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ROLE OF CAVEOLIN-1 IN THE PROLIFERATION OF SOLID TUMOURS *IN VITRO*

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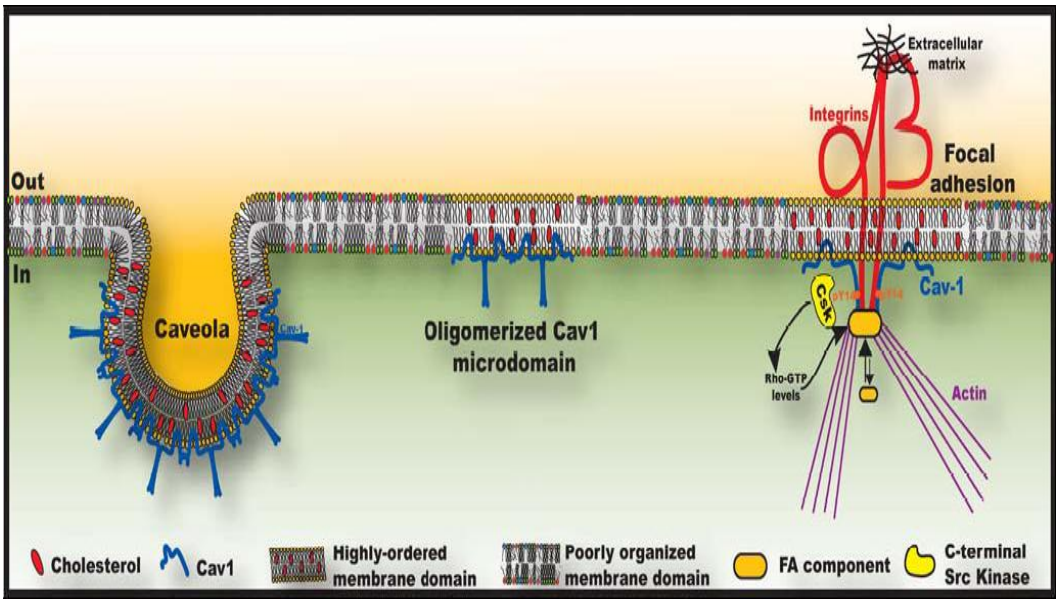
1. INTRODUCTION

1.1 CAVEOLAE AND CAVEOLINS

1.1.1 Caveolae

Caveolae are vesicular invaginations of the plasma membrane of 50-100 nm in size, found in a number of different cell types, identified for the first time in 1953 (Volontè D et al, 2002). They are present primarily in terminally differentiated mesenchymal cells including adipocytes, endothelial cells and fibroblasts (Smart EJ et al,1999).

Caveolae represent a subgroup of lipid rafts that are defined as "small heterogeneous membrane domains enriched in cholesterol and sphingolipids" (Pike L, 2006). The presence of the structural protein caveolin-1 drives the formation of plasma membrane invagination and makes caveolae unique among lipid rafts (Razani B et al, 2000).



Goetz JG et al, Cancer Metastasis Rev, 2008

Caveolae have been implicated in several cellular functions, including signal transduction: a variety of signaling molecules are preferentially localized in lipid rafts and caveolar membranes and interact with caveolin-1scaffolding domain. Cellular metabolism, vesicle trafficking (transcytosis, endocytosis, and potocytosis) and cholesterol homeostasis are also involved: caveolin-1 binds cholesterol and long-chain unsaturated fatty acids and has been implicated in the import and export of cellular cholesterol by caveolae (Razani B et al, 2000; Williams TM and Lisanti MP, 2004; Parton RG and Simons K, 2007).

1.1.2 Caveolins

Three caveolin genes have been identified in mammalian cells: CAV-1, CAV-2 and CAV-3.

CAV-1 is composed by three exons highly conserved in their sequences across species. Two isoforms have been identified, the predominant 178-residue Cav-1α, and the 147-residue Cav-1β derived from an internal translation initiation site (Met^{32}) (Scherer PE et al, 1995).

CAV-2 is composed by three exons that encode a 162-residue protein with a sequence highly homologous to that of Cav-1. Cav-2 α is the full-length protein; two additional truncated isoforms have been identified (Scherer PE et al, 1996).

CAV-3 is composed by two exons that encode a single 151-residue mature protein (Tang Z et al, 1996).

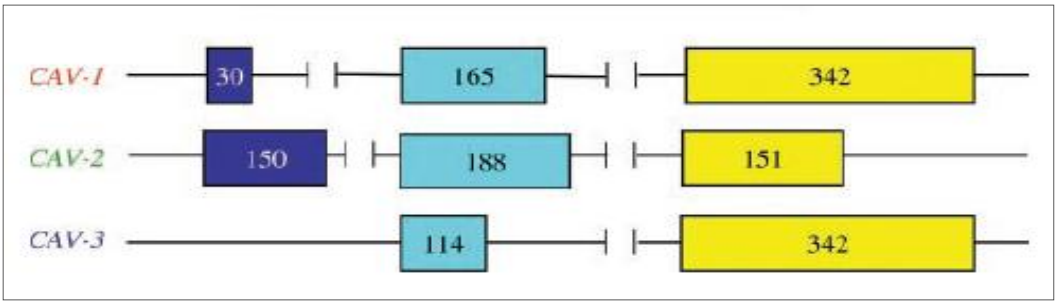
CAV-1 and CAV-2 genes map to 7q31.1 near the D7S522 genetic marker, a known fragile site FRA7G. CAV-3 maps to 3p25.

Caveolin-1 has been the first member of the family to be identified as

a structural component of caveolae and of transport Golgi-derived vesicles. It is ubiquitously expressed, with the highest levels in adipocytes, endothelial cells, fibroblasts, smooth muscle cells and a variety of epithelial cells (Gumbleton AG et al, 2000; Williams TM and Lisanti M, 2004).

Caveolin-2 is closely co-expressed with caveolin-1 but the majority of caveolae require only cav-1 expression for their formation (Tagawa A et al, 2005; Kirkham M et al, 2008).

Caveolin-3 is primarily located in muscle tissues but is also expressed in glial cells where it plays an essential role in caveolae biogenesis (Galbiati F et al, 2001; Capozza F et al, 2005).

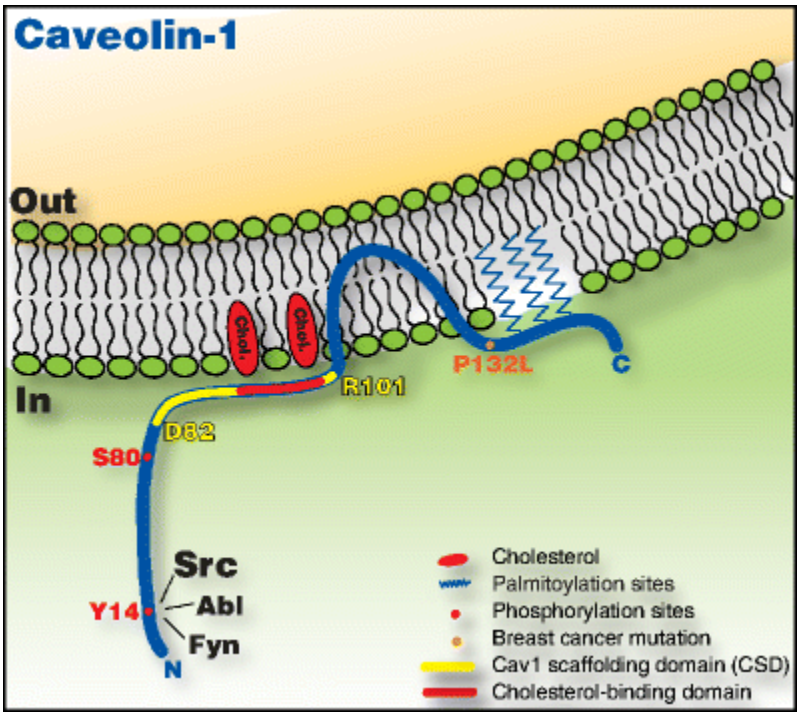


	chromosome	markers	localization
CAV-1	7q31.1	D7S522,WI5336, MET	Endothelial cells, fibroblasts, adipocytes, smooth muscle cells
CAV-2	7q31.1	D7S522,WI5336, MET	
CAV-3	3p25		Muscle cells, glial cells

1.1.3 Caveolin-1

Cav-1 is an integral membrane protein of 22-24 KDa. It has a membrane spanning hairpin loop structure with both C- and N-terminal regions facing the cytosol. N-terminal region (N-MAD: NH2-terminal membrane attachment domain, amino-acids 82-101) can be phosphorylated by Src on its tyrosine 14 residue (Li S et al, 1996). This modification is associated with its translocation to the close proximity of focal adhesions and has been linked to various cellular phenomena including signal transduction, endocytosis, cell migration and focal adhesion dynamics (Lee H et al, 2000; Kimura A et al, 2002; Maggi D et al, 2002).

C-terminal region (C-MAD: COOH-terminal attachment domain, amino-acids 135-150) can be palmitoylated on Cys 133, Cys 143 and Cys 156 and this modification is necessary for Cav-1 oligomerization (Dietzen DJ et al, 1995; Monier S et al, 1996).



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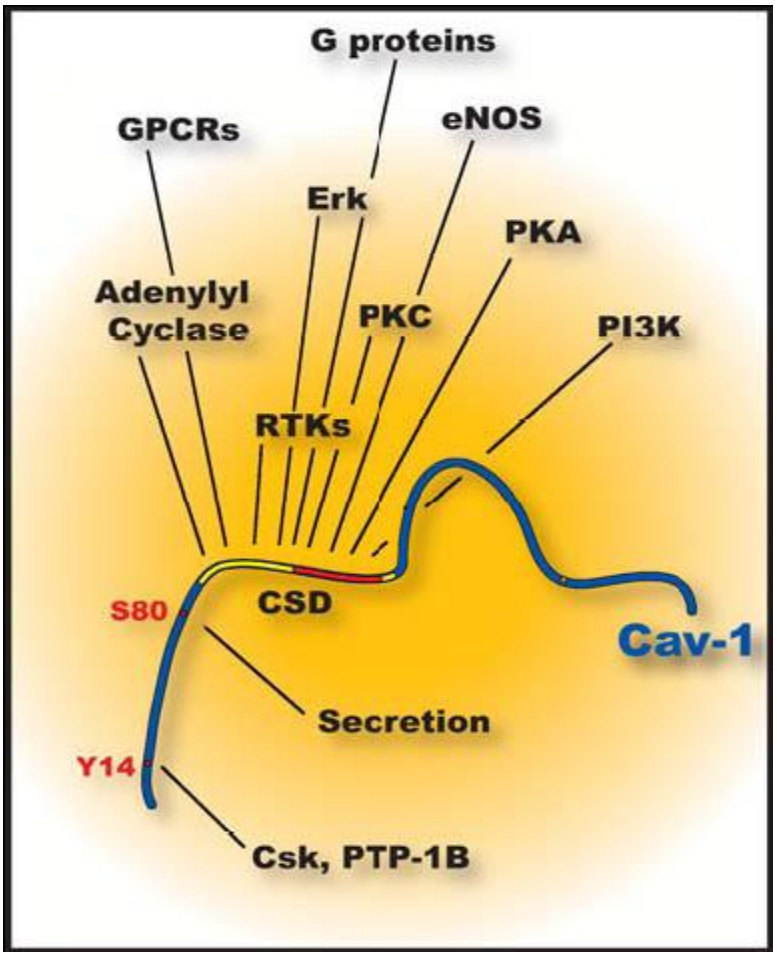
Cav-1 can also undergo serine phosphorylation at Ser80, by which it is converted to a soluble secreted protein (Schegel A et al, 2001).
Other important domains include an oligomerization domain (amino-acids 61-101) and the scaffolding domain (CSD, amino-acids 82-101) (Schegel A and Lisanti MP, 2000). The last one is necessary for Cav-1 to bind to several signalling molecules including Src family tyrosine kinases, growth factors receptors, endothelial nitric oxide synthase, G proteins and G-protein-coupled receptors (Liu P et al, 2002; Razing B et al, 2002) and to regulate multiple cancer-associated processes such as cellular transformation, tumour growth, cell migration and metastasis, cell death and survival, multidrug resistance and angiogenesis (Patel HH et al, 2008).



