (2009). Mammary development meets cancer genomics. Nat. Med. *15*, 842–844.

Rafii, S., Lyden, D., Benezra, R., Hattori, K., and Heissig, B. (2002). Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? Nat. Rev. Cancer *2*, 826–835.

Ram, T.G., Reeves, R., and Hosick, H.L. (1993). Elevated high mobility group-I(Y) gene expression is associated with progressive transformation of mouse mammary epithelial cells. Cancer Res. *53*, 2655–2660.

Reeves, R. (2001). Molecular biology of HMGA proteins: hubs of nuclear function. Gene *277*, 63–81.

Reeves, R., and Adair, J.E. (2005). Role of high mobility group (HMG) chromatin proteins in DNA repair. DNA Repair *4*, 926–938.

Reeves, R., and Wolffe, A.P. (1996). Substrate structure influences binding of the non-histone protein HMG-I(Y) to free nucleosomal DNA. Biochemistry (Mosc.) *35*, 5063–5074.

Reeves, R., Leonard, W.J., and Nissen, M.S. (2000). Binding of HMG-I(Y) imparts architectural specificity to a positioned nucleosome on the promoter of the human interleukin-2 receptor alpha gene. Mol. Cell. Biol. *20*, 4666–4679.

Resmini, G., Rizzo, S., Franchin, C., Zanin, R., Penzo, C., Pegoraro, S., Ciani, Y., Piazza, S., Arrigoni, G., Sgarra, R., et al. (2017). HMGA1 regulates the Plasminogen activation system in the secretome of breast cancer cells. Sci. Rep. *7*, 11768.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinforma. Oxf. Engl. *26*, 139–140.

Rydén, L., Jirström, K., Haglund, M., Stål, O., and Fernö, M. (2010). Epidermal growth factor receptor and vascular endothelial growth factor receptor 2 are specific biomarkers in triple-negative breast cancer. Results from a controlled randomized trial with long-term follow-up. Breast Cancer Res. Treat. *120*, 491–498.

Saba, R., Alsayed, A., Zacny, J.P., and Dudek, A.Z. (2016). The Role of Forkhead Box Protein M1 in Breast Cancer Progression and Resistance to Therapy. Int. J. Breast Cancer *2016*, 9768183.

Scholzen, T., and Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. J. Cell. Physiol. *182*, 311–322.

Scully, O.J., Bay, B.-H., Yip, G., and Yu, Y. (2012). Breast cancer metastasis. Cancer Genomics Proteomics *9*, 311–320.

Sgarra, R., Rustighi, A., Tessari, M.A., Di Bernardo, J., Altamura, S., Fusco, A., Manfioletti, G., and Giancotti, V. (2004). Nuclear phosphoproteins HMGA and their relationship with chromatin structure and cancer. FEBS Lett. *574*, 1–8.

Sgarra, R., Zammitti, S., Lo Sardo, A., Maurizio, E., Arnoldo, L., Pegoraro, S., Giancotti, V., and Manfioletti, G. (2010). HMGA molecular network: From transcriptional regulation to chromatin remodeling. Biochim. Biophys. Acta *1799*, 37–47.

Shah, S.N., Cope, L., Poh, W., Belton, A., Roy, S., Talbot, C.C., Sukumar, S., Huso, D.L., and Resar, L.M.S. (2013). HMGA1: a master regulator of tumor progression in triple-negative breast cancer cells. PloS One *8*, e63419.

Skibinski, A., and Kuperwasser, C. (2015). The origin of breast tumor heterogeneity. Oncogene *34*, 5309–5316.

Soker, S., Takashima, S., Miao, H.Q., Neufeld, G., and Klagsbrun, M. (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell *92*, 735–745.

Sørlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., et al. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc. Natl. Acad. Sci. U. S. A. *98*, 10869–10874.

Sternlicht, M.D. (2006). Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. Breast Cancer Res. BCR *8*, 201.

Sumter, T.F., Xian, L., Huso, T., Koo, M., Chang, Y.-T., Almasri, T.N., Chia, L., Inglis, C., Reid, D., and Resar, L.M.S. (2016). The High Mobility Group A1 (HMGA1) Transcriptome in Cancer and Development. Curr. Mol. Med. *16*, 353–393.

Tanaka, M., Watanabe, T., Tamaki, S., Ichihara, T., Yasushi, T., Abe, T., Masakazu, T., and Nakashima, N. (1993). Revascularization in fibromuscular dysplasia of the coronary arteries. Am. Heart J. *125*, 1167–1170.

Teh, M.-T., Wong, S.-T., Neill, G.W., Ghali, L.R., Philpott, M.P., and Quinn, A.G. (2002). FOXM1 is a downstream target of Gli1 in basal cell carcinomas. Cancer Res. *62*, 4773–4780.

