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SLPI: a novel target of ETS proteins in prostate cancer

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ABSTRACT

Prostate cancer is the most common cancer among men in the world. About 60% of prostate cancer patients exhibit a chromosomal rearrangement involving the promoter region of an androgen-regulated gene (e.g. TMPRSS2) and a member of the ETS transcription factor family; this molecular alteration results in the overexpression of the involved ETS transcriptional factor. The most common chromosomal rearrangement in prostate cancer results in the formation of the TMPRSS2-ERG fusion gene; the translocations involving ETV1, ETV4 and ETV5 genes are less frequent.

The oncogenic role of ETV4 has been previously demonstrated in several human prostate cancer cell lines: ETV4 promotes cell proliferation, anchorage independent growth, migration and cell invasion. In addition, in a transgenic mouse model overexpressing ETV4 at prostate level (ETV4 mice), the overexpression of ETV4 induces prostatic intraepithelial lesion (mPIN) and promotes tumor cell proliferation. The oncogenic mechanisms of ETV4 over-expression have been further investigated by microarray gene expression profiling of prostate tissue comparing wild-type and ETV4 mice. The microarray data have shown that the secretory leukocyte peptidase inhibitor (SLPI) is downregulated in ETV4 mice and this ETV4-mediated down-regulation has been confirmed also in human prostate cell lines. SLPI is a serine protease inhibitor involved in inflammatory processes and that plays a role in oncogenesis. It is intriguing, that the negative regulation of SLPI expression in ETV4 mice is in keeping with the peculiar pattern of SLPI expression in prostate cancer patients: SLPI expression is reduced in the early stage of prostate cancer whereas it is increased in a subset of metastatic prostate cancer patients after androgen deprivation therapy.

The first aim of this PhD project was to investigate if ETV1, another ETS transcriptional factor, behaves as ETV4 in the regulation of SLPI expression, considering that also ETV1 is translocated in about 8-10% of prostate cancer patients. The effect of ETV1 on SLPI expression has been investigated in 2 human prostate cell lines (LNCaP and RWPE cells) by 2 complementary approaches: the stable silencing of ETV1 in LNCaP and its stable over-expression in RWPE demonstrated that also ETV1, as ETV4, regulates negatively SLPI expression.

In addition, the role of androgen/androgen receptor axis in the regulation of SLPI expression has been evaluated using as cellular model the androgen-sensitive and

androgen receptor (AR)-positive LNCaP cells. In this cell line the androgenic stimulus has been able to regulate positively SLPI expression, despite the confounding effect of the androgen-mediated ETV1 over-expression that should reduce SLPI expression. The effect of androgens on the regulation of SLPI expression has been confirmed in another androgen-competent human prostate cancer cell line, 22RV1, that does not express any ETS proteins that could represent a confounding factor. These findings about the androgen/AR axis exert a positive regulation of both SLPI and ETS transcriptional factors combined with the negative regulation of SLPI exerted by the ETS transcriptional factors (ETV4, ETV1) have brought us to hypothesize a model that could explain the peculiar behavior of SLPI expression during the prostate cancer progression.

The second aim of this PhD work was to investigate the contribution of SLPI expression in the determination of the neoplastic features of prostate cancer. We performed several functional analyses in human normal prostate cell line (RWPE) stably transduced with shRNA against SLPI and human prostate cancer cell lines (LNCaP and PC3) stably transduced with a vector expressing SLPI. In normal immortalized RWPE cells, SLPI was found to be able to increase cell migration and invasion ability, and to contribute to the epithelial-mesenchimal transition (EMT) by promoting the "cadherin-switch" and by increasing the expression of several transcriptional factors involved in EMT process. Furthermore, SLPI could play a role in cancer-promotion by inhibiting apoptosis. However, in the cancerous LNCaP and PC3 cells the overexpression of SLPI was not able to provide any additional neoplastic features, likely because the neoplastic phenotype of these metastatic cancerous cell lines is already fully developed.
The last aim of this PhD project was to study the mechanism by which ETS proteins regulates SLPI, since chromatin immuno-precipitation (ChIP) experiments have shown that ETV4 does not bind SLPI promoter. In RWPE cells stably transduced with an expressing vector encoding either ETV1 or ETV4, and in PC3 cells stably transduced with shRNA against ETV4, it has been found that ETV1 and ETV4 downregulate STAT1 expression. STAT1 has been reported to be a positive regulator of SLPI by direct binding to SLPI promoter: we observed that in PC3 cells the overexpression of STAT1 increases the SLPI protein level supporting the notion that the ETV1/ ETV4 mediated regulation of SLPI could be mediated by the downregulation of STAT1.

1. INTRODUCTION

1.1 Epidemiology

Prostate cancer (PCa) is the second most common and fifth most aggressive neoplasm among men in the world.

The Global Cancer Observatory (GLOBOCAN 2020, *http://gco.iarc.fr)* estimates 1,414,259 new cases of prostate cancer in 2020 and that 375,304 men died because PCa (Figure 1). Among European men, prostate cancer is ranked first for incidence and it ranks third cause of cancer death, with an estimate of 473,344 new cases and over 100,000 deaths. It has been predicted an increase of 2,235,568 (+58,1%) of prostate cancer cases and of 720,661 (+92%) number of deaths worldwide by 2040.

The incidence and mortality rate of prostate cancer are different between developed and developing countries. The difference in incidence is likely due to a higher number of prostate-specific antigen (PSA) screening tests in developed countries (Bellamri, et al., 2019). In keeping with this explanation, it has been observed that the larger use of PSA screening and subsequent biopsy in European men during the last years has raised the percentage of prostate cancer from about 11% of all cancers in 1995 to about 22% nowadays (Bray, et al.; 2017).

The limited access to the state-of-art treatments is responsible of the high prostate cancer specific mortality in the developing countries: Caribbean, sub-Saharan Africa, parts of South America and Eastern Europe (Nyame, et al., 2020).

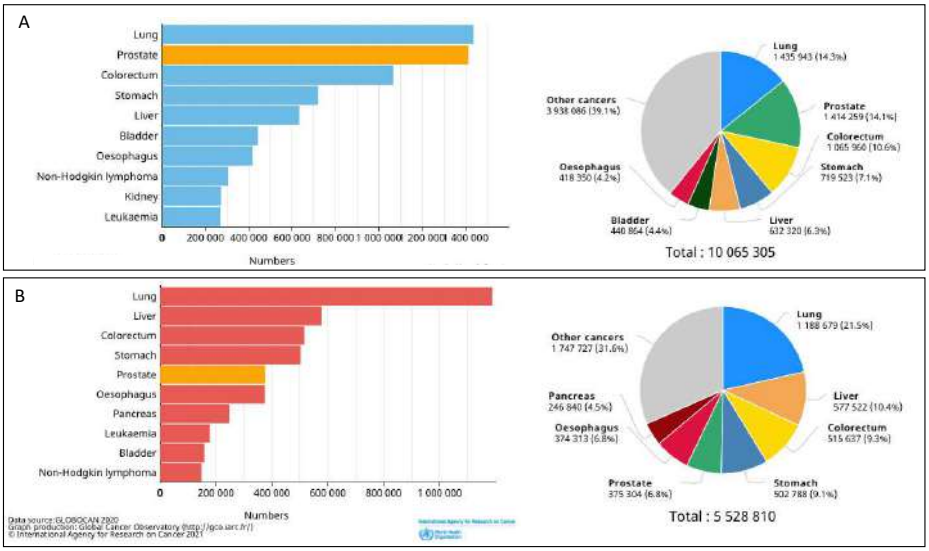


Figure 1: Estimated number of incident cases and deaths worldwide, males, all age. (A) *bar plot showing estimated number of incident cases worldwide, males, all age; pie chart showing estimated number of new cases in 2020, worldwide, males, all age.* ***(B)*** *bar plot showing estimated number of deaths worldwide, males, all age; pie chart showing estimated number of deaths in 2020, worldwide, males, all age.*
Data obtained from Globocan 2020 (http://gco.iarc.fr)

Prostate cancer is a heterogeneous disease ranging from indolent form of the disease to the aggressive tumor. Epidemiologic studies revealed that several factors can contribute to the development and to clinical outcome, including survival, of prostate cancer. The risk factors could be different in the PCa types; thus, it is important to differentiate the risk factors between total prostate cancer and advanced or lethal tumor (Jahn JL, et al., 2015; Pernar CH, et al., 2018).

The recognized risk factors for total prostate cancer are age, race/ethnicity and family history. The incidence rate of prostate cancer increases dramatically over the age of 55 reaching a peak at the age of 70-74 (Gann PH., 2002). Prostate cancer is rare among men younger than 40s; however, taking into account its long induction period, many men may display incipient lesions in their 20s and 30s (Gann PH., 2002; Pernar CH, et al., 2018).

The incidence rate of prostate cancer is about 60% higher in African Americans than in Caucasians and also the mortality rate is approximately double in African Americans than in Caucasians (Gann PH., 2002). Moreover, prostate cancer incidence and mortality rates are lower among Asian/Pacific Islanders, American Indian/Alaskan

Natives, and Hispanic men in comparison with non-Hispanic white men (Pernar CH, et al., 2018). These differences in mortality may be due in part to differences in socioeconomic status and tumor stage at diagnosis, or they could involve genetic factors, environmental factor or a combination of both (Gann PH., 2002).

A family history positive for prostate cancer increases the risk of prostate cancer (Hemminki K, et al., 2002). In fact, a man with prostate cancer whose father or brother died for prostate cancer have twofold risk of death than a man affected by prostate cancer without a positive family history (Brandt A, et al., 2012).

The main risk factors for advanced or lethal prostate cancer include diet rich in fats, dairy consumption and calcium intake, obesity, smoking, and low physical activity.

Several lifestyle modifications may reduce the risk to develop aggressive form of cancer or may offer benefits in patients with prostate cancer (Gann PH., 2002; Pernar CH, et al., 2018).

Currently, most cases at diagnoses are localized cancers that are treated with surgery, radiotherapy or active surveillance. For patients with locally advanced prostate cancer or metastatic disease the main treatment is the androgen-deprivation therapy (ADT). ADT therapy allows the remission of the disease that is evidenced by the reduction of PSA levels in about 90% of patients. However, after 18-24 months some patients may experience disease progression accompanied by an increase of serum PSA, tumor size, metastatic spread and recurring disease-related symptoms. This represents the lethal phenotype of the disease known as castration-resistant prostate cancer (CRPC) (Karantanos, et al.,2013; Lonergan, et al.,2011).

1.2 Anatomy and histology of the human and murine prostatic gland

The human prostate gland is described as a walnut-size tissue surrounding the urethra, it is positioned in the sub peritoneal compartment, between the pelvic diaphragm and the peritoneal cavity.

The prostate has a pyramidal shape and is located inferior to the urinary bladder, posterior to the pubic symphysis and anterior to the rectum; the lower apex of the gland, corresponding to the apex of the pyramid, is in contact with the penile urethra, while the upper base contacts the bladder.

The human prostate is subdivided into three different histological zones: central zone (CZ), transition zone (TZ) and peripheral zone (PZ) (Figure 2).

These three zones have different embryologic origins, histological and anatomical features, biologic functions and susceptibility to pathologic disorders.

The CZ surrounds the ejaculatory ducts, it has a cone shape with the base positioned in correspondence of the prostate's base and the apex in correspondence of the verumontanum. Generally, the CZ does not represent a site of origin of any pathology, although it may be involved in late cancer (Ittmann M, 2018).

The TZ surrounds the prostatic urethra and represents about 5% of the prostatic gland. The TZ is considerably affected by benign prostatic hyperplasia (BPH), a common non-malignant condition of older man (Ittmann M, 2018).

The PZ represents the zone of origin of most prostate adenocarcinomas, this zone is the most voluminous, about 70% of prostate tissue, and extends around the outer portion of the prostate (Ittmann M, 2018).

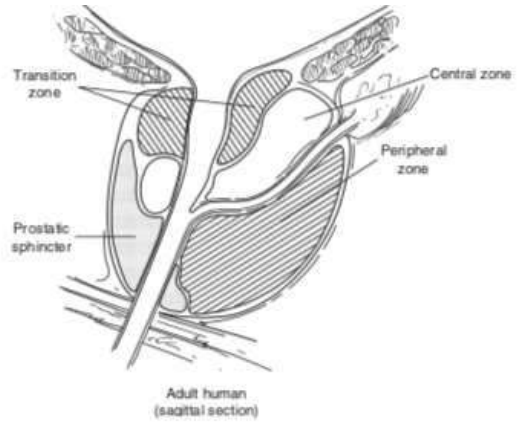


Figure 2: Schematic illustration of human prostate. *Human prostate and location of the three different histological zones: central zone (CZ), peripheral zone (PZ) and transition zone (TZ) (Ittmann M, 2018).*

The murine prostate consists of 4 distinct lobes: ventral, anterior, dorsal and lateral lobes; these last two lobes are considered as a unique dorso-lateral lobe (Figure 3).

These 4 lobular structures surround the urinary bladder and the urethra, more specifically, the ventral lobe is a single structure located ventrally in the midline and dorsally to the urethra, the dorsal prostate is located dorsally near the urethra and at the base of the seminal vesicles; the lateral lobes are located on both sides of dorsal

prostate, the anterior lobes, also known as the coagulating gland, are located along the lesser curvatures of seminal vesicles.

The anterior lobes are analogous to the CZ of human prostate; the dorso-lateral prostate is analogous to the PZ, while murine ventral prostate has not anatomic and histologic analogies with the human prostate (Lee CH, et al., 2011).

Although spontaneous prostate cancer is extremely rare in normal mice, there are several transgenic mouse models in which the prostate cancer occurs mainly in the dorso-lateral lobe.

At histological levels, both mouse and human prostate present a pseudostratified epithelium composed by three types of cells: luminal, basal and neuroendocrine.

Luminal cells constitute a continuous layer of polarized columnar cells. These cells produce a variety of products secretions, including PSA and AR, which contribute to the formation of seminal fluid.

Basal cells are located under the luminal epithelium, adjacent to the basement membrane. Basal cells express low or undetectable levels of AR.

The neuroendocrine cells are rare cells of unknown function, these cells are AR-negative.

Some prostate adenocarcinomas show a strong luminal phenotype and they are considered more aggressive and with a worst prognosis compared with basal-cell-derived prostate cancer (Wang ZA, et al., 2013). However, Smith BA et al., have shown that prostate cancers with basal stem cells signature are more aggressive (Smith BA, et al., 2015).

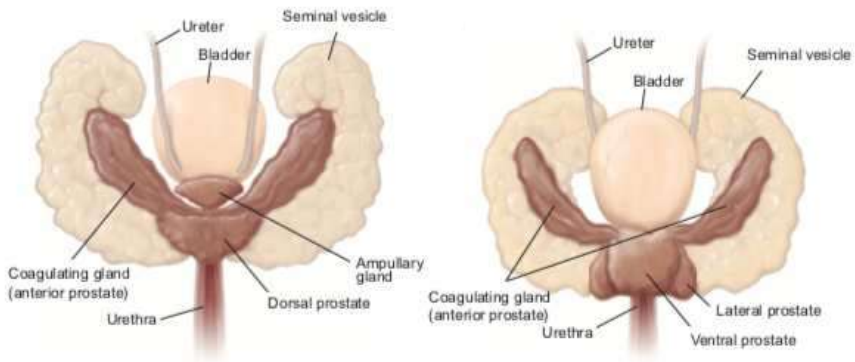


Figure 3: schematic illustration of mouse prostate. *The mouse prostate is subdivided in 4 distinct lobes: ventral lobe, anterior lobe, dorsal lobe and lateral lobe.*

1.3 Diagnosis and treatment of prostate cancer

Most prostate cancer are diagnosed in patients reporting lower urinary tract symptoms (LUTS) such as visible hematuria, dysuria, or erectile dysfunction; nevertheless, a significant fraction of men spends their lives and die with undiagnosed prostate tumor (Merriel SWD, et al., 2018).

The use of PSA blood screening test has allowed the early detection and diagnosis of prostate cancer in asymptomatic men. However, this diagnostic test has some limitations; serum PSA levels can increase in a variety of benign prostate conditions such as, BPH, prostatitis, ejaculation and exercise providing false positive results; this approach can also cause false negative results since, the PSA levels is within the normal range in about 25% of patients with prostate cancer (Schmid HP, et al., 2003).

Other diagnostic methods are used for prostate cancer detection: the Transrectal ultrasound (TRUS) scan, a morphological imaging technique which can be used for guided biopsy and the Color doppler ultrasound, a technique that evaluate the blood flow through prostatic vessels and since micro vessels density is higher in the malignancy, is capable to differentiate the prostate carcinoma from benign hyperplasia (Schatten H., 2018; Sen J, et al., 2008).

Other techniques are used to determine the stage and the possible spreading of cancer: the Positron-Emission Tomography (PET), able to detect the prostate cancer metastasis in different areas of body and thus very useful to evaluate treatments; the Multiparametric Magnetic Resonance Imaging (MRI) to determine the extent and aggressiveness of prostate cancer and an enhancement of the MRI to check lymph nodes state (Schatten H., 2018).

On biopsy tissues it is possible to investigate tumor aggressiveness using novel molecular biomarkers: Decipher, Prolaris and Oncotype DX. The analysis of 31 genes can predict clinical progression and prostate cancer mortality, another assay on 17-gene can predict the risk of adverse pathology at prostatectomy, biochemical recurrence, and metastases and another molecular test based on 22-marker genomic can quantify metastatic risk and also provides prognostic information. These and other molecular biomarkers may help distinguish between an indolent and an aggressive tumor (Litwin MS, et al., 2017).

Although most prostate cancers have an indolent course, many patients with localized or metastatic diseases succumb to the disease in spite of the treatment, (Teo MY, et al., 2019).

Men with a diagnosis of localized disease have 3 different options of treatment:

•Wait-and-see management: disease monitoring without therapy until any sign of disease progression.

•Surgery: that nowadays in the specialized centers includes robotic radical prostatectomy.

•Radiation.

Despite these approaches result curative in most cases, a subset of patients with high Gleason score and high PSA levels at the diagnosis presents a biochemical recurrence rate exceeding 50% at 5-years; the main treatment for these patients with recurrent disease, considering the androgen dependence of prostate cancer, remains the androgen deprivation therapy (ADT) (Teo MY, et al., 2019).

The ADT results in a tumor regression through the induction of tumor cells apoptosis and senescence (Gamat M, et al., 2017).

This treatment approach provides a decrease of serum androgens or of their activity by 1) surgical resection of one or both testicles; 2) gonadotropin releasing hormone (GnRH or LHRH) agonist or an antagonist that decrease hormone production and thus reduces the gonadal production of testosterone; 3) nonsteroidal anti-androgens acting by blocking androgen receptor. The two pharmacological treatments can be used also in combination (Gamat M, et al., 2017).

However, the majority of patients at least 2-3 years after ADT progress to castration-resistant disease (CRPC) (Chandrasekar T, et al., 2015). For the pharmacological treatments for CRPC are used chemotherapeutic agents such as Docetaxel and Cabazitaxel. These chemotherapeutic agents bind tubulin in microtubules, stabilizing them and preventing their depolymerisation that is required for mitosis, resulting, eventually, in the induction of apoptosis. In particular, Docetaxel leads also to phosphorylation of Bcl-2 with consequent caspases activation and apoptosis *in vivo* and *in vitro* (Chandrasekar T, et al., 2015).

Between 2011 and 2012 emerged new hormonal therapies for the treatment of CRPC that include Abiraterone and Enzalutamide using for the first-line treatment of asymptomatic or low symptomatic metastatic castration-resistant prostate cancer

(mCRPC), and for second-line treatment of symptomatic mCRPC in which Docetaxel treatment failed (Mansinho A, et al., 2018).

1.4 Prostate tumor staging

TNM staging system for prostate cancer was introduced in 1992 by the American Joint Committee on Cancer (Beahrs OH, et al., 1992), and it was revised in 1997 (Fleming ID, et al., 1998) and in 2002 (Greene FL, et al., 2002).

The most recent TNM staging system for prostate cancer is based on 3 key pieces of information:

•**Tumor** (**T**): extent of primary tumor that can be either clinical or pathological:

- **Clinical T (cT):** is the best estimate of the extent of the disease based on the result of digital rectal exam, prostate biopsy and imaging tests.
- **Pathologic T (pT):** is more accurate than cT but it can be performed only after the surgical resection because is based on laboratory's exam of the resected prostate.

•**Nodes** (**N**): state of nearby lymph nodes.

•**Metastasis** (**M**): presence of distant metastasis.

The TNM staging is improved by adding two elements: the PSA levels at diagnosis and the grade group based on the Gleason score.

In 1966 Donald F Gleason introduced a classification system for prostate cancer based on architectural pattern of the cancer. In the past two decades the Gleason grading system has reported several important modifications.

Each tumor is classified in a stage (I, II, III, IV) on the basis of spread grade. In addition, the biopsies can be classified as T0 that means there is no sign of cancer.

The Gleason system describes 5 histological growth pattern termed grade; Gleason 1 is referred to nodular lesions circumscribed with uniform, compacted, discrete well-differentiated and normal size glands. Gleason 2 is characterized by lesions that show irregularity at the periphery with glands that display variation in size and an increase of stroma around it. Gleason 3 is the most common pattern, composed by neoplastic gland of generally small but often with variable size that infiltrate in the stroma among normal glands. Gleason 4 is characterized by irregular fused glands or cribriform structures. Finally, Gleason 5 includes solid growth or infiltration by individual tumor cells with the loss of any gland's formation. The Gleason score is the sum of primary pattern

assigned to the predominant histological pattern and the secondary highest patterns present in the scenario (Chen N., et al., 2016).

1.5 Tumorigenesis

The onset of prostate cancer is due to a multistep process that starts as prostatic intraepithelial neoplasia (PIN) followed by localized prostate adenocarcinoma with local invasion that may end in metastatic prostate cancer (Figure 4).

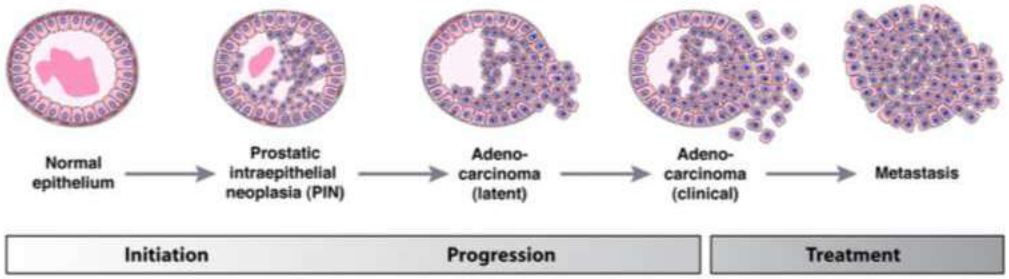


Figure 4: schematic representation of human prostate cancer progression *(Abate-Shen and Shen, 2000).*

1.5.1 Prostatic intraepithelial neoplasia

PIN develops in peripheral zone and is characterized by a proliferation and dysplasia of luminal epithelial cell layer into the pre-existent gland spaces (Brawer M. K., 1992). At the histological level, PIN shows a hyperplasia of luminal epithelial cells with enlarged nuclei and nucleoli, cytoplasmic hyperchromasia, nuclear atypia and a reduction of the basal cell layer that is lost in prostate carcinoma (Shappel et al.; 2004; Ayala AG; 2007).

PIN is classified as low- or high-grade lesions; low grade PIN is not considered a precursor of carcinoma, whereas high grade PIN (HGPIN), which is the *in situ* carcinoma, represents the precursor lesion for the most of invasive PCa (Knudsen B.S., et al.; 2010). HGPIN is citologically characterized by macronucleoli sized > 2-3 µm; moreover, typical of this lesion is that the increasing of severity leads to a progressively loss of basal cells layer integrity (Bostwick DG, et al.; 1993; Bostwick DG, et al.; 1996).

1.5.2 Prostate adenocarcinoma

More than 95% of prostate cancers are classified pathologically as adenocarcinoma that displays luminal phenotype (Shen M.M., et al.; 2010).

Tumor progression from PIN lesion to adenocarcinoma is marked by accumulation of histological changes in invasive epithelial cells characterized by a cytokeratin profile typical of luminal cells; particularly it is observed an excessive branching morphogenesis, loss of basal cells and cytologic atypia with enlargement of nuclei and nucleoli (Knudsen B.S., et al.; 2010).

Prostatic adenocarcinoma shows a multifocal origin, most of 80% of prostates presented two or more separate tumor at the time of clinical diagnosis (Greene D.R., et al.; 1991).

Multifocality of prostate cancer is due to the simultaneous development of HGPIN lesions at multiple sites, lesions that are histologically independent and often also genetically distinct (Knudsen B.S., et al.; 2010).

The biological mechanism for prostate cancer pathogenesis may involve a combination of inherited genetic predisposing factors that could create a “field effect” and acquired factor, such as environmental exposure to chemical and biological carcinogens (Zaridze DG, et al.;1987; Bostwik DG, et al.; 1998), that could lead to the generation of various precursor lesions (Cheng L., et al.; 1998) from which, through an independent clonal expansion, separate and distinct cancers are formed (Figure 5).

The appearance of distinct histological foci of cancer has been observed in prostatic samples of healthy men in their 20s to 40s, this suggests that the cancer initiation may occur at an early age (Sakr WA, et al.; 1994).

Multifocality of prostate cancer leads to latent disease that may not progress in a clinical disease.