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1.1.4 Caveolin-1 in human tumours

The role of Cav-1 in cancer initiation and progression is multidimensional. Cav-1 is highly expressed in terminally differentiated or quiescent cells, suggesting a possible role for it as a negative regulator of cell proliferation. In support of this prediction, several authors have demonstrated a role for Cav-1 in modulating cell cycle progression in mammalian cells (Glaciate F et al, 2001; Lee SW et al, 1998; Scheel J et al, 1999). Galbiati and colleagues (2001) have shown that Cav-1 expression levels are negatively regulated by growth factor stimuli and that recombinant over-expression of Cav-1 can inhibit cellular proliferation, by mediating cell cycle arrest in G_0/G_1. In addition, Cav-1(–/–) null mice embryonic fibroblasts (MEFs) show increased proliferation rates, concomitant with increased S-phase fractions and decreased G_0/G_1 fractions, as well as altered expression of several cell cycle-related proteins (Razani B et al, 2001; Williams TM et al, 2004). Interestingly, Cav-1 has been shown to interact with PI3-kinase and Cav-1 over-expression sensitizes fibroblasts to ceramide-induced death, by a PI3-kinase-dependent mechanism (Zundel W and Giaccia A, 1998). Furthermore, Cav-1 expression sensitizes both NIH-3T3 fibroblasts and T24 bladder carcinoma cells to cell death initiated by staurosporine, a chemical inducer of apoptosis (Liu J et al, 2001).

In contrast, disruption of caveolae by cholesterol-sequestering agents has been shown to block IL-6 and IGF-1-induced activation of the PI3-kinase/Akt signaling pathway (Podar K et al, 2003). Therefore, caveolae and Cav-1 are required to mediate proper survival signals by the PI3-K/Akt pathway. Moreover, Cav-1 over-expression in Rat1A cells and human LNCaP prostate cancer cells or Cav-1 up-regulation

in androgen-insensitive LNCaP clones render these cells more resistant to apoptosis (Timme TM et al, 2000; Tso CL et al, 2000). In addition, the consequence of antisense mediated down-regulation of Cav-1 is a higher sensitivity of cells to apoptosis (Li L et al, 2001; Li L et al, 2003). Finally, Li and colleagues (2003) have demonstrated that Cav-1 over-expression mediates cell survival by sustaining Akt activation.

The cell type-specificity of Cav-1 effects may explain the pro-apoptotic or anti-apoptotic functions of this protein.

An inverse relation between Cav-1 expression and malignant transformation has been clearly established. During the initial characterization of Cav-1, it has been shown that Cav-1 levels are reduced in transformed NIH-3T3 cells and that the level of residual Cav-1 is inversely related to soft agar growth (Koleske AJ et al, 1995). Interestingly, reduction of Cav-1 expression by an antisense approach is sufficient to induce a transformed phenotype into NIH-3T3 cells, allowing these cells to grow in soft agar and to form tumours in athymic (nude) mice (Galbiati F et al, 1998). The utilization of Cav-1(–/–) null mice served for definitive genetic evidence to support the idea that Cav-1 normally works as a "transformation suppressor" gene. Capozza et al (2003) have shown that the skin of Cav-1(–/–) mice is more susceptible to chemical carcinogenic treatment, resulting in the formation of epidermally derived tumours. In addition, genetic ablation of Cav-1 in MEFs render these cells more susceptible to transformation and in vivo tumorigenesis mediated by transforming oncogenes (Wiliams TM et al, 2004). Taken together, these results indicate that Cav-1 normally has a role to negatively regulate the growth and transformation of mammary epithelial cells, as well as to suppress the development of advanced mammary tumours and

metastasis.

CAV-1 gene is located in chromosome 7 (7q31.1), close to a fragile site (FRA7G) frequently deleted in a variety of human cancers, including carcinomas of the breast, colon, kidney, prostate, ovary, head and neck (Engleman JA et al, 1998). These findings have led to suggest that CAV-1 may represent a tumour suppressor in this fragile genomic region. It is a fact that Cav-1 protein levels are low in many primary human cancers, ovarian, lung, and mammary carcinomas, as well as mesenchymal sarcomas. Importantly, sequence analysis of CAV-1 in human tumours has revealed sporadic mutations. In a cohort of patients with primary breast cancer, Hayashi and colleagues (2001) have detected a sporadic P132L mutation in up to 16% of the cases examined. Furthermore, this mutation induces cellular transformation, acts in a dominant negative manner by causing the mislocalization and intracellular retention of wild-type Cav-1, and causes ERK-1/2 hyperactivation (Hayashi K et al, 2001; Lee H et al, 2002).

On the other hand, many cancer cell lines exhibit high expression of Cav-1: it is consistently up-regulated in bladder, esophagus, papillary thyroid and prostate carcinomas, with few exceptions (Pflug BR et al, 1999). In addition, the levels of Cav-1 are elevated in highly metastatic prostate cancer cells (Yang G et al, 2000) and in multidrug resistant cancer cells (Cohen AW et al, 2004).

These evidences may suggest that Cav-1 expression is correlated with tumour stage and grade in several types of cancer and that Cav-1 may act as a pro-survival and tumour-promoting protein in advanced cancer.

The positive correlation between tumour progression and metastasis and Cav-1 expression has been first demonstrated in prostate cancer.

Immunohistochemical analysis has indicated that the proportion of Cav-1 positive samples increases from 29% in T3N1 primary cancer to 56% in lymph node metastasis, whereas in normal and hyperplastic epithelia the percentage is 8% and 18% respectively (Yang G et al, 1998). In addition, Cav-1 is highly expressed in primary and metastatic human prostate cancer after androgen ablation therapy, while antisense-mediated down-regulation of Cav-1 in prostate cancer cells *in vitro* reduces their metastatic phenotype (Li L et al, 2001; Tahir SA et al, 2001). In other reports (Ho CC et al, 2002; Joo HJ et al, 2204; Kato K et al, 2002; Nestl A et al, 2001), Cav-1 over-expression has been correlated with metastasis in esophageal squamous cell carcinoma, clear cell renal cell carcinoma, mammary adenocarcinoma and metastatic cell lines derived from lung adenocarcinoma. These results have implications for those human cancers where Cav-1 is consistently down-regulated and suggest that the up-regulation of Cav-1 may represent an acquired feature that contributes to a metastatic phenotype. This concept of a "biphasic" expression pattern for Cav-1 has been elegantly demonstrated in colon carcinoma cells after selection for metastatic variants (Bender FC et al, 2000). This notion of two opposite roles for a protein, depending on the stage of tumour progression, is not unique. In early stages of transformation and carcinogenesis, transforming growth factor- (TGF) demonstrates tumour suppressive activity, by inducing cell cycle arrest and apoptosis, thereby inhibiting primary tumour growth (Benson JR et al, 2004; Roberts AB and Wakefield LM, 2003). However, at later stages of tumour progression, increased secretion of TGF- by tumour and stromal cells promotes tumour invasion, angiogenesis, and metastasis, as well as immunosuppression of host surveillance mechanisms. Therefore, the role of TGF- in

tumorigenesis can be either suppressive or promoting, depending on the tumour developmental stage. Siegel et al (2003) have definitively demonstrated these contrasting roles for TGF- in breast cancer by interbreeding transgenic mice expressing either constitutively activated or dominant negative forms of TGF- to MMTV-Neu/ErbB2 transgenic mice.

Sloan EK et al (2004), in an orthotopic model of spontaneous breast cancer metastasis, have shown that Cav-1 is expressed in low and non-metastatic primary breast cancer, but at much lower levels in highly metastatic 4T1.2 and 4T1.13. Exogenous expression of Cav-1 at moderate levels in 4T1.2 cells is sufficient to suppress primary tumour growth after inoculation of cells into the mammary gland. In addition, expression of high levels of Cav-1 also inhibits subsequent metastasis to distant organs. Cells expressing high levels of Cav-1 show reduced capacity to invade Matrigel, diminished response to laminin-1 stimulation and decreased metastatic capacity to lung and bone. This study provides the functional evidence that Cav-1 negatively regulates tumour growth and spontaneous metastasis of breast cancer.

Three distinct mechanisms that can serve to functionally inactivate the tumour suppressor function of Cav-1 have been described: tyrosine phosphorylation, serine phosphorylation and a dominant-negative point mutation, P132L. These findings may explain why Cav-1 has been suggested to work both as a tumour suppressor and as an oncogene, depending on the tumour type and/or tumour stage.

Tyrosine Phosphorylation: The dually contrasting roles for Cav-1 in tumour progression may be partly explained by the observation that it possesses several peptide domains with opposing roles. A molecular dissection of the Cav-1 protein has revealed distinct regions that may counteract the effects of the growth-inhibitory CSD. First, Tyr14 at

the extreme NH_2 terminus is important for the binding and recruitment of a c-Src/Grb7 signaling complex (Lee H et al, 2001). This residue is constitutively phosphorylated in v-Src- and v-Abl-transformed cells, transiently phosphorylated during growth factor stimulation in other cells, and localizes to focal adhesions, which are the predominant sites of tyrosine kinase signalling. Functionally, tyrosine 14-phosphorylated Cav-1 binds Grb7 and enhances both anchorage-independent growth and EGF-stimulated cell migration (Lee H et al, 2001). Thus tyrosine phosphorylated Cav-1 may have a role as a growth factor receptor that recruits SH2 domain-containing proteins to the plasma membrane. Cav-2 also undergoes phosphorylation (at Tyr19 and Tyr27) and similarly recruits SH2 domain-containing proteins, such as c-Src, Nck, and Ras-GAP (Lee H et al, 2002).

Serine Phosphorylation: A second region of Cav-1 also appears to have growth-stimulatory properties. Serine phosphorylation of Cav-1 changes its topology, thereby converting Cav-1 from an integral membrane protein to a secreted protein product. Normally, the majority of Cav-1 is associated with the plasma membrane. However, Cav-1 also appears to be secreted, especially in exocrine cell types, where it is packaged into secretory vesicles (Liu P et al, 2002; Schlegel A et al, 2001). Phosphorylation of Cav-1 at Ser80 directly regulates its conversion to a secreted protein. As an example, mutation of Ser80 to glutamate (S80E, which mimics chronic phosphorylation) results in preferential targeting of Cav-1 to the ER membrane and directs its subsequent packaging for secretion (Schlegel A et al, 2001). Furthermore, phosphorylation at Ser80 is necessary for a proper secretion because its mutation to alanine (S80A; which abrogates phosphorylation) results in no detectable secretion of Cav-1 (Schlegel A et al, 2001), concomitant with its intracellular accumulation.

Mechanistically, in terms of cellular transformation, shunting Cav-1 for secretion to the extracellular environment would subvert its normal intracellular tumour suppressive functions. Essentially, these changes in Cav-1 membrane topology have the same consequences as a loss of Cav-1 expression.

In addition, the secretion of Cav-1 results in a protein product that possesses autocrine or paracrine tumour-promoting functions. Tahir and colleagues (Tahir SA et al, 2001) have demonstrated that Cav-1 is secreted in androgen-insensitive human prostate cancer cells and that secreted Cav-1 acts in an autocrine/paracrine fashion, directly stimulating prostate tumour cell growth and survival.

Dominant-Negative Point Mutations: The previously mentioned identification of Cav-1 (P132L) mutations in up to 16% of human breast cancers provides a third distinct mechanism to inactivate the tumour suppressor function of Cav-1. This mutation drives cellular transformation in NIH 3T3 cells. Briefly, NIH 3T3 cells expressing Cav-1 (P132L) show augmented growth in soft agar, as well as increased invasiveness, and increased chemotaxis (Hayashi K et al, 2001).

1.2 LUNG CANCER

1.2.1 Epidemiology

Lung cancer was a rare disease in the 19th century, representing only 1% of all tumours; at the end of the 20th century, lung cancer incidence began to increase and now this tumour is the leading cause of cancer death in most industrialized countries, both in men and women (Jemal A et al, 2009).

The main environmental risk factor is exposure to cigarette smoking, associated with more than 90% of cases of lung cancer, with active smoking causing the majority of these cases but with passive smoking also heavily contributing to the lung cancer burden (Parkin DM et al, 2005; Jemal A et al, 2009).