Timms, J.F., White, S.L., O'Hare, M.J., and Waterfield, M.D. (2002). Effects of ErbB-2 overexpression on mitogenic signalling and cell cycle progression in human breast luminal epithelial cells. Oncogene *21*, 6573–6586.

Tobia, C., De Sena, G., and Presta, M. (2011). Zebrafish embryo, a tool to study tumor angiogenesis. Int. J. Dev. Biol. *55*, 505–509.

Tong, R.T., Boucher, Y., Kozin, S.V., Winkler, F., Hicklin, D.J., and Jain, R.K. (2004). Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res. *64*, 3731–3736.

Tonon, F., Zennaro, C., Dapas, B., Carraro, M., Mariotti, M., and Grassi, G. (2016). Rapid and cost-effective xenograft hepatocellular carcinoma model in Zebrafish for drug testing. Int. J. Pharm. *515*, 583–591.

Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., and Jemal, A. (2015). Global cancer statistics, 2012. CA. Cancer J. Clin. *65*, 87–108.

Ueda, Y., Watanabe, S., Tei, S., Saitoh, N., Kuratsu, J.-I., and Nakao, M. (2007). High mobility group protein HMGA1 inhibits retinoblastoma protein-mediated cellular G0 arrest. Cancer Sci. *98*, 1893–1901.

Vargo-Gogola, T., and Rosen, J.M. (2007). Modelling breast cancer: one size does not fit all. Nat. Rev. Cancer *7*, 659–672.

van 't Veer, L.J., Dai, H., van de Vijver, M.J., He, Y.D., Hart, A.A.M., Mao, M., Peterse, H.L., van der Kooy, K., Marton, M.J., Witteveen, A.T., et al. (2002). Gene expression profiling predicts clinical outcome of breast cancer. Nature *415*, 530–536.

Wang, I.-C., Chen, Y.-J., Hughes, D., Petrovic, V., Major, M.L., Park, H.J., Tan, Y., Ackerson, T., and Costa, R.H. (2005a). Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. Mol. Cell. Biol. *25*, 10875–10894.

Wang, I.-C., Chen, Y.-J., Hughes, D., Petrovic, V., Major, M.L., Park, H.J., Tan, Y., Ackerson, T., and Costa, R.H. (2005b). Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. Mol. Cell. Biol. *25*, 10875–10894.

Wang, X., Quail, E., Hung, N.J., Tan, Y., Ye, H., and Costa, R.H. (2001). Increased levels of forkhead box M1B transcription factor in transgenic mouse hepatocytes prevent age-related proliferation defects in regenerating liver. Proc. Natl. Acad. Sci. U. S. A. *98*, 11468–11473.

Wang, X., Kiyokawa, H., Dennewitz, M.B., and Costa, R.H. (2002). The Forkhead Box m1b transcription factor is essential for hepatocyte DNA replication and mitosis during mouse liver regeneration. Proc. Natl. Acad. Sci. U. S. A. *99*, 16881–16886.

Wang, Z., Banerjee, S., Kong, D., Li, Y., and Sarkar, F.H. (2007). Down-regulation of Forkhead Box M1 transcription factor leads to the inhibition of invasion and angiogenesis of pancreatic cancer cells. Cancer Res. *67*, 8293–8300.

Watanabe, M., Ni, S., Lindenberger, A.L., Cho, J., Tinch, S.L., and Kennedy, M.A. (2013). Characterization of the Stoichiometry of HMGA1/DNA Complexes. Open Biochem. J. *7*, 73–81.

Webber, C., Gospodarowicz, M., Sobin, L.H., Wittekind, C., Greene, F.L., Mason, M.D., Compton, C., Brierley, J., and Groome, P.A. (2014). Improving the TNM classification: findings from a 10-year continuous literature review. Int. J. Cancer *135*, 371–378.

Wei, D., Wang, L., He, Y., Xiong, H.Q., Abbruzzese, J.L., and Xie, K. (2004). Celecoxib inhibits vascular endothelial growth factor expression in and reduces angiogenesis and metastasis of human pancreatic cancer via suppression of Sp1 transcription factor activity. Cancer Res. *64*, 2030–2038.

Weidner, N., Folkman, J., Pozza, F., Bevilacqua, P., Allred, E.N., Moore, D.H., Meli, S., and Gasparini, G. (1992). Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J. Natl. Cancer Inst. *84*, 1875–1887.

Weigel, M.T., and Dowsett, M. (2010). Current and emerging biomarkers in breast cancer: prognosis and prediction. Endocr. Relat. Cancer *17*, R245-262.

Weigelt, B., Peterse, J.L., and van ’t Veer, L.J. (2005). Breast cancer metastasis: markers and models. Nat. Rev. Cancer *5*, 591–602.

Weigelt, B., Mackay, A., A’hern, R., Natrajan, R., Tan, D.S.P., Dowsett, M., Ashworth, A., and Reis-Filho, J.S. (2010). Breast cancer molecular profiling with single sample predictors: a retrospective analysis. Lancet Oncol. *11*, 339–349.

