

FINAL MASTER'S PROJECT

Implication of Hakai-regulated proteins in cancer

Implicación de proteínas reguladas por Hakai en cáncer

Implicación das proteínas reguladas por Hakai en cancro

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1. ABSTRACT

1.1 Abstract

Most human malignant tumours are carcinomas, and the most common cause of mortality is due to metastasis. The E3 ubiquitin-ligase Hakai protein is responsible of the degradation of the E-cadherin at post-traslational level, and in consequence the activation of the epithelial-to-mesenchymal transition programme (EMT). In this study, I analysed the expression levels of the heat shock proteins Hsp70 and Hsp90, previously identified as Hakai-regulated proteins and notoriously related to cancer. I analysed the expression of these proteins in healthy colon tissues, adenoma and different TNM-tumour stages. Then, the expression levels of Hsp90 were analysed by transiently overexpressing Hakai in colon epithelial tumour cells. The Hsp90 levels increase in the transition from healthy and adenoma to carcinoma in human colon adenocarcinoma progression. Furthermore, by immunofluorescence it was detected an increase Hsp90 protein expression in Hakai-transfected LoVo cells.

1.2 Resumen

La mayoría de los tumores malignos humanos son carcinomas y la mayoría de las muertes por cáncer son debidas a las metástasis. La proteína E3 ubiquitin-ligasa Hakai es responsable de la degradación de E-cadherina a nivel postraducciona, y en consecuencia se activa el programa de transición epitelio-mesénquima (EMT). En este estudio, analicé los niveles de expresión de las proteínas de choque térmico Hsp70 y Hsp90, previamente identificadas como proteínas reguladas por Hakai y con un papel relevante en cáncer. Analicé la expresión de estas proteínas en tejidos de colon sanos, adenomas y diferentes estadios TNM de progresión tumoral. A continuación, los niveles de expresión de Hsp90 fueron analizados tras la sobreexpresión transitoria de Hakai en células epiteliales tumorales de colon. Mis resultados indican que los niveles de Hsp90 se incrementan en la transición de tejidos sanos y adenoma a carcinoma en la progresión tumoral de adenocarcinoma humano. Además, por inmunofluorescencia detecté un incremento en los niveles de expresión de proteína Hsp90 en las células LoVo transfectadas con Hakai.

1.3 Resumo

A maioría dos tumores malignos en humanos son carcinomas e a maioría das mortes por cancro son debidas á metástase. A proteína E3 ubiquitín-ligasa Hakai é responsable da degradación de E-cadherina a nivel postraduccional, e en consecuencia actívase o programa de transición epitelio-mesénquima (EMT). Neste estudo, analicei os niveis de expresión das proteínas de choque térmico Hsp70 e Hsp90, previamente identificadas como proteínas reguladas por Hakai e con un papel relevante no cancro. Analicei a expresión destas proteínas en tecidos de colon sans, adenomas e diferentes estadios TNM de progresión tumoral. A continuación, os niveis de expresión de Hsp90 foron analizados tra a sobreexpresión transitoria de Hakai en células epiteliais tumorais de colon. Os meus resultados indican que os niveis de Hsp90 incrementanse na transición de tecidos sanos e adenoma a carcinoma na progresión tumoral de adenocarcinoma humano. Ademais, por inmunofluorescencia detectei un incremento dos niveis de expresión da proteína Hsp90 nas células LoVo transfectadas con Hakai.

2. INTRODUCTION

Cancer is one of the main causes of morbidity and mortality worldwide. The phenomenon that gives rise to the formation and development of cancer is called carcinogenesis. Through carcinogenesis, healthy cells are transformed into cancer cells by genotypic and phenotypic changes. Carcinogenesis is characterized by a series of genetic and cellular modifications that lead the cell to divide in an uncontrolled way. Malignant tumour formation requires the conversion of a benign hyperplasia (condition that results in an increase of the number of cells) to a malignant state (Kufe *et al*, 2003). In most cases, genetic alterations involve activation of oncogenes and/or deactivation of tumour suppressor genes leading to uncontrolled cell cycle progression and inactivation of apoptotic mechanism (Sarkar *et al*, 2013). A malignant tumour, however, is capable of both invading surrounding normal tissue and spreading throughout the body via the circulatory or lymphatic systems, these features, respectively called, “invasion” and “metastasis” are manifestations of further genetic and epigenetic changes. Only malignant tumours are properly referred to as cancers, and are characterized by their ability to invade and metastasize in a distant organ (Cooper., 2000).

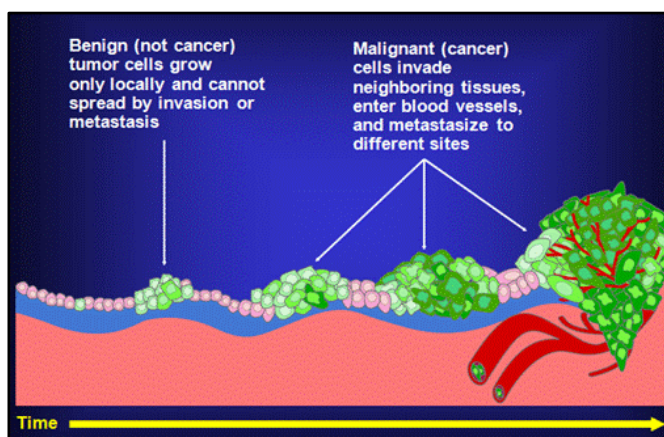


Figure 1. Schematic representation of the basic differences between a benign tumour and a malignant tumour (cancer). (Source: National Cancer Institute)

Most human malignant tumours are carcinomas and almost 90% of cancer-associated mortality is due to metastasis. Carcinomas arise from the epithelial cells transformation (Hanahan *et al*, 2000). The canonical mechanisms involved in generation of carcinomas are mutation, chromosomal translocation or deletion, and dysregulated expression or activity of signalling pathways. These events may activate genes that promote deregulated cell cycling and/or inactivate apoptotic pathways (Fearon *et al*, 1990; Vogelstein *et al*, 2004). The development and progression of cancer are part of a multistep process in which growth control is increasingly impaired. The presence of several genetic alterations in cancer cells strongly indicated that those alterations accumulate in the cells in a stepwise manner during tumour

progression (Yokota, 2000). Cancer is the outcome of an evolutionary process involving successive generations of cells, which are progressively further advanced towards cancerous growth (Yoshida *et al*, 2000).

During tumour progression continuously additional mutations occur within cells of the tumour population resulting in an increased amounts of particular proteins in the malignant cells. This process allows to tumour cells to evolved on the basis of its increased growth rate or other properties (survival, invasion and metastasis) that confer a selective advantage. This phenomenon, called “clonal selection”, continues throughout cancer development so tumours continuously become more rapid-growing and increasingly malignant (Nowell, 1986). Malignant carcinomas may arise from the benign adenomas, when tumour cells start to invade through the basal lamina into underlying connective tissue occurs. Then, cancer cells then continue to proliferate and spread through the connective tissues. Eventually the cancer cells penetrate the wall of neighbouring organs and invade blood and lymphatic vessels to finally metastasize throughout the body. Important number of studies have provided clear evidence that specific stages in malignant transformation can occur discretely (Stracke *et al*, 1992).

2.1 Epithelial-to-mesenchymal transition during tumour progression

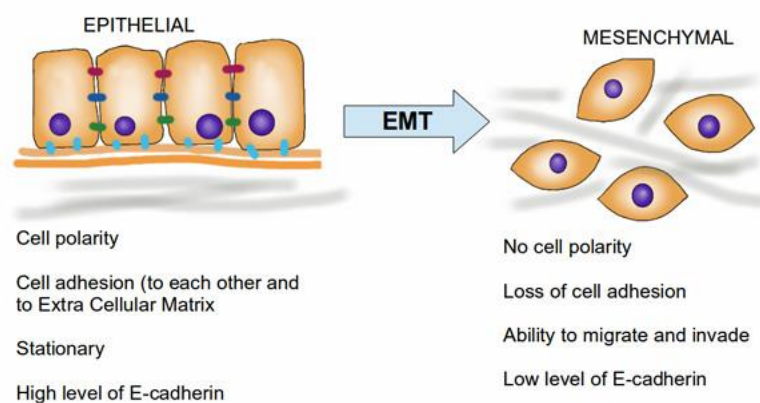
A feature that allows benign tumours to become malignant tumours is acquisition of the invasive characteristics. In fact, a benign tumour tends to expand by compressing the surrounding tissues while malignant tumours tend to expand by infiltrating them. The term “invasion” refers to the direct extension and penetration of cancer cells into neighbouring tissues. The infiltration process requires the active ability to penetrate through the tissues, with the degradation of the extracellular matrix and the disruption of the organ's architecture, including disruption of cell-cell contacts and cell-substratum adhesions (Brabletz *et al*, 2018).

Metastasis are malignant cells that break away from the original tumour and spread to distant organs where they can reproduce and generate new tumours. Metastatic process is defined as “dissemination of cancer cells from their origin to a distant organ” (Lugassy *et al*, 1997). Massive metastatic lesions lead to the development of severe organ failure and, therefore, a patient's death (Tracey *et al*, 2013). However, at the early stage of primary tumour progression, the cells are not invasive and metastatic. Then, new clones with invasiveness and metastatic capability appear as a result of further accumulation of genetic alterations in the cells (Yokota, 2000).