In 2002, it has been estimated that 1,35 million people throughout the world have been diagnosed with lung cancer and that 1,18 million per year died of it. In more developed countries, incidence and mortality rates are generally declining among males and are starting to plateau for females, thus reflecting previous trends in smoking prevalence. In contrast, there are some populations in emerging and less developed countries where increasing lung cancer rates are predicted to continue, due to endemic use of tobacco (Youlden DR et al, 2008).

Some studies (Zang EA and Wynder EL, 1996; Wynder EL,1998; Baldini EH and Strauss GM, 1997; Shiver SP et al, 2000) have suggested that women are more susceptible than men to the carcinogenic effects of cigarette smoking, taking into account baseline exposure, body weight, height and body mass index. In a case-control study, Kruezer M et al (2000) haven't found increased odds ratio for female smokers compared to male smokers: there may be differences in baseline exposure that may alter the interpretation of these results.

However, a large disproportion of female-to-male cases in the never-smoking group has been observed: a 10% of lung cancers occur in never-smokers and the majority of these occur in women (Egleston BL et al, 2009); in addition, a large proportion of these women presents a lung adenocarcinoma, which is a secretory tumour (Scagliotti GV et al, 2009). Estrogens may play a causative role in this phenomenon of secretory types of differentiation, known to be more prominent in female lung cancer (Marquez-Garbon DC et al, 2007; Lim RH and Kobzik L, 2008). However, the role of oestrogen in lung cancer is unclear: studies indicated a positive correlation between post-menopausal oestrogen replacement therapy, smoking and lung adenocarcinoma, suggesting a role for oestrogen in lung cancer risk (Taioli E and Wynder EL, 1994); on the other hand, the higher survival rates in women than in men with NSCLC in a study of Moore KA et al (2003) may indicate a protective effect of oestrogen.

Current treatment options include surgical resection, chemotherapy and radiotherapy alone or in combination. Although advances in therapy have provided some improvement in overall survival, outcomes remain poor, with a five years survival for less than 15% of patients with lung carcinomas (Jemal A et al, 2006). Several genetic alterations have been recently identified in lung cancer, yet the investigation of new molecular markers and candidate genes that might be helpful for therapy remains one of the central focuses in cancer research.

1.2.2 Histological classification

The World Health Organization (WHO) has divided lung cancers in four principal histological types: adenocarcinomas (30-40%), squamous cell carcinomas (25-40%), small cell carcinomas (20-25%) and large cell carcinomas (5%). However, there is a different classification based on biological features and manner, which divides lung cancers into two major histological types: small cell lung cancers (SCLC) and non-small cell lung cancers (NSCLC).

1.2.3 Caveolin-1 in lung cancer

In normal adult lung, Cav-1 is expressed in alveolar type I epithelial cells, endothelial cells and fibroblasts. Levels of this protein are dynamics in tumorigenesis and these changes in the Cav-1 expression may affect tumour progression by influencing cell signalling. Little is known about the regulation of Cav-1 transcription in lung tumorigenesis, if it depends on cellular context and which environmental factors may influence its expression.

However, a positive correlation between Cav-1 expression and tumour stage/grade has been reported in human lung cancers: in lung adenocarcinomas, nearly all metastatic lymph nodes present increased Cav-1 expression in tumour cells by immunochemistry (Ho CC et al, 2002; Yoo SH et al, 2003; Kato T et al, 2004); on the other hand, in most primary lung tumours, Cav-1 expression is exceedingly low (Wikman H et al, 2002; Powell CA et al, 2003; Ho CC et al, 2002; Kato T et al, 2004). In addition, induced CAV-1 gene expression in a low-invasive lung adenocarcinoma cell line increases cell migration and invasiveness (Ho CC et al, 2002); instead, shRNA induced reduction of Cav-1 protein levels inhibits cell growth and reduces matrix invasion and cell migration in H129 NSCLC cells (Shatz M et

al, 2010).

Previous studies reported that Cav-1 is expressed in 21-36% of resected primary NSCLCs and that Cav-1 expression is associated with poor prognosis in primary squamous cell lung cancer (Racine C et al, , 1999; Razani B et al, 2000; Heighway J et al, 2002; Wikman H et al, 2002). Moreover, Sunaga N et al (2004) suggested different roles for Cav-1 between SCLC and NSCLC: it can act as a tumour suppressor in SCLCs, whereas in NSCLC it appears to be necessary for tumour growth.

1.3 OSTEOSARCOMA

1.3.1 Epidemiology

Osteosarcoma is a high-grade malignant tumour composed of mesenchymal cells producing osteoid and immature bone with a peak incidence in the second decade of life (Campanacci M, 1999). In Italy, primary bone tumour incidence rate is approximately 0,8-1 case per 100.000; around 500 new cases per year have been estimated and 20-25% of these are osteosarcomas.

Although current treatment modalities, which include surgery and neoadjuvant multidrug chemotherapy, significantly improved the 5-year disease-free survival from 10% to 60%-70% (Bracci G et al, 2005), no significant drugs or treatment approaches have been developed in the last 10 years, and the percentages of curative therapy remain unacceptably low for high-risk patients.

The causes of osteosarcoma onset are still unknown. The correlation between early age and tumour appearance in the majority of patients, suggests that the increased osteoblastic activity, which is typical during high bone remodelling, may be a predisposing factor to the osteosarcoma onset.

Exposure to radiation is a known causal factor: approximately 4% of osteosarcomas is due to previous radiotherapy for different tumours.

A relevant involvement of genetic alterations in oncosuppressor genes has been demonstrated: complete or partial deletions of Rb gene have been found in approximately 60% of osteosarcomas. The loss of Rb gene, localized in the 13q14 region, is responsible of retinoblastoma onset, a malignant eye tumour of childhood. It may represent an unilateral (mostly sporadic) or bilateral (hereditary) disease. It has been demonstrated that patients with hereditary retinoblastoma have a

500-fold higher risk to develop osteosarcoma in the second decade of life than the healthy population. Moreover, mutations or deletions of p53 gene have been found in 30% to 50% of the patients with osteosarcoma. Mutations of p53 gene are also associated to Li-Fraumeni syndrome which is characterized by a high incidence of some tumours such as osteosarcoma.

1.3.2 Histological classification

In the majority of cases, primary bone tumours are sarcomas, divided into osteosarcomas or chondrosarcomas on the basis of their origin cell type. Osteosarcomas derive from bone tissue and chondrosarcoma from bone cartilage. Ewing's sarcoma is a highly aggressive and undifferentiated tumour that predominantly affects bones of children and young adults. Metastatic bone tumours are highly frequent, in particular those originating from breast, lung and prostate cancers.

Osteosarcomas may represent either low-grade or high-grade malignancies but the most frequent form is the high-grade central osteosarcoma.

1.3.3 Caveolin-1 and osteosarcoma

Cav-1 is highly expressed in normal osteoblasts (Solomon KR et al, 2000) and some experimental studies have indicated that Cav-1 can act as an osteoblast regulator through the modulation of endothelial nitric oxide synthase enzyme activity (Lofthouse RA et al, 2001). This enzyme leads to the production of nitric oxide, an important mediator in the regulation of osteoblastic cell functions localized in caveolae where it binds to Cav-1 scaffolding domain. By binding to this enzyme, Cav-1 can modulate the production of nitric oxide and consequently osteoblast activity (Lofthouse RA et al, 2001).

Cav-1 also impacts on osteoblasts differentiation: Rubin J et al (2007) have indicated that Cav-1 helps maintaining a less differentiated state of osteoblast progenitor cells, while its absence causes bone to undergo maturation more rapidly. Moreover, Cav-1 is involved in the regulation of osteoblast-mediated calcification of extracellular matrix inhibiting its mineralization as well as with the inhibition of osteoblasts differentiation.

In addition to its involvement in bone production, Cav-1 also appears implicated in calcium homeostasis regulation and influences bone resorption (Jung SY et al, 2005).

Compared to osteoblasts, fewer results have been reported in connection with the presence and functional activity of caveolins in osteoclasts. An extremely low expression of Cav-1 and Cav-2 in human osteoclasts has been observed (Luegmayr E et al, 2004); however, the same authors have provided evidence of its possible involvement in osteoclastogenesis, through its influence in cholesterol transport. In fact, osteoclast formation and survival are highly dependent on exogenous sources of cholesterol.

In conclusion, on the basis of these evidences, although Cav-1 is undoubtedly involved in bone development and maturation, its role in these processes is still undefined and scarcely known. On one side, it acts as an inhibitor of osteoblastic differentiation and of bone maturation and mineralization. On the other, by interacting with calcium-sensing receptors, it can enhance bone formation and reduce osteclast-mediated bone resorption. The evidence emerging from the first study by Wiecken K et al (2001) that Cav-1 is generally down-regulated in malignancies compared to normal cells, have suggested its role as an oncosuppressor in sarcomas (Wiechen K et al, 2001; Williams TM et al, 2004; Sloan EK et al, 2004). A better overall

survival rate has been observed for osteosarcoma expressing a level of Cav1 similar to osteoblasts (Wiechen C et al, 2001; Cantiani L et al, 2007). Modulation of its expression in osteosarcoma cell lines greatly modifies the pattern of anchorage-indipendent growth, migration, invasion, adhesion to extracellular matrix components and, more importantly, their metastatic capacity. Furthermore, osteosarcoma cells over-expressing Cav-1 also exhibit reduced *in vitro* malignancy and decreased *in vivo* metastatic capacity (Zucchini C et al, 2004; Manara MC et al, 2006). This oncosuppressor role of Cav-1 may proceed through the modulation of c-Src family tyrosine kinases: in fact, Cav-1 is known to bind and sequester Src family kinases in an inactive configuration (Li S et al, 1996; Wei Y et al, 1999). Cav-1-deprived osteosarcoma cells exhibit a marked increase in c-Src family tyrosine kinase activity, which significantly contributes to their migration and anchorage-indipendent growth (Solomon KR et al, 2000). In contrast with these evidences, Cav-1 has been found to be necessary for tumorigenesis of Ewing's sarcoma (Tirado OM et al, 2006). These apparently contradictory findings may reflect the different origin and genetic features of osteosarcoma and other mesenchymal tumours compared with Ewing's sarcoma and support the need to consider CAV-1 gene studies in its appropriate cellular context. Moreover, Cav-1 expression levels can be reduced, unchanged or up-regulated depending on the osteosarcoma cell lines *in vitro*. Thus, Cav-1 has been certainly proved to play relevant roles in differentiation and functions of bone and in musculoskeletal tumour development, however its involvement is cell context-dependent: it might play an oncosuppressor or a tumour promoter role by releasing the full oncogenic or antioncogenic properties of the proteins sequestered in caveolae in each tumour cell type.

2. MATERIALS

- Dulbecco's Modified Eagle's Medium (DMEM) (**SIGMA-ALDRICH**)
- Ham's F12 (**GIBCO-INVITROGEN**)
- Foetal bovine serum (FBS) (**GIBCO-INVITROGEN**)
- Penicillin/streptomycin (**SIGMA-ALDRICH**)
- L-glutamine (**SIGMA-ALDRICH**)
- Mycoplasma plus™ PCR Primer Set (**STRATAGENE**)
- Trypsin-EDTA solution (**SIGMA-ALDRICH**)
- Trypan Blue (**SIGMA-ALDRICH**)
- Leupeptin (**SIGMA-ALDRICH**)
- Aprotinin (**SIGMA-ALDRICH**)
- 4- (2-aminoethyl) benzenesulfonyl fluoride (AEBSF) (**SIGMA-ALDRICH**)
- BCA Protein Assay reagent Kit (**PIERCE**)
- Albumin from bovine serum (BSA) (**SIGMA-ALDRICH**)
- Markers: Seeblue Plus2, Magic Mark XP Western standard (**INVITROGEN**)
- NuPage Novex Bis-Tris Gel 12% acrylamide-bisacrylamide (**INVITROGEN**)
- Running buffer: NuPage MOPS SDS 20x (**INVITROGEN**)
- Antioxidant: NuPage (**INVITROGEN**)
- Transfer buffer: NuPage 20x (**INVITROGEN**)
- Chromatography paper 3MM (**WHATMAN**)
- Immobilon-P transfer polyvinylidene fluoride membrane (PVDF) (**MILLIPORE**)
- Tween 20 (polyoxyethylene sorbitan monolaurate) (**SIGMA-ALDRICH**)
- Primary antibodies: β-Actin (**SIGMA-ALDRICH**), caveolin-1 (**BD TRANSDUCTION LABORATORIES**), cyclin D1, cyclin E, CDK2, CDK4,

pRb, Akt/pAkt, ERK/pERK, STAT3/pSTAT3, p21 (**SANTA CRUZ**).

