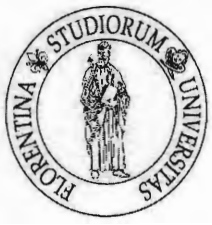

Università degli Studi di Firenze

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DOTTORATO DI RICERCA IN

"Biochimica e biologia applicata"

CICLO XXV

COORDINATORE Prof. Donatella Degl'Innocenti

THE ROLE OF ETV4 IN PROSTATE CANCER

Settore Scientifico Disciplinare BIO/10

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TABLE OF CONTENTS

ABSTRACT

Prostate cancer is the second leading cause of deaths in the male population. An important advance in understanding the molecular basis of prostate cancer has been the discovery of translocations that involve one of the genes of the ETS family (ERG, ETV1, ETV4, ETV5, FLI1). Each one of these translocations results in deregulated expression of one of the ETS proteins, expression that is likely to play a direct role in prostate cancer pathogenesis.

The role of ERG and ETV1 has been thoroughly investigated. This project has focused on the investigation of the mechanism whereby over-expression of the ETV4 gene mediates oncogenesis in the prostate.

We have show that ETV4 inhibition by siRNA in the PC3 and DU145 cancer cell lines reduces cell proliferation, cell cycle progression, anchorage-independent growth in soft agar, tumor growth in a xenograft model. In addition, ETV4 inhibition reduces cell mobility and invasiveness only in the PC3 cells. Conversely, upon ETV4 over-expression in the non–malignant cell line RWPE we have observed increased anchorage-independent growth, cell mobility and cell proliferation, which is probably mediated by down-regulation of p21, producing accelerated progression through the cell cycle. In addition, we have observed that ETV4 over-expression induces a set of morphological changes associated with changes in the expression pattern of epithelial (E-cadherin) and mesenchymal (N-cadherin and vimentin) markers: these changes are characteristic of the so-called epithelial to mesenchymal transition (EMT). In RWPE cells over-expressing ETV4 we have shown that the EMT may result from the increased expression of EMT-specific transcription factors such as Twist1, Slug1, Zeb1 and Zeb2. These results indicate that ETV4, as other ETS proteins (ERG, ETV5 and ETV1), plays a major role in invasiveness and cell migration. In addition, it emerges as unique in that it increases at the same time also the rate of proliferation of prostate cancer cells.

To investigate in vivo the effects of ETV4 expression in prostate we have developed a mouse model: FVB mice in which the truncated form of ETV4, present in patients with TMPRSS2-ETV4 translocation, is expressed under the control of a modified rat probasin promoter that drives high levels of expression only in the prostate (ETV4-FVB mice). ETV4-FVB mice and control wild-type mice have been followed-up and studied by standard H&E staining. No prostate lesions have been found in any of the 6 months old mice, whereas prostatic intraepithelial neoplasia (PIN) lesions have been found in 57% of 10 months old ETV4-FVB mice (n=10) and in none of control mice.

Pten loss is one of most common additional genetic alteration found in prostate cancer. Thus,

we have investigated the possible effect of the interaction of ETV4 expression with Pten loss by crossing our prostate-specific FVB-ETV4 mice with C57 black PTEN+/– mice: Pten+/-|ETV4 mice. In 7 months old animals we have observed neither prostatic hyperplasia nor PIN in wild type controls and FVB-ETV4 mice, PIN lesion in 33% of Pten +/- mice (n=3), and multifocal PIN lesions in all Pten+/-|ETV4 mice (n=9). These transgenic mice models suggest that ETV4 over-expression is sufficient to initiate neoplastic transformation and cooperates with Pten loss in promoting prostate cancer progression.

The work performed during project has proven that ETV4 induces, both in vitro and in vivo, most and perhaps all of the features that make a tumor aggressive. In addition, we have generated an mouse model of prostate cancer that will provide further insight ETV4 in biology of prostate cancer and that might be useful to test the effectiveness of new therapeutic approaches.

INTRODUCTION

1.1 Prostate cancer

Prostate cancer is the most common cancer in males and the second highest cause of cancer-related mortality in developed countries. The incidence of prostate cancer have steadily increased in the last decades, likely because the aging of the population (Jemal et al., 2006). Indeed, the age is one of the most important risk factors. The probability to develop the prostate cancer is very low under the age of 40 years, but increase considerably after the age of 50 years and in most cases the age of diagnosis is about 70 years. Another strong risk factor is the familiarity: the risk to develop the prostate cancer for a person who has a consanguineous affected by the disease is double respect to a person with no affected relatives. Additional risk factors are the high-fat diet, obesity and the reduced fitness. The incidence of prostate cancer changes considerably among different geographic regions and among different ethnic groups. Indeed, the incidence in South-east Asia is lower than in Western countries; however, it increases in Asians that live in Western countries. These data indicate that, in addition to inherited factors, there are environmental factors that may be directly involved in the development of this pathology (Nelson et al., 2003).

Despite its high prevalence, the molecular mechanisms that induce prostate cancer progression are poorly understood. Tumor progression is driven by stepwise processes that involve the genetic alteration of critical genes, resulting in their altered expression and function (Hanahan and Weinberg, 2000).

Prostate cancer originates from glandular epithelial cells. Initial neoplastic lesions shows histological features similar to those observed in others *in situ* cancers: these are called prostatic intraepithelial neoplasia (PIN). The tumor usually grows very slowly, remaining confined to the organ and the patient may not suffer from any symptom for years. When the cancer progress, it first invades the capsule spreading locally to the near tissues and then it can metastasizes to lymph nodes, to bones, or to other organs, such as lung and liver. Localized intracapsular

prostate cancer can be cured by radical prostatectomy. However, about one third of cases do relapse (Van Poppel et al., 2009), and the rate of relapse increases significantly if the tumor has invaded the capsule (Carver et al., 2006).