Weis, S.M., and Cheresh, D.A. (2011). Tumor angiogenesis: molecular pathways and therapeutic targets. Nat. Med. *17*, 1359–1370.

Wierstra, I., and Alves, J. (2007). FOXM1, a typical proliferation-associated transcription factor. Biol. Chem. *388*, 1257–1274.

Wonsey, D.R., and Follettie, M.T. (2005). Loss of the forkhead transcription factor FoxM1 causes centrosome amplification and mitotic catastrophe. Cancer Res. *65*, 5181–5189.

Xue, J., Lin, X., Chiu, W.-T., Chen, Y.-H., Yu, G., Liu, M., Feng, X.-H., Sawaya, R., Medema, R.H., Hung, M.-C., et al. (2014). Sustained activation of SMAD3/SMAD4 by FOXM1 promotes TGF-β-dependent cancer metastasis. J. Clin. Invest. *124*, 564–579.

Yang, C., Chen, H., Tan, G., Gao, W., Cheng, L., Jiang, X., Yu, L., and Tan, Y. (2013). FOXM1 promotes the epithelial to mesenchymal transition by stimulating the transcription of Slug in human breast cancer. Cancer Lett. *340*, 104–112.

Yao, K.M., Sha, M., Lu, Z., and Wong, G.G. (1997). Molecular analysis of a novel winged helix protein, WIN. Expression pattern, DNA binding property, and alternative splicing within the DNA binding domain. J. Biol. Chem. *272*, 19827–19836.

Ye, H., Kelly, T.F., Samadani, U., Lim, L., Rubio, S., Overdier, D.G., Roebuck, K.A., and Costa, R.H. (1997). Hepatocyte nuclear factor 3/fork head homolog 11 is expressed in proliferating epithelial and mesenchymal cells of embryonic and adult tissues. Mol. Cell. Biol. *17*, 1626–1641.

Yie, J., Merika, M., Munshi, N., Chen, G., and Thanos, D. (1999a). The role of HMG I(Y) in the assembly and function of the IFN-beta enhanceosome. EMBO J. *18*, 3074–3089.

Yie, J., Merika, M., Munshi, N., Chen, G., and Thanos, D. (1999b). The role of HMG I(Y) in the assembly and function of the IFN-beta enhanceosome. EMBO J. *18*, 3074–3089.

Zachary, I., and Gliki, G. (2001). Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovasc. Res. *49*, 568–581.

Zhang, Y., Zhang, N., Dai, B., Liu, M., Sawaya, R., Xie, K., and Huang, S. (2008). FoxM1B transcriptionally regulates vascular endothelial growth factor expression and promotes the angiogenesis and growth of glioma cells. Cancer Res. *68*, 8733–8742.

Zhao, Y., and Adjei, A.A. (2015). Targeting Angiogenesis in Cancer Therapy: Moving Beyond Vascular Endothelial Growth Factor. The Oncologist *20*, 660–673.

Zhao, K., Käs, E., Gonzalez, E., and Laemmli, U.K. (1993). SAR-dependent mobilization of histone H1 by HMG-I/Y in vitro: HMG-I/Y is enriched in H1-depleted chromatin. EMBO J. *12*, 3237–3247.

Publications

During my PhD, I contributed to the following work:

"HMGA1 regulates the Plasminogen activation system in the secretome of breast cancer cells"

Giulia Resmini, Serena Rizzo, Cinzia Franchin, Rossella Zanin, Carlotta Penzo, Silvia Pegoraro, Yari Ciani, Silvano Piazza, Giorgio Arrigoni, Riccardo Sgarra and Guidalberto Manfioletti, 2017 Scientific Reports

Cancer cells secrete proteins that modify the extracellular environment acting as autocrine and paracrine stimulatory factors and have a relevant role in cancer progression. The HMGA1 oncofetal protein has a prominent role in controlling the expression of an articulated set of genes involved in various aspect of cancer cell transformation. However, little is known about its role in influencing the secretome of cancer cells. Performing an iTRAQ LC–MS/MS screening for the identification of secreted proteins, in an inducible model of HMGA1 silencing in breast cancer cells, we found that HMGA1 has a profound impact on cancer cell secretome. We demonstrated that the pool of HMGA1–linked secreted proteins has pro–migratory and pro-invasive stimulatory roles. From an inspection of the HMGA1–dependent secreted factors it turned out that HMGA1 influences the presence in the extra cellular milieu of key components of the Plasminogen activation system (PLAU, SERPINE1, and PLAUR) that has a prominent role in promoting metastasis, and that HMGA1 has a direct role in regulating the transcription of two of them, i.e. PLAU and SERPINE1. The ability of HMGA1 to regulate the plasminogen activator system may constitute an important mechanism by which HMGA1 promotes cancer progression.