- Secundary antibodies: anti-mouse IgG-HPR, anti-rabbit IgG-HPR (**SANTA CRUZ BIOTECHNOLOGY**), anti-goat IgG-HPR (**PIERCE**)
- Western Blotting Luminol Reagent (**SANTA CRUZ BIOTECHNOLOGY**)
- Restore ™ Western Blot Stripping Buffer (**THERMO SCIENTIFIC**)
- High performance chemiluminescence film: Amersham Hyperfilm™ ECL (**GE HEALTHCARE**)
- Developer and replenisher (**KODAK GPX**)
- Fixer and replenisher (**KODAK GPX**)
- SiRNA duplex oligoribonucleotides (**INVITROGEN**)
- Oligofectamine Transfection Reagent (**INVITROGEN**)
- OPTI-MEM (**GIBCO-INVITROGEN**)
- DEPC-treated Water (**INVITROGEN**)
- 4-hydroxytamoxifen (4-OHT) (**SIGMA-ALDRICH**)
- 17β-estradiol (**SIGMA-ALDRICH**)

3. METHODS

3.1 Cell cultures

The human RAL (NSCLC) and SCLC-R1 cell lines were obtained from metastatic lesions of lung adenocarcinoma (Gasperi-Campani A et al, 1998a) and small cell lung carcinoma respectively (Gasperi-Campani A et al, 1998b).

The cells were cultured in H/H medium (a mixture containing equal parts of Dulbecco's modified Eagle's medium and Ham's medium) supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine, 50IU/ml penicillin, 50μg/ml streptomycin and non-essential aminoacids 1% . The human osteosarcoma cell lines MG-63 and HOS were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine, 50IU/ml penicillin and 50μg/ml streptomycin.

Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The Mycoplasma Plus PCR Primer set was employed for mycoplasma testing.

3.2 siRNA transfection

Inhibition of Cav-1 expression in lung carcinoma and osteosarcoma cells was performed using small interfering RNA (siRNA). Three Cav-1 Stealth Select RNAi were designed to target specific sequence of Cav-1:

UCGACCUGGUCAACCGCGACCCUA/UUAGGGUCGCGGUUG ACCAGGUCGA,

CCCACUCUUUGAAGCUGUUGGGAAA/UUUCCCAACAGCUU CAAAGAGUGGG,

UCCGCAUCAACUUGCAGAAAGAAAU/AUUUCUUUCUGCAA GUUGAUGCGGA .

Cells were seeded in 6-well culture palates, grown to 30%-40% confluence and transfected with Cav-1 siRNA reconstituted in siRNA dilution buffer (DEPC- treated Water). Non-specific siRNA was used as negative control. For each plate, 4 µl of siRNA from the stock (40nM) was diluted into 171 µl of transfection medium (OPTI-MEM) and 8 µl of transfection reagent (oligofectamine reagent) was diluted into 17 µl of OPTI-MEM in separate tubes. After incubating for 5 minutes at room temperature, the diluted siRNA was mixed with diluted transfection reagent and further incubated at room temperature for 15 minute to allow complex formation. The complex was added to the plate containing cells with 800 µl of OPTI-MEM. Cells were incubated at 37° for 4 hours. Thereafter, 1ml of culture medium containing 20% serum was added and cells were incubated for further 48 or 72 hours. Cav-1 expression was assayed by immunoblot analysis.

3.3 Growth inhibition assay

Growth inhibition was determined using trypan blue dye exclusion method. Briefly, cells were seeded in 6-well culture plates at a density of 50.000 cells/well (MG-63, SCLC-R1 and RAL) or 15.000 cells/well (HOS), grown for 24 hours and then transfected for 48 or 72 hours. After silencing, transfection mixtures were replaced with fresh medium and the cells were maintained in culture for further 24h, 48h and 72 hours.

At each time point, cell number and viability were determined by trypan blue dye exclusion method.

All data are presented as means of triplicate cultures obtained from at

least three independent experiments.

3.4 Western blot analysis

Cells were lysed by buffer containing: 1mM Na_3VO_4, 1mM $Na_4P_2O_7.10H_2O$, 10mM NaF, 1mM 4-(2-Aminoethil)-benzenesulfonylfluoride, 1% Nonidet P-40, 10μg/ml aprotinin, 10μg/ml leupeptin. Protein concentrations were quantified by bicinchoninic acid (BCA) protein assay (Smith PK et al, 1985). Proteins were separated by SDS-PAGE: an equal amount of protein samples (10 μg) was resolved on 12% SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 20 minutes with 5% BSA in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T). Incubation with the primary antibody was carried out at 4°C overnight, then the membranes were incubated with horseradish peroxidase-labelled secundary antibody for 1h at room temperature.

β-Actin levels were determined as a loading control. Immunological complexes were visualised by an ECL detection system and quantified by densitometric scanning. Whenever required, the blots were stripped by incubating the membrane at room temperature for 30 minutes in stripping buffer. Membranes were washed thoroughly with PBS-T and reprobed with required antibodies.

3.5 Pharmacological treatments

- Estradiol treatments: lung cancer cells (SCLC-R1 and RAL cell lines) were grown in culture plates for 24 hours and then the treatment with 10 nM, 100 nM or 1 μM of 17β-estradiol (E_2) was carried out in H/H phenol red-free medium supplemented

with 5% FBS for 5 days. E_2 was prepared as a 20 µM/ml stock solution in absolute ethanol and stored at -20°. The drug was diluted freshly to the appropriate concentration in the culture medium before each treatment.

- 4-hydroxytamoxifen treatments: lung cancer cells were grown in culture plates for 24 hours, followed by 24 hours in H/H phenol red-free medium without FBS and then the treatment with 1 µM, 5 µM or 10 µM of 4-hydroxytamoxifen (4-OHT) was carried out in H/H phenol red-free medium supplemented with 10% FBS for 24, 48 and 72 hours. 4-OHT was prepared as a 25 mM stock solution in 95% ethanol and stored at -20°. The drug was diluted freshly to the appropriate concentration in the culture medium before each treatment.

At the end of each treatment, cell number and viability were determined by trypan blue dye exclusion method.
All data are presented as means of triplicate cultures, obtained from at least three independent experiments.

3.6 Statistical analysis

All the results are expressed as means ± standard error (S.E.). Statistical analysis of the data was performed using the Student's t-test. *P* values of ≤ 0.05 were considered as significant.

4. RESULTS

Recent data obtained in the laboratory where I performed my Doctoral three-year period showed a high expression of Cav-1 in human cancer cell lines derived from metastatic lung cancer and from osteosarcoma. The cell lines were from small cell lung carcinoma (SCLC-R1), adenocarcinoma (RAL) and osteosarcoma (MG-63 and HOS) respectively.

The starting point for the results presented here is the consideration of the complex role exerted by Cav-1 in human malignancies, which seems to be dependent on histotype, stage and grade of each single neoplastic disease, so that it may act as an oncosuppressor gene in some tumours *in vivo* like ovary carcinomas, sarcomas and leukemia, whereas it looks like a promoter of tumour growth in thyroid, prostate, bladder and pancreas carcinomas, where it associates with high risk of metastasis, chemoresistance and apoptosis suppression.

The aim of the prosecuting study during my doctoral period was that to analyse the effects of Caveolin-1 gene silencing on lung cancer and osteosarcoma cell growth and proliferation. These tumours were chosen due to previous results obtained in the laboratory, indicating the down-regulation of Cav-1 protein as a novel target of a group of anticancer drugs.

4.1 Lung cancer

- ✓ ***siRNA-mediated knock-down of Cav-1 in lung cancer in vitro***

To assess the role of Cav-1 in metastatic lung cancer cell growth and proliferation, we knocked-down the expression of Cav-1 protein by small interfering RNA (siRNA) duplex either in the RAL or in the SCLC-R1 metastatic cell lines. Western Blotting analysis showed a highly significant reduction of protein levels in each cell line: in particular, the inhibition of Cav-1 protein expression reached 100% in SCLC-R1 and 80% in RAL lung cancer cell lines (fig.1). 72 hours after transfection, fresh medium was added to cells which were maintained in culture for further 72 hours. As shown in figure 2, the inhibition of Cav-1 expression remained stable up to the maximum time of 72 hours after transfection tested.

- ✓ ***Growth inhibition of lung cancer cells by knock-down of Cav-1***

The Cav-1 knock-down obtained either in the SCLC-R1 and in the RAL cell lines gave rise to the arrest of cell cycle progression in both cell lines, identified immediately at the end of transfection and maintained stable up to the maximum time of 72 hours tested (fig.3 and fig.4).

- ✓ ***Cav-1 silencing affects the expression of cell cycle regulatory proteins***

To elucidate the specific cell cycle regulatory proteins responsible for the growth inhibition mediated by Cav-1 knock-down, we analysed the expression of several molecules involved in cell proliferation. As shown in figure 5, cells transfected with Cav-1 siRNA showed a

marked reduction of cyclin D1 and CDK4 expression. Consequently, protein levels of phospho-Rb (on ser^{795} and ser^{780}) were evaluated, which are known to be regulated primarily by the complex of cyclin D1 associated with CDK4. A significant reduction of pRb on ser^{795} and ser^{780} expression was detected either in the RAL or in the SCLC-R1 cells treated with cav-1 siRNA (fig.5).

- ✓ ***The knock-down of Cav-1 by siRNA inhibits STAT3 signalling pathway***

It is known that Cyclin D1 is a target gene for STAT3 transcription factor activity. For this reason, we evaluated the expression of STAT3 and phospho-STAT3 (Tyr^{705}) and results indicated a significant decrease of STAT3 and phospho-STAT3 (Tyr^{705}) expression, consistent with growth inhibition due to the Cav-1silencing (fig.6). As a consequence, we examined the expression of the kinase proteins Akt and ERK, which are known to be regulators of STAT3 activation. As shown in figure 6, p-Akt and p-ERK were down-regulated in the cells treated with Cav-1 siRNA, whereas the expression of total Akt and ERK remained unchanged.

4.2 Osteosarcoma

According to the results obtained in metastatic lung cancer cell lines, the study proceeded with the evaluation of the effect of Cav-1 silencing on osteosarcoma cell lines proliferation.

- ✓ ***siRNA-mediated knock-down of Caveolin-1 in osteosarcoma in vitro***

To assess the role of Cav-1 in osteosarcoma cell growth and proliferation, we knocked-down the expression of Cav-1 protein by small interfering RNA (siRNA) duplex either in the MG-63 or in the HOS cell lines. Western Blotting analysis showed a highly significant reduction of protein levels in each cell line: in particular, the inhibition of Cav-1 protein expression reached 100% in HOS and 80% in MG-63 osteosarcoma cell lines (fig.7). 48 hours after transfection, fresh medium was added to cells which were maintained in culture for further 72 hours. As shown in figure 8, the inhibition of Cav-1 expression remained stable up to the maximum time of 72 hours after transfection tested.

- ✓ ***Growth inhibition of osteosarcoma cells by knock-down of Cav1***

The Cav-1 knock-down obtained either in the MG-63 or in the HOS cell lines gave rise to the arrest of cell cycle progression in the MG-63 cell line, identified immediately at the end of transfection and maintained stable up to the maximum time of 72 hours tested (fig.9). As regards the HOS cell line, the proliferation was reduced at the time of transfection and remained stable up to 24 hours, then began to grow and returned near to normal levels at 72 hours after transfection

(fig.10).

- ✓ ***Cav-1 silencing affects the expression of cell cycle regulatory proteins***

To elucidate the specific cell cycle regulatory proteins responsible for the growth inhibition mediated by Cav-1 knock-down, we analysed the expression of several molecules involved in cell proliferation. As shown in figure 11, cells transfected with cav-1 siRNA showed a reduction of cyclin E and CDK2 expression more evident at increasing times after transfection tested, whereas the expression of cyclin D1 remained unmodified. Consequently, protein levels of phospho-Rb (on ser^{795} and ser^{780}) were evaluated, which are known to be regulated by the complex of cyclin E associated with CDK2. A reduction of pRb on ser^{795} expression, more evident at increasing times after transfection tested, was detected in MG-63 and HOS cells treated with Cav-1 siRNA (fig.11).