Most of prostate cancers arise from androgen-dependent secretor epithelial cells and androgen receptor (AR) signaling is a common element that affects both the development and progression of prostate cancer. Thus the androgen deprivation is the standard treatment for advanced prostate cancer that has been used for over half a century (Huggins and Hodges, 2002). In physiological conditions, local androgen metabolism maintains a balance between the proliferation and apoptotic cell death of prostatic epithelial cells, while in a tumor conditions, this balance is perturbed and androgen will drive proliferation and survival of the cancerous cells (Isaacs WB et al., 1994). Androgen deprivation is an effective therapy for prostate cancer, but prostate cancer may progress becoming resistant to the androgen deprivation (so called, castration-resistant prostate cancer): this progression has been found associated with several alteration of the androgen receptor (Visakorpi et al., 1995; Dehm et al., 2008).

The most used method for Prostate Cancer screening is the measurement of prostate specific antigen (PSA) in the serum. However, the PSA levels is not a specific marker of prostate cancer; in fact, it can be found increased also in patients suffering from prostatic infection or from benign prostatic hyperplasia (Gleason, 1966; Papsidero et al., 1985). Nevertheless, the monitoring of PSA levels in patient with known prostate cancer may be useful to monitor the relapse or the progression of the disease

The prostate cancer patients is classified according to both TNM system and Gleason score (Epstein et al., 2006). TNM classification evaluates the size of the tumor (T), lymph node metastasis (N), and distal metastasis (M). The Gleason score evaluate the grade of structural changes of morphology of prostate glandular morphology. The score is given by the sum of the primary and secondary grades of the glandular differentiation. Gleason grades range from 1 (mild structural changes) to 5 (full disappearance of glandular structure). Thus, the sum of scores ranges from 2 to 10. A high Gleason score predicts a poorer prognosis for the patient (Epstein et al., 2006). Although this classification works very well in clinical practice, the prognosis can be inaccurate in individual cases. For this

reason the identification of molecular mechanisms responsible of the onset and the progression of prostate cancer, could be extremely important for the clinical practice.

1.2 Genetic lesions in prostate cancer

The accumulation of a discrete set of somatic mutations is a driving force in the transformation of a normal cell into a neoplastic cell. In this respect, prostate cancer is not different from others cancer, it results from the accumulation of genetic and epigenetic alterations that transform normal glandular epithelium to preneoplastic lesions and to invasive carcinoma. Recent advances in molecular research highlight the role of both oncogenes and tumor suppressor genes in the development and progression of the disease. Although the familiarity increases the risk to develop prostate cancer, the large majority (about 90%) of prostate cancers are sporadic (Gronberg et al., 1997). The sporadic tumors are characterized by clinical heterogeneity due to different genetic mechanisms and multiple genetically controlled factors involved in the evolution of tumors. (Brothman et al., 1999).

The alterations described in prostate cancer patients are mutation, deletion, rearrangement, amplifications, promoter hypermethylation and haplo-insufficiency in genes to modify their protein products with oncogenic consequences (Table 1).

Recently it has been found a high frequency of ETS genetic rearrangements in prostate cancer patients (see below); it is plausible that over-expression of ETS factors may represent a crucial event in prostate tumorigenesis.

Table 1: *Summary of genetic abnormalities in prostate cancers (ETS translocations not included)*

Genes identified	**Gene abnormality**	**Chromosomal abnormality**
NKx3.1	Expression suppression	
PTEN/MMAC1	Mutation or deletions	
SPINK1	Over-expression	
AR	Mutations and amplifications	
MCM7	Over-expression	Amplification
EZH2	Over-expression	
PSCA, MYC and eIF3	Over-expression	Amplification
p16 and p14	Hypermethylation and deletion	Deletion
E-cadherin	Hypermethylation or deletions	Deletion
RB1	Hypermethylation or deletions	Deletion
P53	Mutation or deletions	
p27	Expression suppression	Deletion
p21	Expression suppression	
GSTpi	Hypermethylation	
KAI1	Hypermethylation	
CD44	Hypermethylation	
KLF6	Mutation	
ras	Mutation	
BRCA1	Mutation	LOH
CSR1	Methylation	
ANX7	LOH	
PCD1	Over-expression	LOH
Endothelin b receptor	Hypermethylation	
Annexin II	Expression suppression	
TNFRSF6	Expression suppression	
Myopodin	Deletion	
N33	Expression suppression	Deletion
FEZ1	Mutation	
Dermatin	Deletion	

Modified and updated from Yu 2007

1.3 Mouse models of prostate cancer

Mouse models have been proven to be extremely useful to understand biology and to model the spectrum of human diseases. However, the modeling of human prostate cancer is difficult because of the great differences between mouse and human prostate. First, mice do not develop prostate cancer in spontaneous manner. Second, the life of mice is much shorter than human. Third, cancer metastasis has a tendency to initiate from mesenchymal cells in mice compared to epithelial cells in humans (Valkenburg and Williams, 2011). Moreover the human prostate consists in one lobe, while the mouse prostate has four lobes. In fact, in contrast to the human prostate, the murine prostate is composed of multiple lobes arranged at the base of the bladder, surrounding the urethra (Figure 1). These lobes are named for their anatomical position and they are: the anterior prostate right and left (AP R, AP L), ventral prostate (VP) and dorsal prostate (DP). The ductal morphology, shape, histology and secretion patterns of each lobe are unique (Cunha et al., 1987). For example, histologically the anterior prostate displays extensive epithelial-infolding, while the dorsal prostate has less infolding and the ventral prostate shows minimal infolding (Cunha et al., 2004). Analogy between murine lobular structures and zonal structures of the human prostate remain elusive; nevertheless, it has been shown that organogenesis and steroid responsiveness of both murine and human prostates are similar (Meeks and Schaeffer, 2011).