During the transition from adenoma to carcinoma, cells may acquire several characteristic phenotypes, including enhanced cell motility and invasion, and altered cell–cell and cell–substratum adhesions (Figuroa *et al*, 2009). Through a process named epithelial-to-mesenchymal transition (EMT), epithelial cells lose their cell–cell contacts, cell–substratum adhesions and apical–basal polarity, accompanied by the reorganization of the cytoskeleton and acquisition of a migratory or mesenchymal phenotype (Yang *et al*, 2008). A number of distinct molecular processes are engaged in order to initiate an EMT. These include activation of transcription factors, expression of specific cell-surface proteins, reorganization and expression of cytoskeletal proteins, production of extracellular matrix degradation enzymes, and changes in the expression of specific microRNAs (Weinberg *et al*, 2009). EMT is a highly controlled program that was firstly reported during embryogenesis (EMT type I), and also during wound healing and tissue repair (EMT type II). Apart from these two last mentioned physiological EMT, this program can also occur during pathological conditions such as tumour progression (EMT type III) (Aparicio *et al*, 2012).

The best characterized protein and hallmark of the EMT process is the loss of E-cadherin. E-cadherin (epithelial cadherin) is a crucial transmembrane protein important to maintain stable cell-cell contacts in epithelial cells. It is well known that the loss of E-cadherin protein is associated with the disruption of cell-cell contacts and can promote invasive and metastatic behaviour in many epithelial tumours (Birchmeier *et al*, 1994).

Figure 2. Epithelial mesenchymal transition (EMT) and basic features. During the EMT, cells lose cell polarity, cell adhesion and acquire migratory and invasion capabilities, accompanied by the loss of E-cadherin protein expression. (Source: *The Hallmarks of Cancer 6: Tissue Invasion and Metastasis*, Buddhini S.)



E-cadherin expression may be controlled at different levels of regulation. The first post-translational regulator reported of the E-cadherin protein stability is Hakai. Hakai is an E3 ubiquitin-ligase that binds to E-cadherin cytoplasmic domain in a phosphorylation-dependent manner and induce its internalization and subsequent degradation which in consequence it is produce the alteration of cell-cell contacts (Fujita *et al*, 2002).

2.1.1 Role of Hakai

In 2002, Fujita *et al* identified a protein interacting with tyrosine phosphorylated E-cadherin by a modified yeast 2-hybrid screen. This protein was named Hakai, “destruction” in Japanese (Fujita *et al*, 2002). Hakai protein is encoded by *CBLI1* gene and is composed by 491 aminoacids. It is an E3 ubiquitin-ligase that can bind to a tyrosine-phosphorylated E-cadherins to mediate the internalization and subsequent ubiquitin-dependent substrate degradation (Pece *et al*, 2002). In the ubiquitination pathway three types of enzyme are involved: the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2) and a variety of ubiquitin-ligases (E3). These E3 ubiquitin-ligases (E3) are responsible for the specificity of the interaction with the target protein to degrade. Tyrosine phosphorylation of the E-cadherin complex by v-Src kinase (phosphoprotein involved in the initiation and the maintenance of neoplastic transformation) is necessary for the binding of the SH2 domain of Hakai. The Src kinase family have a crucial role in the regulation of many biological functions associated with cellular morphology changes, cellular plasticity and modulation of the intercellular adhesion during EMT.

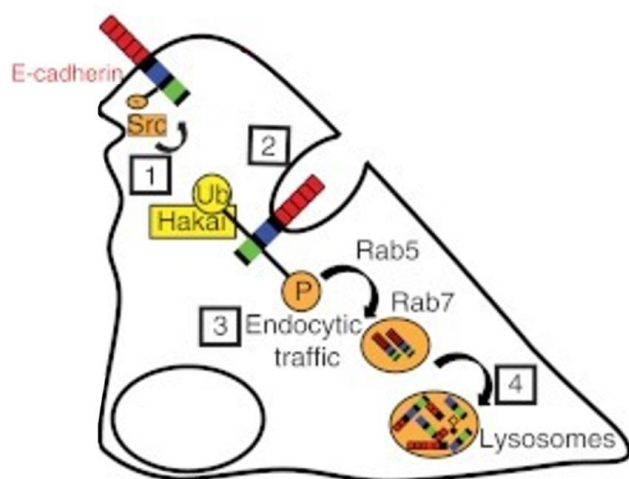


Figure 3. E-cadherin degradation by Hakai.

(1) After tyrosine phosphorylation by Src of the cytoplasmic domain of E-cadherin, (2) Hakai induces the ubiquitination of E-cadherin, (3) following endocytosis into vesicles through the action of Rab5 and Rab7 (GTPases involved in intracellular vesicle trafficking) (4) and finally degrade into lysosomes which in consequence it is altered the integrity of cell-cell contacts. (Modified image from source: Biological influence of Hakai in cancer: a 10-year review. Aparicio *et al*, 2012)

The tyrosine residues, by which the phosphorylation is mediated by Hakai, are only present in E-cadherin and not in N- or OB-cadherin, other members of cadherin family (Fujita *et al*, 2002). Therefore, it is clear the specific role of Hakai on E-cadherin proteins in epithelial cells. By northern blot analysis it is revealed that Hakai is ubiquitously expressed in tissues, included in tissues such as spleen and skeletal muscle, where E-cadherin is absent, suggesting that Hakai may have different substrate or different functions (Figuerola *et al*, 2009). The E3 ubiquitin-ligase Hakai possesses a pTyr-binding (HYB) domain consists of a homodimer where each monomer consists of two zinc-finger domains: a RING domain and a minimum pTyr-

binding domain that incorporates an atypical zinc coordination motif, on which both domain play key roles in the dimerization (Mukherjee *et al*, 2012). Therefore Hakai is active in a dimeric form, since its dimerization allows the formation of a phosphotyrosine-binding pocket that recognizes specific phosphorylated tyrosines and flanking acidic amino acids of Src substrates, such as E-cadherin, Cortactin or DOK1 (Mukherjee *et al*, 2012).

Our group has recently published how protein expression is altered during epithelial-to-mesenchymal transition in a Hakai-dependent manner (Diaz-Diaz *et al*, 2017). By proteomic analysis using a gel-free approach and employing isobaric tags for relative and absolute quantitation (iTRAQ) methodology, it was compared protein expression from non-transformed MDCK epithelial cells with protein profiles from Hakai-transformed MDCK cells, whereby the EMT program is induced. Some of the identified Hakai-regulated proteins using iTRAQ technique and validated independently by Western Blotting were Hsp90 and Hsp70, which are molecular chaperons. Molecular chaperones play essential roles in the folding of newly synthesized proteins and the refolding of denatured proteins (Bukau *et al*, 2006; Young *et al*, 2004).

2.1.2 Hsp90 and cancer

Hsp90, a 90-kDa heat shock protein, is a molecular chaperone that facilitates the stabilization and activation of around 350 “client proteins” (Taipale *et al*, 2012), abundant in the cytosol and exert distinct functions in the protein folding pathway (Nollen *et al*, 2002; Wegele *et al*, 2004). Although Hsp90 assists in the productive folding of proteins, this chaperone appears to have more specialized functions in the folding/assembly pathways (Csermely *et al*, 1998; Pratt and Toft, 2003; Wegele *et al*, 2004). For instance, members of the cyclin dependent kinases family (e.g. Cdk4, Cdk6) are stabilized by Hsp90 before forming functional complexes and in their final enzymatic forms (Vaughan *et al*, 2006). Furthermore, many Hsp90 client proteins are known to be associated with cancer phenotypes. Akt, Src, Raf1, Bcr-Abl, and ErbB2 are Hsp90-dependent protein kinases involved in signal transduction pathways required for abnormal cancer cell growth (Neckers *et al*, 2002). Hsp90 regulates the activity of the tumour suppressor protein p53 (Muller *et al*, 2004) and regulates the degradation of hypoxia-inducible factor 1, a transcription factor important for tumour cell survival under hypoxic conditions (Isaacs *et al*, 2002). These observations indicate that Hsp90 plays crucial roles in cancer cell growth and survival by chaperoning tumorigenic proteins. It has been proposed that given that Hsp90 client

proteins include a number of oncogenic proteins, using specific inhibitors targeting Hsp90 could be a promising anticancer strategy (Neckers *et al*, 2002).

Interestingly, Hsp90 is constitutively expressed at 2–10-fold higher levels in tumour cells than their normal counterparts (Ferrarini *et al*, 1992), and it appears to be required for malignant transformation (Workman, 2007). Neoplastic cells frequently overexpress Hsp90, and a number of oncogenic signalling proteins depend on Hsp90 function, including ErbB2, Src family kinases, Akt, Raf-1, mutated p53, and HIF-1 (Neckers, 2002). Increased expression of Hsp90 has been reported in a range of solid tumours originating from the oesophagus, lung, breast, and pancreas (Shirota *et al*, 2015). Hsp90 is present in multiprotein complexes that are predominant in cancer cells but not in normal cells. The presence of these Hsp90 complexes in tumour cells is consistent with a major role for Hsp90 as the cancer chaperone-buffering and stabilizing metastable and potentially damaging mutant proteins during the evolution and progression of malignancy. This is consistent with the idea that Hsp90 is absolutely required for malignant progression (Workman, 2004).