- ✓ ***The Cav-1 silencing induces p21-mediated negative signalling pathway of cell cycle***

It is known that Cyclin E-CDK2 complex may be inhibited by p21, a relevant negative regulator of cell cycle. For this reason we evaluated the expression of p21 by Western blotting analysis and the results showed a significant increase in p21 expression levels in each osteosarcoma cell line transfected with Cav-1 siRNA (fig.11).

On the basis of these results, we evaluated the expression of Akt, a protein kinase which is known to interact with caveolin-1 scaffolding domain and to down-regulate p21. A reduction of phospho-Akt expression was detected either in the MG-63 or in the HOS cells

treated with Cav-1 siRNA, more evident at increasing times after transfection tested (fig.12).

4.3 Sensitivity of metastatic lung cancer to estrogen stimulation *in vitro*

During my three-year period of Doctorate in Oncology I have been involved also in a second part of the laboratory projects, regarding the investigation of the role of estrogens in lung cancer, which has not been yet clarified, and the functional cross-talk between Cav-1 and estrogens/estrogen receptors in it.

To begin, we evaluated the effect of 17β-estradiol on the proliferation of lung cancer *in vitro*, and to establish whether estrogens could modulate cell proliferation of metastatic lung cancer cells from small cell lung cancer (SCLC-R1) and from lung adenocarcinoma (RAL), cells were exposed to increasing concentrations of 17β-estradiol (E_2) and the effect of the treatment on cell proliferation was tested. Results indicate a significant increase in cellular growth in both cell lines in the range of concentrations used (fig.13 and fig.14).

The second step was the evaluation of the effect, if one, of 4-hydroxy-tamoxifen (4-OHT) on the growth of both SCLC-R1 and RAL cell lines. Results indicate a growth inhibitory effect exerted by 4-OHT either in the SCLC-R1 or in the RAL cell line (fig.15 and fig.16), in a dose- and time-dependent manner.

5. DISCUSSION

Recent data obtained in the laboratory where I performed my Doctoral three-year period showed a high expression of Cav-1 in human cancer cell lines derived from metastatic lung cancer and from osteosarcoma. The cell lines were from small cell lung carcinoma (SCLC-R1), adenocarcinoma (RAL), which have been stabilized and characterized in the same laboratory (Gasperi-Campani A et al, 1998a; Gasperi-Campani A et al, 1998b) and osteosarcoma (MG-63 and HOS). These last two cell lines are different as regards presence (HOS) or absence (MG-63) of TP53 gene.

The starting point for the results presented here is the consideration of the complex role exerted by Cav-1 in human malignancies, which seems to be dependent on histotype, stage and grade of each single neoplastic disease, so that it may act as an oncosuppressor gene in some tumours *in vivo* like ovary carcinomas, sarcomas and leukemia, whereas it looks like a promoter of tumour growth in thyroid, prostate, bladder and pancreas carcinomas in vivo, where it associates with high risk of metastasis, chemoresistance and apoptosis suppression. Lung cancer and osteosarcoma were chosen here as experimental models where to analyze the effects of Caveolin-1 gene silencing on cell growth and proliferation due to previous results obtained in the laboratory, indicating the down-regulation of Cav-1 protein as a novel target of a group of anticancer drugs (unpublished data).

Emerging evidence indicates a close relation between Cav-1 protein and the development of lung cancer (Yoo SH et al, 2003). Recent studies reported Cav-1 expression in approximately 30% of resected primary NSCLCs and association of it with poor prognosis of primary squamous cell lung cancer (Racine C et al, 1999; Razani B et al, 2000;

Heighway J et al, 2002; Wikman H et al, 2002). Sunaga et al, 2004, hypothesized different roles for Cav-1, as a suppressor gene in SCLCs and as a growth promoter in NSCLCs, where it appears necessary for survival and growth.

In the present study, we first of all analyzed the role of Caveolin-1 in lung cancer cell lines, SCLC-R1 and RAL, which have a metastatic origin. Our data show that Cav-1 silencing blocks the metastatic proliferation either in the RAL or in the SCLC-R1 cells, suggesting a proliferation/survival promoting action of cav-1 in both cell lines.

In different tumours, there is evidence suggesting that Cav-1 promotes cancer growth via inhibition of apoptosis through induction of cyclin D1 (Nagajyothi F et al, 2006), which belongs to a family of proteins that promote progression through the G1-S phase of cell cycle by binding to cyclin-dependent kinase (CDK)-4 and as a consequence phosphorylating the retinoblastoma pRb protein, with release of the E2F transcription factor to permit progression in the cell cycle. Several cancers, including breast, colon and prostate, overexpress the cyclin D1 gene (Jares P et al, 1997; Drobnjak M et al, 2000; Massague J et al, 2004; Arnold A and Papanikolaou A, 2005; Barbieri F et al, 2004). Our results indicate both pRb on ser^{795} and pRb on ser^{780} down-regulation in the Cav-1 silenced RAL and SCLC-R1 metastatic cells, so suggesting that the knock-down of Cav-1 may arrest cell proliferation by regulating pRb phosphorylation. A significant reduction of CDK4 and cyclin D1 expression also has been detected in RAL and SCLC-R1 cells treated with Cav-1 siRNA, indicating that the regulation of phospho-Rb induced by down-regulation of Cav-1 is mediated possibly *via* the inhibition of cyclin D1.

Several studies (Darnell JE, 1997; Levy DE and Darnell JE, 2002; Benekli M et al, 2003) indicated that cyclin D1 is the primary target

gene of STAT3. STATs are a family of transcription factors, which may be activated by phosphorylation of a conserved tyrosine residue in response to extracellular signalling molecules, such as cytokines and growth factors (Benekli M et al, 2003; Adach A et al, 2009; Aktinson GP et al, 2010). There is evidence (Bromberg JF et al, 1999) showing that aberrant activation of STAT3 signalling plays a critical role in oncogenesis. STAT3 resides in fact in the cytoplasm of quiescent cells and may be activated by growth factors and cytokines via the Janus Kinase (JAK) family of proteins. Upon activation, phospho-STAT3 forms homodimers or heterodimers and translocates to the nucleus to induce gene transcription. Constitutively active STAT3 and its over-expression have been detected in a wide variety of human malignancies (Turkson J et al, 2000; Yu H et al, 2004) and aberrant active STAT3 promotes uncontrolled growth and survival through an up-regulation of downstream targeted genes encoding apoptosis inhibitors and cell-cycle regulators, such as cyclin D1 (Grandis JR et al, 2000; Epling-Burnette PK et al, 2001; Benekli M et al, 2003; Diaz N et al, 2006). These abnormal changes in gene expression result in the inhibition of apoptosis or in the dysregulation of cell cycle. On the other hand, selective inactivation of STAT3 may lead to the inhibition of cell proliferation and to the induction of apoptosis in various cancer cell lines (Niu G et al, 1999; Ni Z et al, 2000; Liu PS et al, 2010). All these findings suggest that STAT3 may represent a promising target for anticancer therapy. Consistent with growth inhibition by down-regulation of Cav-1, a significant decrease of STAT3 and phosphorylated STAT3 (on tyr^{705}) expression levels was observed here in the RAL and SCLC-R1 cells where Cav-1 had been silenced.

A recent study (Guruswamy S et al, 2009) demonstrated that the Cav-

1 dependent cell survival signals are mediated through Akt activation as well as through its down-stream effectors, among which ERK and STAT3. Akt, a protein kinase associated with the scaffolding domain of Cav-1, is involved in several signal transduction pathways of cell proliferation and apoptosis in different tumours. This protein is able to facilitate growth factor-mediated cell survival and to block apoptotic cell death. After its phosphorylation, Akt promotes the activation of ERK and subsequently STAT3 in different tumours (Datta SR et al, 1999; Kortylewski M et al, 2003). A significant reduction of pAkt expression was observed here in the RAL and SCLC-R1 cells where Cav-1 had been silenced, together with a significant decrease in the phosphorylation of ERK. The ERK signalling cascade is critical for transducting signals essential for cell survival and activated ERK can phosphorylate a variety of transcription factors regulating expression genes such as STAT3.

In summary, the results obtained here in metastatic lung cancer *in vitro* indicate the activation of Akt by its interaction with the scaffolding domain of Cav-1, and this promotes the ERK and STAT3 phosphorylation and subsequently the transcription of cyclin D1 gene. The activation of cyclin D1 expression and its interaction with CDK4 lead to pRb phosphorylation and then to progression through the cell cycle. This is the first report of a block of metastatic lung cancer proliferation obtained by the Cav-1 silencing, in the same time suggesting a new cell cycle inhibiting pathway, mediated by Cav-1 knock-down, in lung cancer and providing new insights into the molecular mechanisms underlying the pro-survival and tumour-promoting functions of Cav-1.

As regards osteosarcoma, it is known that Cav-1 frequently acts as an oncosuppressor in sarcomas *in vivo* (Wiechen K et al, 2001; Williams

TM et al, 2004; Sloan EK et al, 2004). However, its role in bone development and maturation as well as in osteoblast transformation and sarcoma progression is still undefined. Recently Cav-1 has been found necessary for tumorigenesis of Ewing's sarcoma (Tirado OM et al, 2006).

Here we demonstrate that the suppression of Cav-1 obtained in the osteosarcoma MG-63 and HOS cell lines by siRNA results in the block of MG-63 growth and in the slowing down of HOS growth, suggesting a proliferation/survival-promoting action of Cav-1, different in the cell lines analysed. We are now studying in the lab the possible involvement of TP53 in this result.

It is known that cyclins, in particular cyclin D1 and cyclin E, by binding to cyclin-dependent kinases (CDKs), regulate the cell cycle promoting progression through the G1-S phase in osteosarcoma also (Nurse PM, 2002; Sherr CJ and Roberts JM, 2004). In our hands, in the MG-63 and HOS cells treated with Cav-1 siRNA, the cyclin E expression was significantly decreased, whereas cyclin D1 expression remained unmodified, indicating that the inhibition of proliferation by Cav-1 knock-down is mediated here possibly *via* the inhibition of cyclin E.

It is known that the cyclin E-CDK2 complex regulates pRb phosphorylation in osteosarcoma also, which represents a critical step in the G1 to S phase transition (Nevins JR et al, 1997). The results presented here indicate that both CDK2 and pRb (on ser^{795}) are down-regulated in transfected MG-63 and HOS cells, so suggesting that the knock-down of Cav-1 affects cell proliferation by regulating pRb phosphorylation.

Two different cyclin-dependent kinase inhibitors (CDKI) are known: the INK4 family and the CIP/KIP family which are able to stop the

cell cycle progression in response to appropriate regulatory signals. The first group is composed by four members, p15, p16, p18 and p19, which inhibit the kinase activity of the CDK4/cyclinD1 complexes; the CYP/KIP family is composed by three members, p21, p27 and p57, that hinder the kinase activity of CDK2/cyclinE complexes. Consistent with growth inhibition or slowing down by down-regulation of Cav-1, a significant increase of p21 expression has been observed in MG-63 and HOS cells where Cav-1 had been silenced. As already stated above, it has been demonstrated that Akt down-regulates p21 expression (Viglietto G et al, 2002; Andreu EJ et al, 2005; Motti ML et al, 2005) and that Akt is a tyrosine kinase which interacts with Cav-1 scaffolding domain and plays a relevant role in tumorigenesis, affecting the growth and survival of cancer cells through the phosphorylation and relocalization of key regulatory molecules such as p21 (Zhou BP et al, 2001). In the present study, a significant reduction of phospho-Akt expression has been detected in Cav-1 knocked-down MG-63 and HOS cells.

In summary, we show in the present study that the activation of Akt, by its interaction with the scaffolding domain of Cav-1, promotes the down-regulation of p21 and thus the activation of cyclin E-CDK2 complex which lead to Rb phosphorylation and to progression through cell cycle. We show also that the suppression of Cav-1 expression obtained by siRNA, inhibiting this signalling cascade, affects the MG-63 and HOS osteosarcoma cell lines growth, causing the arrest of cell proliferation in MG-63 cells and a slowing down of it in the HOS cells. The study is in progress to understand the role that TP53 may have in this respect.

As for the results obtained in metastatic lung cancer cells, those obtained in osteosarcoma cells suggest a new cell cycle inhibitor

pathway, Cav-1 knock-down mediated, and provide new insights into the molecular mechanisms underlying the pro-survival and tumour-promoting role of Cav-1 in osteosarcoma.