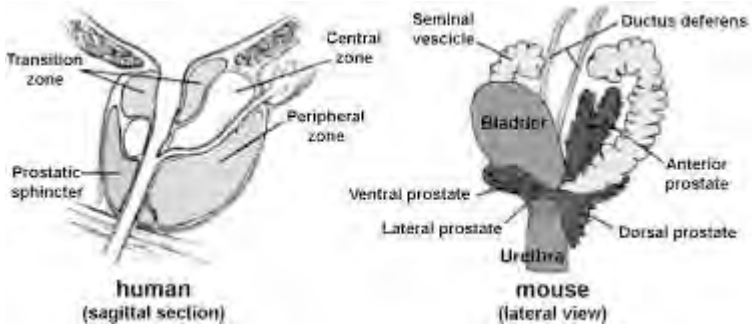

FIGURE 1: *Comparison between the Structure of human and prostate (modified from McNeal, 1969 and Cunha et al., 1987)*

Despite these differences between human and mice, their genomes are ~95% identical with structurally similar genes that are easy to manipulate (de Jong and Maina, 2010). Furthermore, mice are small, easy to handle and typically produce

large litters making generation within a reasonable amount of time attainable. Although the listed limitations, several transgenic or Knock Out (KO) mouse models of prostate cancer have been generated and have provided significant contributions in identifying molecular mechanisms involved in the disease initiation and progression.

A number of transgenic models have been generated with the SV40 Large-T and small-t antigens. One of the best characterized of these models is the TRAMP model that displayed progressive forms of prostatic disease resembling human prostate cancer, from mild intraepithelial hyperplasia to large multinodular malignant neoplasia (Greeneberg et al., 1995).

In most of the transgenic mice, the high levels of prostate specific expression of the transgenes have been obtained by using the PSA promoter, the rat probasin promoter (PB) and its derivative ARR2PB promoter.

The over-expression of Androgen Receptor (AR) driven by the PB promoter resulted in lesions similar to human PIN in 4/11 animals after 12 months, showing that increased AR levels appear sufficient to promote moderate to severe dysplasia, but not metastasis (Stanbrough et al., 2001). Transgenic model of mouse prostate cancer have been obtained also by over-expressing growth factors that are critical in many processes, including tumorigenesis: mice over-expressing insulin-like growth factor-1 (IGF-1) under the control of the bovine keratin 5 (BK5) promoter showed hyperplasia after 6 months and HGPIN after 14 months of age with metastasis into the surrounding tissue (Culig et al., 1994). Among the oncogenes implicated in Prostate Cancer development, the over-expression of c-myc resulted in HGPIN with locally invasive adenocarcinoma (Ellwood-Yen et al., 2003), whereas the over-expression of either Ras or fos resulted only in epithelial hyperplasia with stromal proliferation, but no evidence of PIN. (Barrios et al., 1996; Voelkel-Johnson et al., 2000).

Several KO mice models of tumor suppressor genes have been produced. The loss of p53 expression did not affect prostate development, however, the mice were more susceptible to spontaneous tumor formation (Colombel et al., 1995) and the mice expressing the p53 His273 mutant driven by PB promoter showed hyperplasia/dysplasia at 12 months of age, but further progression was not observed (Elgavish et al., 2004).

The homeobox gene Nkx3.1, can contribute to prostate carcinogenesis, acting as tumor suppressor gene. The KO of a single Nkx3.1 allele was sufficient to generate low grade PIN and similar phenotype was observed in homozygotes Nkx3.1-/- mice and in mice where prostatic Nkx3.1 expression was conditionally eliminated (Schneider et al., 2000; Abdulkadir et al., 2002). Thus, the loss of Nkx3.1 expression initiates the development of early-stage disease.

Another tumor suppressor gene whose inactivation has been associated with Prostate Cancer is the phosphatase and tensin homologue (PTEN). The gene, localized on 10q23, encoded for a protein that suppresses the effects of PI3K (Phosphoinositide-3 kinase) by dephosphorylating PIP3 (phosphatidylinositol 3,4,5-trisphosphate), a lipid anchor of AKT, a growth factor-regulated protein kinase. PTEN, counteracting the action of PI3K on PIP3, inhibits the AKT cascade, a central survival and proliferation pathway. Somatic mutations of PTEN have been detected at high frequency in many sporadic cancers, including glioblastoma, endometrial cancer and prostate cancer (Bonneau and Longy, 2000). Complete PTEN inactivation has been found in 15% of primary prostate tumors, and in up to 60% of prostate cancer metastases (Cairns et al., 1997). Although the PTEN-/- genotype is embryonically lethal, heterozygote PTEN+/- mice are viable. In this model it was observed increased epithelial proliferation, hyperplasia and low grade PIN, indicating that PTEN haploinsufficiency was sufficient to initiate carcinogenesis, but not to promote metastasis (Podsypanina et al.,1999; Di Cristofano et al., 2001). In another mice model the embryonic lethality was circumvented by inactivating PTEN expression only in the prostate through a Cre/LoxP system. Mice with the loss of a single PTEN allele developed only PIN; mice with the homozygous loss PTEN developed hyperplasia, PIN, invasive adenocarcinoma and metastasis at 12 weeks of age. The mice with homozygous PTEN loss showed larger glands and higher proliferation indexes than wild-type, confirming that loss of PTEN expression induce the proliferation (Wang et al., 2003). The mouse models demonstrate that disrupting the expression of a single gene leads, in most cases, to low grade PIN and high grade PIN but not to metastatic disease. Therefore, a number of bigenic models have been generated to determine whether at least two genetic events will promote metastasis. For example, although the loss of Nkx3.1 expression causes hyperplasia and dysplasia,

the PTEN+/- Nkx3.1-/- mice showed high grade PIN and early carcinoma lesions (Kim et al., 2002). These bigenic models clearly demonstrate that prostate cancer progression requires more than a single genetic event.