Clinical prostate cancer likely initiates from a different pathogenic program than latent prostate cancer, otherwise most of latent foci could not undergo to critical activating events that give rise to clinical disease or could remain under active suppression that maintain these foci in an indolent state (Shen M.M., et al.; 2010).

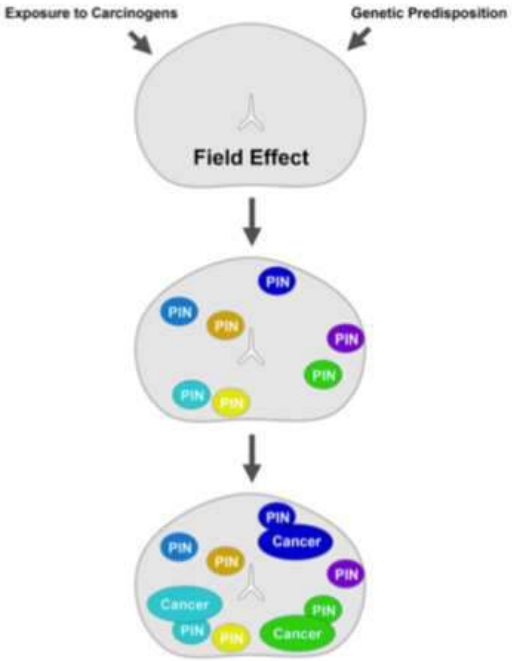


Figure 5: carcinogenesis of prostate cancer. *Representation of multifactorial etiology leading to a field effect and progression from PIN to multifocal prostate carcinoma*.

1.5.3 Metastatic disease

Despite the heterogeneity of prostate cancer, molecular and cytogenetic analysis demonstrate that metastases of the same patient are clonally related, indicating that metastases arise from a single clone of localized disease during cancer progression (Mehra R, et al.; 2008).

Average age of metastatic disease at diagnosis is 77 years and 91,1% of patients showed bone metastases (Gandaglia G., et al. 2015); other common site of prostate cancer metastases are lymph nodes, lung, liver and pleura (Bubendorf L., et al.; 2000). Metastatic prostate cancer to bone with skeletal metastases results in significant complications, such as bone pain, pathological fracture, compromised mobility, spinal cord compression and symptomatic hypercalcemia that noticeably reduces the quality of life in affected patients (Coleman RE, 1997). Despite the progress in the diagnosis and treatment of prostatic carcinoma, the metastatic disease to the bone remain incurable; the only possible therapies are mostly palliative treatments that include hormonal therapy, pharmacological management of bone pain, radiotherapy for pain and spinal

cord compression, chemotherapy and the use of bisphosphonates to inhibit osteoclast activity (Keller ET. et al., 2001; Szostak MJ, et al., 2000; Papapoulos SE., et al., 2000).
Prostate cancer bone metastases occur as a mixture of osteolytic and osteoblastic lesion with a predominance of the second over the first. Bone metastases in prostate cancer are characterized by the presence of several osteoblasts close to prostate cancer cells, whereas osteoblasts are commonly absent in normal bone or in bone metastases of other cancers (breast, lung and kidney) that, usually, contain osteoclasts (Logothetis CJ, et al., 2005).
Regarding the metastatic process from prostate cancer to bone metastases, the epithelial mesenchymal transition (EMT) plays a critical role for the spread of prostate cancer cells through the circulation.

1.6 Genomic alterations in prostate cancer

It is estimated that about a 5-10% of all prostate cancers in the population are "familial" and may be defined as "hereditary" prostate cancer. These hereditary forms represent the major proportion of disease among younger population.
Hereditary prostate can be differentiated from the most common "sporadic" tumor when are present at least one of the so-called Johns Hopkins criteria:
1) three or more first-degree relatives with prostate cancer.
2) three successive generations with prostate cancer, within paternal or maternal lineage.
3) two siblings with prostate cancer diagnosed at a relatively young age, before age 55 years (Brandão A, et al., 2020; Stanford JL, et al., 2001).
However, hereditary tumor represents only a small fraction of prostate cancers, and the initiation of sporadic prostate cancer is due by accumulation of *de novo* genetic changes in the prostate gland during the lifetime of the individual (Knudsen B.S., et al.; 2010).
Several genomic analyses have identified numerous somatic genetic changes and epigenetic events involved in prostate carcinogenesis. Extensive genomic studies conducted in both primary prostate tumor and metastatic disease have established recurrent DNA copy number changes, mutations, chromosomal rearrangement and gene fusion, summarized in Figure 6 (Grasso CS, et al., 2012; Taylor BS, et al., 2010).

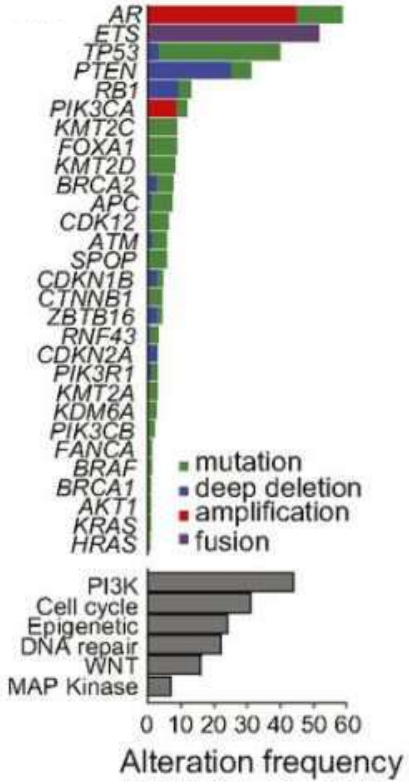


Figure 6: Genomic alterations in prostate cancer in order of frequency.

GLUTATHIONE-S-TRANSFERASE P1 (GSTP1)

Epigenetic changes resulting in a hypermetilation and consequent silencing of GSTP1 expression occurs in up to 70% of PIN lesions and in 90-95% of prostate cancer (Lee WH, et al., 1994).

GSTP1 is an enzyme that reduces oxidative damage in cells; thus, the GSTP1 expression reduction results in an increase of oxidative damage in luminal cells and this will result in an accumulation of genetic changes with the consequent possible neoplastic transformation (Knudsen B.S., et al.; 2010).

NKX3.1

The primary tumor frequently presents a down-regulation of a PSA and androgen-regulated homeobox gene, named NKX3.1 (Wang G, et al., 2018; Gurel B, et al., 2010); it is localized within chromosome 8p21.2 a region that displays loss-of-heterozygosity in up to 12% in PIN and up to 85% in prostate carcinoma (Bethel CR, et al., 2006).

NKX3.1 is expressed in prostate luminal epithelial cells and is important for normal differentiation of epithelial cells (Abate-Shen C, et al., 2008). The reduction of its expression causes a defective epithelial cells differentiation that can promote neoplastic transformation (Knudsen B.S., et al.; 2010).

SPINK1

In silico bioinformatics analyses demonstrated the outlier-expression of SPINK1 in a subset of cancers negative for the presence of ETS rearrangement (about 10% of total cases). The overexpression of SPINK1 is correlated with aggressive cancer and reduced progression free survival (Tomlins SA, et al., 2008; Leinonen K.A., et al., 2010).

SPOP

The point mutation of SPOP in prostate is an early clonal event mutually exclusive to ETS fusion (Barbieri CE, et al., 2012). SPOP mutations occur in 6-15% of cases and it has been found in localized and metastatic prostate cancer and most recently, SPOP mutations have been found also in High grade PIN (Arora K et al., 2018).

MYC

MYC is a proto-oncogene located on chromosome 8q24, this chromosomal region is somatically amplified in 30-40% of primary prostate cancers and in 90% of metastatic disease. Increased MYC copy number correlates with poor clinical outcome (Knudsen B.S., et al.; 2010; Sato H, et al., 2006). However, recent studies showed that the over-expression of MYC may be found in 76% of PIN lesions in absence of gene amplification (Gurel B, et al., 2008).

PTEN

PTEN is a tumor suppressor deleted or mutated in several human tumor, including prostate cancer (Salmena L, et al., 2008). PTEN is located on human chromosome 10q23 a locus that is highly susceptible to mutation in primary tumor (Salmena L, et al., 2008; Steck PA, et al., 1997). The monoallelic loss at this locus occurs into 50-80% of sporadic tumors, including prostate cancer (Salmena L, et al., 2008). Approximately 5-27% of localized and 30-60% of metastatic prostate carcinoma exhibit PTEN mutation (Karan D, et al., 2003).

The data reported from the studies of both human tumor and mouse model of prostate cancers, suggest that PTEN has an important role in the origination of lethal form of prostate cancer (Knudsen B.S., et al.; 2010).

ANDROGEN RECEPTOR (AR)

The AR gene is located on chromosome X at the locus Xq11-Xq12 and encodes for a 110 kDa nuclear receptor that belongs to the steroid hormone group of nuclear receptors (Tan MH, et al., 2015).

Point mutations and amplification of AR gene are common events that occur in 1% of primary PCa and in about 60% of CRPC metastases. However, in depth-studies of AR-androgens axis revealed alterations in 56% of primary PCa and 100% of CRPC metastases, confirming that this pathway is the most frequently altered in prostate cancer (Aurilio G, et al., 2020; Nyquist MD, et al., 2013).

In AR gene have been identified 1029 mutations, 159 of these *AR* mutations have been found in PCa tissue and almost all of them are single-base substitutions due to somatic rather than germline mutations (Gottlieb B, et al., 2012).

STRUCTURAL REARRANGEMENTS: overexpression of ETS family proteins

About 60% of all prostate cancers present a rearrangement that involves the promoter of androgen-regulated gene and one of the ETS genes with the consequent overexpression of a specific ETS transcription factor (Kumar-Sinha C., et al.; 2008).

Intra and inter-chromosome rearrangements may be the result of DNA double-stand breaks that occur during both replication and transcription process (Wallis CJ, et al. 2015).

1.7 ETS transcription factor family

First member of ETS transcription factors family was identified over 30 years ago as a component of retrovirus E26 isolated from a case of avian leukosis and described in 1962 (Ivanov X et al., 1962).

E26 genome consists of a unique tripartite structure that includes the sequences of two cellular oncogenes, one is v-myb, that have already been identified in avian myeloblastosis virus, whereas the second is the sequence v-ets (E-twenty-six transformation-specific sequence). Subsequently c-ets1, the cellular homologue of v-ets, has been identified in chickens (Leprince D, et al., 1983).

Since then, several cellular homologs were isolated from *C. elegans* or *Drosophila melanogaster* to humans (Hart AH, et al., 2000; Hsu T, et al., 2000).

All genes belonging to ETS family, share an evolutionary-conserved sequence of ~ 85 amino acids, the ETS binding domain, that encodes the DNA-binding domain and recognizes the core motif 5'-GGAA/T-3' on the DNA (Graves BJ, et al., 1998).

In humans, ETS family of transcription factors, consist of 28 members classified in 12 subfamilies on the basis of the structure and of homology in the ETS domains (Watson, et al., 2010) (Figure 7).

The majority of ETS family members present the ETS domains in their C-terminal region, however many ETS family proteins have the ETS domains at N-terminal region.

Moreover, in addition to ETS domains, a portion of ETS family members present another evolutionary-conserved DNA-binding domain defined pointed domain (PNT) at their N-terminal regions, that forms a helix-loop-helix (HLH) structure that has a role in protein-protein interaction (Kim CA, et al., 2001).

The ETS domains structure present three α-helixes and four-stranded antiparallel β-sheet that forms a winged helix-turn-helix (wHTH) structural motif (Kodandapani R, et al., 1996), in particular the third α-helix is responsive to contact the major groove of the DNA (Oikawa T, et al., 2003).

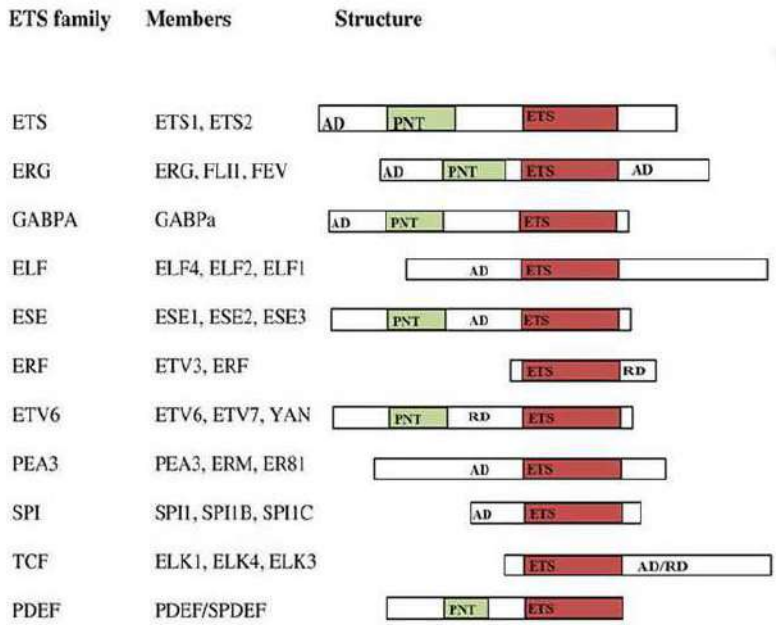


Figure 7: Subfamilies and members of ETS transcription factor family and their structure. *The structure of the members of ETS family present DNA-binding (ETS) domain, pointed domain (PNT), activation domain (AD), repression domain (RD).*
(Kar A., et al., 2013).

ETS transcription factors positively or negatively regulate the genes expression; a 2013 review reports that over 700 ETS target genes have been identified because the presence of an ETS binding site in their regulatory region (Findlay VJ, et al., 2013), and many more have been added since then.

The target genes of ETS family members are involved in signaling pathways, development, cell proliferation, differentiation, hematopoiesis, migration, apoptosis, invasion and metastasis, tissue remodeling, ECM composition and angiogenesis (Watson, et al., 2010) (Figure 8). ETS factors can undergo point mutations, gene amplification, loss or rearrangement resulting in altered ETS gene expression which break up the regulated control of many biological processes promoting tumorigenesis (Seth A, et al., 2005). Altered expression of ETS genes are correlated with several human carcinomas, such as thyroid, pancreas, liver, prostate, colon, lung, breast and leukemia (Seth A, et al., 2005).

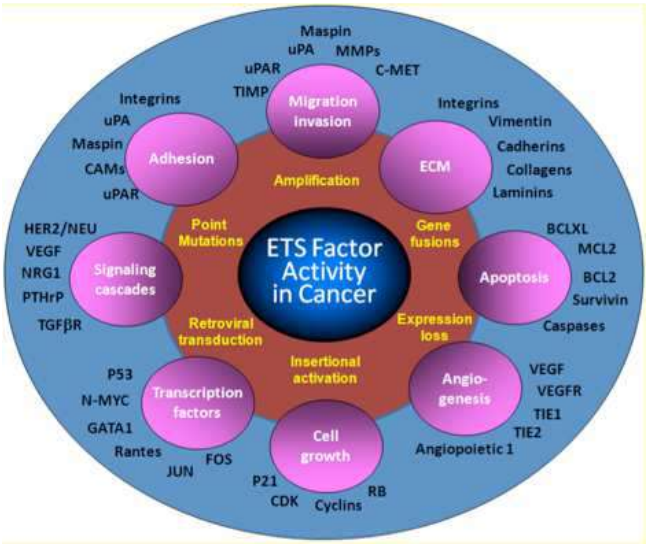


Figure 8: ETS transcription factors regulate the expression of genes associated with cancer progression.

1.7.1 ETS and prostate cancer

Recurrent chromosomal rearrangements have been described first in hematological malignant diseases (leukemia and lymphomas) and soft tissue sarcomas (Rowley JD, 2001), subsequently they have been found among epithelial cancers, mainly in the Prostate Cancer.

ERG (ETS-related gene) and ETV1 (ETS variant gene 1), two members belonging to ETS transcription factor family, were identified as outlier in several prostate cancer profiling studies using a new bioinformatic approach, the Cancer Outlier Profile Analysis (COPA) (Tomlins SA, et al., 2005). Physiologically, the members of ETS transcription factors family are not expressed in normal adult prostate cells; thus, the over-expression of ERG or ETV1 could represent an important molecular event in a subset of prostate cancer patients (Kumar-Sinha C, et al. 2008; Oikawa T, et al., 2003; Sorensen PH, et al., 1994).

Tomlins and colleagues demonstrated that the over-expression of ERG or ETV1 in prostate cancer was due to recurrent chromosomal rearrangements: they found that the 5' ends of ERG or ETV1 are replaced with the 5' untranslated region of a prostate-specific, androgen responsive, transmembrane serine protease gene (TMPRSS2)

(Tomlins SA, et al., 2005). Later on, these ETS proteins were then found fused also to other promoters of genes highly expressed in the prostate (Nicholas TR, et al., 2019).

The observed TMPRSS2-ETS fusions very rarely generate a chimeric protein, instead they result in a massive overexpression of 5' truncated or full-length ETS transcription factors under the control of these different promoter elements (Kumar-Sinha C, et al. 2008; Mesquita D, et al., 2015).

Furthermore, Tomlins and colleagues identified also the gene fusions of ETV4 or ETV5 with TMPRSS2 (Tomlins SA, et al., 2006; Helgeson BE et al., 2008) or with the promoter of other genes.

Both ERG and ETV1 genes were already known for their involvement in gene fusions in Ewing's sarcoma. In prostate cancer, as well as in Ewing's sarcoma the overexpression of the ETS proteins is mutually exclusive. However, it has been observed that separate cancer foci in a single prostate patient may show different translocation of ETS that occurred independently (Clark JP, et al., 2009).

The most common molecular alteration in prostate cancer is the fusion gene TMPRSS2-ERG, found in about 50% of Caucasians, whereas is less frequent in African Americans and even less common in Asians (Fry EA, et al., 2018). The translocations involving ETV1 are found in 8-10% of prostate cancer, instead those involving ETV4 occurs in 2-5% of cases and translocation with ETV5 are less frequent (Nicholas TR, et al., 2019) (Figure 9).

ETS genes translocation is an event that occur in the early stage of prostate carcinogenesis, it is found in about 20% of pre-cancerous lesion and HGPIN (Mesquita D, et al., 2015); however, these events alone are not sufficient to develop prostatic neoplasia and other genomic alterations, such as PTEN or TP53 loss, are needed (Nicholas TR, et al., 2019; Attard G, et al., 2016).

The whole transcriptome expression profile studies have identified genes differentially expressed in the ETS-positive cancers from ETS-negative tumors, such as F5 and SLC2A12, whose expression levels could differentiate between the ETV4 (also called PEA3)-positive and ETS-negative tumor and other genes over-expressed only in a specific ETS-positive cancer, such as CADPS2 and TMEFF2, that can distinguish ETV4-positive from ERG-positive tumors (Mesquita D, et al., 2015).

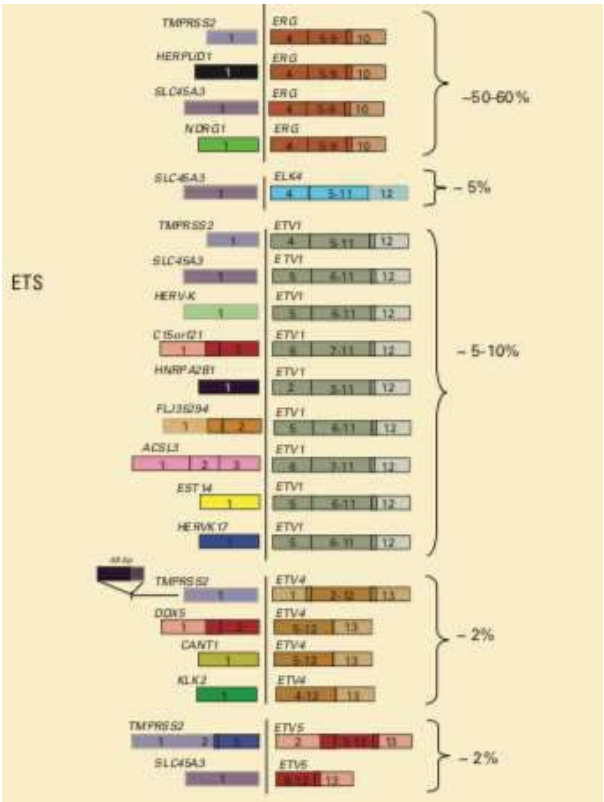


Figure 9: classification of chromosomal rearrangement in prostate cancer.

1.7.2 ETS TRANSLOCATION VARIANT 4

ETS translocation variant 4 (ETV4), also termed E1AF for adenovirus E1A enhancer-binding protein or PEA3 (polyomavirus enhancer activator 3), is a member of PEA3 subfamily of the ETS transcription factors.

All members of this subgroup show more than 95% identity in the ETS binding domain, more than 85% identity in the acidic domain (AD) located in the N-terminal region and about 50% identity in the final 61 residues related to the C-terminal tail (Ct); both these last domains (AD and Ct) represent two unique and independent transactivation domains, in addition ETV4 exhibits DNA-binding autoinhibitory sequences placed on both sides of ETS domain (de Launoit Y, et al., 1997; Hollenhorst PC, et al., 2011).

ETV4 is located on chromosome 17q21 (Isobe M, et al., 1995) and contains 13 exons, the thirteenth exon is the largest exon (901 bp) and contains the ETS domain, the C-terminal domain and the 3'-untranslated region, the other exons varied from 48 (exon 4) to 266 (exon 8) bp (Coutte L, et al., 1999; Yasuyoshi Miyata, 2010).

ETV4 plays a key role in growth and development of normal neuronal axonal, promotes the development of normal kidney and is closely related to fertility in both males and females (Qi T, et al., 2020) (Figure 10).

Figure 10: schematic representation of ETV4. *ETS DNA-binding domain is colored in blue, and in green the two distinct transcriptional activation acidic domains (Oh et al., 2012).*

ETV4 is overexpressed in several tumors such as gastric cancer, hepatocellular carcinoma, lung cancer, colorectal cancer, breast cancer, malignant melanoma, and it is associated with tumor progression, poor prognosis and drug resistance (Qi T, et al., 2020; Yasuyoshi Miyata, 2010); moreover, ETV4 is overexpressed in a small fraction of prostate cancer; in about 6% of primary prostate cancer, in 6% of patients with lymph-nodes metastasis and in 4% of those with distant metastasis (Shaikhibrahim Z, et al., 2012). A fraction of these patients shows a chromosomal rearrangement between ETV4 gene and the promoter region of a gene that drives the aberrant expression of ETV4 in prostate cells (Qi M, et al, 2015). The more frequently promoter involved in the translocation, as in others ETS translocations, is that of TMPRSS2 gene; nevertheless, other 5' fusion partners have been found: *SLC45A3*, *CANT1*, *KLK2*, *DDX5*, *HERVK17*, and *UBTF* (Kumar-Sinha C, et al., 2008; Barros-Silva JD, et al., 2013; K.G. Hermans et al., 2008); most of these 5' partners are androgen responsive, but also androgen insensitive and androgen repressed fusion partner have been found (Nicholas TR, et al., 2019).

The high levels of ETV4 have been correlated with high Gleason score (P=0,0045) high pathological tumor stage (P=0,041) and poor prognosis of patients with prostate cancer (Qi M, et al, 2015; Shaikhibrahim Z, et al., 2012). In fact, the analysis of the Kaplan-Meier curves, showed that the group of patients with the overexpression of ETV4 have higher rate of mortality than the group of patients that do not present the overexpression of ETV4 (P=0,004), thus ETV4 overexpression represents a significant prognostic predictor of prostate cancer survival (Qi M, et al, 2015).

In vitro studies in several prostate cancer cell lines demonstrated the oncogenic role of ETV4 overexpression. ETV4 has been found over-expressed in two different prostate

cancer cell lines, in PC3 at high levels and DU-145 at lower levels, and in another MDA-PCa-2b prostate cancer cell line (Pellecchia A, et al., 2012; Hollenhorst PC, et al., 2011; Mesquita D, et al., 2015).

The reduction of ETV4 expression levels in PC3 and DU-145 cell lines by specific shRNA, decreased the cell growth in adherence and also in the absence of anchorage surface and also the ability of prostate cancer cells to migrate and invade (Pellecchia A, et al., 2012). The oncogenic role of ETV4 in cell proliferation, migration, invasiveness and anchorage independent growth has been also confirmed in RWPE treated with two different expression vectors: one encoding the full-length protein of ETV4 and one containing the common TMPRSS2-ETV4 fusion gene (Pellecchia A, et al., 2012).

ETV4 regulates the expression of matrix metalloproteinases (MMPs), in particular MMP1, MMP3, MMP7 and MMP9 expression (Pellecchia A, et al., 2012; Maruta S, et al., 2009). Furthermore, ETV4 silencing in PC3 cells results in a decrease of the expression levels of urokinase plasminogen activator (uPA) and its receptor uPAR, two proteins that play a crucial role in cancer invasion and metastasis, because the binding of uPA to uPAR promotes the conversion of plasminogen to plasmin with the consequent activation of MMPs (Qi M, et al, 2015; Kumano M, et al., 2009; Noh H, et al., 2013). The regulation of uPA occurs through the direct binding of ETV4 to the uPA promoter (Qi M. et al., 2015).

Finally, the overexpression of ETV4 decreases also the expression of the epithelial markers, such as E-cadherin and Zonula-occludens 1, whereas increases the expression of mesenchymal markers, such as vimentin, N-cadherin and cadherin 11 (Pellecchia A, et al., 2012; Qi M, et al, 2015); thus, ETV4 is capable to induce in prostate cancer cells Epithelial-mesenchymal transition (EMT), a dynamic cellular process that plays a key role during the development of epithelial tumor and metastases.