As for the second part of the laboratory projects, in which I have been involved, this regards the investigation on the role of estrogens in lung cancer, which has not jet been clarified, and the functional cross-talk between Cav-1 and estrogens/ estrogen receptors in it.

It has been recently reported in the literature the 2-fold higher frequency of lung cancer in never-smoking women than in never-smoking men, suggesting the involvement of gender-dependent factors, like estrogens, in the etiology of this tumour (Omoto Y et al, 2001.)

However, the role of estrogens in lung cancer is unclear: several studies on sex differences in lung cancer risk and disease presentation have suggested that estrogen-signalling pathways may play a key role in the genesis and in controlling the growth of lung cancer (Stabile LP and Siegfried JM, 2003); on the other hand, the higher survival rates for women with non-small cell lung cancer (NSCLC) in respect to men seems to indicate a protective effect of estrogens (Moore KA et al, 2003.)

The cellular response to estrogens is mediated by estrogen receptor α (ERα) and β (ERβ) and the orphan receptor GPR30 (G protein-coupled receptor-30), which has been recently implicated in rapid and specific estrogens binding in mediating the action of several estrogenic compounds (Madeo A et al, 2010).

Dougherty SM et al, in nine different human adenocarcinoma cell lines from male or female lung cancer patients, demonstrated that although no differences in the levels of ERα and ERβ could be

detected, there are significant differences in cellular responses to 17β-estradiol (E_2) and to the anti-estrogens 4-hydroxytamoxifen (4-OHT) between cell lines from males and females. In his study, whereas the proliferation of the cell lines from females is stimulated by E_2 and blocked by concomitant administration of 4-OHT, the cell lines from males do not respond to these treatments (Dougherty SM et al, 2006).

In the present study, we evaluated the effect of 17β-estradiol (E_2) on the proliferation of lung cancer *in vitro*, to establish whether oestrogens could modulate cell proliferation of metastatic lung cancer cells from small cell lung cancer (SCLC-R1) and from lung adenocarcinoma (RAL) in a manner dependent or not by the original gender of the patients from which the cells had been derived. The cell lines express ERβ and GPR30 and not ERα (previous data from this laboratory). We could observe a significant increase in cellular growth in both cell lines in the range of E2 concentrations used, independently of the different male (SCLC-R1) or female (RAL) origin.

On the bases of these results, we proceeded investigating the growth inhibitory effect, if one, exerted by 4-OHT either in the SCLC-R1 or in the RAL cell lines and could observe that both cell lines are sensitive to 4-OHT antiproliferative effect; additional studies are now in progress in the lab to a better understanding of the ERs (ERα, ERβ, GPR30) role in these tumours.

F. Pancotti, L. Roncuzzi, M. Maggiolini, A. Gasperi-Campani

Caveolin-1 silencing blocks metastatic lung cancer proliferation *in vitro* (2010 nov, submitted)

6. FIGURES

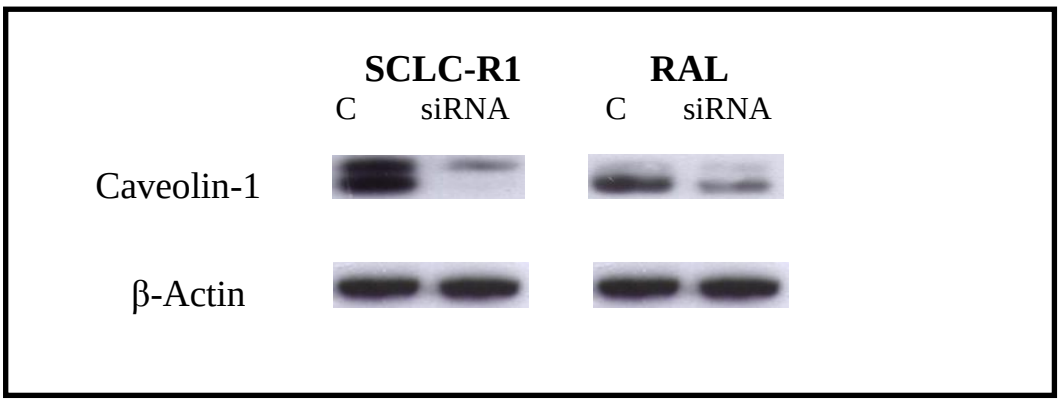

Figure 1. siRNA-mediated down-regulation of Cav-1 expression in lung cancer SCLC-R1 and RAL cell lines: cells were transfected with siRNA targeted against Cav-1 (*siRNA*) or a random sequence (*C*) for 72 hours and the protein expressions were examined by Western blot analysis. β-Actin was used as a protein loading control. The results are representative of 3 independent experiments.

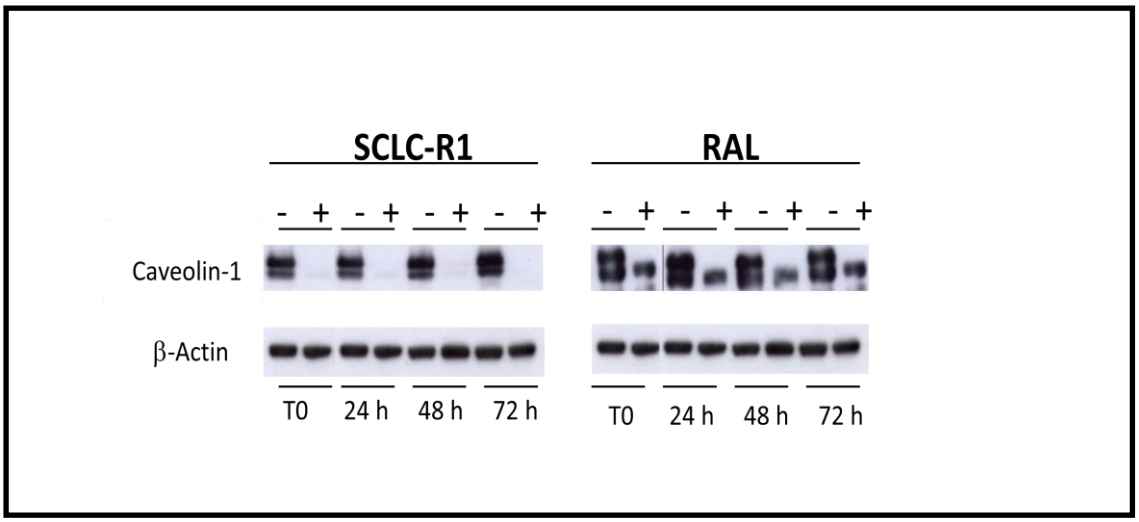

Figure 2. siRNA-mediated down-regulation of Cav-1 expression in lung cancer SCLC-R1 and RAL cell lines: cells were transfected with siRNA targeted against cav-1 (+) or a random sequence (-). 72 hours after transfection (T0) and after 24, 48 and 72 hours, the protein expressions were examined by Western blot analysis . β-Actin was used as a protein loading control. The results are representative of 3 independent experiments.

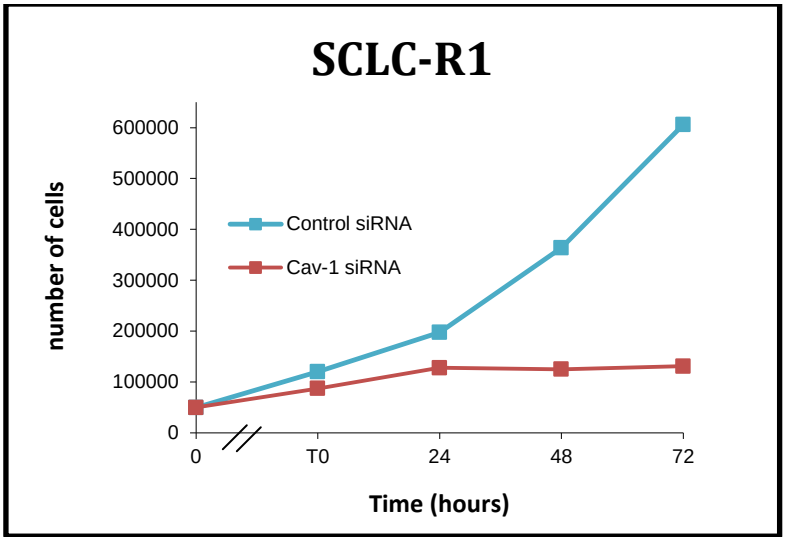

Figure 3. Effect of siRNA-mediated down-regulation of Cav-1 on the proliferation of human lung cancer SCLC-R1 cell line: 72 hours after transfection (0-T0), cells were cultured for 24, 48 and 72 hours, the cell proliferation was analyzed and subsequently the growth curves were plotted. The results are representative of 3 independent experiments.

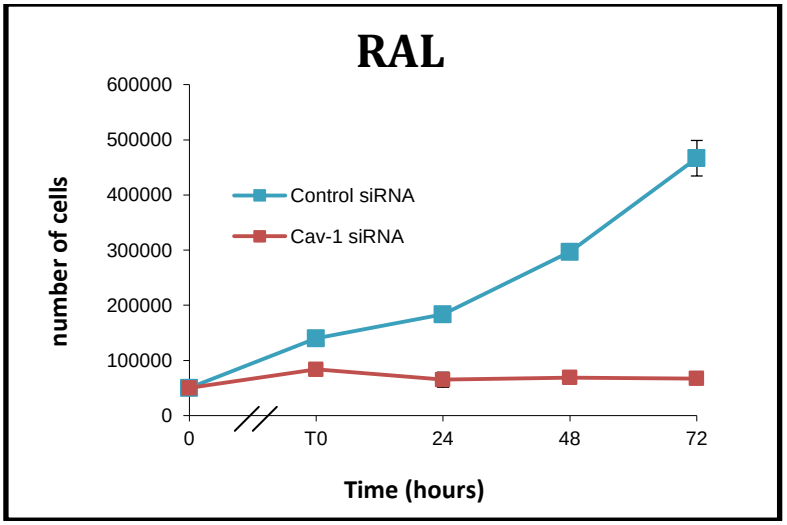

Figure 4. Effect of siRNA-mediated down-regulation of Cav-1 on the proliferation of human lung cancer RAL cell line: 72 hours after transfection (0-T0), cells were cultured for 24, 48 and 72 hours, the cell proliferation was analyzed and subsequently the growth curves were plotted. The results are representative of 3 independent experiments.

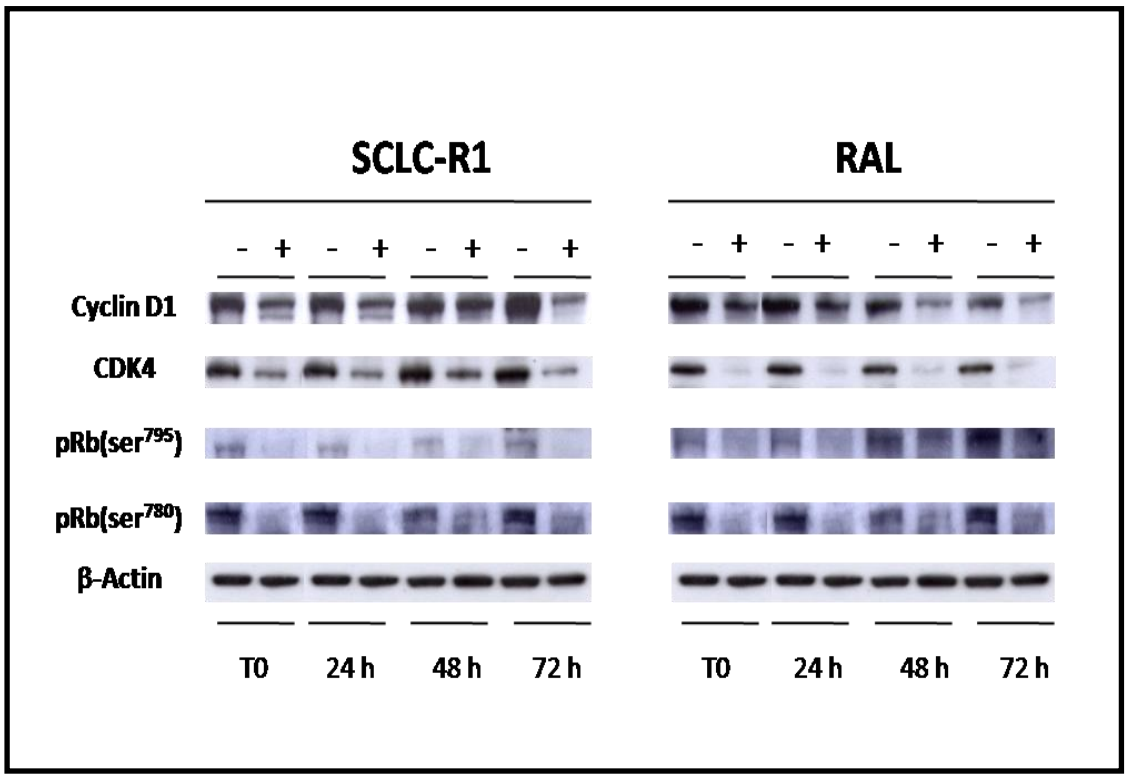

Figure 5. Cav-1 inhibition affects the expression of cell cycle regulatory proteins: SCLC-R1 and RAL cells were treated with Cav-1 siRNA (+) or control siRNA (-) for 72 hours. Immediately after transfection (T0) and after 24, 48 and 72 hours, cyclin D1, CDK4, pRb(ser^{795}) and pRb(ser^{780}) protein expressions were examined by Western blot analysis. β-Actin was used as a protein loading control. The results are representative of 3 independent experiments.