1.4 Chromosomal translocations and prostate cancer

Chromosomal translocations are frequently associated with human leukemias and sarcomas. Recently, it has been found that chromosomal translocation can be also present in solid tumors. An important advance in the understanding of prostate cancer pathogenesis has been the recent finding that a chromosomal translocation is present in 50-70% of patients, depending on the clinical cohorts investigated (Tomlins et al., 2005 and 2007; Saramäki et al., 2008 reviewed by Kumar Sinha et al., 2008; Tomlins et al., 2009). These chromosomal translocations juxtapose the promoter of an androgen-regulated gene or the promotor of a gene highly expressed in the prostate to the coding region of an ETS gene (Tomlins et al., 2005, 2006 and 2007; Helgeson et al., 2008; Paulo et al., 2012). (Figure 2).

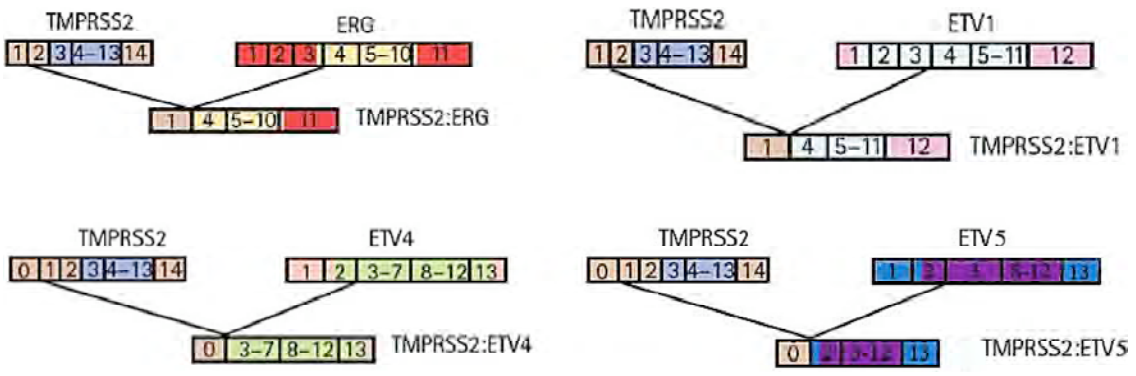

FIGURE 2: *Common chromosomal translocations involving the promoter of the gene TMPRSS2 and some ETS transcriptional factors (modified from Tomlins et al., 2005; Tomlins et al., 2006 and Helgesons et al., 2008)*

The most common translocation is derived from the fusion between the exon 1 or 2 of the transmembrane protease serine 2 (TMPRSS2) and the coding sequence of the v-ets erythroblastosis virus E26 oncogene homolog (ERG) (Tomlins et al., 2005 and 2007). These two genes are about 3 Megabase apart on the chromosomal

region 21q22.3 (Figure 3) and thus the TMPRSS2-Erg fusion gene is the result of the deletion of the intervening DNA sequence, but may also occur by a translocation (Yoshimoto et al., 2006). TMPRSS2 is an androgen-regulated gene which is highly expressed in the normal and pathological prostate. The gene encodes a serine protease that contains a type II transmembrane domain, a receptor class A domain, a scavenger receptor cysteine-rich domain and a protease domain. Serine proteases are known to be involved in many physiological and pathological processes. (Lin et al., 1999; Vaarala et al., 2001). The first two exons of TMPRSS2 are not coding thus the protein derived from the deletion/translocation is a truncated form of the Erg protein.

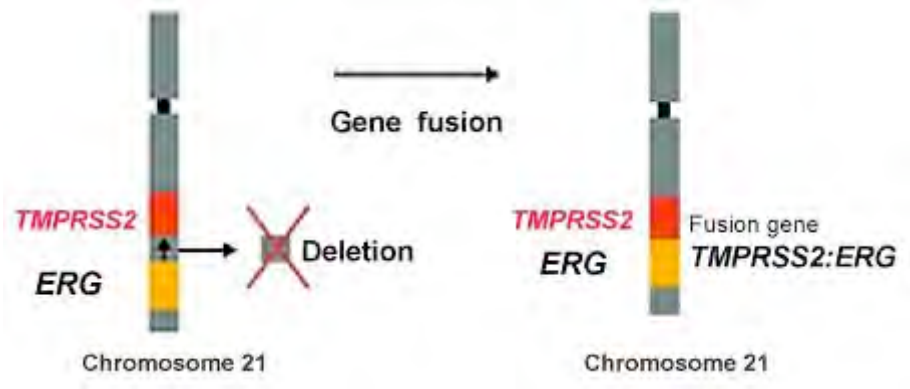

FIGURE 3: *TMPRSS2:ERG fusion is the result of a deletion of a part of DNA present between the two genes (from Martinez-Piñeiro L, 2010).*

In about 5% of patients, the translocation involve the TMPRSS2 promoter with another ETS proteins: ETV1, ETV4, ETV5, ElK4 and FLI1 (Rubin et al., 2011; Paulo et al., 2011). In few patients the translocation involve the promoter of other genes that either (1) have an androgen dependent expression or (2) have a constitutive expression (reviewed in KumarSinha et al., 2008; Rubin et al., 2011). As for TMPRSS2, the final result is the abnormal expression of a specific ETS protein in prostatic tissue (Figure 4; Table 2), (Sreenath et al., 2011).