2.1.3 Hsp70 and cancer

Hsp70 is a member of highly conserved Hsp70 family of molecular chaperones, which are found in every membranous organelle of all cells (Bukau *et al*, 2006). Unlike Hsp90, there are no truly specific ‘client’ proteins for Hsp70. Instead, this protein binds to stretches of exposed hydrophobic residues on unfolded and misfolded proteins, holding them in an intermediately folded state and preventing their aggregation. Furthermore, different to Hsp90, Hsp70 can directly unfold misfolded proteins, in an adenosine triphosphate (ATP)-dependent fashion (Murphy, 2013). The accumulated data on Hsp70 strongly argue that this chaperone can play a crucial role in cancer initiation. There are extensive evidences that Hsp70 is overexpressed in cancer, and that high expression of this chaperone correlates with increased tumour grade and poor prognosis. For instance, Hsp70 overexpression is a marker of early hepatocellular and prostate cancer (Chuma *et al*, 2003; Abe *et al*, 2004).

It is generally held that elevated Hsp70 expression in transformed cells protects these cells from apoptosis, from the stress associated with aneuploidy, accumulation of mutated protein and from the proteotoxic stress associated with abnormally rapid proliferation (Murphy, 2013). Though Hsp70 is overexpressed in most human cancers, there are some tumour types where lower expression of Hsp70 is observed compared to adjusted normal tissues, and

correlation between and survival is lacking. Moreover, an inverse correlation between Hsp70 expression and patient's prognosis is observed in some cancers, such as squamous cell carcinoma, cholangiocarcinoma, oral, or lung cancer (Sherman *et al*, 2014).

2.2 Colon cancer progression

Colorectal cancer provides a good model for the study of morphological and genetic stages in cancer progression (Kinzler *et al*, 2002). Adenomatous polyp (or colon adenoma) represents a tumour of benign neoplastic epithelium with variable potential for malignancy and it is widely accepted that more than 95% of colorectal cancers arise from adenomatous polyps (Bujanda *et al*, 2010). A number of different staging criteria have been used to estimate the depth of cancer penetration in the colon as well as the extent of extra-colonic disease involvement. Nowadays, the most used staging method for colon cancer is based on the TNM (Tumour-Node-Metastasis) system as delineated by the American Joint Committee on Cancer (Compton *et al*, 2012).

In colorectal cancer, in stage 0 (carcinoma *in situ* stage), abnormal cells are found in the mucosa (innermost layer) of the bowel wall, these abnormal cells may become cancer and spread. At stage I the cancer cells are growing in the colon or rectum walls, histologically denotes a well-differentiated intestinal-type adenocarcinoma and well-formed glands with open lumina or with more than 95% glandular differentiation. It has not spread into nearby tissue or lymph nodes. In the stage II, cancer has grown through the wall of the colon or rectum, through the layers of the muscle to the lining of the abdomen, called the visceral peritoneum or through the wall of the colon or rectum and has grown into nearby structures. However, it has not spread to nearby tissue or to the nearby lymph nodes. Histologically adenocarcinoma is moderately differentiated intestinal-type with 50%-95% of glandular differentiation. At stage III cancer grows beyond the colon or rectum walls until to reach neighbouring tissues or lymph nodes. A poorly differentiated intestinal-type carcinoma can be seen through histologically analysis. Finally, in the stage IV cancer has spread to other parts of the body producing metastasis and it is seen an undifferentiated tumour which have less than 5% glandular differentiation (Bujanda *et al*, 2010).

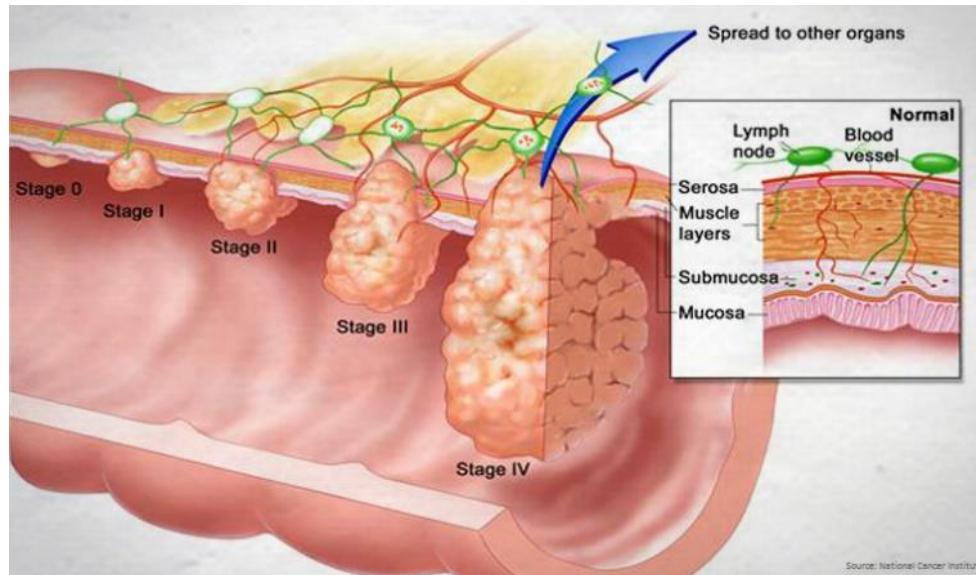


Figure 4. Schematic colon cancer staging. (Source: National Cancer Institute)

2.3 Inflammation

The malignant tumour microenvironment consists of tumour, immune, stromal, and inflammatory cells which produce cytokines, growth factors, and adhesion molecules that promote cancer progression and metastasis (Lewis *et al*, 2006). In 1863, for first time Virchow hypothesized that the origin of cancer was at sites of chronic inflammation, in part based on his hypothesis that some classes of irritants, together with the tissue injury and ensuing inflammation they cause, enhance cell proliferation (Balkwill *et al*, 2001). Several types of inflammation (differing by cause, mechanism, outcome, and intensity) can promote cancer development and progression, however, not all inflammatory response increase cancer risk (Grivennikov *et al*, 2010). Tumour-associated macrophages and their mediators affect key elements in the multistep process of invasion and metastasis, from interaction with the extracellular matrix to the construction of a premetastatic niche. Several evidences indicate that inflammatory mediators affect genetic stability and cause persistent epigenetic alterations suggesting that inflammatory components of the tumour microenvironment impacts on fundamental mechanisms responsible for the generation of metastatic variants (Mantovani *et al*, 2010).

It has been reported several cytokines and growth factors that are important in the tumour microenvironment to promote cancer progression such as IL-1 β , TGF β 1, CD68, CD163, COL1A1, CCL5 and CCL20. For instance, IL-1 β (Interleukin 1 beta) is a pleiotropic

cytokine with numerous roles in both physiological and pathological inflammatory states. It is known to be up regulated in many tumour types and it has been implicated as a factor in tumour progression via the expression of metastatic and angiogenic genes and growth factors (Lewis *et al*, 2006). On the other hand, in normal and premalignant cells, TGF β 1 (Transforming Growth Factor beta 1) enforces homeostasis and suppresses tumour progression directly through cell-autonomous tumour-suppressive effects (cytostasis, differentiation, apoptosis) or indirectly through effects on the stroma (suppression of inflammation and stroma-derived mitogens). However, when cancer cells lose TGF β 1 tumour-suppressive responses, they can use TGF β 1 to their advantage to initiate immune evasion, growth factor production, differentiation into an invasive phenotype, and metastatic dissemination or to establish and expand metastatic colonies (Massaguè, 2008). TNF (Tumor Necrosis Factor- α) also stimulates proliferation, survival, migration, and angiogenesis in most cancer cells that are resistant to TNF-induced cytotoxicity, resulting in tumour promotion. CD68 (Clusters of Differentiation 68) is a glycoprotein, and CD163 (Clusters of Differentiation 163) is a member of the scavenger receptor family that functions as innate immune sensor for bacteria, both are monocyte/macrophage markers. Finally, COL1A1 (Collagen type I alpha 1) expression appeared to be associated with metastasis, furthermore it was recently found to be associated to a variety of tumour types, and the expression of COL1A1 was high in tumour tissues and cells (Tian *et al*, 2015). CCL5 and CCL20 are two pro-inflammatory chemokine (small signalling protein) and their main function consists in the activation and recruitment of the leucocytes in the sites of inflammation.

3. MATERIALS AND METHODS

3.1 Immunohistochemistry

Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded sections of tumours. Sections were stained for Hsp90 using a polymer-based method (Envision + Dual Link System-HRP; Dako).

Sections were deparaffinized in xylene and rehydrated through a graded ethanol series (100%–70%) and antigen retrieval was performed by heating in a Target Retrieval Solution, citrate pH 6 (Dako) in a pressure cooker at 121°C for 15 min. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide solution (Dako) for 15 min and then washed with PBS/Tween-20 buffer (sodium chloride 0.15 M, potassium chloride 0.0027 M, Tween20 0.05%, sodium phosphate buffer 0.010 M). To minimize non-specific interactions of the antibody samples were blocked for 30 min at room temperature with 2% BSA and 1% TX-100. Then the samples were incubated overnight at 4 °C in a wet chamber with the primary antibody diluted in Dako antibody diluent (Dako). As the negative control slides were only incubated with the antibody diluent, in absence of the primary antibody. After washing in PBS/Tween-20 buffer for three times, the sections were incubated with the peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins in Tris-HCl buffer for 1 hour at room temperature, then washed in PBS/Tween-20 buffer three times. The samples were incubated with the DAB+ substrate-chromogen buffer solution (DakoReal Envision kit) for 5 min then washed for three times with distilled water. Finally, the tissue sections were counterstained with Gill's Hematoxylin, dehydrated through a graded ethanol series (70%–100%), then in xylol and mounted with DePeX (Serva).