In vivo study demonstrated the engagement of ETV4 in prostate carcinoma onset; in fact, in a transgenic mouse model in which ETV4 is expressed only in the prostate, the overexpression of the transgene induces prostatic intraepithelial neoplasia (mPIN) in about two-third of ETV4 mice at 10 months of age. Nevertheless, the progression in prostate cancer does not occur even in older mice, suggesting that additional genetic alterations are necessary for the development of the neoplasia (Cosi et al. 2020).

Furthermore, ETV4 regulates *in vitro* and *in vivo* tumor cells proliferation through the inhibition of two different cell-cycle regulating proteins, p21 and p27, that belong to

Cip/Kip family and that are encoded by cyclin-dependent kinase inhibitor 1A (Cdkn1a) and cyclin-dependent kinase inhibitor 1B (Cdkn1b), respectively (Cosi et al. 2020).

1.7.3 ETS TRANSLOCATION VARIANT 1

ETV1, ETS-translocation variant 1, also known as ER81 for ETS-related 81, belongs, together with E1AF/ETV4 and ERM/ETV5 to the PEA3 subfamily of ETS family of transcription factors. The gene is located on chromosome 17p21 and encodes for 7 protein isoforms containing from 374 to 477 amino acids. ETV1 gene has 13 exons, of which the latest is the largest and includes the end of ETS domain, the C-terminal region and a part of the 3'-untransleted region, the reminder 12 exons ranging from 48bp (exon 4) to 248bp (exon 8) (Coutte L, et. al., 1999).
ETV1, as well as other members of PEA3 subfamily, presents two distinct transcriptional activation acidic domains (AD) both at N- and C- terminus, and the conserved ETS domain located in the carboxy terminal region. The central region of ETV1 has an inhibitory effect on transactivation (Janknecht R. et al., 1996; Oh S, et al., 2012) (Figure 11).

Figure 11: schematic representation of ETV1. *ETS DNA-binding domain is colored in blue, and in green the two distinct transcriptional activation acidic domains (Oh et al., 2012).*

ETV1 is expressed in several tissues, high levels of expression have been found in heart, brain and lung, moderate levels are present in the spleen, pancreas, intestine and colon, low in liver and skeletal muscle (Janknecht R. et al., 1996).
It has been demonstrated that ETV1 plays pleiotropic roles in motor coordination, since ETV1 knock-out mice die roughly one month after birth and show a reduced direct connection of muscle sensory neurons and spinal motor neurons resulting in limb ataxia and abnormal flexor-extensor posturing; additionally, they display defect in muscle spindle formation and also, these mice do not develop the Pacinian corpuscle limb mechanoreceptors (Arber S, et al., 2000; Sedý J, et al., 2006).

The oncogenic properties of ETV1 have been observed for the first time in Ewing's sarcoma in which ETV1 is involved in a gene fusion with EWSR1 (Ewing's sarcoma breakpoint region 1) gene; this chromosomal rearrangement generates a chimeric protein representing a constitutively activated form of ETV1 capable of inappropriate upregulation of ETV1 target genes (Shin S, et al., 2008).

Among the known ETV1 target genes there are enzymes promoting cell invasion and metastases such as MMP1-7 and heparinase. Others ETV1 targets are the vascular endothelial growth factor (VEGF), the major regulator of tumor angiogenesis; the telomerase reverse transcriptase, whose upregulation is responsible for the immortalization of over 90% of tumor cells; Smad7, a TGFβ family members involved in cell signaling (Shin S, et al., 2008) and HER2/Neu, an oncoprotein associated with breast cancer (Bosc DG, et al., 2002).

ETV1 results expressed in some tumor cell lines, such as those of teratocarcinoma and prostatic adenocarcinoma, whereas it is not expressed in cervix or hepatocarcinoma cells, this suggest that ETV1 may be involved in malignant transformation of only specific cells (Janknecht R. et al., 1996).

About 8-10% of prostate cancer patients display ETV1 gene rearrangements (Nicholas TR, et al., 2019), in which ETV1 has as 5' fusion partners *TMPRSS2*, *SLC45A3*, *HERV-K*, *HERVK17*, *C15ORF21*, *HNRPA2B1*, *OR51E2*, *EST14*, *FLJ35294*, *FOXP1*, and *ACSLS* (Nicholas TR, et al., 2019).

In about half of ETV1 positive patients, the ETV1 protein translated from gene fusion is truncated, lacking the acidic transactivation domain at N-terminal region (dETV1) (Hermans KG, et al., 2008), whereas in another subset of patients the high levels of full-length ETV1 expression are due to the translocations of the whole gene from chromosome 7 to chromosome 14. (Gasi D, et al., 2011; Hermans KG, et al., 2008). In *in vitro* studies, both the truncated and full length ETV1 proteins did not display significant difference in cell proliferation, migration and invasion and both forms increased the expression of MMP1, MMP3 and MMP7; however, the full-length ETV1 stimulated the anchorage independent growth much more than the truncated one (Hermans KG, et al., 2008).

About 75% of transgenic mice overexpressing the truncated version of ETV1 in the prostate developed mPIN at 12-14 weeks of age with the presence in all prostatic lobes of nuclear atypia, including stratification, hyperchromasia and macronucleoli (Tomlins

SA, et al., 2007). Also in this mouse model it has not been observed the development of invasive tumor.

1.8 SLPI: SECRETORY LEUKOCYTE PEPTIDASE INHIBITOR

Human SLPI belongs to the whey-acidic protein (WAP) family characterized by four-disulfate core domains. The SLPI gene is mapped to chromosome 20q12-13.2 and includes 4 exons and 3 introns spanning 2.6 kb (Kikuchi T, et al., 1998; Stetler G, et al., 1986). The protein is composed by 132 amino acids and has a molecular weight of 11,7 kDa. The protein structure contains two homologues WAP domains that are characterized each one by 8 cysteine residues forming four intramolecular disulfate bonds; the WAP II domain of the protein is located in C-terminal region, it is responsible for the inhibitory activity of SLPI against proteases; regarding the biological function of WAP I (N-terminal) domain, this is poorly understood, although is thought that the antimicrobial activity of SLPI is provided by this region (Nugteren S, et al., 2021; Majchrzak-Gorecka M, et al., 2016) (Figure 12).

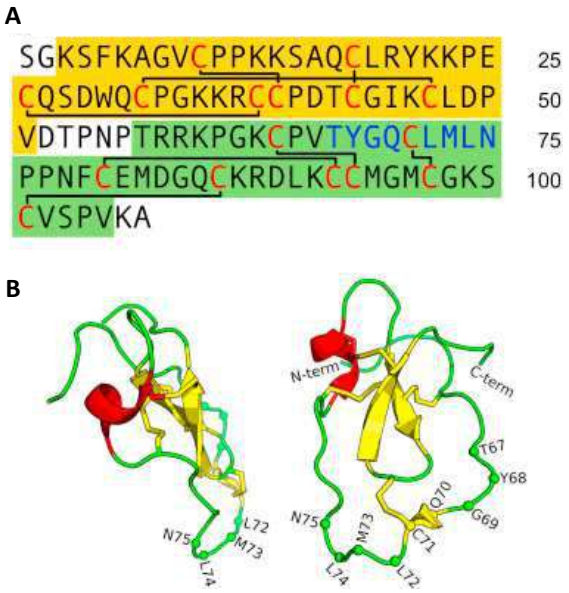


Figure 12: Amino acidic sequence of human SLPI protein and 3D structure of WAP II domain. A. *in yellow is shown the amino acidic sequence of WAP I domain, whereas the WAP II domain is colored in green; in red are shown the cysteine residues and the black lines represent the intradomain disulfide bridges. The fragment of the WAP II domain responsible for*

interaction with proteases is represented in blue. ***B.*** *two orthogonal views of 3D structure of the WAP II domain (Majchrzak-Gorecka M, et al., 2016).*

SLPI is produced by epithelial cells, including those lining the reproductive, respiratory and digestive tracts, but also in the cells of the parotid glands, skin, breast and kidney (Majchrzak-Gorecka M, et al., 2016). SLPI is also expressed by innate immune cells such as neutrophils, macrophages, mast cells and fibroblasts (Nugteren S, et al., 2021). SLPI plays an important role in the protection of the epithelial barriers from excessive activity of inflammatory immune response. SLPI counteracts the inflammation response through the inhibition of serine proteases activity, this action is able to limit the tissue damage. The main role of SLPI is the reversible inhibition of neutrophil elastase (NE), since the binding of SLPI with NE is the strongest among all other proteases (Majewski P, et al., 2016). SLPI, besides its role in the inhibition of enzymatic activity of proteases, has the capability to suppress the synthesis of proteases, such as MMPs (Zhang Y, et al., 1997); moreover, SLPI through its binding to annexin A2 blocks the plasminogen activation and, consequently, interferes with plasmin generation; since plasmin generation at the cell surface is associated with detachment, invasion and metastases of tumor cells, SLPI may have an antitumoral effect (Majchrzak-Gorecka M, et al., 2016; Wen J, et al., 2011).

The anti-inflammatory activity of SLPI is also due to its role in the prevention of the production of several pro-inflammatory cytokines and in the consequent recruitment of immune cells (Nugteren S, et al., 2021).

SLPI also possesses an antimicrobial propriety against fungi (*Candida albicans*, metabolically active *Aspergillus fumigatus*, *A. fumigatus conidia*), viruses (human immunodeficiency virus type 1) and several intracellular and extracellular bacteria (*Salmonella typhimurium, group A Streptococcus, Mycobacterium bovis and Mycobacterium tuberculosis, Neisseria gonorrhoeae*) (Majchrzak-Gorecka M, et al., 2016). The interaction of SLPI with the surface of mycobacteria helps the phagocytosis of the pathogens (Gomez SA, et al., 2009).

Finally, SLPI has a role in wound-healing, a multi-step process that ends with the formation and remodeling of new tissues. In fact, SLPI deficient mice display a delay in tissue repair caused by an increased and prolonged inflammatory response and to a delay in matrix accumulation (Ambrosi N. et al., 2015).

1.8.1 SLPI in cancer

SLPI results over-expressed in several cancers: ovarian, lung, gastric, pancreatic and papillary thyroid. On the contrary, SLPI is expressed at low levels in squamous cell carcinoma of the head and neck (HNSCC), in cervical, bladder, nasopharyngeal cancer, in early phase of prostate carcinoma. It is expressed at low levels also in some breast carcinoma, but SLPI overexpression correlates with more-invasive form of the disease (Bouchard D, et al., 2006; Nugteren S, et al., 2021).

The role of SLPI in cancer is still unclear: several studies suggest that SLPI is correlated with tumor aggressiveness and metastatic potential, others have pointed out that some functions of SLPI may have antitumoral proprieties (Nugteren S, et al., 2021).

In breast cancer, some authors have suggested that SLPI has an anti-apoptotic and cell growth inhibitory role (Amiano, et al., 2013; Rosso, et al., 2014); others have described SLPI as an important pro-metastatic component especially for triple negative breast cancers (Sugino T, et al., 2007; Kozin SV, et al., 2017). High levels of SLPI expression in triple negative breast cancer are strongly correlated with poor outcome in patients with Basal/ triple negative breast cancers (Kozin SV, et al., 2017). Sugino and colleagues (Sugino T, et al., 2007) have reported results apparently different in their *in vitro* and *in vivo* studies: *in vitro* SLPI has inhibitory role on the invasion ability of tumor cells through the suppression of MMP1 and MMP9 production while *in vivo* in a xenograft mouse model SLPI promoted tumor cell growth and the formation of spontaneous metastases in the lungs. The authors proposed an invasion-independent pathway where SLPI induces the formation of well-developed sinusoidal vessels in the peri-necrotic area placed at the center of the tumor and through them the cancer cells enter the circulation without the classical vascular invasion. Instead, Rosso et al. demonstrated that SLPI expression triggers apoptosis-related events through the downregulation of E-cadherin and the induction of beta-catenin re-localization (Rosso et al. 2014).

In a murine xenograft model of lung cancer cells, overexpression of SLPI resulted in a reduction in the number of liver metastases compared to control; this protective effect of SLPI on liver colonization should be due to its ability to suppress inflammation induced by cancer cells during the early stages of liver metastases (Wang N, et al., 2006). However, SLPI in other experiments seems to stimulate tumor growth *in vivo* (Jan Treda, et al., 2014) and cells proliferation *in vitro* (Devoogdt, et al., 2003).

In colorectal cancerous tissues SLPI is overexpressed compared to normal tissues (Wei Z, et al., 2020). Furthermore, SLPI is involved in several cellular processes, such as cellular growth, survival and metastases (Amiano 2013; Wei Z, et al., 2020). The pathogenesis of colorectal cancer from SLPI appears to be mediated by regulation of AKT activation in cancer cells (Wei Z, et al., 2020).
Also in gastric, pancreatic and endometrial cancer it has been suggested a role of SLPI in tumorigenesis (Nugteren S, et al., 2021).
SLPI expression shows a totally different pattern in oral squamous cell carcinoma: in fact, it is dramatically reduced in patients with this cancer and it has been observed a progressive decrease of SLPI levels between tissues of healthy normal subjects and patients with oral premalignant lesion tissue followed by a significant decrease in tissues of patients with oral squamous cell carcinoma. Since SLPI reduces the NF- κB transcriptional activity in oral premalignant cell line, therefore the decrease in SLPI production may contribute to a pro-inflammatory state underlying the development of oral squamous cell carcinoma (Yang Y, et al., 2014).
Several mechanisms have been hypothesized to explain the role of SLPI in promoting tumor metastases: not only through the formation of extravascular networks *in vivo* and tubular structures on Matrigel *in vitro*, but also as an anticoagulant favoring the flow of extravascular channels and the consequent intravasation of tumor cells (Wagenblast E, et al., 2015); furthermore, SLPI within the tumor cell nucleus would stimulate the binding of FoxM1 to its target genes associated with tumor growth and metastasis. The activity of FoxM1 is repressed by the interaction with its inhibitory protein Rb; a physical interaction between SLPI and Rb protein has been described in cancer cells and this promotes release of Rb from FoxM1 (Kozin SV, et al., 2017).

1.8.2 SLPI and prostate cancer

SLPI is frequently over-expressed in several cancers, while in prostate cancer its expression levels are bimodal; in fact, it is down-regulated, at the mRNA and protein level, in the epithelial cells of patients with early prostate cancer compared with their normal tissue, whereas it is up-regulated in a subset of metastatic CRPC tumors (mCRPC) (Xuan Q, et al., 2008; Zheng D, et al., 2016).
Since the main role of SLPI is the homeostatic control of inflammation activity and the tumor microenvironment is frequently of an inflammatory nature, it is not surprising the

finding of an increased SLPI expression associated with several aggressive tumors; in prostate cancer the inflammatory stage occurs very early, often appears before the clinical diagnosis, this could likely explain the low level of SLPI expression found in prostate carcinoma compared to normal prostate tissues or benign hyperplasia (Galustian C, et al., 2011).

Zheng and colleagues (Zheng D, et al., 2016) suggested that SLPI expression increases with the progression of CRPC; in fact, they observed different levels of SLPI expression in three different human prostate cancer cell lines derived from LNCaP cells: in the androgen-dependent LNCaP and the CRPC C4-2 cell lines SLPI was at lower level than in the C4-2B cells, a more advanced stage of CRPC. Furthermore, from their results of the RNA interference against AR, they suggested that SLPI expression was AR-dependent, but independent of androgens.

The same group has studied the oncogenic role of SLPI in C4-2B prostate cancer cells in which SLPI have been stably knockdown in presence and absence of androgenic stimuli. The inhibition of SLPI expression reduced cell growth, cell invasion and soft-agar colony formation and increased apoptosis under androgen deprivation condition (Zheng D, et al., 2016). Additionally, SLPI overexpression promotes xenograft tumor growth in immunodeficiency mice after castration, suggesting that SLPI may promote tumor cell survival and growth *in vivo* after ADT (Zheng D, et al., 2016).

The authors suggested that the role of SLPI in tumorigenesis is due to the protection, through SLPI anti-protease activity, of Progranulin (PGRN) from elastase-mediated degradation. PGRN is an epithelial growth factor that plays an important role in the wound healing process through cell proliferation, migration and differentiation and it is over- expressed in several tumors. Further evidences of the role of SLPI-PGNR in cancer are the physical interaction in prostate cancer cells between SLPI and PRGR, demonstrated by co-immunoprecipitation experiments, and the correlation between the levels of PRGR and those of SLPI in CRPC human serum samples (Zheng D, et al., 2016) (Figure 13). The overexpression of both SLPI and PRGR has also been found in ovarian cancers, suggesting that these two proteins may contribute to tumorigenic and malignant activity of different types of cancer (Simpkins FA, et al., 2008).

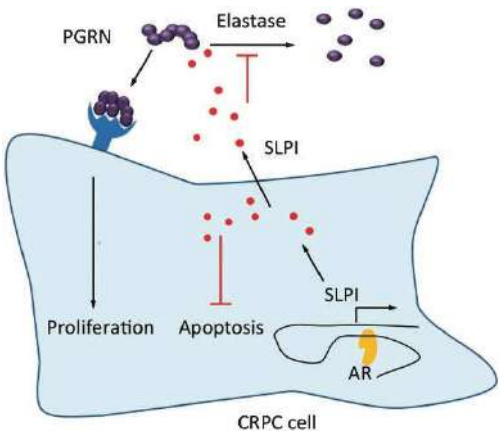


Figure 13: a schematic model of mechanisms that drive CRPC growth mediated by SLPI. *(Zheng D, et al., 2016).*

Recently, Yang Z, et al. investigated the role of AR in vasculogenic mimicry (VM) in prostate cancer; this process occurs in several tumors and is associated with poor clinical outcome and tumor metastases; in prostate cancer it is correlated with high Gleason score. They have observed that the ectopic AR expression in AR-negative PC3 cancer cell line reduced VM ability of these cells and that it is possible to restore their VM ability by adding SLPI. Furthermore, the authors suggested that AR, combined with the transcription factor NFIX, increases miR-525-5p levels that inhibit SLPI expression and thus VM formation and metastasis (Yang Z, et al., 2019).

1.9 SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION (STAT) FAMILY

The Signal Transducer and Activator of Transcription (STAT) family was discovered in 1994; to date, seven mammalian members of the STAT family have been identified: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Verhoeven Y, et al., 2020).

The members of this family have the role to transduce the signals from the cell membrane to the nucleus, where they regulate the expression of several genes involved in many physiological cellular processes, such as proliferation, differentiation, apoptosis, angiogenesis and immune system regulation (Verhoeven Y, et al., 2020).

The STAT proteins display some structurally and functionally conserved regions: the N-terminal domain (ND) that with Src-homology 2 (SH2) domain is responsible for homo- and hetero-dimerization of STAT monomers upon their activation, the coiled-coil domain (CCD) involved in nuclear translocation and in the interactions with regulatory proteins, the C-terminal transcriptional activation domain (TAD) that, after serine phosphorylation by several kinases, recruits additional transcriptional activators that intensify transcriptional activity of STAT, the DNA-binding domain (DBD) responsible for the direct bind with specific regulatory sequences of target genes (Verhoeven Y, et al., 2020) (Figure 14).

Figure 14: STAT protein structure. *(Ebersbach C, et al., 2021)*

The inactive STAT proteins are located in the cytoplasm as monomers or unphosphorylated dimers, their activation is provided by a multitude of extracellular signaling proteins, including cytokines, interferons (IFNs), interleukins (ILs), growth factors and hormones that bind to specific membrane receptors. The main mechanism of STAT proteins activation is the Janus kinase (JAK)-STAT pathway: following binding of a ligand to its receptor, receptor-associated JAKs are activated. STAT proteins are then activated by tyrosine phosphorylation by JAK kinases, allowing their dimerization and following transport to the nucleus by importins where they bind to specific DNA response elements on target genes (Verhoeven Y, et al., 2020).

Aberrant regulation of STAT proteins results in inhibition of apoptosis, increased cell proliferation, migration and invasion, deregulation of immune surveillance (Halim CE, et al., 2020) and correlates with tumor progression, metastasis and resistance to therapy. Constitutive active form of STAT1, STAT3 and STAT5 have been found in several human tumors including hematological neoplasms and solid tumors such as breast, lung, prostate and pancreatic cancers (Ebersbach C, et al., 2021; Halim CE, et al., 2020).

Aberrant STAT proteins (STAT1, STAT3, STAT5a-b, and STAT6) expression has also been found in prostate cancer, especially in advanced and metastatic disease and this expression has also been correlated with therapy resistance (Ebersbach C, et al., 2021).

STAT3 has been found constitutively active in primary prostate cancer, and the phosphorylated form of the protein is associated with a higher Gleason score. In advanced and metastatic prostate cancer, the STAT3 expression and activity is elevated especially in bone metastases compared to lymph node and visceral metastases (Ebersbach C, et al., 2021).

STAT5 has been described as a marker of poor outcome in prostate cancer; in fact, the positive STAT5 activation is associated with a significant early prostate cancer recurrence and shorter progression-free survival (Li H, et al., 2005). In addition, the amplification of STAT5A/B genes, identified by FISH analysis, increases significantly in patients with high Gleason score and the STAT5 expression increases during progression of localized prostate cancer to castrate resistant or metastatic disease (Haddad BR, et al., 2013).

Prostate cancer displays an elevated expression of STAT6 compared to benign prostate, it acts as a pre-cancerous factor of prostate cancer, and is described as an essential factor in metastatic disease (Ebersbach C, et al., 2021).

In conclusion, the members of STAT family represent potential biomarkers and viable therapeutic targets for advanced and metastatic prostate cancer.

1.9.1 Signal transducer and activator of transcription 1 (STAT1)

STAT1 gene is located on chromosome 2q32.2 and, together with STAT2, is the first member of STAT family discovered in the INF signal transduction pathway in mammalian cells (Verhoeven Y, et al., 2020).

STAT1 has two isoforms: the full length STAT1α and the truncated STAT1β; they are due to alternative splicing and show different biological properties. The longer STAT1α (91 kDa) is the transcriptionally active form of STAT1 whereas the STAT1β isoform (84 kDa), that lacks a part of C-terminal transactivation domain and the conserved serine 727 (S727) phosphorylation site, is transcriptionally inactive and acts as a negative inhibitor of STAT1α activation by inhibiting the phosphorylation of tyrosine 701 (Y701) and the binding to DNA (Baran-Marszak F, et al., 2004).

In addition, the unphosphorylated form of STAT1 (U-STAT1) is able to bind DNA and regulate gene expression (Yang J, et al., 2008).

Usually, STAT1 is considered a tumor suppressor, however in some human cancers STAT1 acts as an oncoprotein.

The tumor suppression functions of STAT1 are due to the inhibition of cell cycle progression, the induction of pro-apoptotic genes and suppression of angiogenesis acting on both endothelial and cancer cells. Furthermore, STAT1 plays an important role in immunosurveillance by regulating the adaptive and innate immune responses against cancer cells. In addition, STAT1 upregulates major histocompatibility complex class I (MHC class I) promoting an efficient recognition and elimination of cancer cells by cytotoxic T lymphocytes. The STAT1 tumor-promoting properties are due to its role in the promotion of an immunosuppressive tumor environment, in the increase of invasiveness and metastasis and in the conferring resistance to irradiation and chemotherapy (Meissl K, et al., 2017).

In several human tumors, such as ovarian, colorectal cancer, hepatocellular, esophageal, pancreatic carcinoma, sarcoma, oral squamous cell carcinoma and melanoma, elevated levels of STAT1 are associated with good prognosis and longer overall survival compared to low or negative expression levels (Meissl K, et al., 2017; Zhang J, et al., 2020); on the contrary, in other solid cancers such as renal cancer, lung, pancreatic adenocarcinoma and lower grade glioma, the overexpression of STAT1 is correlated with a poor prognosis and worst overall survival compared with low expression levels (Zhang J, et al., 2020).

Higher levels of STAT1 have been detected in breast cancer than in normal tissue (Watson CJ, et al., 1995) and the levels of the phosphorylated form of STAT1 (pSTAT1) were increased in highly aggressive breast cancer compared to non-invasive ductal carcinoma *in situ* (Hix LM, et al., 2013). However, in another study high levels of pSTAT1 in breast cancer have been correlated with a positive outcome, although STAT1 mRNA overexpression was still correlated with poor survival (Tymoszuk P, et al., 2014).

In soft tissue sarcoma the increase of cytoplasmatic unphosphorilated STAT1 (U-STAT1) levels have been associated with the reduction of disease-specific survival whereas the high levels of nuclear pSTAT1 to the increase in disease-specific survival (Zimmerman MA, et al., 2012).

1.9.2 STAT1 in prostate cancer

In prostate cancer it has been found that STAT1 may play the role of either tumor-suppressor or oncogene. In fact, higher levels of STAT1 in localized disease are correlated with prolonged cancer-specific survival and in advanced disease the absence of STAT1 expression is associated with biochemical recurrence and poor prognosis.
Hatziieremia S, et al. suggested that the loss of STAT1 expression in a specific subgroup of patients with metastatic disease is associated with low nuclear AR expression, although they have demonstrated in LNCaP cells that STAT1 expression is not regulated by AR and androgens.
However, in accordance with observation in patients, they have demonstrated that the silencing of STAT1 promotes proliferation and cell viability in AR-negative PC3 cells but not in AR-positive LNCaP cell line. In PC3 cells the silencing of STAT1 also enhances the clonogenic capacity and cell migration in the wound-healing assay (Hatziieremia S, et al., 2016).
In prostate cancer, as in other tumors (head and neck squamous cell carcinoma, breast cancer and gliosarcoma), STAT1 activated by irradiation, plays a cytoprotective role against radiotherapy (Ebersbach C, et al., 2021). *In vitro* studies in human metastatic prostate cancer cell line (DU145), demonstrated that the docetaxel treatment induces the increase of STAT1 expression levels, and its activation causes the increase of clusterin protein expression that inhibits the docetaxel-induced apoptosis.