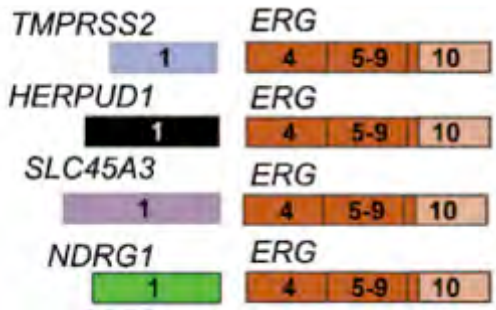

FIGURE 4: *Chromosomal translocations involving promoters of androgen-dependent genes and ERG found in patients with prostate cancer (from Rubin MA, 2012).*

TABLE 2 *: Promoters involved in translocation with ETS genes.*

GENES AT 5'	***GENES AT 3'***
Androgen-Dependent	
TMPRSS2	ERG[1], ETV1[1], ETV4[2], ETV5[3], FLI1[4]
SLC45A3	ERG[5], ETV1[6], ETV4[11], ETV5[3] , ELK4[11]
HERVK22q11.23	ERG[10] e ETV1[6]
KLK2	ERG[10] e ETV4[7]
CANT1	ERG[10] e ETV4[5]
DDX5	ETV4[5]
NDRG1	ERG[8]
HERPUD1	ERG[9]
ACSL3	ETV1[11]
EST14	ETV1[7]
Herv17	ETV1[7]
Constitutives	
C15orf21	ETV1[6]
HNRPA2B1	ETV1[6]

References: 1) Tomlins et al., 2005; 2) Tomlins et al., 2006; 3) Helgeson, 2008; 4) Paulo et al., 2011; 5) Han et al., 2008; 6) Tomlins et al., 2007; 7) Hermans et al., 2008; 8) Pflueger et al., 2009; 9) Maher et al., 2009; 10) Tomlins et al., 2008; 11) Rubin et al., 2011.

1.5 The ETS family of transcription factors

The ETS family is a large family of the transcriptional regulators that have several functions and activity, some of them have activator function (as ERG and ETV4), others have inhibitory function (as Tel) and others have both functions (as ESE1/-2/-3) (Sharrocks, 2001; Oikawa and Yamada, 2003). The ETS family members are involved in the regulation of various signaling cascades that control diverse biological process: proliferation, differentiation, development, transformation, apoptosis, migration, invasion and angiogenesis in various cell types, as well as in different neoplastic cells (Isaacs et al., 2005; Warren et al., 2002; Patikoglou and Burley, 1997). In humans, the ETS family consists of 28 members, divided in 12 subfamilies (Figure 5) characterized by an highly conserved DNA-binding domain, the ETS domain, which consist of 85 amino acids with 4 tryptophan repeats (Papavassiliou et al., 1995).

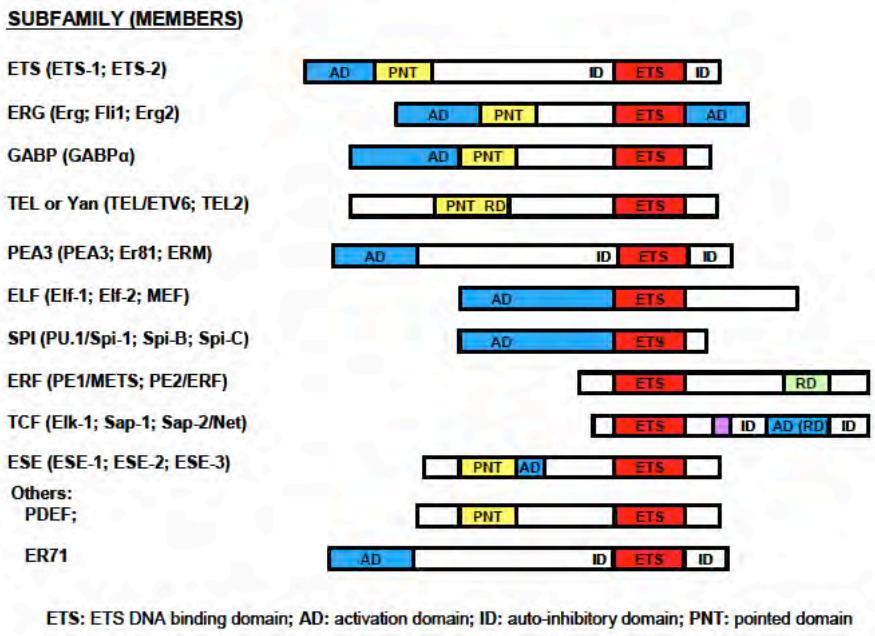

FIGURE 5: *Subfamilies of ETS protein and their functional domains (modified from Hollenhorst et al., 2011)*