Sixteen formalin-fixed and paraffin-embedded (FFPE) colon cancer tissues (4 µm) corresponding to the different stages of colon cancer progression (TNM I-IV, four per each stage), four adenomas and the corresponding adjacent normal colon tissues were used for immunohistochemistry with anti-Hsp90 antibody. Five pictures of each section were taken using an Olympus BX61 microscope and the images were quantified with ImageJ software (H-DAB settings).

Colon cancer biopsies were obtained from the Pathological Anatomy department from the Complejo Hospitalario Universitario A Coruña (CHUAC), under informed consent from all patients signed and research investigation was approved by the Research Ethics Committee

from A Coruña-Ferrol. Paraffin samples were provided by CHUAC Biobank integrated in the Spanish Hospital Platform Biobanks Network.

3.2 Antibodies

For immunochemistry the mouse monoclonal anti-Hsp90 antibody (ab13495) and anti-Hsp70 antibody (ab53496) were from Abcam.

For the western blot analysis the antibodies used, the mouse anti-GAPDH and rabbit anti-Hakai were obtained from Invitrogen™, anti-Hsp90 antibody was from Abcam. The secondary antibody used were Amersham ECL Mouse IgG HRP-linked whole antibody (from sheep) and Amersham ECL Rabbit IgG, HRP-linked whole antibody (from donkey), both from GE Healthcare Life Sciences.

Finally, for immunofluorescence analysis rabbit polyclonal anti-HSP90- α antibody (NB120-2928) from Novus Biologicals (Colorado, USA) and mouse monoclonal anti-GFP antibody [9F9.F9] (ab1218) from Abcam were used. The secondary antibodies were Labelled Donkey Anti-Rabbit IgG Antibodies from Invitrogen™ and Alexa Fluor® 488 Goat anti-mouse IgG from Abcam.

3.3 Statistical analysis

Immunohistochemistry quantification did not follow a normal distribution therefore was used Kruskal-Wallis with Tukey correction test. Significance of the Student t-test and Kruskal-Wallis with Tukey correction test among the experimental groups indicated in the figure X is shown as * $P < 0.05$ was made with GraphPad Prism software.

3.4 Cell lines

In this study, LoVo (ATCC® CCL-229™) cell line, colon rectal carcinoma cells derived from left supraclavicular region metastatic site, was used. Cell cultures were obtained from frozen cell lines in freezing medium containing 90% fetal bovine serum (-FBS-, from Life Technologies, USA) and 10% DMSO (Sigma-Aldrich, Germany), maintained in cryovials at -196 ° C.

The LoVo line was cultivated in F12 medium (ThermoFisher, USA) supplemented with 10% FBS, 1% penicillin (100 U/ml, Gibco) and streptomycin (100 µg/ml, Gibco).

LoVo cells were cultivated in 100 mm culture plates (Sigma-Aldrich, Germany) and the cultures were maintained at 37°C in a 5% atmosphere in CO₂ and 95% humidity, keeping passes each time they reached a confluence of between 80-90% of the total plate. Furthermore, Mycoplasma test was checked by PCR during culture routinely using Taq PCR kit (New England BioLabs, USA).

3.5 Transfection

LoVo cells were transfected with pcDNA-GFP-Hakai, using Lipofectamine[®] 2000 reagent (Invitrogen, USA), with a DNA (µg) to Lipofectamine 2000 (µl) ratio of 1:2, following the manufacturer instructions.

3.6 Western blot

3.6.1 Protein extraction from cells

Medium was removed from the confluent cell culture and washed twice with PBS 1X pH 7.6 (MPBiomedicals, USA). Cells of the plate were “scraped” using 1mL of PBS and cell suspension was collected in an Eppendorf tube, then centrifuged at 5000 rpm for 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in 40µL of TritonX-100 1X lysis buffer (20mM Tris-HCl pH 7.5, 150 mM NaCl and 1% of TritonX-100) supplemented with phenylmethylsulfonyl fluoride (PMSF) 1X and a mix of 1X protease inhibitors (Sigma-Aldrich, Germany). Protein extracts were incubated for 20 minutes at 4°C and then centrifuged at 12,000rpm for 10 minutes at 4°C in a refrigerated centrifuge (Microfuge[®] 22R Centrifuge, Beckman CoulterTM, USA). The supernatant obtained was recovered in a new Eppendorf tube and stored at -20°C.

3.6.2 Protein quantification by BCA method and samples preparation

To determine protein concentration of each sample the bicinchoninic acid assay (BCA assay), using PierceTM BCA protein assay kit (Thermo Scientific, USA) was used.

BCA method is a colorimetric assay based on the reduction of Cu⁺² to Cu⁺ in the presence of proteins, in a medium with high pH (alkaline). The Cu⁺ ions generated as a consequence of the

reduction of Cu^{+2} bind to the bicinchoninic acid, which acts as a chelant, and as a consequence a purple compound is formed. The union of the Cu^{+} ions to the BCA causes them to vary their electronic structure, being able to absorb light at 562 nm. Under the conditions of the reaction, the absorbance of the compound is proportional to the concentration of protein present. Proteins present in the solution were quantified by measuring the absorption spectra and comparing with BSA (bovine serum albumin) curve, on which the concentration dilution is known. Nanoquant Infinite M200 (Tecan, Switzerland) was used to measure absorbance.

Twenty μg of protein from each sample were loaded and 2 μl of loading buffer 5X (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0,004% bromophenol blue, 0.125 M Tris-HCl) was added. Finally, samples were incubated at 95° for 10 min (to denature proteins) in a thermoblock (ThermoFisher, UniEquip, Germany) and stored at -20°C until its later use.

3.6.3 Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

The resolving gel was prepared at 10% acrylamide. As follows: Tris/HCl 1,5M (pH 8.8) (Trizma Base, Sigma-Aldrich, Germany), 10% glycerol (Sigma-Aldrich, USA), 10% acrylamide/bis-acrylamide (NZYTech, Portugal), 0,1% SDS (Sigma-Aldrich, USA), 0,15% TEMED (NZYTech, Portugal) and 0,03% APS (Sigma-Aldrich, Germany).

The stacking gel was prepared at 4% acrylamide. As follows: Tris/HCl 0,5M (pH 6.8) (Trizma Base, Sigma-Aldrich, Germany), 40% acrylamide/ bis-acrylamide (NZYTech, Portugal), 0,1% SDS (Sigma-Aldrich, USA), 0,1% TEMED (NZYTech, Portugal) and 0,1% APS (Sigma-Aldrich, Germany).

The solutions were introduced in a vertical electrophoresis apparatus (Criterion™ Vertical Electrophoresis Cell, BioRad, USA) and the running conditions used were 80V for 20 minutes and 200V for 1 hour, at room temperature. Samples were loaded and the molecular weight marker used was *NZYColour Protein Marker I* (Nzytech, Portugal).

3.6.4 Electroblothing

To perform the electroblothing (protein transfer onto a membrane) PVDF (polyvinylidene difluoride) membrane (Millipore Immobion-P, USA) was used. Firstly, PVDF membrane was activated with methanol for 15 seconds and moistened in water. The assembly of the transfer was carried out following the instructions of the Criterion™ blotter (BioRad, USA). The device

is designed to perform the transfer in humidity and 1X transfer buffer (Trizma base 0.025 mM, glycine 0.192 M and 20% methanol in water) was used. The conditions of the transfer were: 200mA for 1 hour, on ice.

3.6.5 Immunodetection

The membranes were incubated for 1 hour at room temperature in a blocking buffer: TBST 1X (10% Tris buffered saline 10X + 0.05% Tween 20 in water) + 5% Skim Milk Powder (Sigma-Aldrich, Germany). After blocking, the membrane was incubated at 4°C overnight and in agitation with the primary antibody (to detect a specific protein), diluted in the blocking buffer. Antibody dilution used for primary antibody was 1/1000, except for GAPDH which was used at a dilution 1/10000. After incubation with the primary antibody, membranes were washed three times in TBST 1X for 10 minute in agitation. Then the membrane was incubated at room temperature for 1 hour with the secondary specific antibody diluted in blocking buffer (1/2000 dilution). The secondary antibodies are conjugated with horseradish peroxidase (HRP), which allows its subsequent detection by a chemiluminescence reaction. Finally, by adding Amersham ECLTM Western Blotting Analysis System (GE Healthcare Life Sciences, UK), a specific compound which increases the intensity of the light emitted in the chemiluminescence, the protein was detected in the Amersham Imager 600 (GE Healthcare Life Sciences, UK).