2. AIM OF THE STUDY

One of the most frequent genetic alterations in prostate cancer are the chromosomal translocations between an ETS transcription factor and a promoter of a gene highly expressed in the prostate, resulting in ectopic expression of the specific ETS factor in the prostate.

The laboratory where I carried out my PhD has engineered a transgenic mouse model in which human ETV4 is specifically over-expressed in the prostate tissue. ETV4 over-expression in prostate cells is associated with the acquisition of a neoplastic phenotype. The oncogenic mechanisms resulting from ETV4 over-expression have been investigated by microarray, comparing RNA from prostate tissue of wild-type mice with RNA from prostates of mice that over-express ETV4.

One of the genes found down-regulated in ETV4 mice was SLPI, a gene that encodes for a protease inhibitor. SLPI is involved in inflammation and seems to have a role in oncogenesis: in fact, it is over-expressed in most cancers, whereas it is reduced in patients with early prostate cancer (Thompson *et al.*, 2008; Zheng *et al.*, 2016).

The data on the SLPI expression levels from microarray analysis have been confirmed by quantitative Real-Time PCR (qRT-PCR), immunohistochemistry and Western Blot. In addition, *in vitro* experiments suggested that ETV4 negatively regulates SLPI expression levels; in fact, we found that ETV4 silencing in PC3 cells results in increased levels of SLPI, whereas their levels were reduced upon the overexpression of ETV4 in RWPE cells. However, through luciferase experiments and ChIP assay we have not been able to demonstrate the direct binding of ETV4 to the SLPI promoter.

The first aim of my PhD thesis was to verify whether also ETV1, another of the ETS proteins found over-expressed in human prostate cancer, and androgens, hormones with a key role in the prostate cancer onset and progression, regulate the expression of SLPI.

The second aim was to analyze the phenotypic alteration due to silencing of SLPI in normal cell line with high SLPI levels and to the overexpression of SLPI in cancer cell lines with low SLPI expression levels.

The third aim was to evaluate the mechanism by which ETS proteins downregulate SLPI expression by focusing on STAT1 as possible intermediate since it has been reported that STAT1 upregulates SLPI expression by direct interaction with its promoter (Meyer M, et al., 2013)

3. MATERIAL AND METHODS

Cell Culture

The immortalized human prostate epithelial cell line RWPE was maintained in Keratinocyte serum-free medium (Gibco™) supplemented with 2.5 µg of human recombinant epidermal growth factor (rEGF), 25 mg of bovine pituitary extract (BPE) and 1% penicillin/streptomycin. The human prostate cancer cell line PC3 derived from bone metastases of a grade IV prostatic adenocarcinoma was maintained in Ham's F12 (Carlo Erba) supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine and 1% penicillin/streptomycin. The human prostatic cell line LNCaP derived from lymph node metastases was cultured with RPMI 1640 (EuroClone) containing 20% fetal bovine serum (FBS), 2% L-glutamine and 1% penicillin/streptomycin.

The human prostatic cancer cell line 22RV1 derived from a xenograft was cultured in RPMI 1640 (EuroClone) containing 10% fetal bovine serum (FBS), 2% L-glutamine and 1% penicillin/streptomycin. All cells were cultured at 37° in a humidified atmosphere containing 5% CO_2.

Vectors and transfection

RWPE cells were stably transduced with pLK0.1 (Addgene) vector containing short hairpin (sh) RNAs against SLPI: ShSLPI-1 5'-ATGCAACACTTCAAGTCACGC-3' or ShSLPI-2 5'-ATTTCTTAGGAGGACAGACTC-3', whereas as control we used the same vector containing an irrelevant shRNA 5'-GCCTATTTACGCCTGACAA-3'. The RWPE cells after transduction were selected with 0,5 µg/ml of puromycin (Sigma).

LNCaP cells were stably transduced with pLK0.1 vector containing shRNAs against ETV1: ShETV1-1 5'-TTGTGTTCATACACTGGGTCG-3' or shETV1-2 5'-AAACTTGTAGACATATCTCTC-3', and the pLK0.1 vector containing a shRNA against AR 5'-ATCCTGGAGTTGACATTGGTG-3' and the control vector containing the irrelevant shRNA. PC3 cells were transduced with two different shRNA vectors against ETV4 (shETV4-1 CCCTGTGTACATATAAATGAA and shETV4-2 GGCGCTTCCCAACTTCATA) and with the control vector. The LnCap and PC3 transduced cells were selected with 1 µg/ml of puromycin (Sigma).

The expression lentiviral plasmids were obtained cloning the human ETV4; ETV1, SLPI and STAT1 cDNA, amplified from PC3 (ETV4), LNCap (ETV1) and RWPE (SLPI and STAT1) cells, in a pLX304 vector (# 118625 from Addgene), in which we inserted a short sequence containing the recognition sequence of EcoRV and MluI sites. The primers for the RT-PCR contained the EcoRV and MluI recognition sequence to allow the cloning.

The cells stably transduced with these expression vectors and with an empty vector as control were then selected with 10 μg/ml of Blasticidin (Sigma).

For the ChIP experiments the RWPE cells were transfected with a vector containing the human ETV4 sequence and the FLAG sequence at C terminus, whereas as control the RWPE cells were transfected with an empty vector containing the FLAG sequence.

Protein extraction

Cells at 70% of confluence were lysed in RIPA Buffer containing protease and phosphatase inhibitors. Total protein concentration was measured by bicinchoninic acid (BCA) assay (Thermo Scientific) and then read with spectrophotometer.

Western blot

The protein extracts were separated on an 8%, 10% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred on polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking membranes, they were incubated overnight at 4°C with appropriate dilutions of specific primary antibodies. Then the membranes were hybridized with horseradish peroxidase conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) and detected using ECL (Superfemto Amersham, Piscataway, NJ) and the ImageQuant 350 chemoluminescence reader (GEHealthcare, Pittsburg, PA, USA); the data were then processed with the Quantity One (BIO-RAD) software.

The primary antibodies used are reported in Table 1.

Antigen	Producer
B-actin	Sigma-Aldrich, St Louis, MO, USA
HSP90 a/b	SantaCruz Biotechnologies, Dallas TX, USA
hETV4	Abnova, Atlanta, GA, USA
hETV1	Sigma-Aldrich, St Louis, MO, USA
hSLPI	R&D Systems, Minneapolis, MN, USA
Cleaved caspase 3 (Asp175)	Cell signalling; Boston, MA, USA
Caspase 3	Cell signalling; Boston, MA, USA
Cleaved caspase 7 (Asp198)	Cell signalling; Boston, MA, USA
Caspase 7	Cell signalling; Boston, MA, USA
Cleaved caspase 8 (Asp391)	Cell signalling; Boston, MA, USA
Caspase 8	Cell signalling; Boston, MA, USA
Cleaved caspase 9 (Asp330)	Cell signalling; Boston, MA, USA
Caspase 9	Cell signalling; Boston, MA, USA
MMP-2	Cell signalling; Boston, MA, USA
MMP-3	Cell signalling; Boston, MA, USA
MMP-9	Cell signalling; Boston, MA, USA
E-cadherin	SantaCruz Biotechnologies
N-cadherin	SantaCruz Biotechnologies
SLUG	Cell signalling; Boston, MA, USA
ZEB	Cell signalling; Boston, MA, USA
TWIST-1	Cell signalling; Boston, MA, USA
STAT1	Cell signalling; Boston, MA, USA

Table 1: *primary antibodies used for western blot experiments.*

RNA extraction

The extraction of RNA was performed from cells at 70% of confluences using RNeasy Mini Plus Kit (Qiagen). Total RNA was quantified by a spectrophotometer.

RT-PCR and Quantitative Real Time PCR

1 µg of RNA was reverse transcribed with High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, ThermoFisher Scientific). The retro transcription reaction was performed using the Thermal Cycler (BIO-RAD) with the following protocol: 37°C for 60'and then 95°C for 5'.

Quantitative Real Time PCR was conducted using the CFX96 real time thermocycler (BIO-RAD), with Sso Advanced Universal SYBR Green Supermix (BIO-RAD) diluted 1:2, the cDNA diluted 1:30 and 0.4 µl of each primer 10 µM. The protocol was the following: 98°C for 30'', 45 cycles at 98°C for 6'' and then 60°C for 10'', finally, 60°C for 5''.

The expression level of each gene was normalized to the expression of the housekeeping gene GAPDH and it was measured by relative quantification compared it to the control sample following the 2ΔΔC(T) method developed by Livak and Schmittgen (2001). The Real Time was performed in triplicate and each experiment was executed at least three times.

The primers used for each gene were reported in the Table 2.

Gene	Forward primer	Reverse primer
hETV4	5'-CCACCAGGATCAAGAAGGAG-3'	5'-CTCAGGAAATTCCGTTGCTC-3'
hETV1	5'-AACAGAGATCTGGCTCATGATTCA-3'	5'-TCTGGTACAAACTGCTCATCATTGTC-3'
hSLPI	5'-CCCTTCCTGGTGCTGCTT-3'	5'-TCTAAGGCACTGGGCAGATT-3'
hAR	5'-AATGAGTACCGCATGCACAA-3'	5'-CCCATCCACTGGAATAATGC-3'
MMP2	5'-ACGACCGCGACAAGAAGTAT -3'	5'-ATTTGTTGCCCAGGAAAGTG-3'
MMP3	5'-ATGCAGAAGTTCCTTGGATTGG -3'	5'-GATGCCAGGAAAGGTTCTGAAG -3'
MMP9	5'-GGGCTCCCGTCCTGCTT -3'	5'-CCTCCACTCCTCCCTTTCCT -3'
SLUG	5'-GCCCTCCAAAAAGCCAAACTA-3'	5'-CACAGTGATGGGGCTGTATG-3'
ZEB	5'-GCCAATAAGCAAACGATTCTG-3'	5'-TTTGGCTGGATCACTTTCAAG-3'
TWIST	5'-GCCGGAGACCTAGATGTCATT-3'	5'-CACGCCCTGTTTCTTTGAAT-3'
hGAPDH	5'-AACGGATTTGGTCGTATTGGGC-3'	5'-TTGATTTTGGAGGGATCTCG-3

Table 2: *sequences of primers used for qRT-PCR experiments.*

Chromatin Immunoprecipitation (ChIP)

For ChIP experiment the RWPE cells were transiently transfect with pCMV-ETV4-3Flag and pCMV-3Flag vector (stratagene). The chromatin was extracted using Magna ChIP A/G kit (Millipore), it was sonicated to a fragment size range of 100-500bp and then precipitated over-night at 4°C with Normal Mouse IgG antibody (Millipore, Burlington, MA, USA) for negative control and with an anti-Flag antibody (Cell Signaling Technology, Danvers, MA, USA). After purification the precipitated chromatin was analyzed by Real Time PCR (the primers used are reported in Table 3). ChIP-qPCR data were normalized using the "Percent Input Method", that divided the signals obtained from the ChIP by signals obtained from an input sample. The input sample represents 1% of the amount of chromatin used in the ChIP, thus a dilution factor of 6.644 cycles (log2 of 100) is subtracted from the Ct value of diluted input.

Gene	Forward primer	Reverse primer
h*COX2*	5'-TCCCTCCTCTCCCCTTAAAA-3'	5'-AGTGGGGACTACCCCCTCTG-3'
SLPI1	5'-ACTTCCCAGGCCAATCTCTT-3'	5'-AAGCAGGAAACGTAGCCAGA-3'
SLPI2	5'-CCTATGCAGACTGGGTAGCAA-3'	5'-GGGAAAGCAGCTCATCAGTC-3'
SLPI3	5'-CCAGCCCCTTTTCATTCTTT-3'	5'-CCAGGGGATAATTTGATTTCTCT-3'
SLPI4	5'-CTCCAGGGCTGGCTACATAA-3'	5'-GAGAGAAACTGCCAAAGAAAGTT-3'

Table 3: *sequences of primers used for Chip-qPCR experiments.*

Cell proliferation assay

The RWPE, PC3 and LNCaP were plated in triplicate in 96-well plates at the density of 3000 cells/well of RWPE and PC3 and 2000 cells/well of LNCaP. Each cell line was cultured as described above. The cell proliferation was evaluated using Cell Counting Kit-8 (Sigma-Aldrich). After 24-48 and 72 hours, the CCK-8 was added to attached cells and were incubated for two hours in incubator, and then the absorbance was measured at 450 nm using a plate reader (Victor X5, PerkinElmer)

Cell migration assay

The RWPE, PC3 and LNCaP were seeded in the Culture-Inserts (Ibidi) placed in a 6-well using to create the cell free gap. After 24 hours the Culture-Inserts were removed

to create the cell free gap and the wells were filled with appropriate medium with 2% of FBS for LNCaP or 1% of FBS for PC3 or without supplements for RWPE. In the medium 0,5 µM of mitomycin C was added to prevent cell proliferation. The migration was measured on photographs taken at 0h and 36h.

Cell invasion assay

Transwell membrane inserts with pore of 8 µm (Corning, Lowell, MA USA) were coated with 50 µl of matrigel (BD Biosciences). RWPE, LNCaP and PC3 cells were plated (150.000 RWPE/LNCaP or 70.000 PC3 for well) in 500 µl of the appropriate medium, without Fetal Bovine Serum (FBS) or for RWPE cells without supplements, in the upper chamber, whereas 500 µl of the medium completed with FBS or supplements was loaded in the lower chamber (in LNCaP and PC3 20µg/ml of hEGF were added in the lower chamber). The inserts were incubated at 37° in CO2 incubator for 48h (RWPE) or 72h (PC3) or 5-days (LNCaP).

The invading cells through the Matrigel were stained with DIFF-QUICK (Medicult, Firenze, Italy) and then were counted by microscope.

Apoptosis analysis by flow cytometry

Apoptotic cell death was measured by using "PE-annexin V apoptosis detection kit" (BD Bioscienses). RWPE, PC3 or LNCaP cells were collected with their growth medium, briefly washed with cold PBS, resuspended in 100 µl of 1% Binding buffer and stained with PE-conjugated annexin V and 7-AAD. After 15 minutes of incubation at room temperature in the dark, 400 µl of 1% Binding buffer were added and then the stained cells were analyzed by flow cytometer (Cytoflex, Beckman Coulter).

Statistical analysis

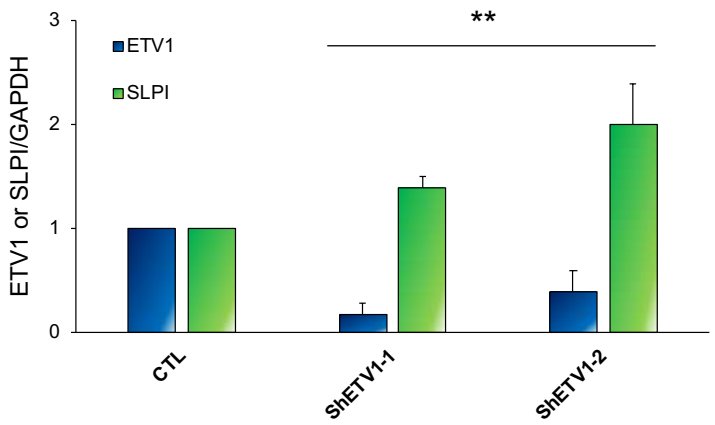
All results were expressed as mean ± standard error. Statistical analysis was performed using t-test. Statistical significance was accepted for $p < 0,05$.

4. RESULTS

4.1 SLPI is down regulated by ETV1 *in vitro*

In the laboratory in which I have worked for my PhD it was previously demonstrated that the Secretory Leukocyte Protease Inhibitor (SLPI) expression was reduced in a murine-model over-expressing ETV4 in the prostate compared to WT mice and this is similar to what has been observed in human prostate cancer patients. Furthermore in *in vitro* experiments we have demonstrated that ETV4 down-regulates SLPI expression. However, ETV4 is only one of the ETS proteins found overexpressed in prostate cancer patients. For this reason, we decided to test if also ETV1, another ETS protein that belongs, as ETV4, to the Pea3 subfamily, down-regulates SLPI expression. We reduced the ETV1 expression levels with two different short hairpin (sh) RNAs in the LNCaP cells, a human prostate cancer cell line in which there are high levels of ETV1 due to the translocation of the entire locus of ETV1 in the last intron of the prostate- specific MIPOL1 gene on chromosome 14 (Tomlins SA, et al., 2007). As control cells we used the LNCaP cells transfected with a scrambled shRNA. In preliminary experiments in our lab, it has been shown that the silencing of ETV1 expression with both shRNAs resulted in an increase of SLPI expression at both mRNA and protein level.
I performed additional experiments that confirmed these results (Figure 15 A-B).

A.



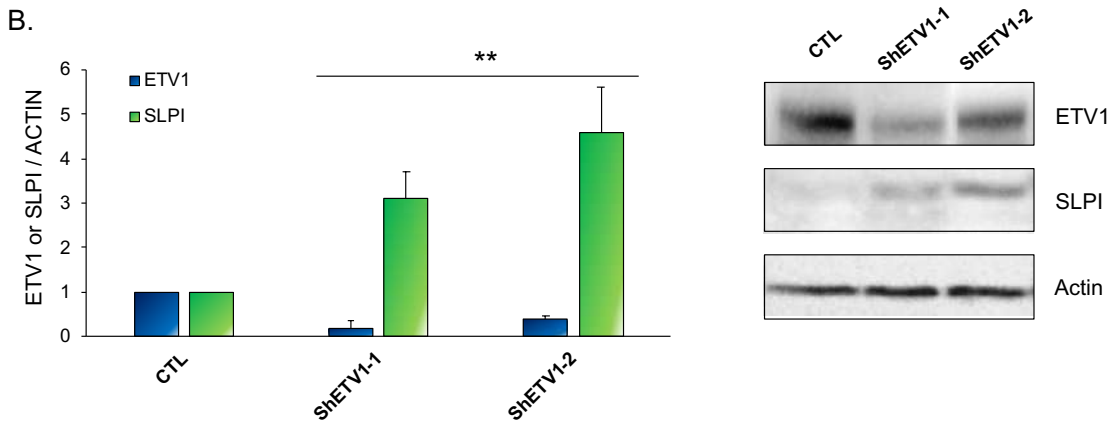
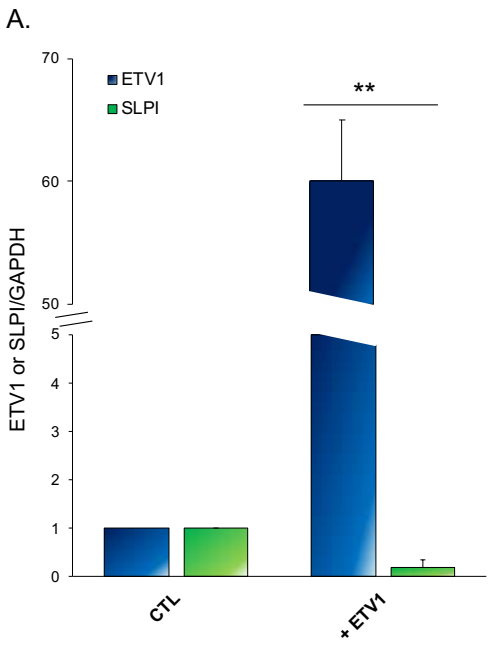


Figure15: *ETV1 and SLPI expression levels in LNCaP cell line stable transduced with two ETV1 shRNA (ShETV1-1 and ShETV1-2).* ***A)*** *mRNA levels of ETV1 (blue) and SLPI (green) obtained by qRT-PCR. The statistical significance is indicated by ** for P≤ 0,01* ***B)*** *Bars diagram (left) and representative western blot image (right) of ETV1 and SLPI protein level. In the bar diagram in blue are reported the levels of ETV1 and in green the levels of SLPI. The statistical significance is indicated by ** for P≤ 0,01*

In addition, we stably transduced the RWPE, a human no-malignant prostate cell line, with an expression vector encoding the human ETV1. The cells transduced with ETV1 showed an increase of ETV1 compared with control cells (RWPE cells transduced with an empty vector) of 60-fold at mRNA and 90-fold at protein levels. The overexpression of ETV1 resulted in a strong reduction of SLPI mRNA and protein levels (Figure16 A-B).



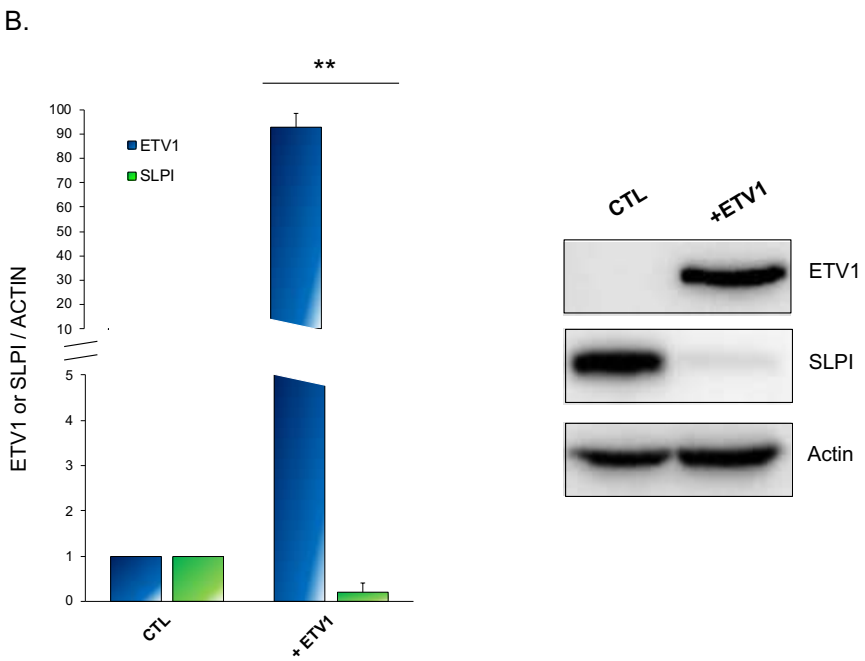


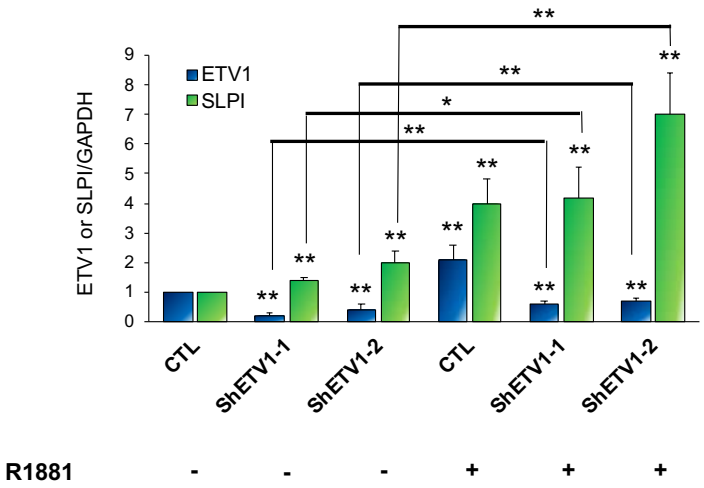
Figure 16: ETV1 and SLPI expression level in RWPE cells stably transduced with ETV1 expressing vector. **A)** *ETV1 and SLPI expression assessed by qRT-PCR are indicated by blue and green bars, respectively. The ** represents the statistically significance variations (P ≤ 0,01).* ***B)*** *Bars diagram (left) and representative western blot image (right) of ETV1 and SLPI protein level. In the bar diagram in blue are reported the levels of ETV1 and in green the levels of SLPI. The statistical significance is indicated by **for P≤ 0,01*

4.2 Androgens induce the expression of SLPI in prostate cells

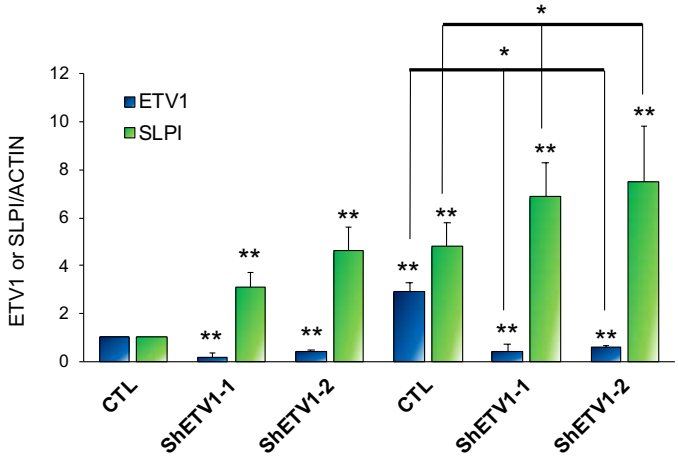
Since LNCaP cells are an androgen-sensitive cell line, we decided to test the effect of androgenic stimulus on the regulation of SLPI expression. The LNCaP cells were grown in an androgen-free Charcoal stripped FBS medium without phenol red since its structural similarity with steroid hormone may mimic androgens. The experiments were performed in presence or absence of the synthetic androgen R1881 (Methyltrienolone). To evaluate the efficiency of androgenic stimulus we used as positive control the PSA, a pivotal target gene of the androgen receptor (AR) signaling.