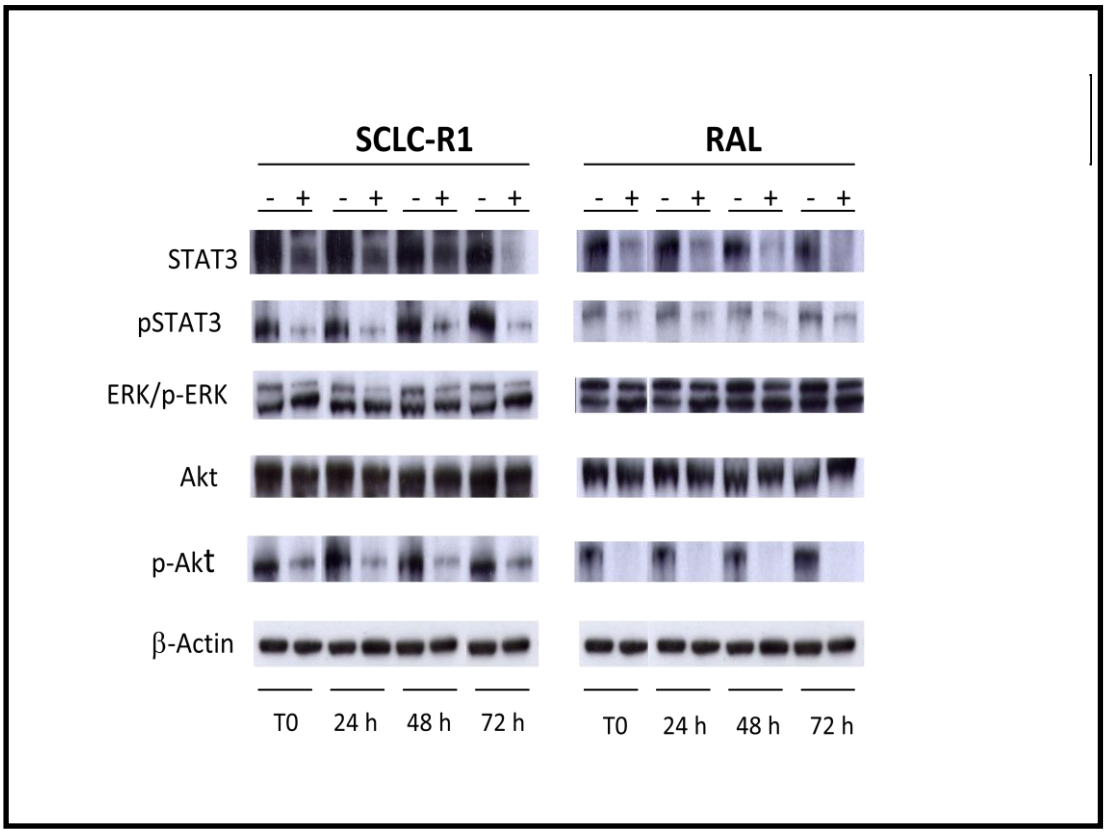

Figure 6. Effect of down-regulation of Cav-1 on STAT3 signalling pathway: SCLC-R1 and RAL cells were treated with Cav-1 siRNA (+) or control siRNA (-) for 72 hours. Immediately after transfection (T0) and after 24, 48 and 72 hours, STAT3, p-STAT3, ERK, p-ERK, Akt and p-Akt protein expressions were examined by Western blot analysis. β-Actin was used as a protein loading control. The results are representative of 3 independent experiments.

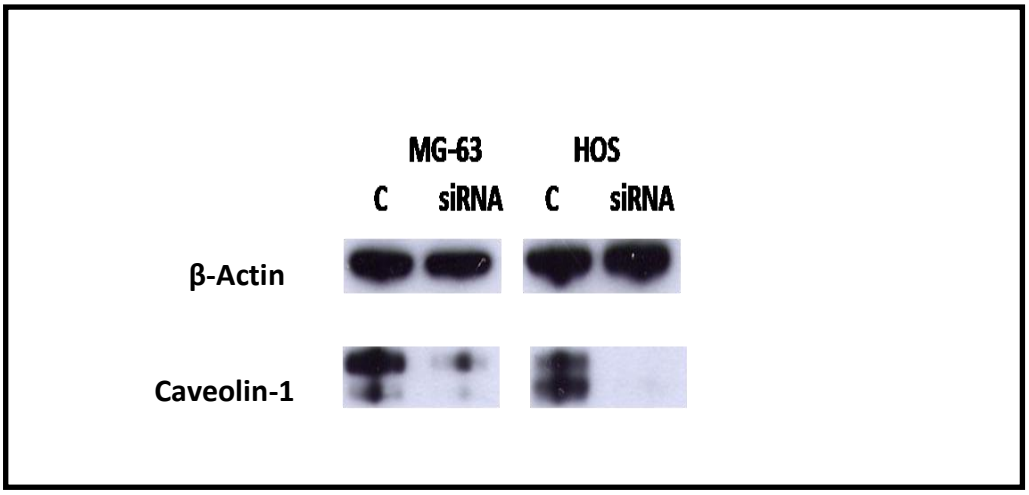

Figure 7. siRNA-mediated down-regulation of Cav-1 expression in human osteosarcoma MG-63 and HOS cell lines: cells were transfected with siRNA targeted against Cav-1 (*siRNA*) or a random sequence (*C*) for 48 hours and the protein levels were examined by Western blot analysis. β-Actin was used as a protein loading control. The results are representative of 3 independent experiments.

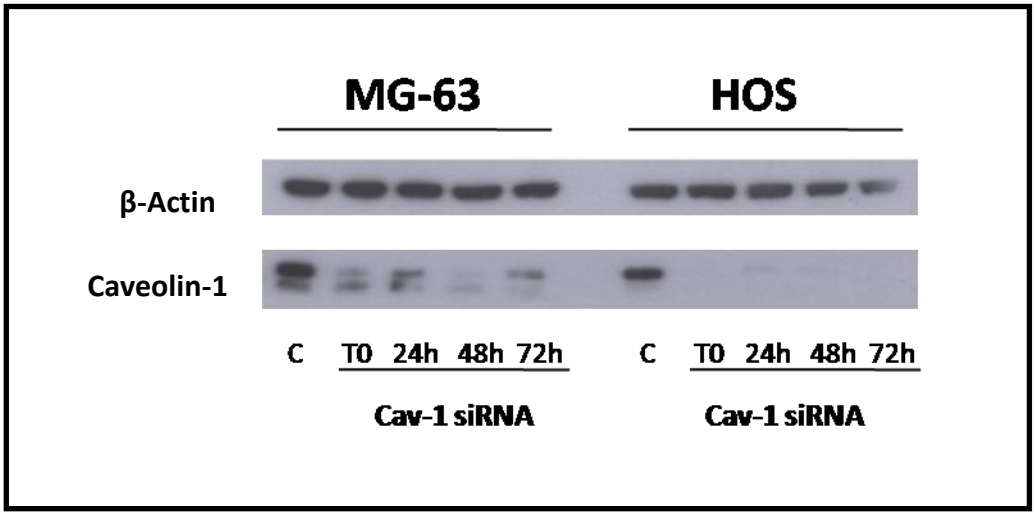

Figure 8. siRNA-mediated down-regulation of Cav-1 expression in human osteosarcoma MG-63 and HOS cell lines: cells were transfected with siRNA targeted against Cav-1 (*Cav-1 siRNA*) or a random sequence (*C*). 48 hours after transfection (T0) and after 24, 48 and 72 hours, the protein expressions were examined by Western blot analysis. β-Actin was used as a protein loading control. The results are representative of 3 independent experiments.

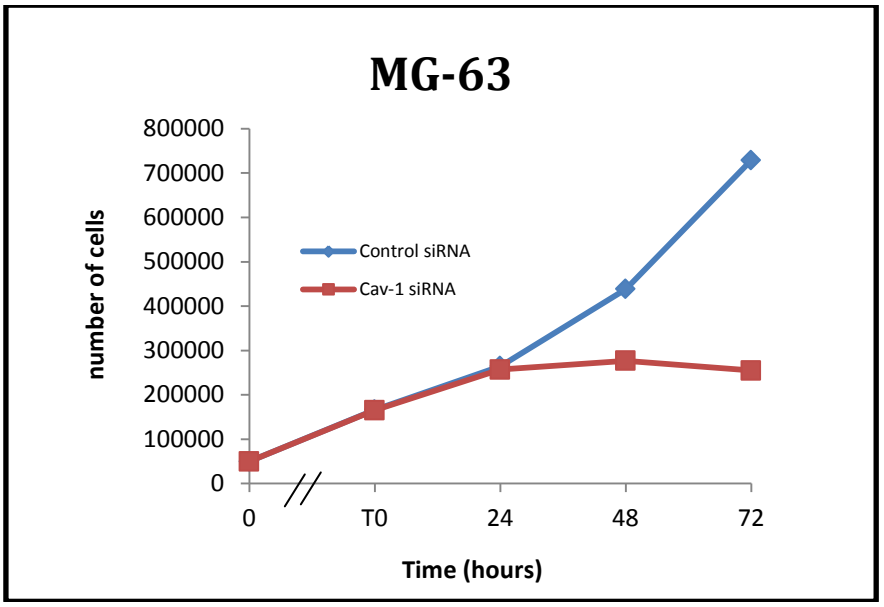

Figure 9. Effect of siRNA-mediated down-regulation of Cav-1 on the proliferation of human osteosarcoma MG-63 cell line: 48 hours after transfection (0-T0), cells were cultured for 24, 48 and 72 hours, the cell proliferation was analyzed and subsequently the growth curves were plotted. The results are representative of 3 independent experiments.

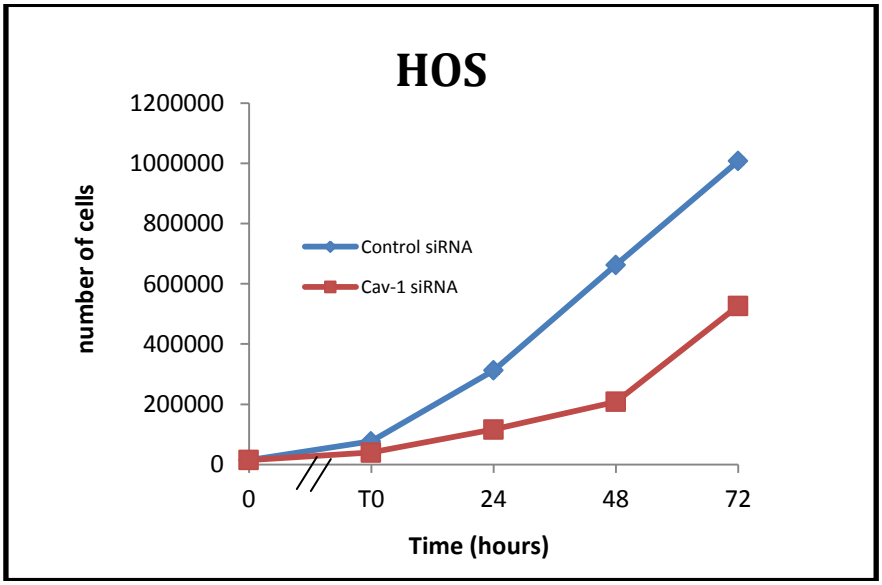

Figure 10. Effect of siRNA-mediated down-regulation of Cav-1 on the proliferation of human osteosarcoma HOS cell line: 48 hours after transfection (0-T0), cells were cultured for 24, 48 and 72 hours, the cell proliferation was analyzed and subsequently the growth curves were plotted. The results are representative of 3 independent experiments.