3.7 Immunofluorescence

For the immunofluorescence, LoVo cells were seeded on sterile glass coverslips previously placed on wells (Corning® CellBIND® Multiple Well Plate). After 24 hours of incubation at 37 °C, in an atmosphere of 5% CO₂ and 95% humidity, the cells were washed twice with PBS (1x PBS 0.1% Tween 20 pH 7.4), and then fixed in a 4% solution of paraformaldehyde (PFA) in PBS for 15 minutes at room temperature. After fixation, the PFA was removed and washed with PBS three times, and then cells were permeabilized using Triton X-100 / 0.25% PBS for 15 minutes at room temperature, to allow the access of the antibody into the cell. Once removed the Triton X-100 / 0.25% PBS, the cells were blocked incubating with F12/10% FBS for 1 hour, to reduce unspecific binding of the antibodies. Then, the samples were incubated for 2 hours at room temperature with the primary antibody (GFP-antibody, 1:500 dilution; Hsp90-antibody, 1:200 dilution), diluted in block buffer (F12/10% FBS). After washing with PBS twice, the cells were incubated for 1 hour at room temperature and in the dark with the specific secondary

antibody, diluted in block buffer (F12/10% FBS). The samples were washed 3 times with PBS, then incubated for 5 min, in the dark, at room temperature with the Hoechst 33342 (Life Technologies, USA) in PBS (1/2000 dilution) to stain the nuclei. After washing with PBS, a final wash with distilled water was performed to remove excess of salts. Finally, the crystals were mounted on clean, pre-labelled slides using ProLong[®] Gold Antifade Mountant (Thermo Fisher Scientific, USA) and store in the dark at 4°C.

The immunofluorescence visualization was performed by Motorized reflected Fluorescence System (Olympus, Japan).

3.8 RNA extraction from formalin-fixed and paraffin-embedded (FFPE) tissues and qRT-PCR

3.8.1 Deparaffinization of FFPE tissue and total RNA purification

For inflammatory mRNA levels study, sections of formalin-fixed and paraffin-embedded tissues (4 µm) obtained from xenografts previously injected subcutaneously into the flank with five million MDCK cells or Hakai transformed MDCK cells were used.

In order to remove paraffin, FFPE tissue sections were put in a 1,5mL microcentrifuge tube, on which 1mL of 100% xylene was added. After vortexing and centrifuging (13,400 rpm/min for 2 min), xylene was removed and added 1mL of ethanol (96-100%) to the pellet, and again vortexing and centrifuging (13,400 rpm/min for 2 min). Ethanol was removed and the precipitate tissues were were dry up at room temperature for 10 min (to remove any residue of ethanol). Samples were resuspended into 240µl of Buffer PKD (proteinase K digest buffer, QIAGEN) and 10µl of proteinase K, mixed by vortexed. Samples were incubated at 56° for 15 min, then at 80° for 15 min.

For total RNA purification, samples were incubated on ice for 3 min, centrifuged for 15 min at 13,400 rpm/min, and the supernatants were transferred to a new microcentrifuge tube. Samples were incubated with 25µl of DNase Booster Buffer (QIAGEN) and 10µl of DNase I for 15 min at room temperature, and then 500µl of RBC Lysis Solution (QIAGEN) and 1200µl of ethanol (100%) were added and mixed by pipetting. The samples were transferred to RNeasy MinElute spin column (QIAGEN) and centrifugated for 15 sec at 10,000 rpm/min. Two washing steps were performed adding 500µl of Buffer RPE to the RNeasy Min Elute spin column, and centrifuged for 15 sec and 2 min, respectively. The RNeasy Min Elute spin column

were centrifuged at 13,400 rpm/min for 5 min to eliminate the residual ethanol. Finally, the RNA was eluted by add 30µl RNase-free water directly to the spin column following by centrifugation at 13,400 rpm/min for 1 min.

3.8.2 Nucleic acid quantification

NanoDrop 1000 Spectrophotometer (Termo Fisher Scientific, MA, USA) was used to determine the quantity and quality of RNA samples.

3.8.3 Real-time quantitative PCR (qRT-PCR)

Total RNA extracted was analysed by real-time quantitative PCR (qRT-PCR). The first strand cDNA synthesis was performed using NZY First-Strand cDNA Synthesis Kit (NZYTech). Different amounts of total RNA were added to reach the quantity of 300ng for sample. Then, DEPC-treated water was added up to 20µl total. Samples were mixed and incubate at 25°C for 10 min and next at 50° for 30 min. In order to inactivate the reaction by heating, samples were incubated at 85° for 5 min, and then chilled on ice. Finally, was added 1µl of NZY RNase H (*E. coli*) and incubate at 37°C for 20 min.

cDNA amplification was performed in a Light Cycler 480 II using following master mix: 5µl of LightCycler 480 SYBR Green I Master, 0,7µl forward primer, 0,7µl reverse primer, 2,6µl of distilled water, 1µl of cDNA. Data was analysed by using the comparative CT method (Lightcycler400 software). For primers designing, a Primer3 Input (version 0.4.0) software was used. (Primer Sequences are indicated in table 1).

Table 1. Primers sequences used for inflammatory mRNA analysis

mRNA of interest	Forward sequence	Reverse sequence
GAPDH	5'-GTGCTGAGTATGTCGTGGAGTCTAC-3'	5'-GGCGGAGATGATGACCCTTTTGG-3'
RN18S	5'-CGATGCGGGCGGCGTTATTC-3'	5'-ATCTGTCAATCCTGTCCGTGTCC-3'
CD163	5'-CCTCCTCATTGTCTTCCTCCTGTG-3'	5'-CATCCGCCTTTGAATCCATCTCTTG-3'
CD68	5'-TCCAAGATCCTCCACTGTTG-3'	5'-ATTTGAATTTGGGCTTGGAG-3'
TNF- α	5'-ATGAGAAGTTCCCAAATGGC-3'	5'-CTCCACTGGTGGTTTGCTA-3'
TGF- β 1	5'-CCCTATATTTGGAGCCTGGA-3'	5'-CTTGCGACCCACGTAGTAGA-3'
IL-6	5'-CTCTGGGAAATCGTGAAAT-3'	5'-CCAGTTTGGTAGCATCCATC-3'
IL-1 β	5'-CACAGCAGCACATCAACAAG-3'	5'-GTGCTCATGTCCTCATCCTG-3'
CCL5	5'-AGATCTCTGCAGCTGCCCTCA-3'	5'-GGAGCACTTGCTGCTGGGTAG-3'
COL1A1	5'-GCTGAGATGGCAACAGGGCTA-3'	5'-TTGATGGACGGGAATGAACA-3'
CCL20	5'-CGACTGTTGCCTCTCGTACA-3'	5'-GAGGAGGTTACAGCCCTT-3'

4. RESEARCH OBJECTIVES

The main research objectives established for this work are the following:

1. To evaluate the expression of heat shock protein 90 and heat shock protein 70 in healthy tissues, adenoma and different tumour stages.
2. To determine whether the Hsp90 protein levels is regulated by the E3 ubiquitin-ligase Hakai overexpression in LoVo cells by western-blotting.
3. To determine whether the Hsp90 protein levels are regulated by the E3 ubiquitin-ligase Hakai overexpression in LoVo cells by immunofluorescence.

4.1 Secondary objective

1. To analyse the mRNA levels of some specific inflammatory factors in sections of formalin-fixed and paraffin-embedded tissues obtained from xenografts injected subcutaneously with non-transformed MDCK cells or Hakai transformed MDCK cells.

5. RESULTS

5.1 Analysis of Hsp90 protein expression in different colon cancer TNM stages by immunohistochemistry

The Dra. Figueroa research group, in which I have been doing the TFM internship, has been working for years on the study of colorectal cancer and in particular focusing on role that E3-ubiquitin ligase Hakai in the development of cancer. Our group has recently published a proteomic analysis comparing the protein expression in Hakai overexpressing normal epithelial MDCK cells at compared to normal MDCK cells (Figueroa *et al*, 2017). Among the found differentially expressed proteins, one of the most up-regulated proteins was Heat shock protein 70 and Heat shock protein 90 (Hsp70) (Hsp90). The expression of Hsp90 has been reported in various types of cancer.

In order to analyse whether Hsp90 is differentially expressed in different colon cancer stages, we performed an immunohistochemistry. A total of 30 human colon samples were analysed including: different TNM stages (I-IV) from colon adenocarcinomas, colon adenoma compared to human colon healthy tissues (*figure 5*). In particular, adenoma and healthy colon tissues appear to present low levels of Hsp90 expression (*figure 5a and 5b, respectively*). On the contrary, the tissues belonging to different tumour stages (I-IV) present higher expression (black arrows in *figure 5 c-f*).