The ETS domain (indicated with ETS in Figure 5) consists of three α-helices and antiparallel β-sheet scaffold that form a "winged helix-turn-helix" motif. This domain binds a consensus sequence on DNA spanning from 12 to 15 base pairs,

but it displays sequence preference only for ~9 bp with a central, invariant 5'-GGA(A/T)-3' core. The most important direct interactions of the ETS domain involve the hydrogen binding of the 2 invariant arginines (in the helix H3) with the 2 guanines of the GGA(A/T) core. In addition, the first α-helix, the first β-sheet and the loop between the second and third helixes are involved in the binding with DNA residues close to the core (Hollenhorst et al., 2011). Although all ETS proteins bind sites with the same core sequence, each ETS family member shows a unique biological function, because there are two distinct classes of binding sites (Hollenhorst et al., 2007 and 2009): the "redundant" binding sites, found in the promoters of housekeeping genes, that bind any ETS protein with relatively high affinity; the "specific" binding sites in genes that mediate the specific biological functions of a particular member of the ETS family. These "specific" binding sites are characterized by ETS sequence with low-affinity and are frequently flanked by binding sites for other transcription factors. This is consistent with a model in which low-affinity ETS-binding sites, together with the cooperative interactions with neighboring transcription factors, mediate specific ETS functions.

Other regulatory mechanism are described for the ETS proteins: 1) the presence of cis-acting auto inhibitory modules that are thought to act by blocking promiscuous DNA binding, such as in the transcription factor ETV4 (ETS Translocations Variant 4, also called PEA3), some amino acids around the ETS domain (black region in Figure 6) that can reduce the affinity for the DNA (Laget et al., 1996; Bojovic and Hassell, 2001; Brown et al., 1998; Greenall et al., 2001); 2) phosphorylation that affects the ETS-DNA binding and the association with other transcription factors (Baert et al., 2002; Wu and Janknecht, 2002).

ERG, the most frequently rearranged in prostate tumors, and FLI1 belong to the ERG subgroup of the ETS proteins. This subgroup is characterized by the presence of the pointed (PNT/SAM) domain (Figure 6), that is an helix-loop-helix structure involved in the omo-oligomerization and in the etero-dimerization with co-regulators factors (Lacronique et al., 1997; Sharrocks, 2001; Baker et al., 2001). The others genes rearranged in prostate cancer (ETV1, ETV5, ETV4) belong to the PEA3 subgroup. The PEA3 subgroup proteins bind preferentially the sequence 5'-ACCGGAAGT-3' (Wei et al., 2010); they lack of the PNT/SAM domain, and have two acidic domains, at N- and at C-terminus (grey regions in the Figure 7), that are

strong transactivation domains (Janknecht et al., 1996; Laget et al., 1996). These acidic domains are negatively regulated by the adjacent sequences (Sangphil et al., 2012; Bojovic and Hassell, 2001) in ETV4 and by central sequences in ETV1 (Janknecht et al., 1996).

FIGURE. 6: *ERG subfamily and its functional domains: ETS domain in black and HLH domain (PNT/SAM) in grey (from Oikawa et al., 2003)*

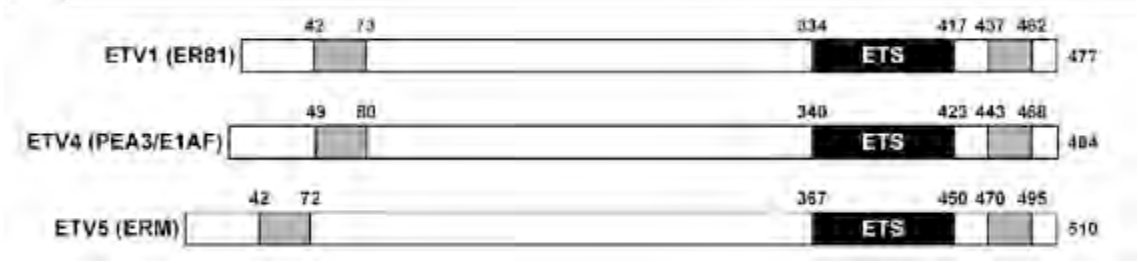

FIGURE 7: *ETS factors that belong to PEA3 subfamily and their functional domain. In black is ETS binding domain, in grey there are acidics domains (from Oh et al., 2012).*

1.6 ETS and cancer

The ETS genes have been found amplified or deleted or located at translocation breakpoints in leukemia and solid tumors. Their altered expression has been observed in various tumors, including thyroid, pancreas, liver, prostate, colon, lung, breast cancers and leukemia. ETS over-expression resulting from a chromosomal translocations has been described in leukemias and sarcomas: (1) the ETS domain of ERG2 translocated downstream the gene TLS/FUS in some cases of chronic myelomonocytic leukemia; (2) the ETV6 translocation downstream the MN1 gene in some cases of acute myeloid leukemia (Seth and Watson, 2005); (3) a fusion protein with the N-terminal region of the EWS gene, an RNA binding protein, and C-terminal portion of ETS proteins such as FLI1, ETV1, Erg in Ewing's sarcoma. These fusion proteins have an increased transactivation

potential in the respect of wild type ETS protein, that is likely contribute to the development of the malignant phenotype (Kovar, 2005).

In other tumors it has been found the over-expression of ETS protein without any chromosomal translocation. In the breast cancer has been described the overespression of either ETV1 and ETV4 (Seth and Watson, 2005). The over-expression of ETV4 has been linked to increased activity of Her2/Neu receptor tyrosine kinase, a predictor of aggressiveness and lethality in breast cancer. Further studies examining ETS factors expression profiles in normal and cancerous breast cells have shown that the up-regulation of multiple ETS factors (ETS-1, ETS-2, ETV4, ETV5 and ETV1) is associated with poor prognosis and metastasis in breast cancer and other ETS proteins (PDEF, ESE-2 and ESE-3) are downregulated during breast cancer progression. Other examples are the ETS1 and ETS2 over-expression found in thyroid cancer, pancreas, liver and lung patients (Kumara et al., 2006; Seth and Watson, 2005). All these examples suggest that the ETS proteins have a key role in oncogenesis and tumor progression.