The addition of androgen results, as expected, in an increase of ETV1 that is controlled by the promoter of the androgen-regulated MIPOL1 gene and in an increase of SLPI expression that was even higher in the cells with reduced ETV1 levels (Figure 17 A-B-C). These experiments have confirmed preliminary experiments performed in our laboratory.

A.


ETV1
SLPI
ETV1 or SLPI/GAPDH
0
1
2
3
4
5
6
7
8
9
**
*
CTL
ShETV1-1
ShETV1-2
CTL
ShETV1-1
ShETV1-2
R1881
- - - + + +

B.


ETV1
SLPI
ETV1 or SLPI/ACTIN
0
2
4
6
8
10
12
**
*
CTL
ShETV1-1
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ShETV1-2
R1881
- - - + + +

C.

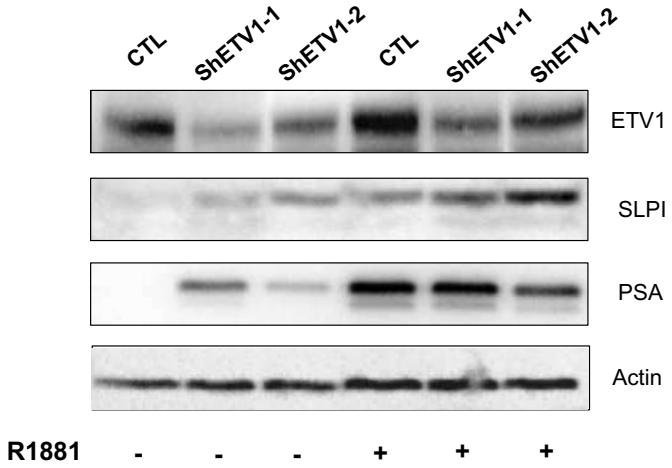

CTL
ShETV1-1
ShETV1-2
CTL
ShETV1-1
ShETV1-2
ETV1
SLPI
PSA
Actin
R1881
- - - + + +

Figure 17: *ETV1 and SLPI mRNA and protein level in two stable ETV1-knocked down (ShETV1-1 and ShETV1-2) LNCaP cell line in presence and in absence of R1881.* ***A)*** *ETV1 (blue bars) and SLPI (green bars) expression with and without R1881. The statistical significance is indicated by * for P ≤ 0,05 and by ** for P ≤ 0,01.* ***B)*** *Bar diagram with the results of ETV1 (blue bars) and SLPI (green bars) protein levels in presence and in absence of R1881 assessed by western blot. The statistical significance is indicated by * for P ≤ 0,05 and by ** for P ≤ 0,01* ***C)*** *Western blot showing the protein expression of ETV1, SLPI and PSA in LNCaP cells with ETV1 silencing both in presence and in absence of R1881.*

In order to confirm the regulation of SLPI expression by androgens, we tested the effects of androgen stimulus in 22RV1, an androgen responsive human prostate cancer cell line that does not express any ETS proteins. Also in this cellular model we observed an increase of SLPI expression following the androgen stimulus (Figure 18). These data suggest that both androgens and the androgen-regulated ETV1 regulate SLPI expression in opposite manner.

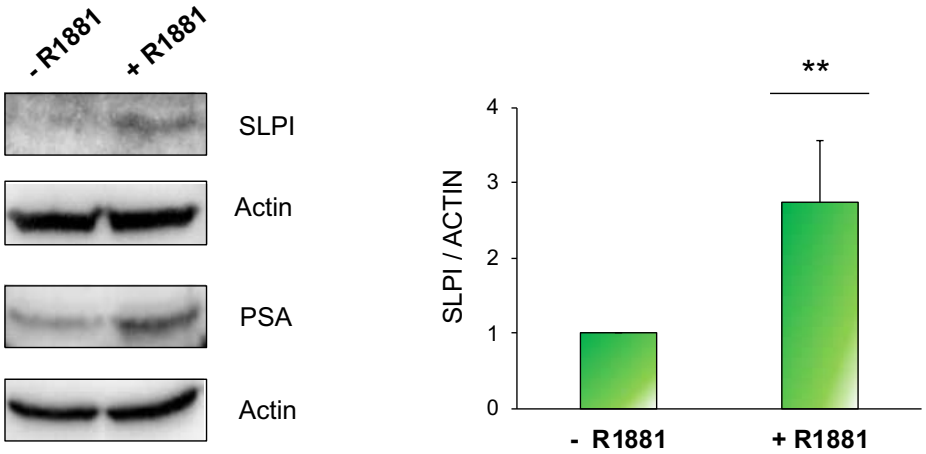


Figure 18: *Western blot image (left) and bar diagram (right) showing the protein expression of SLPI in 22RV1 cell line in presence and in absence of R1881. PSA is used as positive control for the androgen stimulation.*

4.3 SLPI is up regulated by Androgen Receptor (AR) *in vitro*

We also investigated the role of AR in the regulation of SLPI expression in the AR-positive LNCaP cells. When we reduced the AR expression in the LNCaP cell with a specific shRNA we observed the reduction of both ETV1 and SLPI mRNA and protein expression compared to the control cells (Figure 19 A-B).

These data appear even more significant considering the reduction of ETV1 caused by AR knockdown that should increase SLPI expression.

These results suggested that SLPI is up regulated by the androgen/androgen receptor axis.

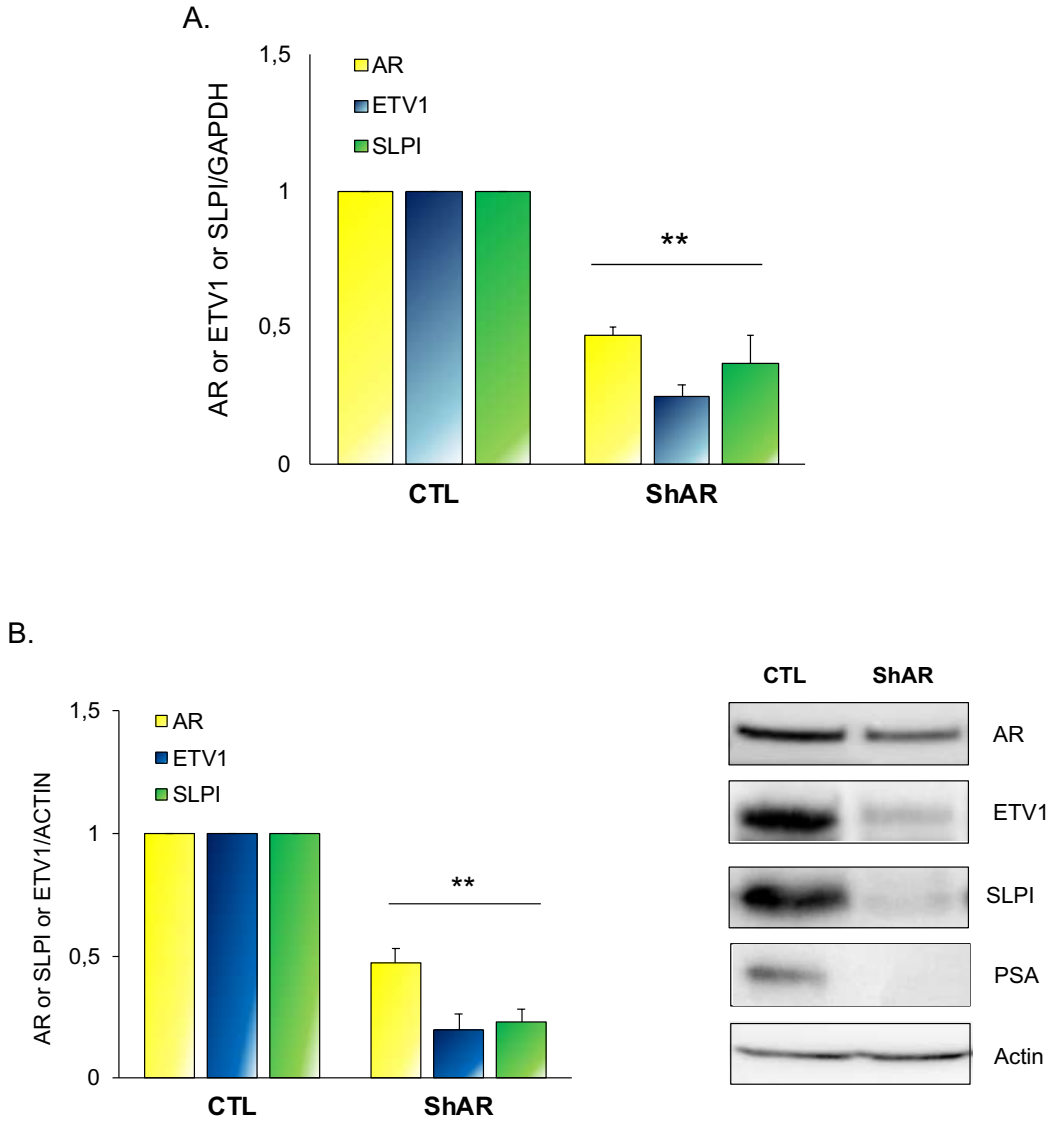


Figure 19: *Androgen Receptor (AR), ETV1 and SLPI mRNA and protein levels in LNCaP cells transduced stably with a sh against AR.* ***A)*** *Expression levels of AR (yellow bars), ETV1 (blue bars) and SLPI (green bars) measured by qRT-PCR.* ***B)*** *protein levels of AR, ETV1 and SLPI were showed by bar diagram (left) and western blot image (right). PSA is used as positive control for AR silencing. The ** indicates the statistically significance variations (P ≤ 0,01).*

4.4 Downregulation of SLPI expression reduces migration, invasion, EMT and induces apoptosis in immortalized human prostate cell line *in vitro*

We measured SLPI expression level in an immortalized normal human prostate cell line, RWPE and we found that the SLPI mRNA and protein levels were much higher than those of LNCaP and PC3 cell lines (Figure 20 A-B).

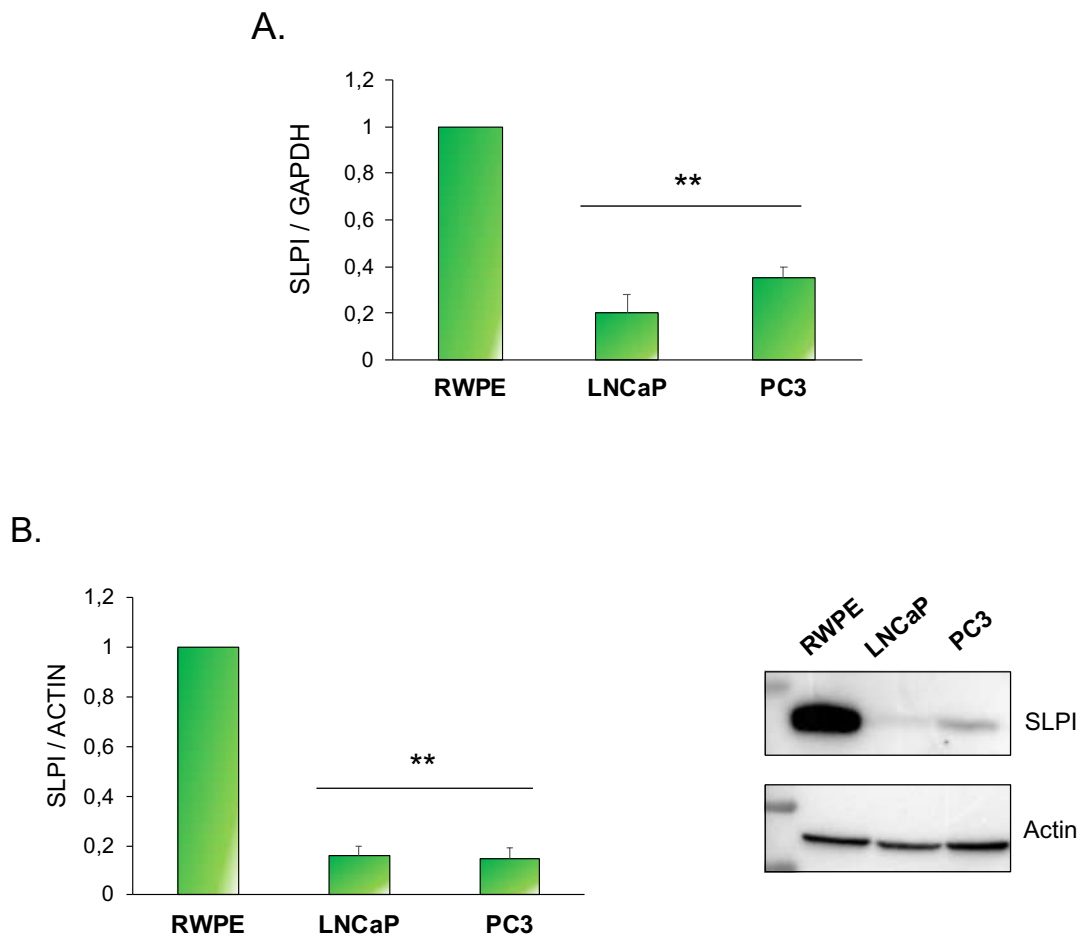


Figure 20: *SLPI mRNA and protein levels in RWPE, LNCaP and PC3 cell line.*
A) *SLPI expression level normalized to the housekeeping gene GAPDH measured by qRT-PCR* ***B)*** *protein levels of SLPI measured by western blot. Beta Actin was used as loading control. The ** indicates the statistically significance variations (P ≤ 0,01).*

In order to study the role of SLPI on the phenotypic hallmarks of prostate cancer, we used as cellular model the RWPE cells stably transduced with two different shRNAs against SLPI. The SLPI levels were about 10-fold lower than RWPE control cells (RWPE transduced with a scrambled negative control shRNA) at mRNA levels and the reduction was even higher at protein level (Figure 21 A-B).

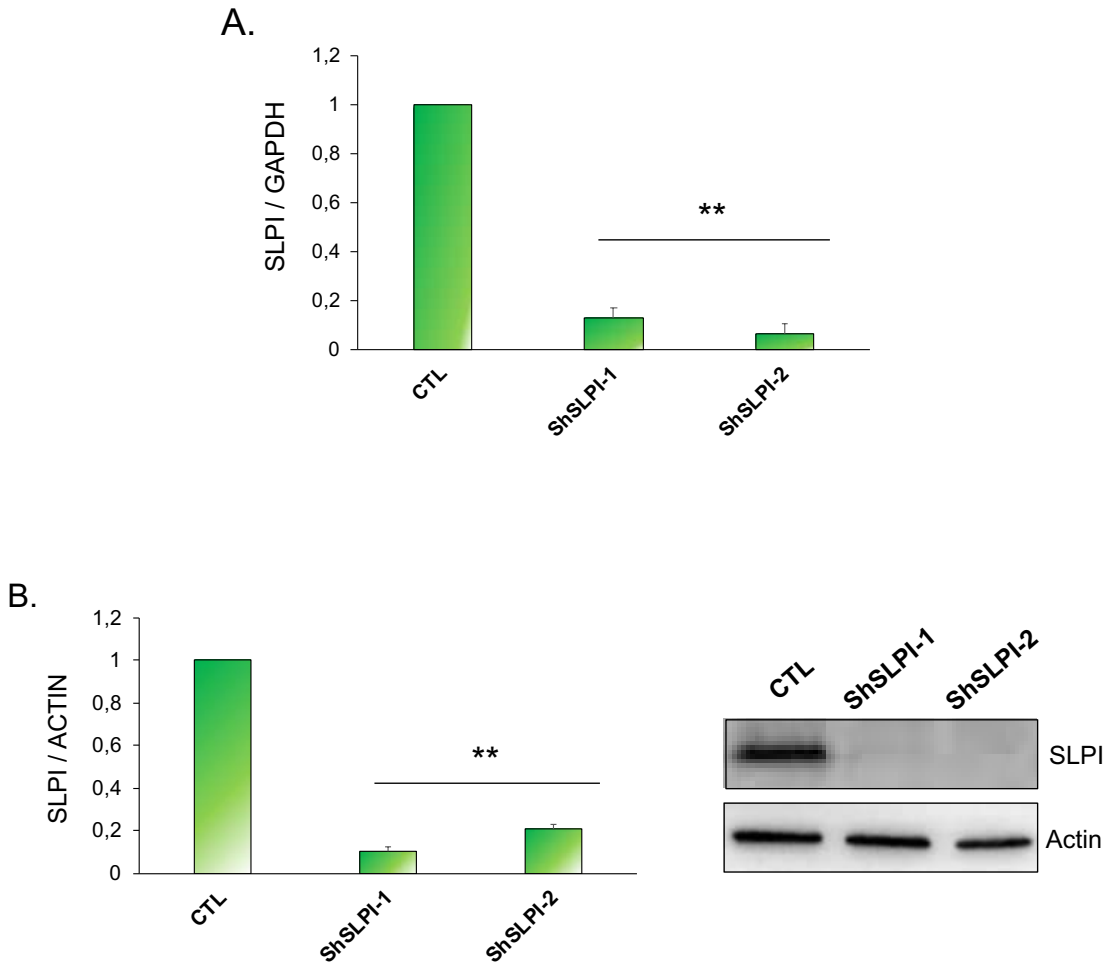


Figure 21: *SLPI mRNA and protein level in two stably SLPI knocked down (ShSLPI-1 and ShSLPI-2) RWPE cell line.* ***A)*** *SLPI expression level normalized to the housekeeping gene GAPDH measured by qRT-PCR.* ***B)*** *protein levels of SLPI assessed by western blot. Beta Actin was used as loading control. The ** indicates the statistically significance variations (P ≤ 0,01).*

Cell proliferation

We first measured the effect of SLPI silencing on the growth of RWPE cells with CCK-8 assay, a colorimetric test capable of determining the number of viable cells through the detection of the amount of the formazan dye produced by the activity of the dehydrogenase in cells that is directly proportional to the number of living cells.

We observed that the reduction of SLPI levels in RWPE cells does not affect the proliferation rate (Figure 22).

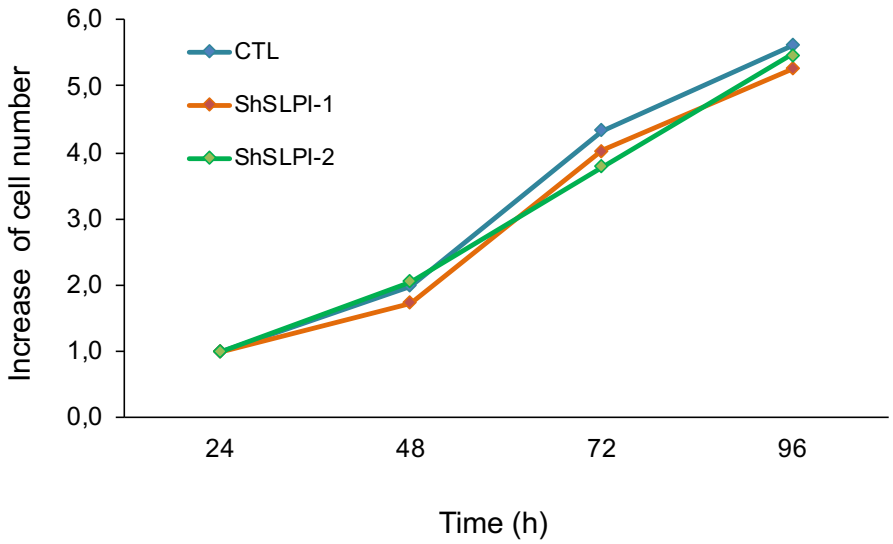


Figure 22: *Analysis of cell proliferation in RWPE cells transduced with two different ShRNAs, orange and green lines, and the control represented by blue line. Each point represents the average of 3 different experiments.*

Cell Migration

The contribution of SLPI on migration was tested using a culture-insert (IBIDI) that produces a cell-free gap. In order to inhibit the cell proliferation, we added mitomycin C to the culture medium. After 36 hours, the RWPE cells with lower SLPI levels showed a reduced ability to migrate of about 5,2-fold compared to control RWPE cells (Figure 23).

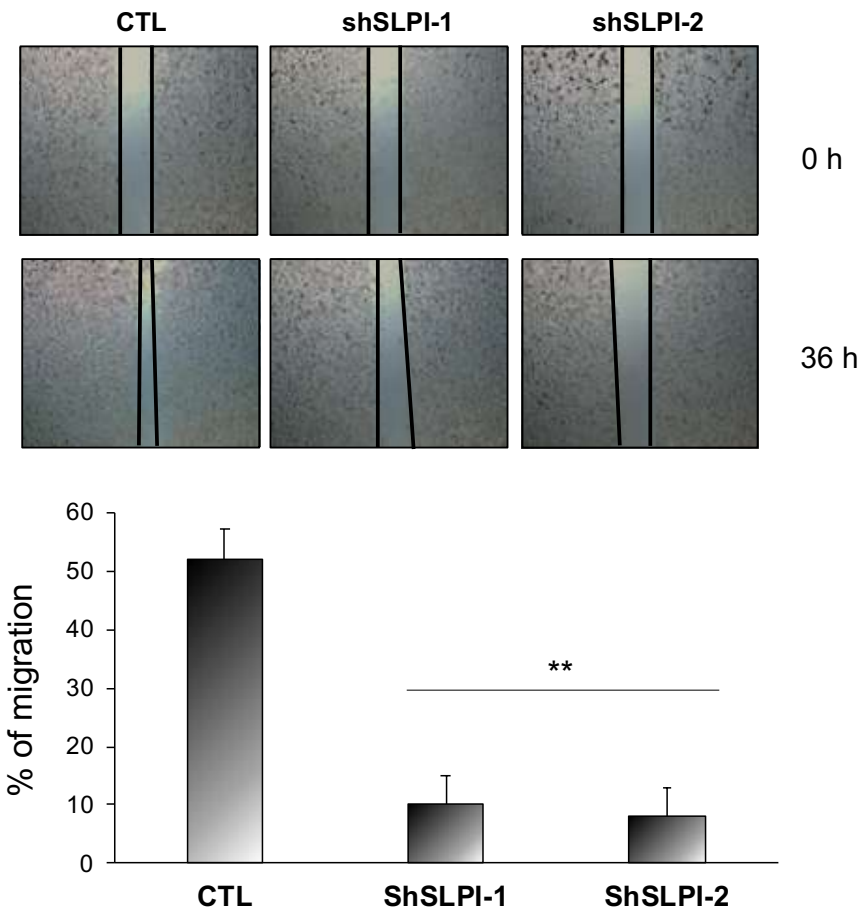


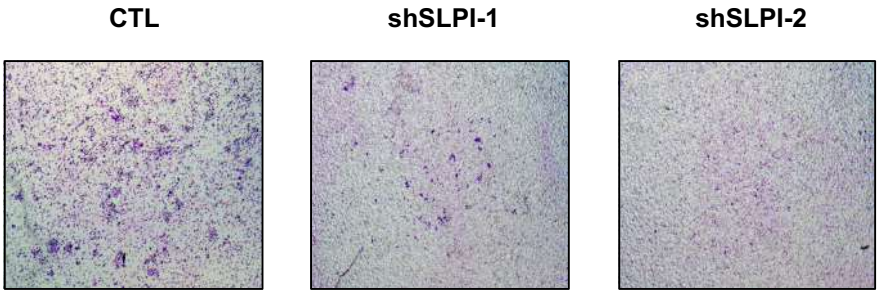
Figure 23: *Cell migration evaluated after 36 hours of RWPE stably transduced with 2 shRNA against SLPI and RWPE control cells (CTL). The ** represents the statistically significance variations (P ≤ 0,01).*

Cell invasion

We also tested the ability of RWPE after reduction of SLPI to invade a Matrigel matrix that mimic *in vitro* the basal membranes of tissues.

The cells were plated in a Transwell insert coated with Matrigel that was inserted in the well of a plate. In both chambers we used the same culture media but only in the lower chamber we added Bovine Pituitary Extract (BPE) and the Human Recombinant Epidermal Growth Factor (rEGF). After 48 hours we examined the ability of RWPE cells to degrade the Matrigel and move through the pores of the membrane, by using an inverted microscopy.

We observed that the number of RWPE cells with reduction of SLPI have a lower capability to invade the Matrigel matrix than control cells (Figure 24).



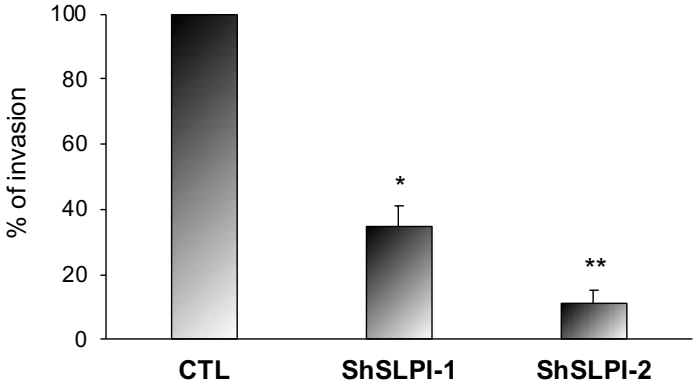


Figure 24: *the percentage of invasion of stably transduced RWPE was detected via Transwell analyses by using an inverted microscope. The ** represents the statistically significance variations (P ≤ 0,01).*

The MMPs play a pivotal role in invasion of cancer cells and tumor dissemination by degrading the extracellular matrix (ECM) components through their proteolytic activity (Niland S, et al., 2021), thus we decided to analyze the effect of SLPI on MMPs mRNA and protein levels.