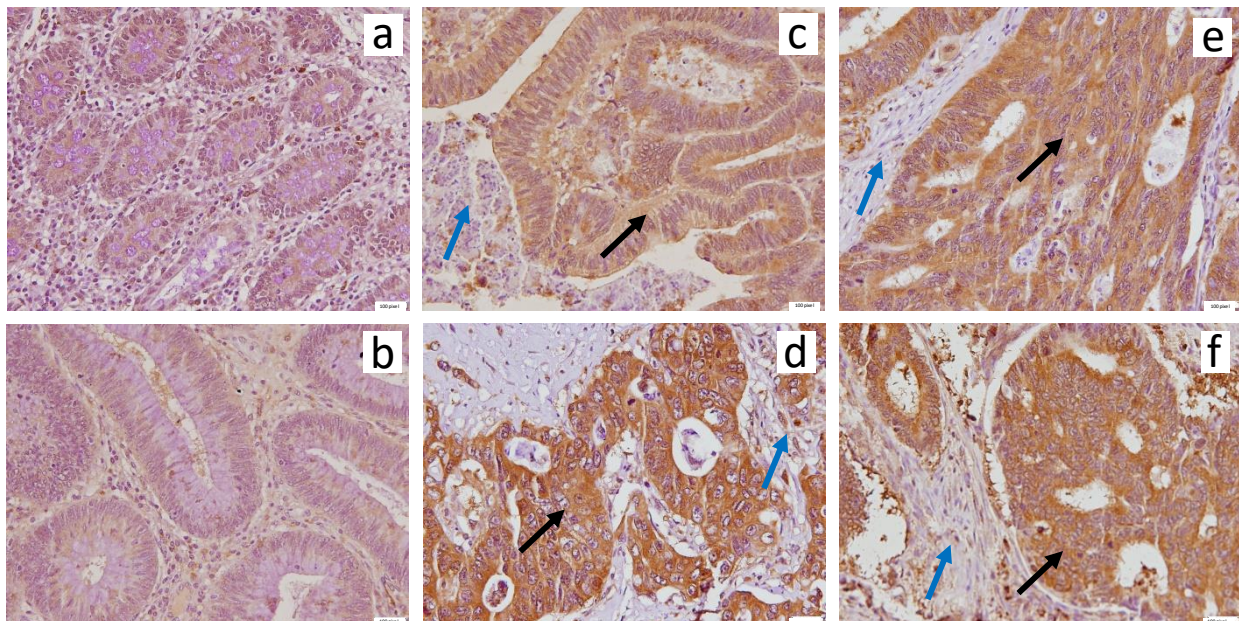


Figure 5. Immunohistochemical analysis of Hsp90 protein expression in human samples from colon cancer patients. (a) Normal healthy colon tissue (b) adenoma (c) colon cancer stages I, (d) colon cancer stages II (e) colon cancer stages III and (f) colon cancer stages IV. Non-staining connective tissue (blue arrows) denotes the specificity of immunoreaction. Olympus BX61 microscope (Magnification of 200X).

The fact that the brown colour is not present on the cells belonging to the connective tissue on the side of the tumour area indicated the specificity staining in epithelial colon tissue (blue arrows in *figure 5: c-d-e-f*). In order to determine whether the different expression levels observed between samples were statistically significant, quantification of the signal intensity of Hsp90 in the immunohistochemistry staining was performed using ImageJ software (*figure 6*). As seen in figure 6, an increase Hsp90 protein expression was detected from carcinoma compared to healthy and in the adenoma stage tissues. Moreover, it is important to note that no differences were detected between different TNM-stages (I-IV).

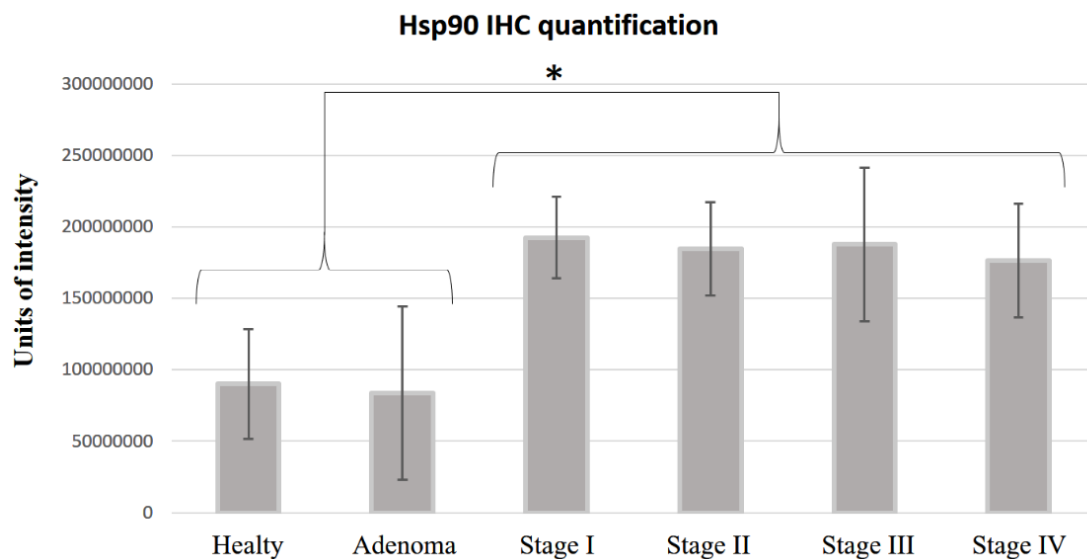


Figure 6. Immunohistochemical quantification of Hsp90. Five pictures of each section (four different patient collections for every healthy/tumor grade tissue) were taken with an Olympus BX61 microscope and the images were quantified with Image J software (H-DAB settings) (*P<0.05).

5.2 Immunohistochemistry of Hsp70 in different tumour stages

Next, we decided to investigate the protein expression of another member of the Heat Shock Protein, the Hsp70. Once again, I have analysed different human tissue samples using biopsies corresponding to adenoma, TNM-stages from adenocarcinoma compared to adjacent colon healthy tissue. The images staining show that there are no differences between protein level of expression of Hsp70 by comparing healthy tissue with adenoma and different stages of cancer from 1 to 4 (*figure 7*)

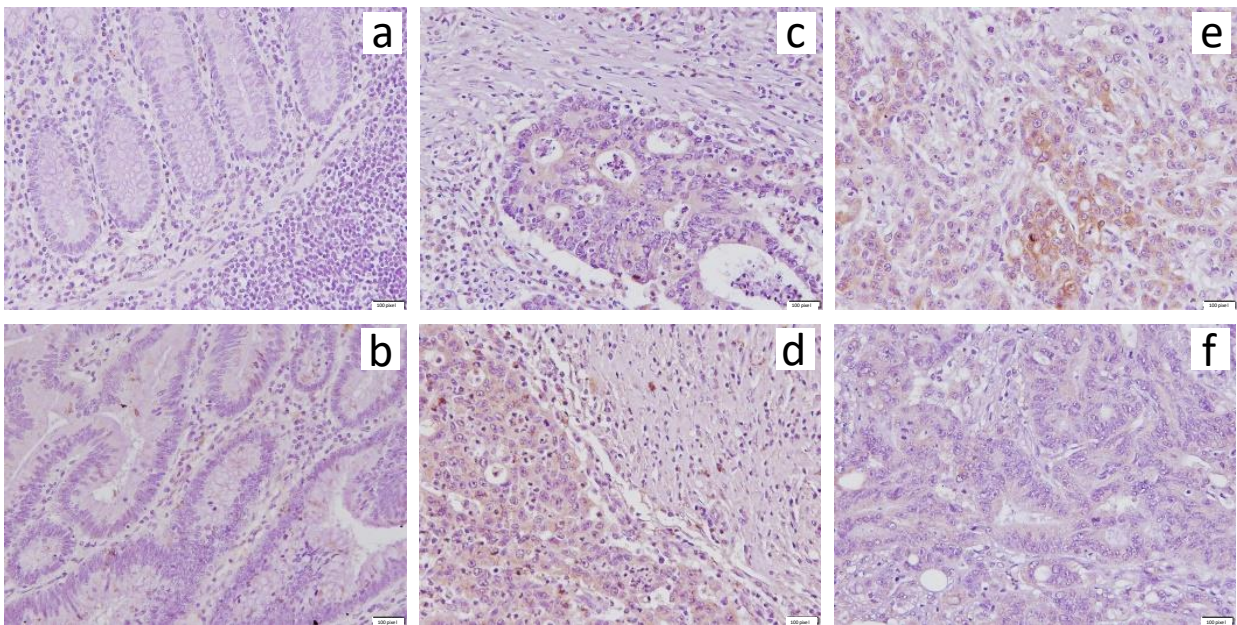


Figure 7. Immunohistochemical analysis of Hsp70 protein expression in human samples from colon cancer patients. (a) Normal healthy colon tissue (b) adenoma (c) colon cancer stages I, (d) colon cancer stages II (e) colon cancer stages III and (f) colon cancer stages IV. Olympus BX61 microscope (Magnification of 200X).

5.3 Western blotting analysis of Hsp90 in Hakai-transfected LoVo cells

Given the interesting results obtained in the differential expression of Hsp90 protein expression in human colon adenocarcinoma, we decided to determine, the possible mechanism of action of Hakai on Hsp90 protein. For this purpose, we decided to transfect GFP-Hakai in LoVo colon cancer epithelial cells. Once we confirmed by western blot analysis that the overexpression of Hakai was detected (*figure 8*), I analysed the effect of Hsp90 protein expression to determine whether the protein regulation had occurred. As shown in figure 8, Hakai shows two different bands and molecular weights in correlation with endogenous Hakai (*figure 8*, lower band) and the correctly transfected GFP-Hakai (*figure 8*, upper band). However, no difference was

detected in Hsp90 protein levels in presence or absence of Hakai overexpression in LoVo transiently transfected cells, using GAPDH as loading control.