1.7 ETS proteins in prostate cancer: clinical studies

TMPRSS2-ETS and other ETS rearrangements are specific of prostate cancer. In fact, *TMPRSS2-ETS* rearrangement has been never found in benign prostatic hyperplasia, or proliferative inflammatory atrophy (De Marzo et al., 2006). Furthermore, the oncogenetic role of ETS rearrangements has been confirmed by the fact that *TMPRSS2-ETS* rearrangement has been detected in neither normal prostate cells nor in stromal cells adjacent to prostate cancer cells carrying the *TMPRSS2-ETS* rearrangement (Perner et al., 2006; Mosquera et al., 2008).

Since the initial discovery of ETS fusions in prostate cancer, several studies have tried to define the timing of the appearance of *TMPRSS2-ETS* during prostate oncogenesis. Some studies have shown that in prostate cancer carrying an ETS rearrangement, the rearrangement was present only in cancers cells but not in the paired PIN lesions from the same patient (Tomlins et al., 2005; Rubin et al., 2011). However, other studies have shown that *TMPRSS2-ERG* fusion is detected in about 20% of high grade PIN that are present in the patient with prostate cancer carrying

the same fusion gene (De Marzo et al., 2006; Cerveira et al., 2006), suggesting that the ETS fusions could mediate the transition from PIN to invasive cancer. In addition, it has been shown that ETS rearrangements have similar frequencies in clinically localized prostate cancer, in hormone-naïve cancer (33 and 46%) (Perner et al., 2007; Lapointe et al., 2007) and in hormone-resistant metastatic cancer (33 and 37%) (Mehra et al., 2008; Saramäki et al., 2008), suggesting that ETS lesions have a role in driving the transformation, but probably have less significance in the progression (reviewed by Tomlins et al., 2009).

Several studies have investigated the association between the presence of an *ETS* fusions with the clinical outcome of prostate cancer. Population-based observational studies in patients conservatively treated for prostate cancer have shown a significant association between the presence of TMPRSS2:ERG fusion with disease-specific death (De Michelis et al., 2007), or with shorter overall survival (Attard et al., 2009). In addition, the duplication of the TMPRSS2-ERG fusion was a strong independent predictor of poor clinical outcome (Attard et al., 2009). Thus, it seems that in untreated patients TMPRSS2-ERG prostate cancer are more aggressive than fusion-negative cancer. On the other hand, retrospective studies investigating the possible association between TMPRSS2-ERG and the outcome of radical prostatectomy have produced conflicting results: some studies reported the association with poor prognosis (Nam et al., 2007; Rajput et al., 2007), whereas other studies reported no association with poor prognosis (Rouzier et al., 2008; Lapointe et al., 2007) or, even, association with better prognosis (Saramaki et al., 2008) or with lower Gleason grade (Gopalan et al., 2009). Thus, in patients treated immediately after the diagnosis there is no consensus about the prognostic role of TMPRSS2-ERG fusion because the data are still insufficient to draw any reasonable conclusion.

1.8 ETS proteins in prostate cancer: Experimental Studies

Several functional studies *in vitro* and *in vivo* on ETS over-expression have been performed in order to address its role in the development of pre or neoplastic lesions.

In vitro studies

The rearrangement TMPRSS2-ERG has been found in the human V-Cap prostate cell line and the rearrangement TMPRSS2-ETV1 has been found in the human Ln-Cap prostate cell line. These rearrangements result, just as in prostate cancer, in the over-expression of either ERG (V-Cap cells) or ETV1 (Ln-Cap cells). Thus, V-cap cell line and Ln-Cap cell line have been extensively used as cellular model to investigate the effect of ETS rearrangements. The reduction of ERG (in V-Cap cells) and of ETV1 (in Ln-Cap cells) expression levels by RNA interference has showed that the over-expression of either one of these genes had a direct role in invasiveness (Tomlins et al., 2007; Tomlins et al., 2008; Kumar-Sinha et al., 2008). The inhibition of these ETS proteins determines a reduction of cell migration and invasion ability. This phenotypic changes were found associated with a reduction of the expression of proteins involved in the extracellular matrix remodeling during the invasiveness, as MMP-1, MMP-3, MMP-7 e MMP-9 metalloproteinases, uPA (urokinase-type plasminogen activator) and its receptor uPAR (Blasi and Sidenius, 2010; Bourboulia and Stevenson, 2010). In keeping, when the immortalized but non transformed prostate cell line RWPE, has been transfected with a vector overexpressing one of the ETS proteins (such as ERG, ETV1 or ETV5) they acquired the ability to migrate and invade associated with the activation of a transcriptional program leading to invasiveness (Tomlins et al., 2007; Tomlins et al., 2008; Helgeson et al., 2008).