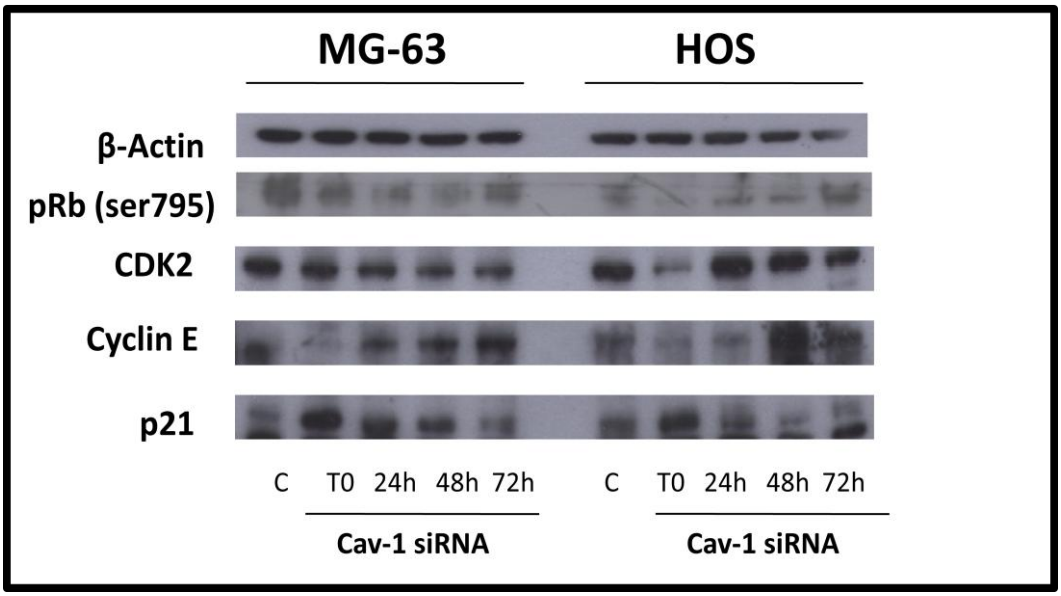

Figure 11. Cav-1 inhibition affects the expression of cell cycle regulatory proteins: MG-63 and HOS cells were treated with Cav-1 siRNA or control siRNA (C) for 48 hours. Immediately after transfection (T0) and after 24, 48 and 72 hours, phospho-Rb (on ser^{795}), cyclin E, CDK2 and p21 protein expressions were examined by Western blot analysis using the respective antibodies. β-Actin was used as a protein loading control. The results are representative of 3 independent experiments.

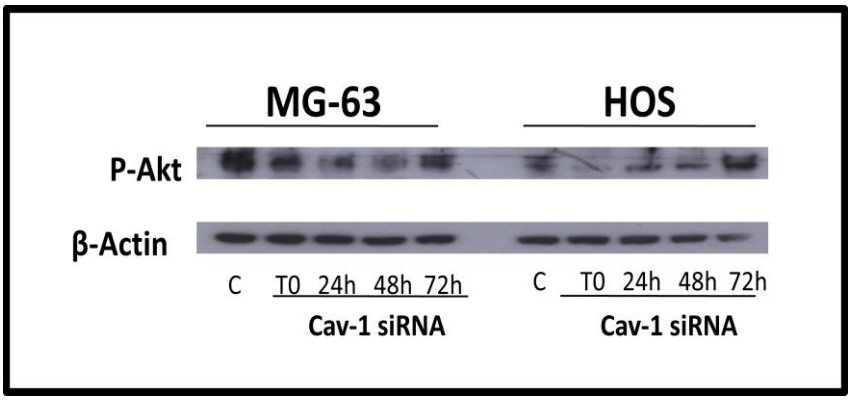

Figure 12. Effect of down-regulation of Cav-1 on p21 signalling pathway: MG-63 and HOS cells were treated with Cav-1 siRNA or control siRNA (C) for 48 hours. Immediately after transfection (T0) and after 24, 48 and 72 hours, Akt /p-Akt protein expressions were examined by Western blot analysis. β-Actin was used as a protein loading control. The results are representative of 3 independent experiments.

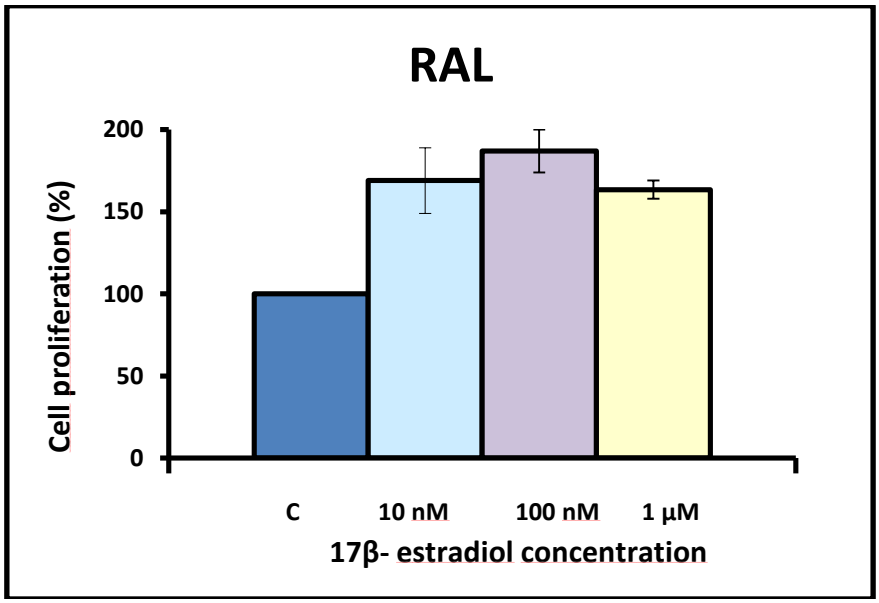

Figure 13. Effect of 17β-estradiol on the proliferation of RAL lung cancer cells: RAL cells were treated with 17β-estradiol. 5 days after treatment, the cell proliferation was analyzed and subsequently the histograms were plotted (C: untreated cells).

The results are representative of 3 independent experiments.

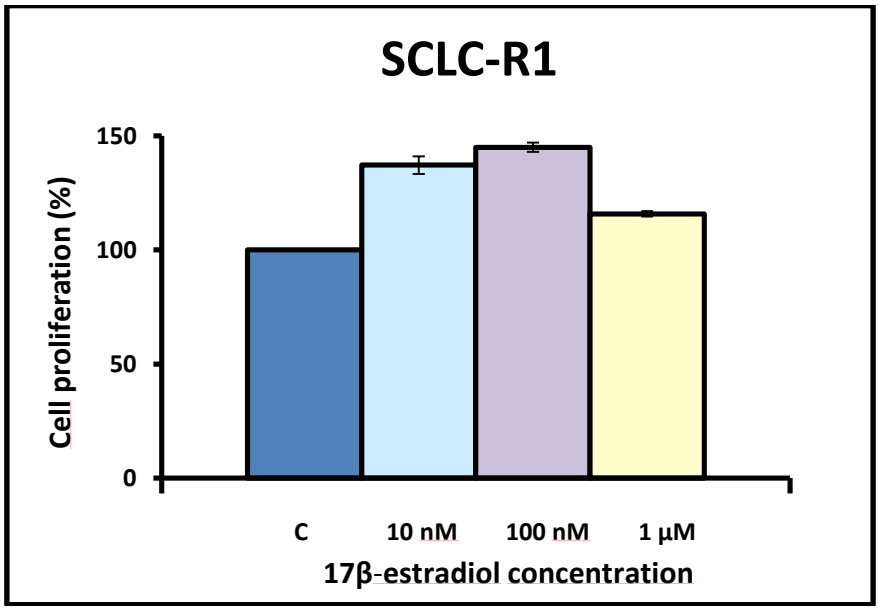

Figure 14. Effect of 17β-estradiol on the proliferation of SCLC-R1 lung cancer cells: SCLC-R1 cells were treated with 17β-estradiol. 5 days after treatment, the cell proliferation was analyzed and subsequently the histograms were plotted (C: untreated cells).
The results are representative of 3 independent experiments.

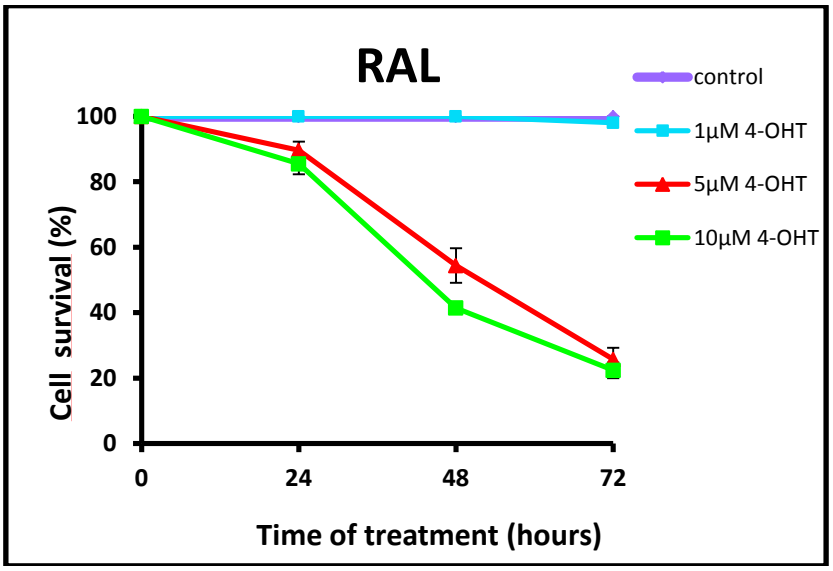

Figure 15. Effect of 4-OHT on the proliferation of RAL lung cancer cells: RAL cells were treated with 4-OHT. 24, 48 and 72 hours after treatment, the cell survival was analyzed and subsequently the growth curves were plotted. The results are representative of 3 independent experiments.

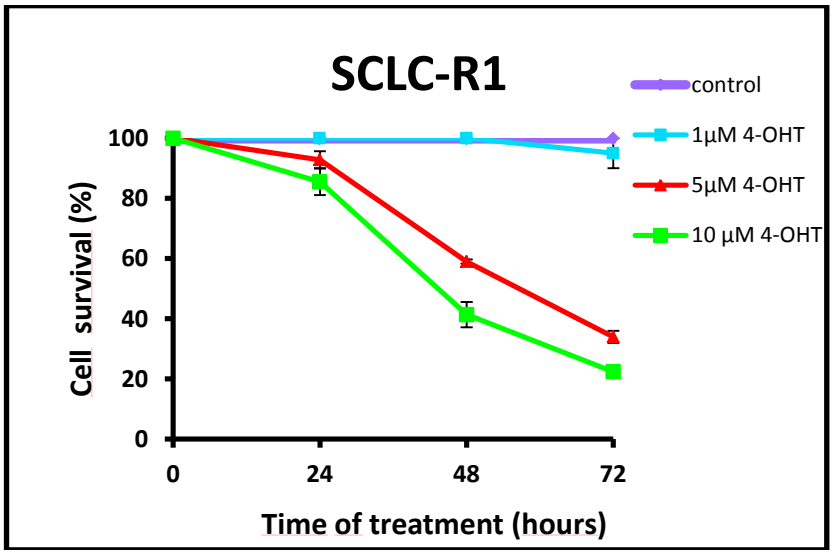

Figure 16. Effect of 4-OHT on the proliferation of SCLC-R1 lung cancer cells: SCLC-R1 cells were treated with 4-OHT. 24, 48 and 72 hours after treatment, the cell survival was analyzed and subsequently the growth curves were plotted. The results are representative of 3 independent experiments.

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