We observed that the silencing of SLPI with both shRNAs results in a reduction of MMP-2, MMP-3 and MMP-9 at mRNA levels (Figure 25 A). A strong reduction was also detected for MMP-2 and MMP-3 proteins (Figure 25 B), whereas we have not been able to detect the MMP-9 protein levels.

A.

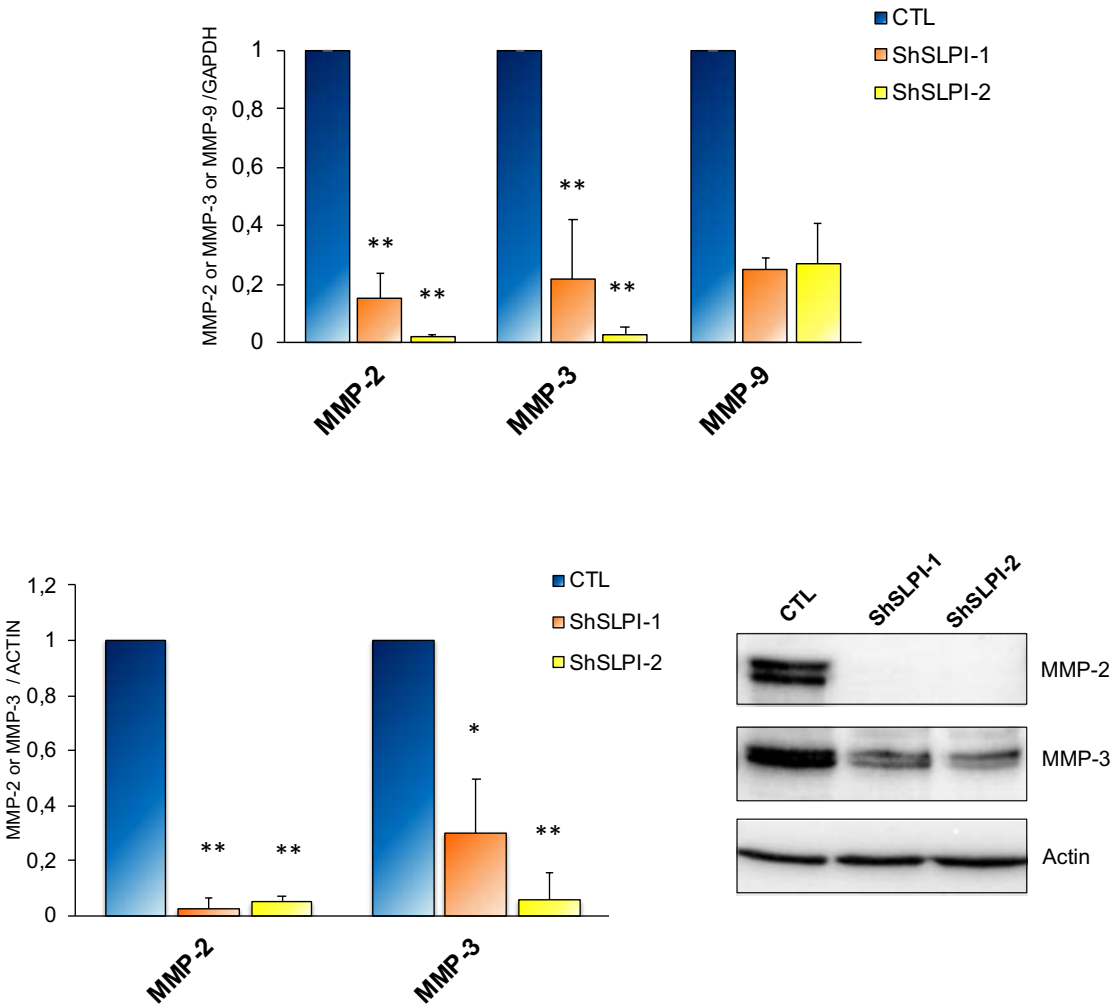


Figure 25: *MMPs expression levels in RWPE with reduced SLPI levels.* ***A)*** *mRNA levels of MMP-2, MMP-3 and MMP-9 in RWPE stably transduced with ShSLPI-1 (orange bar), ShSLPI-2 (yellow bar) and scramble shRNA containing vector (blue bar).* ***B)*** *protein levels of MMP-2 and MMP-3 obtained by western blot experiments were showed by bar diagram (left) and western blot image (right). Beta Actin was used as loading control.*
*The statistical significance is indicated by * for $P \leq 0,05$ and by ** for $P \leq 0,01$.*

Epithelial mesenchymal transition (EMT)

The observation of a role of SLPI in migration and invasion capacity in RWPE cell line suggested to verified if SLPI is able to alter the EMT program, that plays a pivotal role in cell invasion and dissemination.

We first evaluated the expression levels of E-Cadherin and N-Cadherin, two calcium-dependent transmembrane glycoproteins that belong to the family of classical cadherins;

E-cadherin is a marker of epithelial phenotype whereas N-Cadherins represents a mesenchymal marker.

During EMT in cancers, E-Cadherins results downregulated while N-Cadherins is upregulated, this cadherin switching is associated with an improved of migratory and invasive phenotype.

We observed that the silencing of SLPI results in an increase of E-Cadherin expression and a decrease of N-Cadherin expression both through real-time and western blot analyses (Figure 26 A-B).

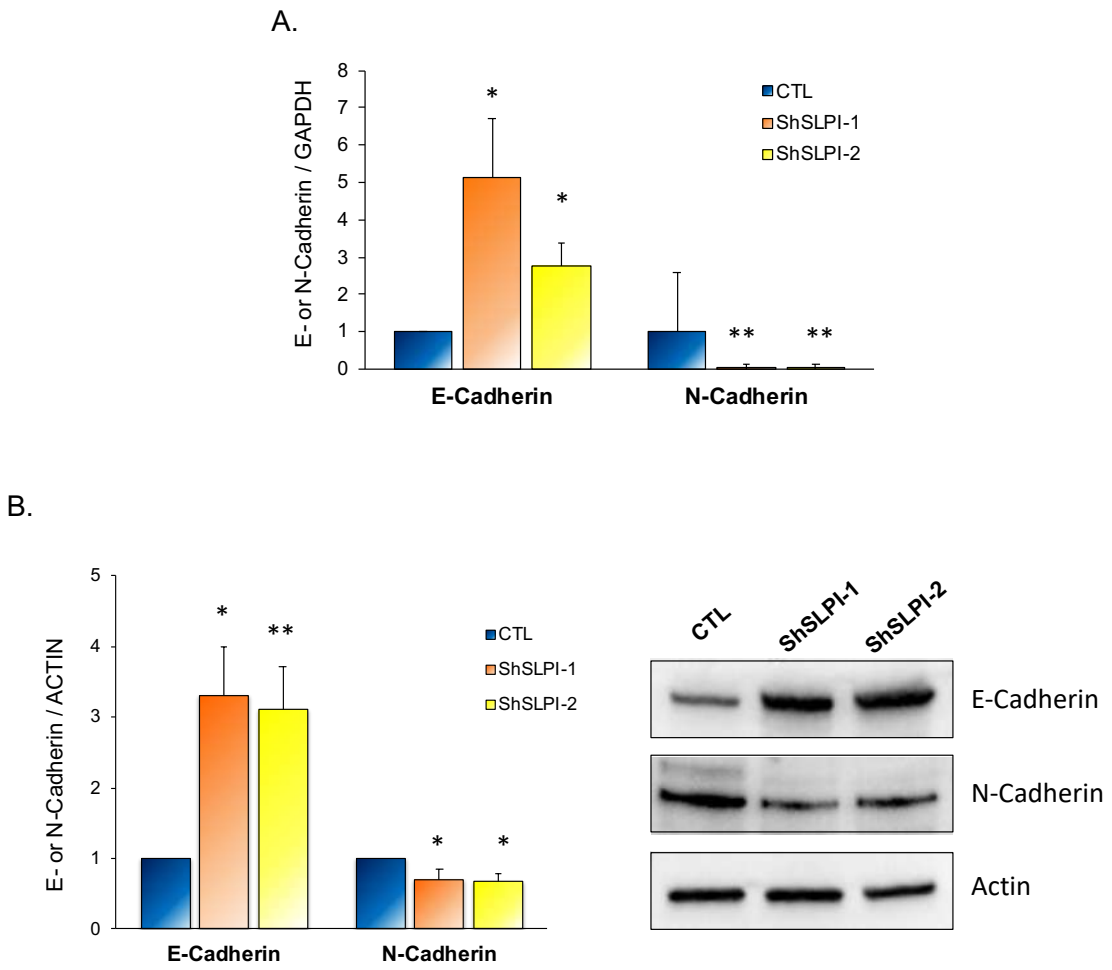


Figure 26: *E-Cadherin and N-Cadherin levels in RWPE after the reduction of SLPI levels.* ***A)*** *mRNA levels of E-Cadherin and N-Cadherin in RWPE stably transduced with ShSLPI-1 (orange bar), ShSLPI-2 (yellow bar) and scramble shRNA containing vector (blue bar), assessed by qRT-PCR.* ***B)*** *protein levels of E-Cadherin and N-Cadherin assessed by western blot. Beta Actin was used as loading control.*

*The statistical significance is indicated by * for $P \leq 0,05$ and by ** for $P \leq 0,01$.*

Next, we also analyzed the expression levels of several transcription factors that promote EMT, such as: SLUG, TWIST, ZEB.

We found that the mRNA levels of SLUG, TWIST and ZEB decrease in the RWPE cells in which SLPI expression is silenced (Figure 27A).

Furthermore, the reduction of SLPI in RWPE cell line induces a decrease of protein levels of SLUG and TWIST and a strong decrease of ZEB expression. (Figure 27 B).

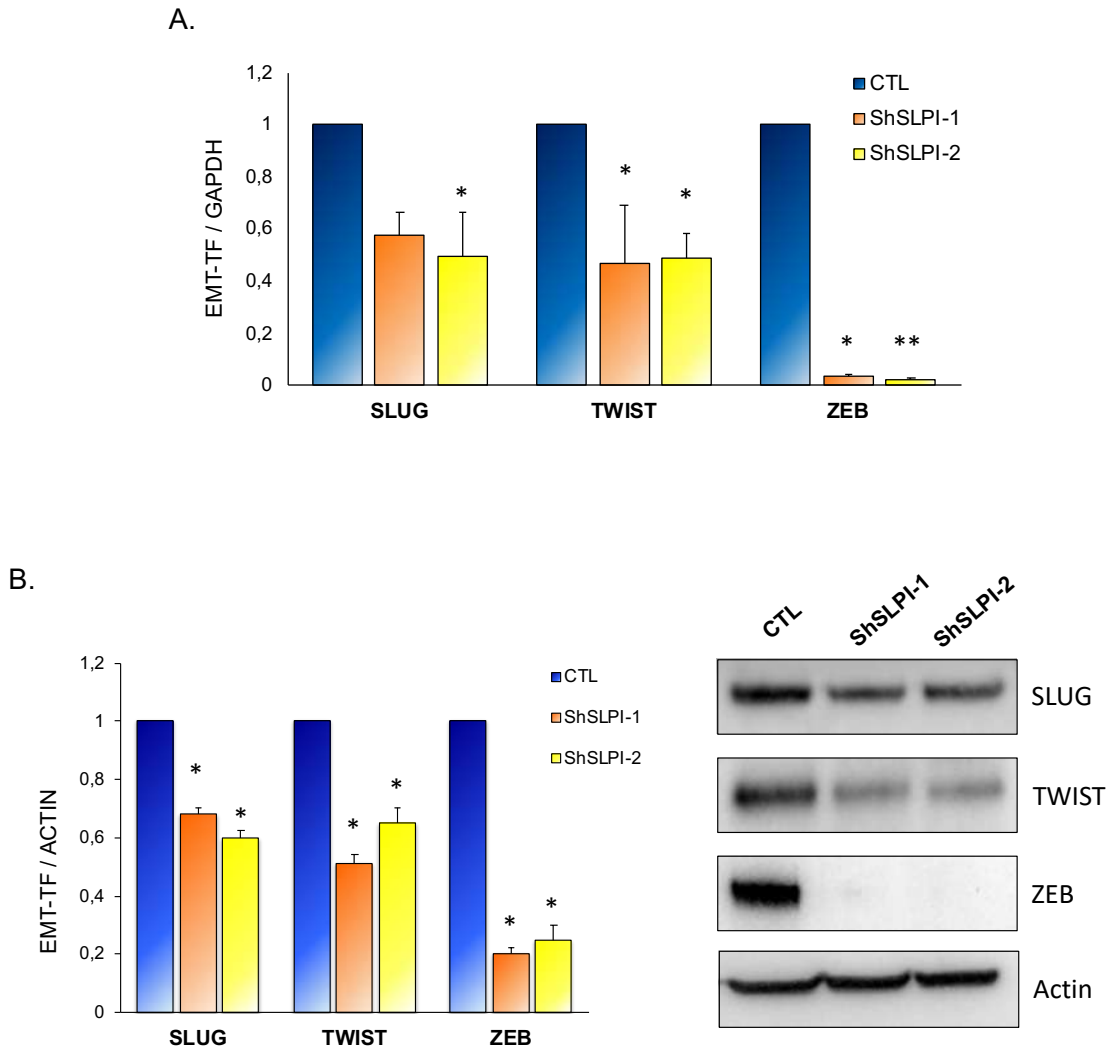


Figure 27: *expression levels of SLUG, TWIST and ZEB after the reduction of SLPI in RWPE cells with two different shRNAs, ShSLPI-1 (orange bar), ShSLPI-2 (yellow bar) and scramble shRNA containing vector (blue bar).* ***A)*** *mRNA levels of SLUG, ZEB and TWIST assessed by qRT-PCR.* ***B)*** *protein levels of SLUG, ZEB and TWIST measured by western blot were showed by bar diagram (left) and representative western blot image (right).*

*The statistical significance is indicated by * for $P \leq 0,05$ and by ** for $P \leq 0,01$.*

Apoptosis

We investigated whether SLPI induces apoptosis in RWPE cells in which SLPI is stably silenced, by performing an Annexin V/ 7-AAD double staining and flow cytometry analyses. In Figure 28 is reported a dot plot obtained by flow cytometry analyses with alive cells (Annexin V- / 7-AAD - cells in the lower left quadrant), cells in early apoptosis (Annexin V + / 7-AAD – at the lower right quadrant), cells in late apoptosis (Annexin V + / 7-AAD + in the upper right quadrant) and death cells (Annexin V - / 7-AAD + in upper left quadrant). We found that the silencing of SLPI induces apoptosis in RPWE cells, in fact we observed an increase of both cells in early or late apoptosis in SLPI silenced RWPE compared to control (Figure 28).

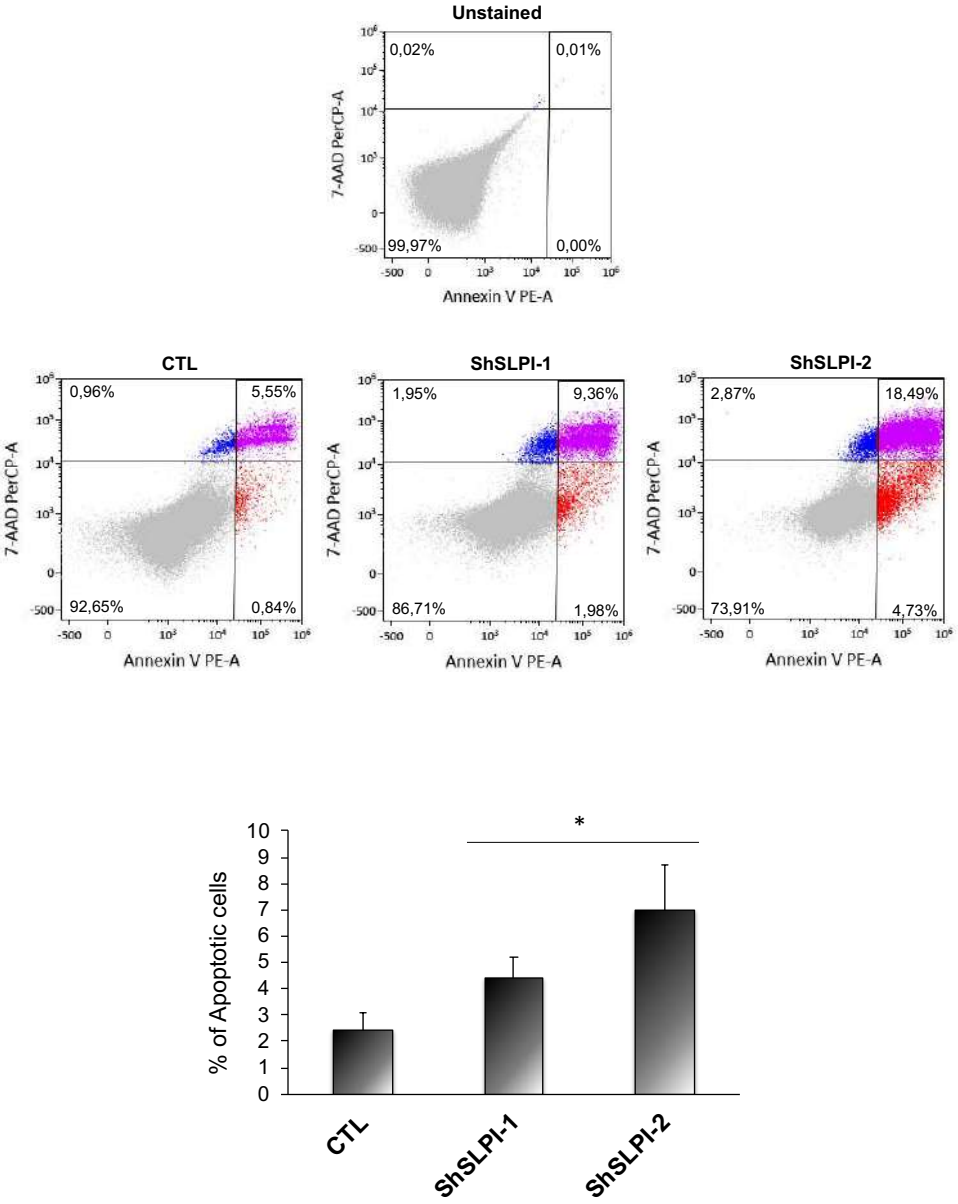
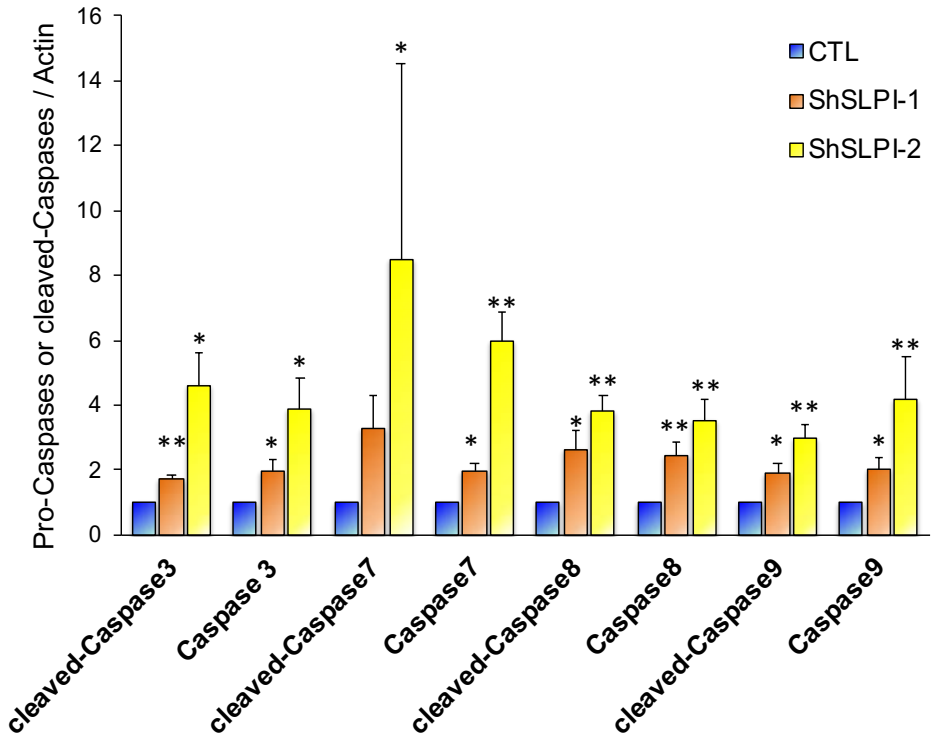


Figure 28: *Flow cytometry analysis of RWPE cells stable transduced with two different shSLPI (shSLPI-1 and shSLPI-2) and control (CTL). RWPE cells double stained with Annexin V and 7-AAD. In grey is reported the viable cells (Annexin V - / 7-AAD -), in red the cells in early apoptosis (Annexin V + / 7-AAD -), in pink the cells in late apoptosis (Annexin V +/ 7-AAD +) and in blue the death cells (7-AAD + / Annexin V -). The * indicates the statistically significance variations (P ≤ 0,05).*

In order to understand how SLPI regulate apoptosis we decided to analyze the Caspases expression levels by western blot experiments. In particular we analyzed the expression levels of the apoptotic initiator of caspases-8 and -9 and the effector caspases-3 and -7. We observed that when SLPI expression is reduced in RWPE the caspases-3, -7, -8 and -9 expression are increased both as unprocessed zymogens (pro-caspases) and as activated cleaved caspases (Figure 29).



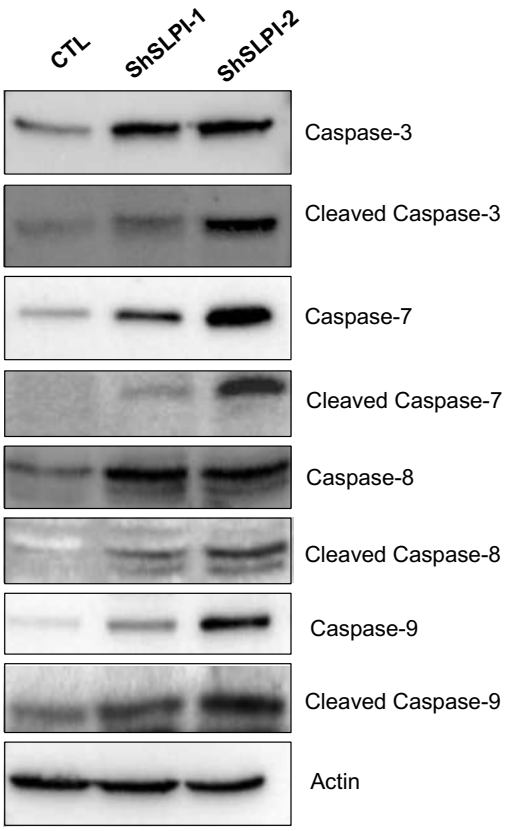


Figure 29: *Protein levels of Caspases-3, -7, -8 and -9 of RWPE stable cells transduced with two different shRNAs, ShSLPI-1 (orange bar), ShSLPI-2 (yellow bar) and control (CTL) represented by blue bar analyzed by western blot analysis.*

*The statistical significance is indicated by * for P ≤ 0,05 and by ** for P ≤ 0,01.*

4.5 Effects of overexpression of SLPI on the phenotype of two human prostate cancer cell lines

In order to study the contribution of SLPI to the phenotype of prostate cancer, we stably transduced the LNCaP and PC3, two human prostate cancer cell lines, with an expression vector encoding for human SLPI and an empty vector as control. In the androgen responsive LNCaP cells we also studied the role of SLPI in prostate tumorigenesis in association with androgenic stimulus.

In both transduced cell lines, we observed a great increase of SLPI levels (2000-fold in PC3 and 1100-fold in LNCaP) compared to control (Figure 30 A- B) and the addition of androgen results in further increase of SLPI mRNA and protein levels compared to unstimulated cells (Figure 30 A-B).