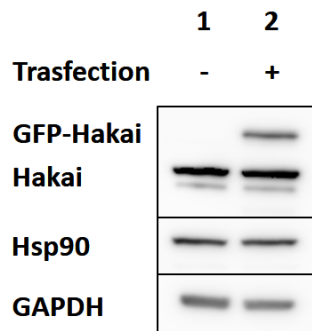


Figure 8. Western blot of Hakai-transfected LoVo cells. LoVo cell lysate (Lane 1) and GFP-Hakai-transfected LoVo cell lysate (lane 2). GFP-Hakai band is only visible in the transfected LoVo cells (lane 2). No difference is detected in Hsp90 bands comparing normal LoVo cell lysate with Hakai-transfected LoVo cell lysate (lane 1 and 2, respectively).

5.4 Immunofluorescence analysis of Hsp90 in Hakai-transfected LoVo cells

Since the low transfection efficiency with the GFP-Hakai vector could affect the result seen in the western blotting, I evaluated whether the negative western blot result was due to a low population of transfected cells. For this purpose, I performed an immunofluorescence staining on GFP-Hakai plasmid transfected LoVo cells, using as negative control an empty GFP plasmid. As seen in figure 9, an increase in Hsp90 expression in correctly GFP-Hakai plasmid transfected cells (*figure 9*, orange arrow). On the contrary, the negative control, containing the GFP plasmid, does not show the same increase in fluorescence (*figure 9*, white arrows).

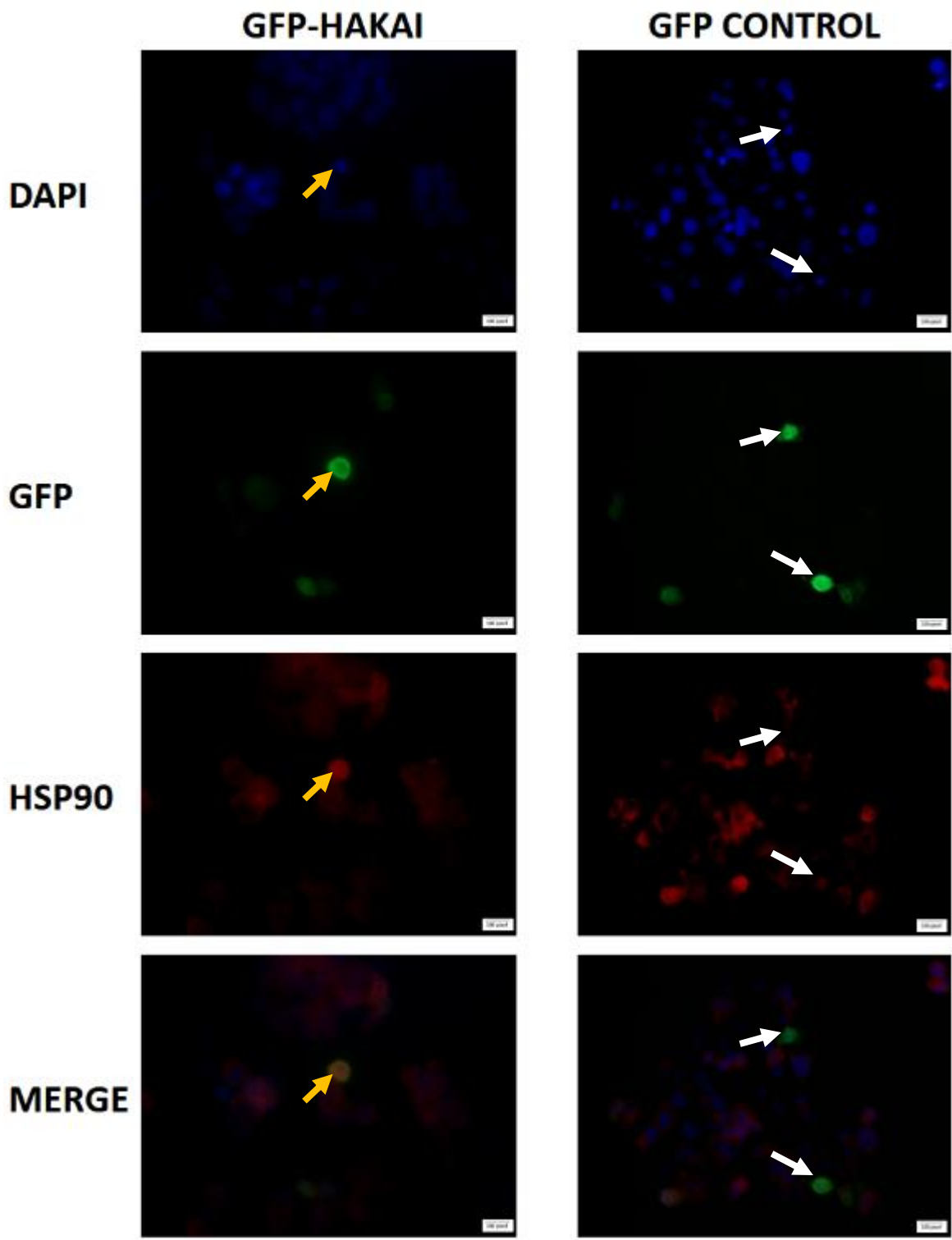


Figure 9. Effect of GFP-Hakai overexpression on Hsp90 in LoVo cells by immunofluorescence. GFP-Hakai (left panel) and GFP empty plasmid (right panel) transfected in LoVo cells. GFP-Hakai transfected cells (yellow arrow) show an increased fluorescence of Hsp90. Olympus BX61 microscope (total magnification of 200X).

5.5 Comparative study of inflammatory mRNA levels from xenografts injected subcutaneously with non-transformed MDCK cells or Hakai transformed MDCK cells

In the recently published proteomic analysis mentioned, while comparing the protein expression in Hakai overexpressing normal epithelial MDCK cells and to normal MDCK cells (Diaz-Diaz *et al*, 2017), we have also found an important number of proteins involved in inflammation process. Therefore, we decided to also analysed, as secondary objective in this project, whether we could find differences in inflammatory mRNA levels in vivo. For this purpose, we used an established mouse model in our lab, using tissue samples obtained from xenografts injected subcutaneously with non-transformed MDCK cells or Hakai transformed MDCK cells. As previously reported, non-transformed MDCK cells injected in immunocompetent mice formed teratomas while injected Hakai transformed MDCK cells formed aggressive tumours (Castosa *et al*, 2018). By using teratoma and tumours from these mice model, we analysed by qRT-PCR the expression levels of different inflammatory mRNAs. The results obtained from the study of the different inflammatory mRNAs indicate that the "teratoma" samples (obtained from teratoma tissues formed in MDCK-injected mice) present a level mRNA expression higher than the "tumour" samples (obtained from tumour tissues formed in Hakai-MDCK-injected mice) in all different inflammatory mRNA that I analysed. Indeed, comparing mRNA levels between tumour and teratoma samples, it was found that CD163 shows a level of 25-fold change expression in teratoma compared to tumours (*figure 10, a*). CD68 shows 10-fold expression higher in teratoma compared to tumours (*figure 10, b*), CCL20 and about 5-fold change in teratoma (*figure 10, c*). Interestingly, TGF- β , COL1A1, and CCL5 show a mRNA level higher in teratoma compared to cancer, around six thousand, forty thousand, and seventy thousand fold increase respectively (*figure 10, d-e-f*). Finally, IL- β and TNF- α show low expression levels in teratoma samples and it is almost absent in the carcinogenic samples (*figure 10, g-h*). All these results are not conclusive and further replicates in xenograft mouse model are necessary in order to conclude whether a differential expression of the inflammatory mRNA are regulated under Hakai influence.