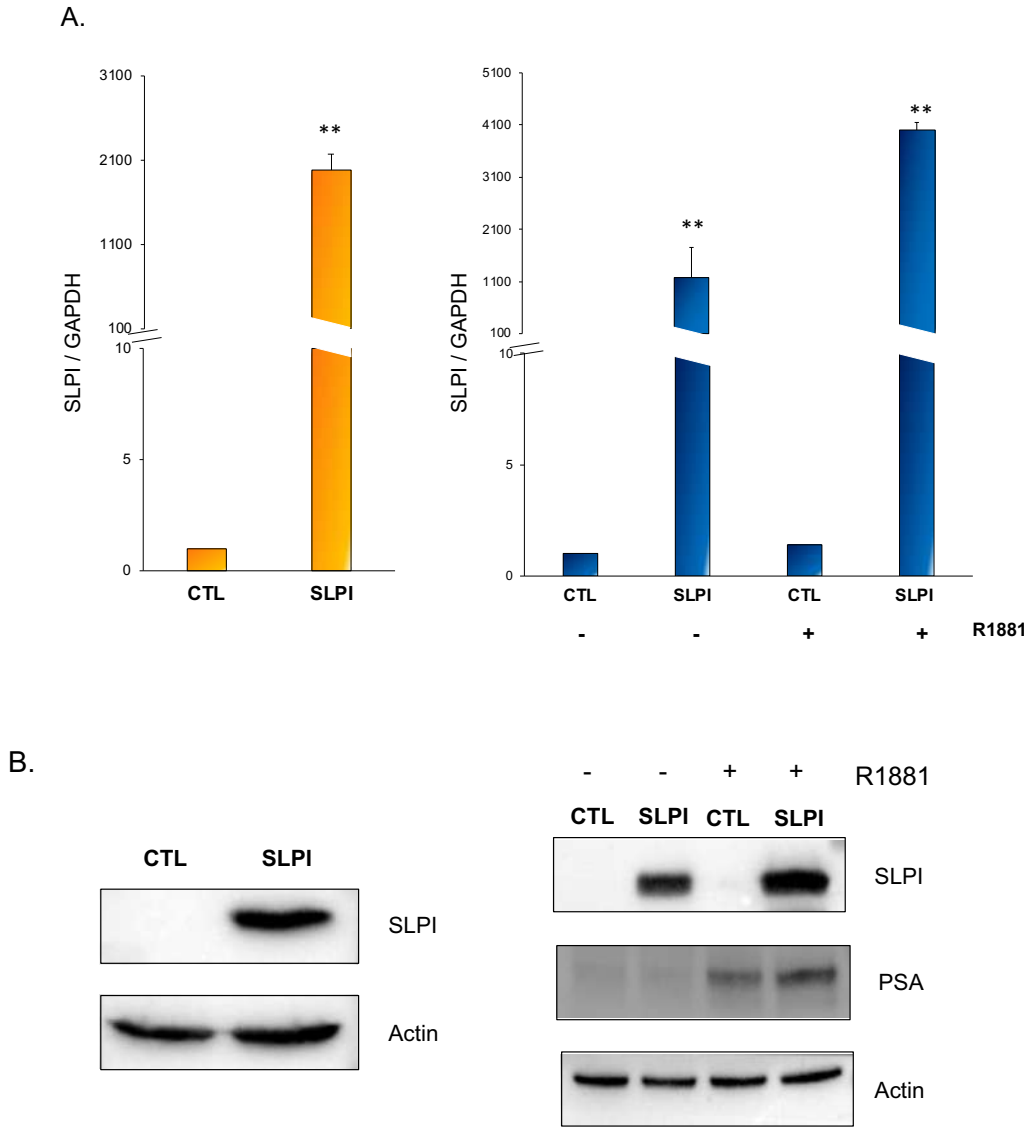


Figure 30: *SLPI in PC3 cell line and LNCaP cells overexpressing SLPI.* ***A)*** *SLPI mRNA levels measured by qRT-PC in PC3 (blue bar) and in LNCap (orange bar) with and without R1881 addition.* ***B)*** *SLPI protein levels in PC3 (on the left) and LNCaP (on the right) cell lines. PSA was used as positive control for androgen stimulus.*
*The * indicates the statistically significance variations (P ≤ 0,05).*

Cell proliferation

In order to evaluate the effect of SLPI in cell proliferation we performed CCK-8 assay in LNCaP and PC3 cell lines stably transduced with SLPI. In both the two cellular models the SLPI overexpression did not modified the proliferation rate (Figure 31A-B). Furthermore, the addition of R1881 in LNCaP cells does not affect the proliferation ability (Figure 31A).

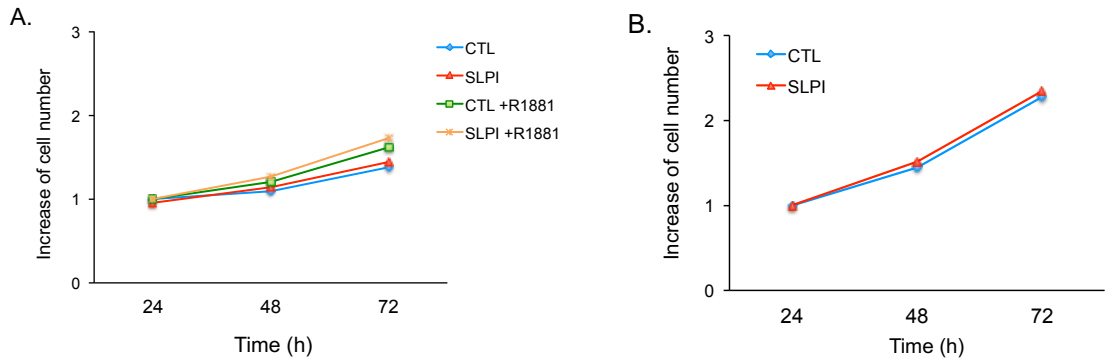


Figure 31: *Cell proliferation rate in PC3 and LNCaP cells transduced with SLPI.* ***A)*** *plot of the proliferation rate in LNCaP cells overexpressing SLPI (SLPI) and control cells (CTL) in presence or in absence of R1881.* ***B)*** *plot of the proliferation rate in PC3 cells overexpressing SLPI (SLPI) and control cells (CTL).*

Cell migration

The role of SLPI in cell migration ability was evaluated by the wound healing assay. This assay showed that in LNCaP cells the overexpression of SLPI did not modify the migration ability either in presence or in absence of androgen (Figure 32 A). Similar results were obtained also in PC3 cells (Figure 32 B)

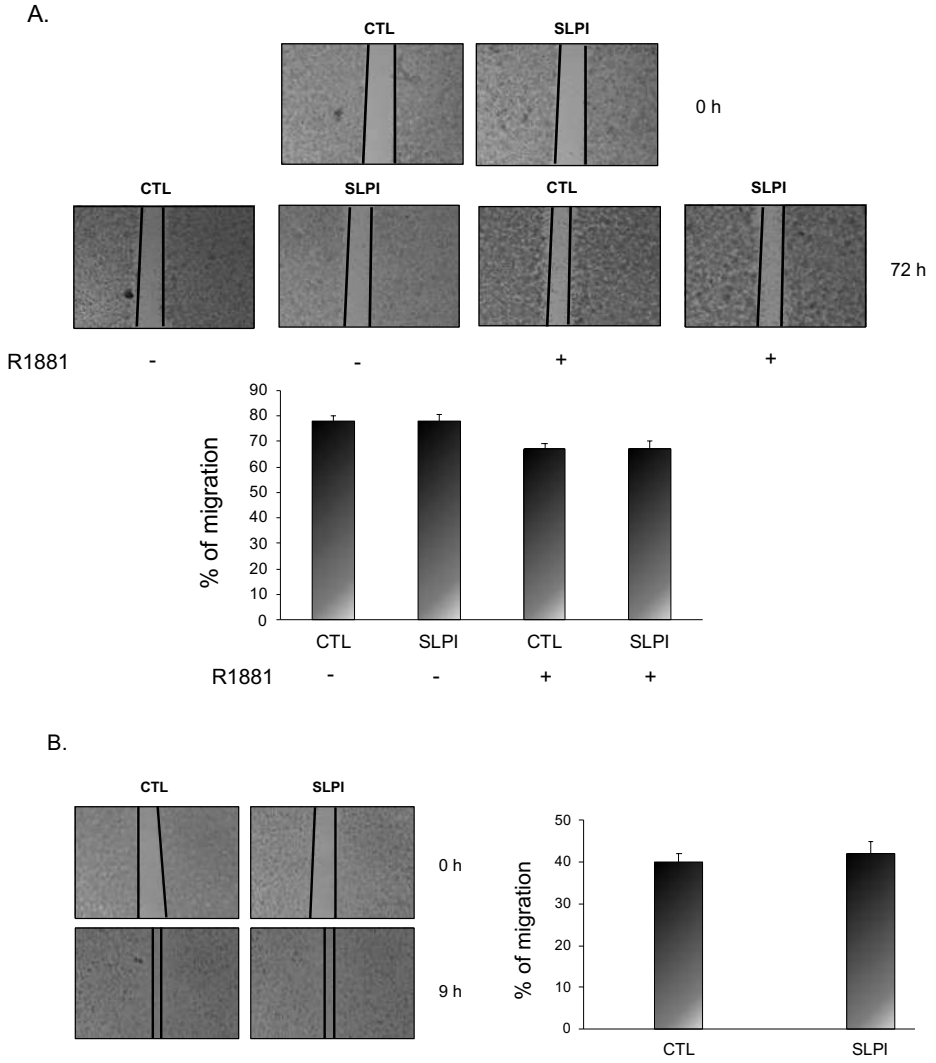


Figure 32: *cell migration of PC3 and LNCaP cells overexpressing SLPI based on wound healing assay.* ***A)*** *representative images and bar diagram of cell migration assay in stable SLPI-overexpressing LNCaP cells in presence and in absence of R1881 at 0 and 72 hours.* ***B)*** *representative images and bar diagram of cell migration assay in stable SLPI-overexpressing PC3 cells at 0 and 9 hours.*

Cell invasion and EMT

We also analyzed the role of SLPI in the invasive capability of PC3 and LNCaP cells. In PC3 cells the overexpression of SLPI does not affect the cell ability to invade through the Matrigel (Figure 33), while we were not able to observe the Matrigel invasion of LNCaP cells in any of tested condition even after 5 days of culture.

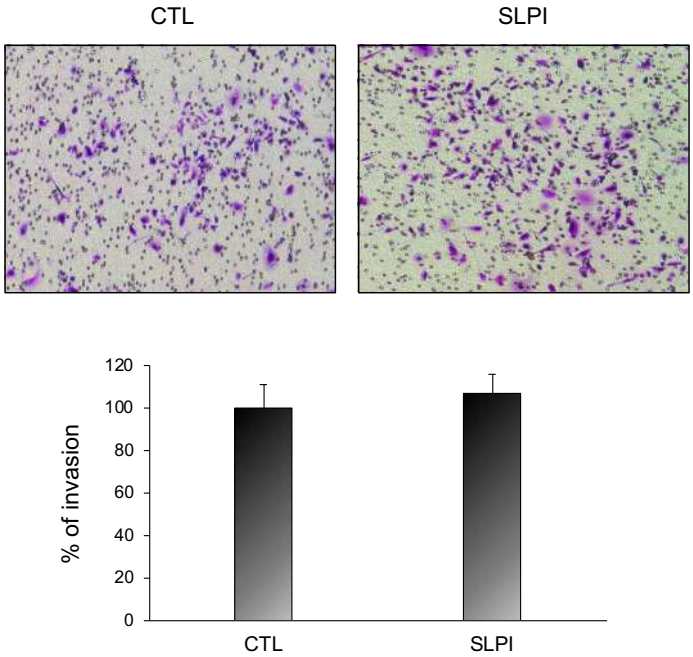


Figure 33: *representative images and bar diagram of Matrigel invasion assay of PC3 overexpressing SLPI.*

According with these results we did not observe any changes in the MMPs mRNA levels in both cellular models: the expression levels of MMPs did not differ in the SLPI-overexpressing cells compared to control (data not shown).

In addition, the expression levels of E-cadherin, N-cadherin and EMT transcription factors (SLUG, ZEB, TWIST) were also not modified after SLPI overexpression in both cellular models (data not shown).

Apoptosis

In order to investigate the effects of SLPI on apoptosis, we analyzed by flow cytometry LNCaP and PC3 cells overexpressing SLPI after the staining with AnnexinV and 7-AAD.

In PC3 cell line the overexpression of SLPI did not alter the apoptosis rate, in fact the percentage of the AnnexinV positive and 7-AAD negative (early apoptotic cells) and AnnexinV/7-AAD positive (late apoptotic cells) is similar in the cells with SLPI overexpression and in the control cells (Figure 34).

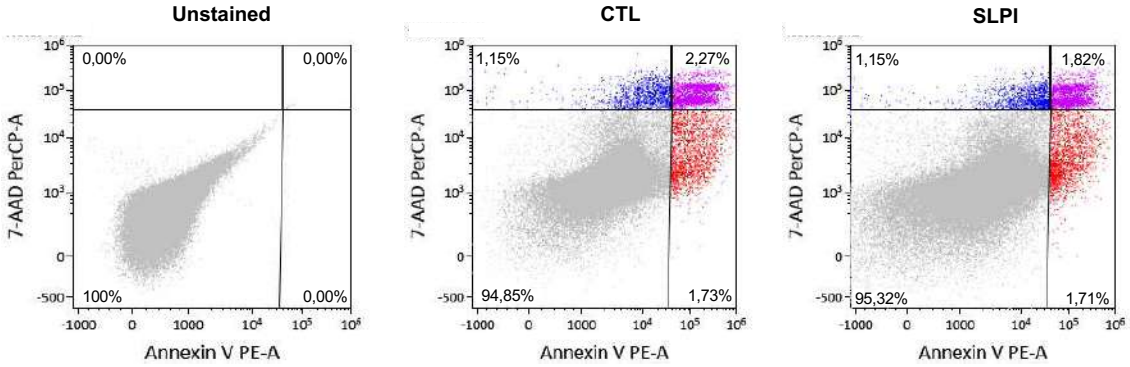


Figure 34: *Apoptosis in PC3 stably transduced with SLPI determined by flow cytometry and Annnexin V/7-AAD double staining. In grey is reported the population of viable cells, in red the population of cells in early apoptosis (Annexin V positive and 7-AAD negative), in pink the population of cell in late apoptosis (Annexin V and 7-AAD positive) and in blue the 7-AAD positive and Annexin V negative population. CTL= control cells; SLPI=cells transduced with a SLPI expressing plasmid.*

In LNCaP cells, after the overexpression of SLPI, we observed a reduction of the late apoptotic population (AnnexinV/7-AAD positive), whereas the percentage of the early apoptotic cells (AnnexinV positive and 7-AAD negative) was similar to that of control cells (Figure 35).

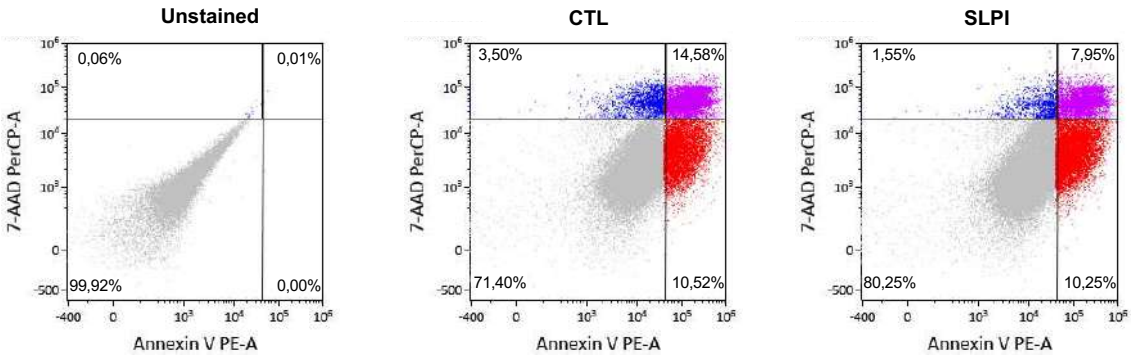


Figure 35: *Apoptosis in LNCaP stably transduced with SLPI determined by flow cytometry and with Annnexin V/7-AAD double staining. In grey is reported the population of viable cells, in red the population of cells in early apoptosis (Annexin V positive and 7-AAD negative), in pink the population of cell in late apoptosis (Annexin V and 7-AAD positive) and in blue the 7-AAD positive and Annexin V negative population. CTL= control cells; SLPI=cells transduced with a SLPI expressing plasmid.*

We verified if the SLPI over-expression in LNCap cells altered the levels of several caspases (caspase 8, 9, 3 and 7). Among them, we found a decrease of cleaved form of caspase-8 when SLPI is overexpressed and this result was mainly observed in LNCaP cells stimulated with R1881. The pro-caspase-8 did not appear modulate in SLPI-overexpressing LNCaP cells (Figure 36 A).

Also, in PC3 cells the cleaved-caspase-8, but not the pro-caspase-8, was reduced after the overexpression of SLPI (Figure 36 B). However, this reduction was not statistically significant.

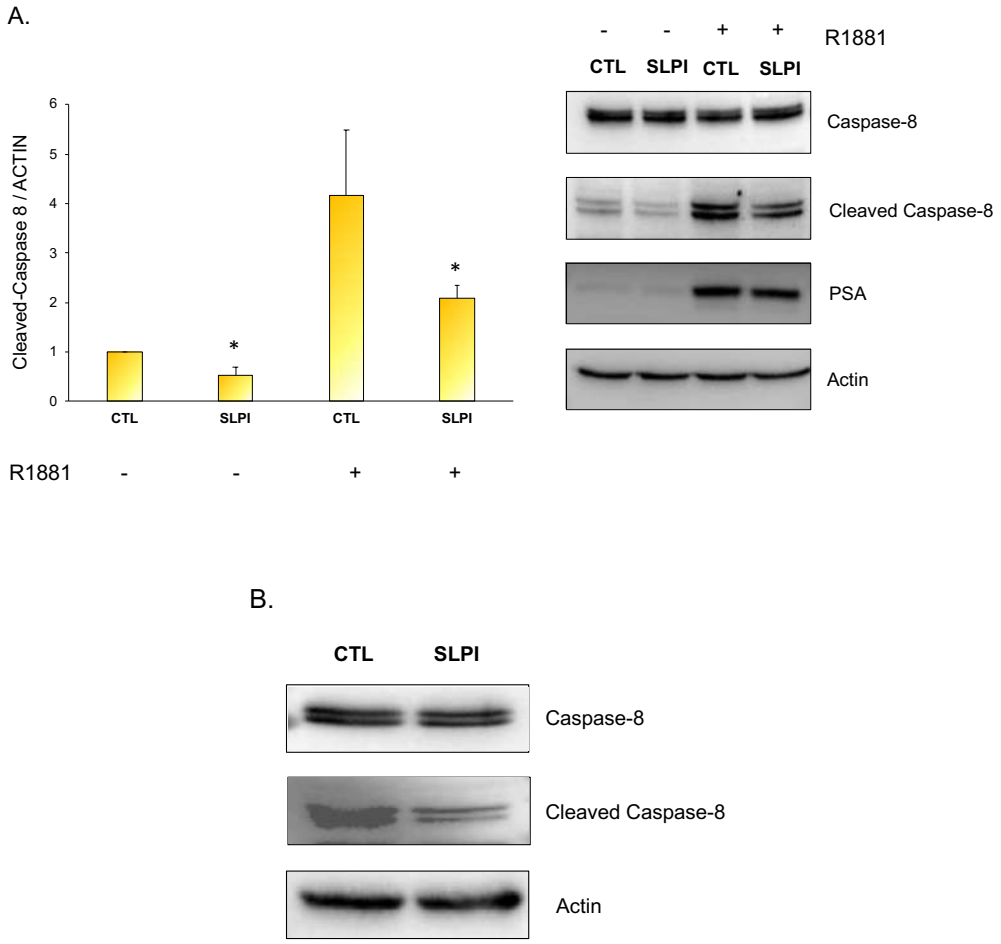


Figure 36: *levels of Caspase-8 protein expression in LNCaP and PC3 cells assessed by western blot.* ***A)*** *on the left the bar diagrm shows the protein levels of cleaved-Caspase 8, whereas on the right a representative western blot image with the result of pro-Caspase 8 and cleaved Caspase 8 in LNCaP cells overexpressing SLPI and control (CTL) cells in presence and in absence of R1881. PSA was used as positive control for androgen stimulus. The * indicates the statistically significance variations (P ≤ 0,05).*
B) *Representative western blot image with the result of pro-Caspase 8 and cleaved Caspase 8 in PC3 cells overexpressing SLPI and control (CTL) cells.*

4.6 ETV4 modulates SLPI expression through an indirect interaction

The laboratory in which I have worked for my PhD has shown, and I have further confirmed, that ETS genes, specifically ETV4 and ETV1, are able to reduce SLPI expression in several *in vitro* models. In addition, it has been shown that ETV4 was able to reduce the expression driven by the SLPI promoter by dual-Luciferase Reporter Assay. In order to determine if ETV4 directly bind SLPI promoter, in collaboration with dr. Irene Cosi, we performed a Chromatin Immunoprecipitation (ChIP) assay. The ChIP experiments were conducted in the normal prostate cell line RWPE in which we transiently transfected a vector containing ETV4-Flag or as control a vector containing only Flag. The analysis *in silico* of the sequence of SLPI promoter showed the presence of four putative ETV4 Binding sites (ETV4-BS) (Figure 37 A). The protein-chromatin complex was immunoprecipitated using the Flag antibody and IgG; the DNA was tested by qRT-PCR using four different pairs of primers, each one was for a specific putative ETV4 binding site and the data has been analyzed using the "Percent Input Method" (see Material and methods). A sequence in the promoter of Cox2, known to be directly regulated by ETV4 (Ratovitski, 2010), was used as a positive control and a sequence in G6PD coding region as negative control.
As expected, when we used the pair of primers specific for Cox2 (positive control) there was an increase of amplified DNA from the RWPE transfected with the ETV4-FLAG expression vector and immunoprecipitated with antibody against Flag compared with the 3 controls (RWPE transfected with Flag control vector and immunoprecipitated with either antibody against Flag or antibody against IgG, and the RWPE transfected with

the ETV4-FLAG and immunoprecipitated with antibody against IgG) (Figure 37 B), whereas we did not observe any increase of amplified DNA (Figure 37 B) from each region around the 4 putative ETV4-BS and this suggests that ETV4 does not bind in direct manner to the SLPI promoter.

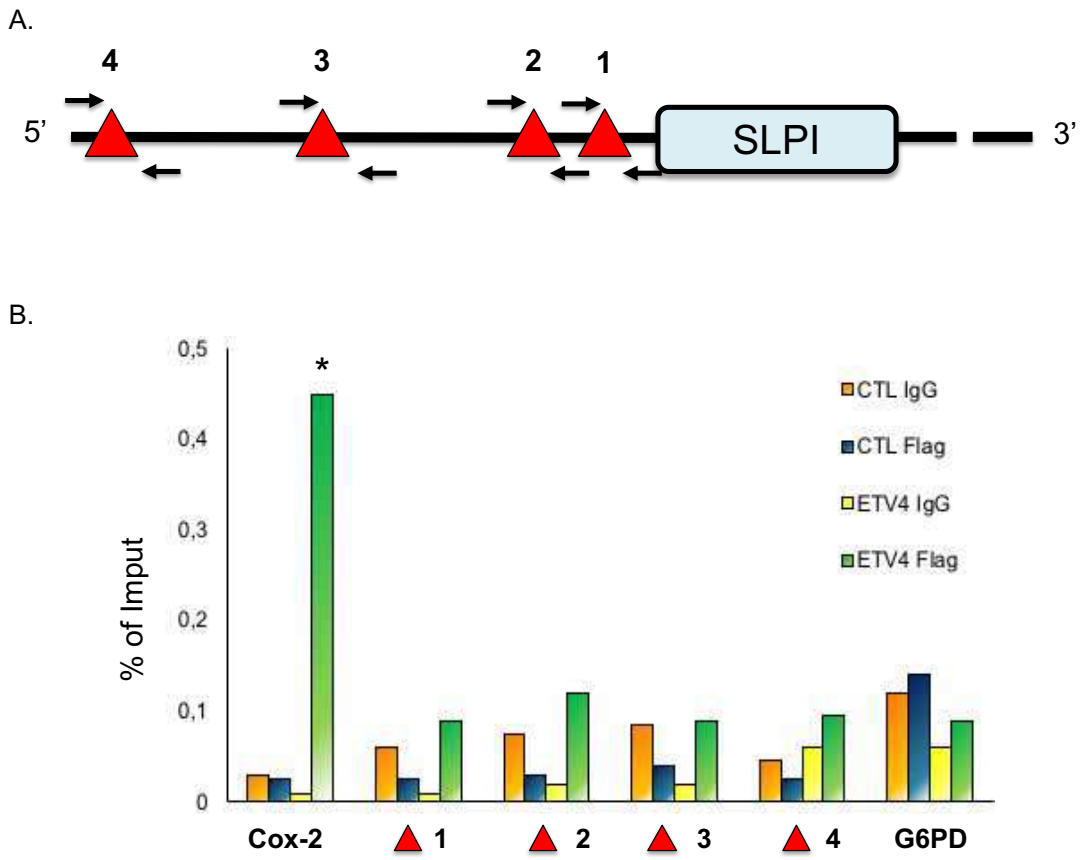


Figure 37: A) *Schematic representation of SLPI promoter. The four putative ETV4 binding sites on SLPI promoter are represented with red triangles.* ***B)*** *Bar plot with the results of the ChIP analysis.*

The results obtained by the precipitation with the anti-Flag antibody are reported in green for ETV4-flag transfected RWPE and in blue for the flag transfected RWPE cells. The other 2 bars indicate the results from the immunoprecipitation with IgG antibody: yellow bars indicate the results from ETV4-flag transfected RWPE and orange bars those from flag transfected RWPE. Cox-2 promotor and G6PD have been used as positive and negative control.

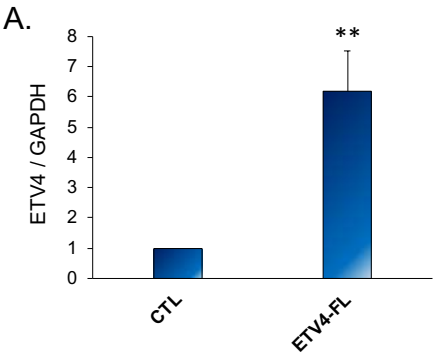
*The * represents the statistically significance variations (P ≤ 0,05).*

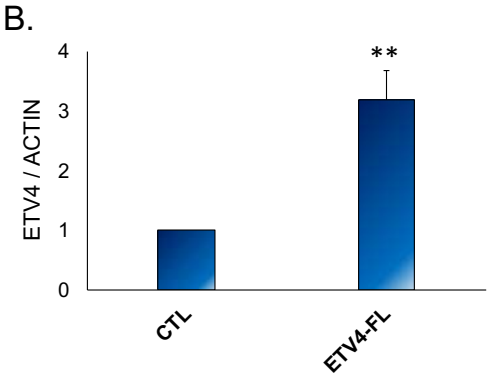
4.7 Preliminary data: STAT1 is downregulated by ETV4 and ETV1 *in vitro*

As described in paragraphs 4.1 and 4.6, ETV1 and ETV4 downregulate SLPI expression, however ETV4 (and maybe also ETV1) does not bind SLPI promoter in direct manner, thus we decided to investigate if STAT1, a positive regulator of SLPI expression in lung (Meyer et al., 2013), could be the intermediate in the regulation of SLPI expression by these ETS proteins.

We used the two specular models used also before: the RWPE cells, with low levels of ETV4 expression, stably transfected with a vector encoding the human full-length ETV4 (ETV4-FL) and with the empty vector as control; the PC3 cells, with high levels of ETV4, stably transduced with two different ShRNAs against ETV4 (ShETV4-1 and ShETV4-2).

In RWPE cells stably transfected with ETV4, the ETV4 mRNA levels were about 6-fold higher than in control cells (Figure 38 A) whereas at protein level the increase of ETV4 was about 3-fold (Figure 38 B).





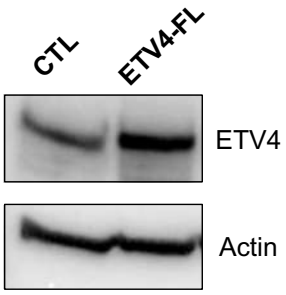


Figure 38: *ETV4 mRNA and protein level in RWPE cell line stably transfected with ETV4* ***A)*** *ETV4 expression level normalized to the housekeeping gene GAPDH measured by qRT-PCR.* ***B)*** *protein levels of ETV4 assessed by western blot. Beta Actin was used as loading control. The statistical significance is indicated by ** for P ≤ 0,01 and by * for P ≤ 0,05.*

In PC3 cells stably transduced with any of two ETV4 shRNAs the expression levels of ETV4 was reduced about 4-fold at mRNA level (Figure 39 A) and about 2-fold at protein level (Figure 39 B) compared to PC3 transduced with a scrambled shRNA vector.

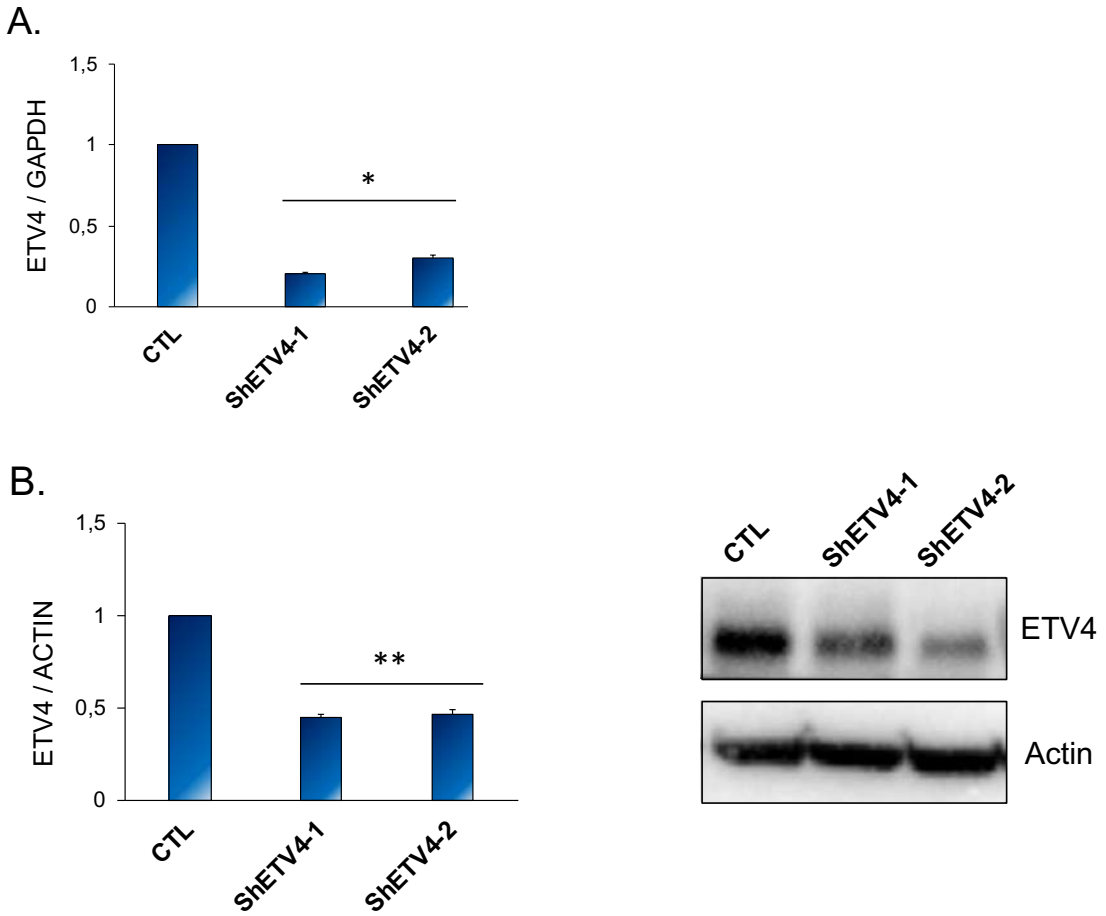


Figure 39: *ETV4 mRNA and protein level in PC3 cell line stable transduced with two different shRNAs vectors against ETV4* ***A)*** *ETV4 expression level normalized to the housekeeping gene GAPDH measured by qRT-PCR. The * represents the statistically significance variations (P ≤ 0,05).* ***B)*** *protein levels of ETV4 assessed by western blot. Beta Actin was used as loading control. The ** represents the statistically significance variations (P ≤ 0,01).*

In both cellular models we observed that ETV4 negatively regulate STAT1 expression. (Figure 40 A and B).

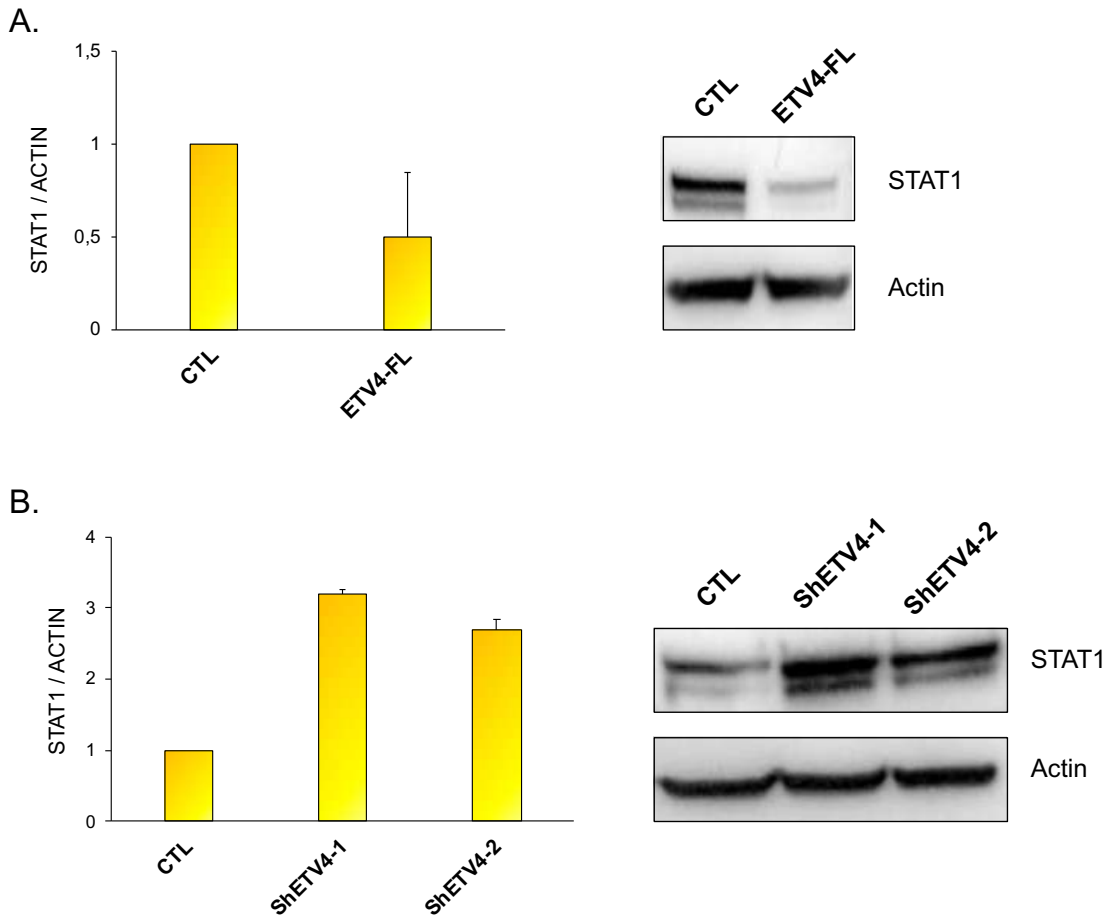


Figure 40: *protein level of STAT1: A) bar diagram (left) and representative western blot image (right) of RWPE cells stably transfected with a vector overexpressing ETV4; the STAT1 expression level is reduced by ETV4 overexpression. B) bar diagram (left) and representative western blot image (right) of PC3 cells stably transduced with two shRNAs against ETV4; the STAT1 expression level is increase after the ETV4 expression reduction.*

We also tested whether ETV1 regulates STAT1 expression and we observed that the overexpression of ETV1, like that of ETV4, decreases STAT1 expression in RWPE cells (Figure 41)

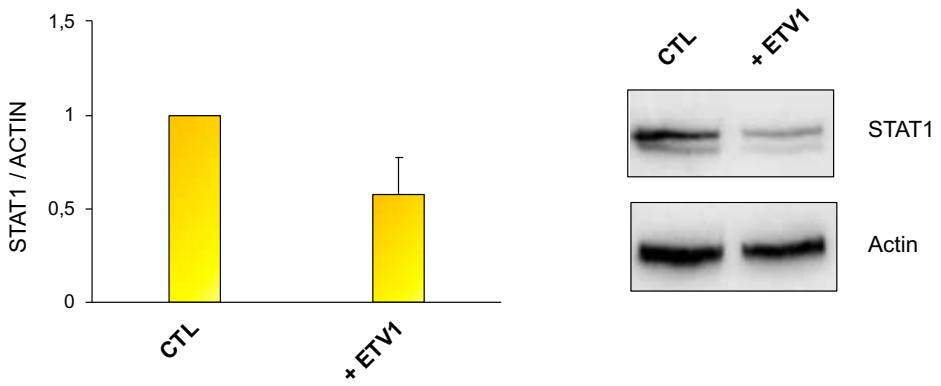


Figure 41: *bar diagram (left) and representative western blot image (right) with the results of STAT1 expression in RWPE cells stably transfected with a vector overexpressing ETV1. β Actin was used as loading control.*

4.8 Preliminary data: STAT1 increases SLPI in PC3 cell line

In order to investigate whether STAT1 also regulates SLPI expression in the prostate, we stably transduced the human prostate cancer PC3 cell line with an expression vector encoding human STAT1 and then we measured the protein level of SLPI by western blot analyses. The PC3 cells after transduction showed high STAT1 expression levels and this increase resulted in an increase of SLPI protein level (figure 42).

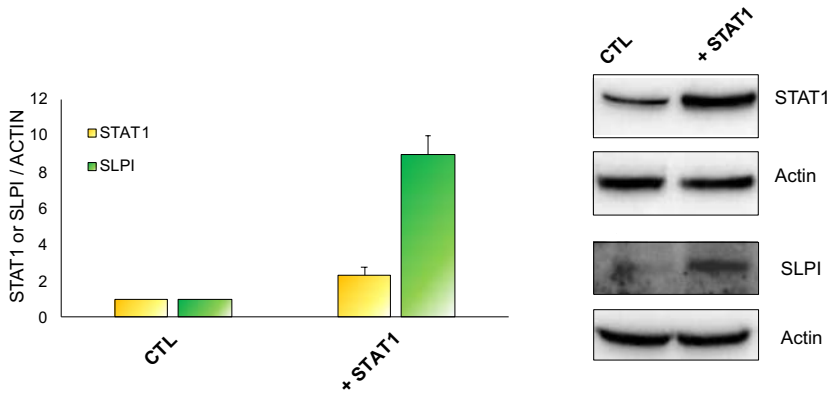


Figure 42: *bar diagram (left) and representative western blot image (right) of STAT1 (yellow bar) and SLPI (green bar) protein levels in PC3 cells stably transduced with a plasmid encoding for STAT1 and the negative control obtained by transducing the cells with an empty vector.*

5. DISCUSSION

Human SLPI is a protease inhibitor synthesized and secreted by various epithelial cells and inflammatory cells, which plays a key role in the protection of tissues during inflammation by inhibiting a wide spectrum of proteases (Williams SE, et al. 2006). In addition, SLPI also shows antimicrobial and anti-viral properties (Nugteren S, et al., 2021).

The increased expression of SLPI has been reported in several tumors: specifically, lung (Ameshima S, et al., 2000), gastric (Cheng WL, et al., 2008), thyroid (Jarzab B, et al., 2005), pancreatic (Zuo J, et al., 2015), ovarian (Hough CD, et al., 2001), uterine cervix (Rein DT, et al., 2004) and endometrial carcinomas (Zhang D, et al., 2002). In addition, high levels of SLPI have been found associated with an aggressive phenotype. However, SLPI expression has been found reduced in a few tumors such as nasopharyngeal carcinoma (Huang C, et al., 2011), bladder tumor, head and neck squamous cell carcinoma, and some breast tumors (Hu Y, et al., 2004; Kluger HM, et al., 2004).

SLPI has a peculiar pattern of expression in prostate cancer: in fact, it is reduced in the early stage of prostate cancer compared to normal prostate tissue (Thompson M, et al., 2008), whereas it is increased in a subset of metastatic CRPC patients after ADT (Zheng D, et al., 2016). It is intriguing that we have found a similar reduction of SLPI in our murine model that overexpress ETV4 at prostate level and develops premalignant prostate lesions (PIN). In keeping with these findings, *in vitro* experiments performed in our laboratory have shown that ETV4 is a negative regulator of SLPI expression.

Taking into account that most patients with early stage of prostate cancer present a reduction of SLPI expression whereas ETV4 is just one of the ETS genes translocated in these patients, we hypothesized that other members of ETS transcription factors family could also downregulate SLPI expression. Specifically, we focused on *ETV1*, a gene that belongs to the same ETS subfamily of ETV4. We have investigated the effect of ETV1 on SLPI expression in two different human prostate cell lines (LNCaP and RWPE). By either ETV1 silencing in LNCaP cells or ETV1 over-expression in RWPE we have demonstrated, confirming previous preliminary data, that ETV1, as ETV4, modulates negatively SLPI expression at both mRNA and protein level. However, the

androgen-mediated increase of ETV1 expression in the androgen-sensitive cell LNCaP cell line did not result in the expected reduction of SLPI expression but, surprisingly, in its increase. This suggests that androgens regulate positively SLPI expression: in fact, when we released ETV1-mediated down regulation of SLPI by ETV1 silencing we observed a very high increase of SLPI expression both at RNA and protein level. This finding is in apparent contrast with Zheng and collaborators (2016), which have shown that SLPI is an androgen-independent AR-dependent gene. The androgen-mediated upregulation of ETV1 in LNCaP cell line represents a potential confounding factor. However, we have confirmed that SLPI expression is upregulated by androgens also in the androgen responsive 22RV1 human prostate cancer cell line that does not express any ETS gene. The difference about the role of androgens in the regulation of SLPI expression between our results and the previous observations (Zheng et al., 2016) may to be due to the fact that we stimulated the cells by using a synthetic androgen more stable than dihydrotestosterone used by Zheng and collaborators.

Our findings about the regulation of SLPI expression by ETS proteins and by androgen/androgen receptor axis could explain the biphasic pattern of SLPI expression found in patients suffering from prostate cancer: reduced expression at diagnosis and increased expression in metastatic CRPC (Zheng et al., 2016). We can hypothesize that in early stage of prostate cancer the increased levels of an ETS gene down regulates SLPI expression overcoming the androgen-mediated upregulation of SLPI. At variance, in metastatic prostate cancer in which, usually, it is present a constitutive and strong activation of AR, similar to the strong activation obtained with the synthetic androgen (methyltrienolone) *in vitro,* this androgenic stimulus overcome ETS-mediated down-regulation with the consequent increase of SLPI expression levels (Figure 43).

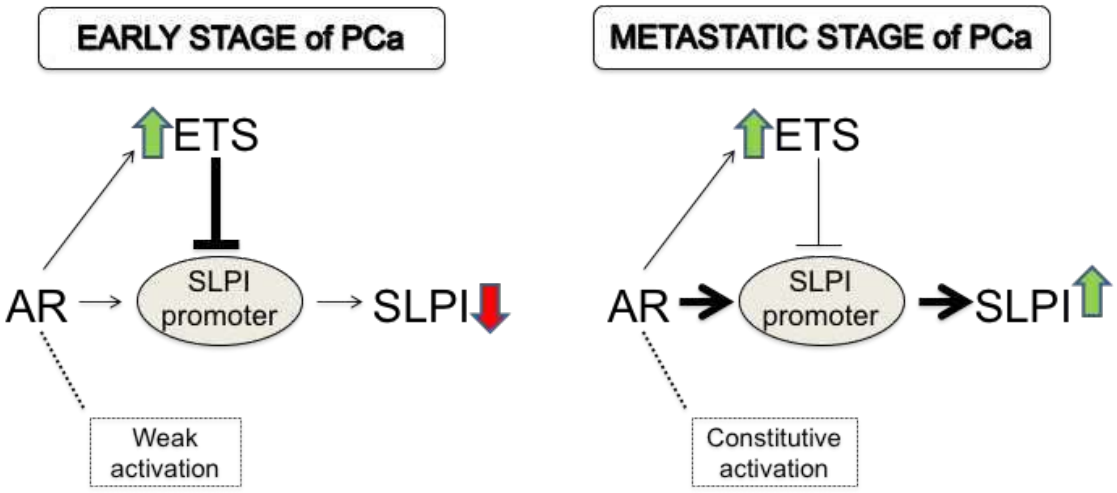


Figure 43: *representative cartoon summarizing the SLPI expression regulation by ETS and androgen/androgen receptor axis in the early sage (left) and in the advanced stage of prostate cancer (right).*

High levels of SLPI expression has been associated with more aggressive forms of cancer (Kozin SV, et al., 20017; Devoogdt N, et al., 2003), also including metastatic prostate cancer resistant to the anti-androgenic therapy (Zheng et al., 2016). SLPI has been described as a pro metastatic factor; however, the role of SLPI expression in determining the aggressive malignant phenotype of prostate cancer has been only poorly investigated. In the present study we have explored the contribution of SLPI expression in determining the malignant phenotype of prostate cancer. We performed the functional analyses of the human prostate normal cells (RWPE) stably transduced with two different shRNAs against SLPI and human prostate cancer cells (LNCaP and PC3) stably transduced with a vector expressing SLPI.

We found that the silencing of SLPI in RWPE cell line significantly reduces the ability of cells to migrate in the wound healing assay just like it has been observed in SLPI-deleted gingival carcinoma Ca9-22 cells (Takamura T, et al., 2017).

We also observed that the silencing of SLPI reduces the ability of the cells to invade through the Matrigel. The effect of SLPI on the invasive phenotype of human prostate cells has been also confirmed by MMPs expression levels, in fact, we found that SLPI

promotes the expression of MMP-2, MMP-3 and MMP-9. Similar results were obtained in gastric tumor in which SLPI promotes the metastasis of SNU638 gastric cancer cells by increasing MMP-2 and MMP-9 expression, indicating that in specific conditions SLPI may play as a signaling molecule and not as a protease inhibitor (Choi et al.; 2011).

In a murine and human model of breast cancer SLPI has been described as a negative modulator of E-cadherin expression (Rosso M, et al., 2014), in keeping with this, we found that the reduction of SLPI levels in RWPE cell line promotes the "cadherin-switch" phenomenon by increasing the E-cadherin expression levels and reducing the expression levels of N-cadherin, phenomenon that in epithelial cells indicates the EMT process. Furthermore, we demonstrate for the first time that SLPI in human RWPE prostate cells modulates at mRNA and protein levels the expression of several transcriptional factors involved in EMT such as SLUG, ZEB and TWIST. Furthermore, we evaluated the antiapoptotic activity of SLPI by measuring the expression levels of caspases and by a quantitative flow cytometry analysis of annexin V/7AAD. We found that the silencing of SLPI significantly increases the expression levels of effector and initiator caspases-3, -7, -8 and -9 and also increases the number of cells in early and late apoptosis. Thus, we have demonstrated that SLPI may play a role in cancer-promotion by inhibiting apoptosis. Similar flow cytometry results were described in pancreatic cell line Bxpc-3 and Panc-1 in which the silencing of SLPI increased the Annexin V/propidium iodide (PI) double staining positive cell population (Zuo J, et al., 2015). Overall, these findings suggest that SLPI acts as pro-tumour molecule in normal immortalized human prostate epithelial cells by inducing cell migration, invasion and by inhibiting cell apoptosis; these data are consistent with previous studies on several solid tumours such as ovarian cancer, gastric cancer, pancreatic cancer and HNSCC (Nugteren S, et al., 2021).

We have also evaluated the effect of SLPI in two metastatic prostate cancer cell lines (LNCaP and PC3) in which we stably overexpressed SLPI. In these cellular models, we found that SLPI expression was not able to further increase any of the neoplastic features of these cells (proliferation, cell migration; invasion). Similar findings have been previously reported by Zheng and collaborators (Zheng D, et al., 2016), which found that SLPI overexpression in LNCaP was not able to exert any additional pro-tumoral effects. Thus, SLPI contributes to the phenotype of a normal immortalized

prostate cellular model but does not provide any additional neoplastic feature to metastatic prostate cancer cell lines in which these features were already present due to other molecular alterations. However, in LNCaP cells we observed a decrease of caspase-8 expression levels upon SLPI expression that may confirm the anti-apoptotic role of SLPI.

Finally, we have tentatively investigated the mechanisms by which ETS genes, specifically ETV4 and ETV1, reduce SLPI expression. It has been previously shown that both ETV4 and ETV1 are able to reduce SLPI expression at mRNA and protein level in several in vitro models and that are able to reduce the expression driven by the SLPI promoter by dual-Luciferase Reporter Assay (Cosi I et al, 2021 personal communication). However, ChIP experiments, confirmed in this thesis, have documented that ETV4 does not bind the SLPI promoter suggesting that the regulation of SLPI expression by ETV4 occurs in an indirect manner by a mechanism that still is not clear.

It is established that in bronchial epithelial cells *in vitro* and in the respiratory epithelium *in vivo* STAT1 increases SLPI expression level binding directly to SLPI promoter (Meyer M, et al., 2014), thus we decide to investigate if STAT1 could be a possible intermediate in the regulation of SLPI by ETV4 and ETV1. In both cellular models the RWPE cells in which we stably overexpressed ETV4 and ETV1 and in human prostate cancer cell line PC3 in which we silenced ETV4 we demonstrated that both these ETS proteins down regulate the expression of STAT1 expression. Furthermore, we observed that, in the prostate as in the bronchial epithelial cells (Meyer et al., 2014), overexpression of STAT1 in PC3 cells increases SLPI at protein level. These preliminary findings suggest that ETS-mediated down regulation of SLPI expression in prostate cells could be mediated by the ETS-mediated down regulation of STAT1 (Figure 44): however, further experiments will be necessary to confirm this mechanism.

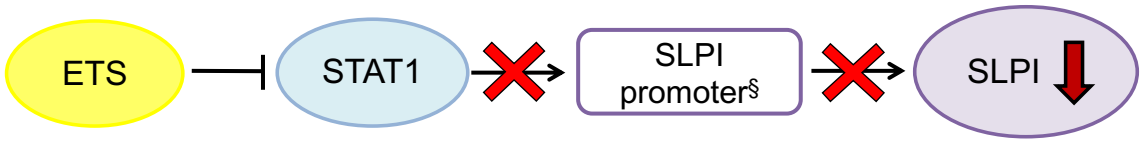


Figure 44: *representative cartoon synthesizing the mechanism by which ETS proteins downregulate SLPI expression through STAT1. (§ Meyer M, et al., 2014).*

In this work we have shown that SLPI expression is down-regulated by ETS proteins, specifically ETV4 and ETV1, and up-regulated by androgen/androgen receptor axis: the relative contribution of these two regulatory axes could explain the biphasic pattern of SLPI expression found in patients suffering from prostate cancer. We have also shown that SLPI may contribute to some neoplastic features of prostate cells, and we have provided preliminary evidence that the ETS regulation of SLPI could be mediated by STAT1.

In the next future, in addition to the thorough investigation of the role of SLPI in prostate cancer it will be interesting to study the role of other ETS genes on the regulation of SLPI expression.

Furthermore, we are planning to perform additional experiments in order to confirm the molecular mechanism about the ETS-mediated downregulation of SLPI through STAT1.

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