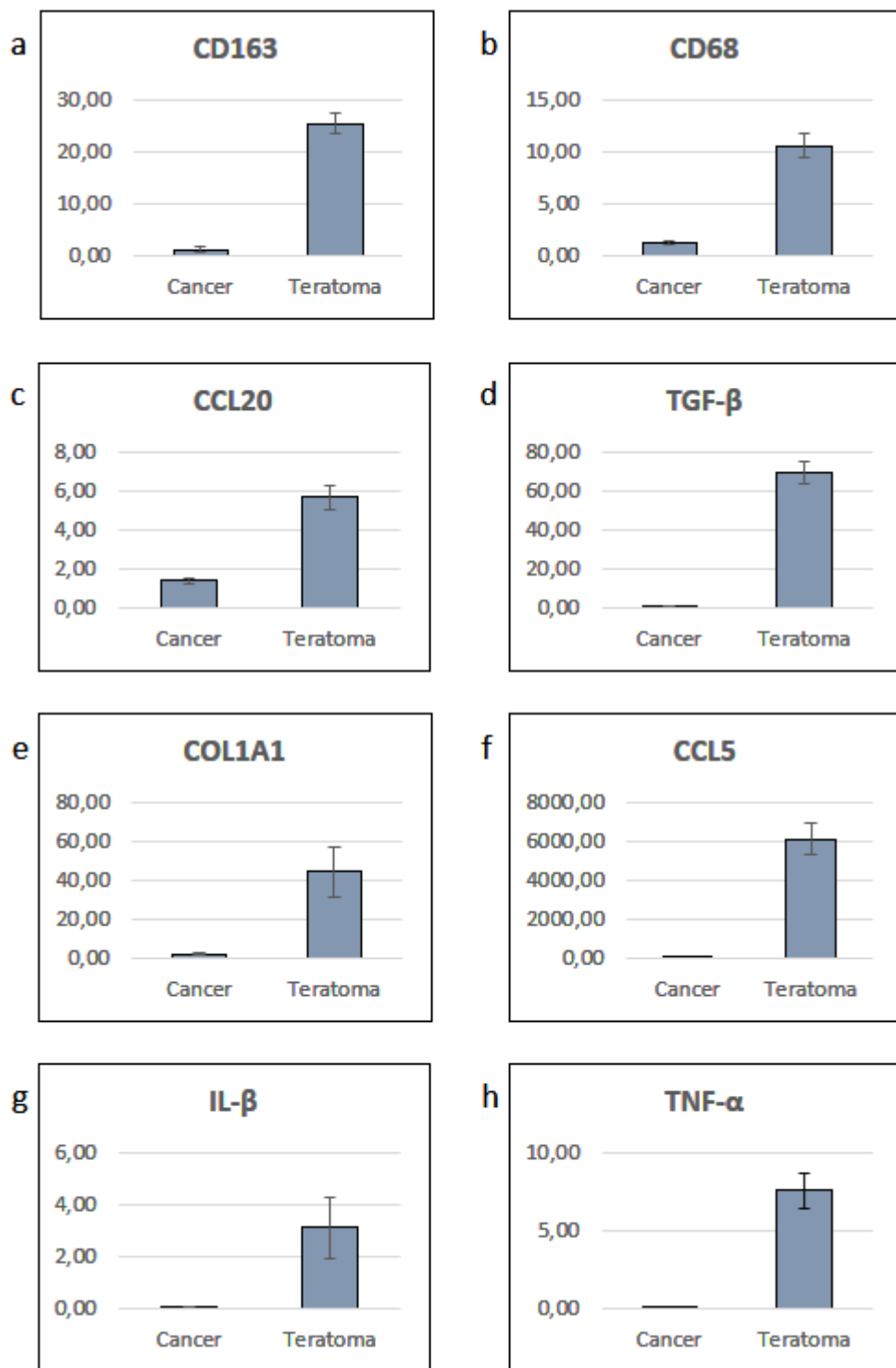


Figure 10. Inflammatory mRNAs of xenografts injected subcutaneously with non-transformed MDCK cells or Hakai transformed MDCK cells. Mean values of (a) CD163, (b) CD68, (c) CCL20, (d) TGF- β , (e) COL1A1, (f) CCL5, (g) IL- β , (h) TNF- α inflammatory mRNAs in tumour and teratoma tissues.

6. DISCUSSION

In a previous study of the Epithelial Plasticity and Metastasis Group, it has been shown that Hakai expression is gradually increased in adenoma and in different TNM stages (I-IV) from colon adenocarcinomas compared to human colon healthy tissues (Castosa *et al*, 2018). Moreover, in MDCK cells that stably overexpressed Hakai, it was detected an increase expression of the Hsp90 and Hsp70 proteins compared to normal, non-transformed MDCK (Diaz-Diaz *et al*, 2017). Given these premises, the differential expression during carcinoma progression in human colon adenocarcinoma was studied for Hsp70 and hsp90.

Collectively the immunohistochemical Hsp90 images (*figure 5*) show that the tissues belonging to the various tumour stages (I-IV) present an increased protein expression compared to healthy or adenoma tissues. An important change of expression level between the benign tumour (adenoma) and the malignant cancerous tumour occurs, as demonstrated by the subsequent quantification of the staining through the software ImageJ (*figure 6*). However, it does not seem to be statistical differences between Hsp90 protein levels between the different TNM-stages, further suggesting that Hsp90 is a relevant protein for the transition from adenoma to carcinoma. Unlike Hsp90, immunohistochemical staining of the Hsp70 (*figure 7*) did not show any statistical differences in human colon adenocarcinoma during tumour progression. Indeed, no changes were detected while comparing healthy tissue with adenoma and different stages of cancer (I-IV).

The apparently increase expression levels of Hsp90 during human colorectal cancer progression may suggest that the existing stress factor such as hypoxia and acidosis at the beginning of colorectal cancer formation contribute to the higher Hsp90 expression level in colorectal cancer tissues compared to normal tissues (Qiu *et al*, 2014). Therefore, it would be interesting to determine whether Hakai and Hsp90 could act together in response to these factors and its impact at early stages tumour progression. Hsp protein family, specially Hsp90 protein, could play a role as “cancer chaperone” to promote cancer development. For instance, cancer cells could rely on the Hsp90 chaperone to protect a set of mutated and overexpressed client oncoproteins from misfolding and degradation (Neckers *et al*, 2012). Thus, Hsp90 could be a critical facilitator of “oncogene addiction” and cancer cell survival by its action on Hakai proteins; opening the possibility to study whether there is a mutual relationship between Hakai and Hsp proteins during cancer development.

A classical tumour marker for gastrointestinal cancers and CRCs is CEA (carcinoembryonic Antigen). CEA marker was discovered almost 50 years ago, in 1965, and it remains the only tumour marker of recognised efficacy in monitoring CRC patients therapy. However, the sensitivity and specificity of CEA for specific tumours is not precise since this protein is expressed widely in gastrointestinal mucosa. In a recent study, it was explored the diagnostic value of the new marker, Hsp90. Authors analysed the combination of tumour markers such as Hsp90 and CEA or CA 19-9 to show the potential diagnostic value of CRC at an early stage and monitoring the plasma Hsp90 pre- and post-surgery (Kasanga *et al*, 2018). They demonstrated that plasma Hsp90 was a powerful marker with better sensitivity and specificity than CEA and CA 19-9 in discriminating the early stage (I and II) cancer supporting our results obtained by immunohistochemistry on which Hsp90 was crucial at early stages of tumour progression.

On the other hand, as previously reported the Hsp70 protein plays a cryptic role within the cancer and it is proposed to have a pro-oncogenic or anti-oncogenic role depending on the type of cancer and genetic background. For instance, in cancer where the inflammatory component seems to be critical for its development, a decrease rather than increase in the levels of Hsp70 would promote cancer. Moreover, in cancer accompanied by a strong inflammation knockout of Hsp70 increased inflammatory cytokines and aggravated cancer (Sherman *et al*, 2016). Although our results do not show any differential expression during human colon cancer progression, it would be interesting to determine whether this protein could be a biomarker in CRC associated to inflammation. Moreover, many authors reported that elevated levels of Hsp70 are critical to cancer cells to combat harsh conditions and suppress apoptosis. These early studies demonstrated that, surprisingly, besides its molecular chaperone function, Hsp70 also plays a special role in apoptotic signal transduction (Rérole *et al*, 2011). Nowadays, Hsp70 and Hsp90 play a crucial role in cancer but their molecular mechanism is still unknown.

Given the promising results, I further investigated whether Hsp90 protein is regulated in LoVo by transiently overexpressing the E3 ubiquitin-ligase Hakai protein by western blot and immunofluorescence analysis. The hypothesis that the Hsp90 protein could be over-expressed in LoVo cells by transiently transfection of Hakai plasmid did not give positive results (*figure 8*), therefore, we speculate that it could be due to a low transfection efficiency. Next, I performed an immunofluorescence to detect Hsp90 in presence or absence of Hakai overexpression. In fact, from this experiment it has emerged that there is an increase expression

of Hsp90 in several Hakai-transfected cells (*figure 9*), suggesting that it could be a relationship between action of Hsp90 and Hakai, however this hypothesis should be deepened and investigated.

Overexpression and knockdown of Hakai seems to affect cell proliferation in some cell lines, thus, it is plausible that Hakai interacts with other proteins than E-cadherin (Figuroa *et al*, 2009). Several publications reported alterations in the regulation of Hsp in many types of cancer and recent studies are investigating the role of Hsp90 localizing at the membrane level and in the extracellular environment, which is still unknown.

HSP is a group of highly conserved proteins synthesized after heat induction. During the growth and development of normal cells, Hsp70 and Hsp90 are constitutively expressed at low or moderately levels, but the expression is dramatically enhanced by stressful conditions (Hosseinzadeh *et al*, 2012). Heat shock proteins can act promoting cancer survival also in a condition of intracellular proteotoxic stress. Intracellular environment is incredibly chaotic (crowding phenomenon) even more in a cancerous cell, on which the protein regulation is lost and some genes are continually expressed. Then, it may be possible to find a correlation between the levels of heat shock protein present in tumour tissue and different cancer staging, such as Hsp90 in colorectal and liver cancer (Kasanga *et al*, 2018; Sourbier, 2017).

7. CONCLUSIONS

1. The Hsp90 levels increase from the transition from healthy and adenoma tissues to carcinoma in human colon adenocarcinoma progression.
2. No significant differences in protein levels of Hsp70 expression were detected by comparing healthy tissue, adenoma and different stages of human colon adenocarcinoma (I-IV).
3. It was not detect differences in expression of Hsp90 protein levels in presence or absence of Hakai overexpression in LoVo transiently transfected cells, by western-blotting.
4. By immunofluorescence analysis, it was detected an increase expression of Hsp90 protein levels in presence of Hakai overexpression in LoVo cells by transiently transfection.
5. Comparative study of inflammatory mRNA levels in xenograft mouse model injected subcutaneously with non-transformed MDCK cells or Hakai-transformed MDCK cells is not conclusive. Further experiments are necessary in order to determine whether the inflammatory mRNA expression is regulated under Hakai influence.

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