Mouse models

The role of the prostate expression of ETS proteins has been investigated also in transgenic mouse models. The role of ERG has been investigated in few transgenic mice models with controversial results. Some studies suggest that ERG over-expression promotes cancer development. Tomlins and co-workers (Tomlins et al.,

2009) showed that transgenic mice expressing the ERG gene fusion product develop mouse prostatic intraepithelial neoplasia (PIN), a precursor lesion of prostate cancer. Similar results where obtained by Klezovitch (Klezovitch et al., 2008): they reported that the over-expression of ERG activates prostate cell invasion and results in the displacement of prostate basal epithelium by the luminal cells and the development of PIN. However, other studies indicate that the over-expression of ERG is not sufficient to induce neither pre or neoplastic lesions, but it can cooperate with other molecular lesions in promoting cancer progression (Carver et al., 2009; King et al., 2009; Casey et al., 2012). In these studies the transgenic mice expressing the ERG fusion gene in the prostate failed to develop any PIN or other neoplastic prostate lesions. When these ERG-expressing mice were crossed with Pten-deficient mice, (Carver et al., 2009; King et al., 2009; Casey et al., 2012) their offspring developed PIN, but only in Carver's study it was observed prostate cancer progression. These studies demonstrated that PTEN haploinsufficiency cooperates with aberrant ERG expression to accelerate initiation and promote progression of prostatic adenocarcinoma. It is intriguing that similar findings have been observed in mice overexpressing ERG together with the overexpression AKT that, as Pten deficiency, is associated with alterations in PI3K signalling (King et al. 2009; Zong et al., 2009).

Similar results have been observed in transgenic mice overexpressing ETV1 in prostate tissue: two different studies have found that ETV1 overexpression did not lead to Prostate Cancer, but is enough for development of preneoplastic lesions (Tomlins et al., 2007; Shin et al., 2009).

These findings suggest that ETS proteins overexpression may have an effect on tumor progression but have to synergize with additional genetic events to determine the carcinoma formation and an invasive behaviour.

Experimental data about the oncogenic role of other ETS proteins in prostate cancer are not available.

The research group in which I have worked has focused on investigating the role in prostate cancer of the over-expression of ETV4 gene.

1.9 ETV4 (ETS Translocation Variant 4)

ETV4 (also called PEA3 or E1AF) is a protein of 484 amino acids and with a molecular weigh of about 54 Kda. Its gene, localized on chromosomal 17q21, is long about 19 kb and has 13 exons. Until now it has been described 2 variants that differs for their 5' UTR (Figure 8).

ETV4 is the prototypic member of PEA3 subfamily of ETS proteins. As all member of the PEA3 subfamily, ETV4 has an ETS binding domain and two acidic domains at the N- and C terminus, each of which constitutes the core of a transcription activation domain with the N-terminal activation domain that appears to be more potent than the C-terminal one (Janknecht et al., 1996; Laget et al., 1996). The systematic analysis of the DNA sequence specificity revealed that ETV4 preferentially bind to 5'-ACCGGAAGT-3', wherein the importance of bases in determining the strength of DNA-binding diminishes with the distance from the 5'-GGAA-3' core (Wei et al., 2010). The ETS domain is regulated in an intramolecular manner: amino acids on both sides of the ETS domain inhibit its DNA-binding ability (Janknecht et al., 1996; Laget et al., 1996; Brown, 1998; Grenall et al., 2001). Interestingly, antibodies can relieve this intramolecular inhibition of DNA-binding (Bojovic and Hassell, 2001). As the ETS domain, also the activation domains are inhibited by their flanking sequences (Bojovic and Hassell, 2001).

ETV4 is normally expressed at low levels in brain, kidney, lung, heart. It regulates many physiologic functions: motor axon guidance, mammary gland and kidney morphogenesis. One emerging role of ETV4 is in branching morphogenesis where primitive epithelial buds bifurcate to generate branched or acinar structures. (Chotteau-Lelievre et al., 2003).

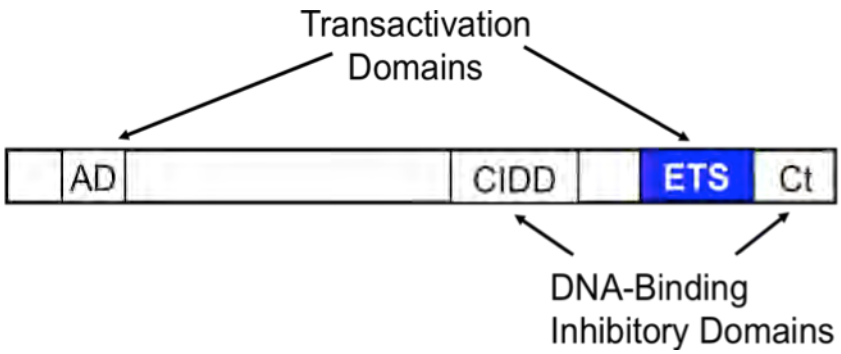

FIGURE 8: *ETV4 structure and its functional domains .*

ETV4 has been found over-expressed in several types of carcinoma, including head and neck, lung and breast cancer (Hida et al., 1997; Hiroumi et al. 2001; Benz et al., 1997). ETV4 over-expression has been associated with metastasis and with poor prognosis (de Launoit et al., 2000). ETV4 expression has been correlated with the activation of cancer-related genes relevant to cell proliferation and to invasiveness (Discenza et al., 2004; Jiang et al., 2007; Kaya et al., 1996; Hiroumi et al., 2001). On the other hand, some reports have suggested that ETV4 may function as a tumor suppressor (Xing et al., 2000; Yu et al., 2006).

ETV4 has been found over-expressed also in prostate cancer patients: in some cases over-expression is associated with translocations of *ETV4* to the promoter of a gene highly expressed in prostate (*TMPRSS2*, *KLK2*, *DDX5*, *CANT1*) (Tomlins et al., 2006; Han et al., 2008; Hermans et al., 2008) (Figure 9); in others it is has been observed without any detectable translocation. (Iljin et al. 2006; Paulo et al., 2011)

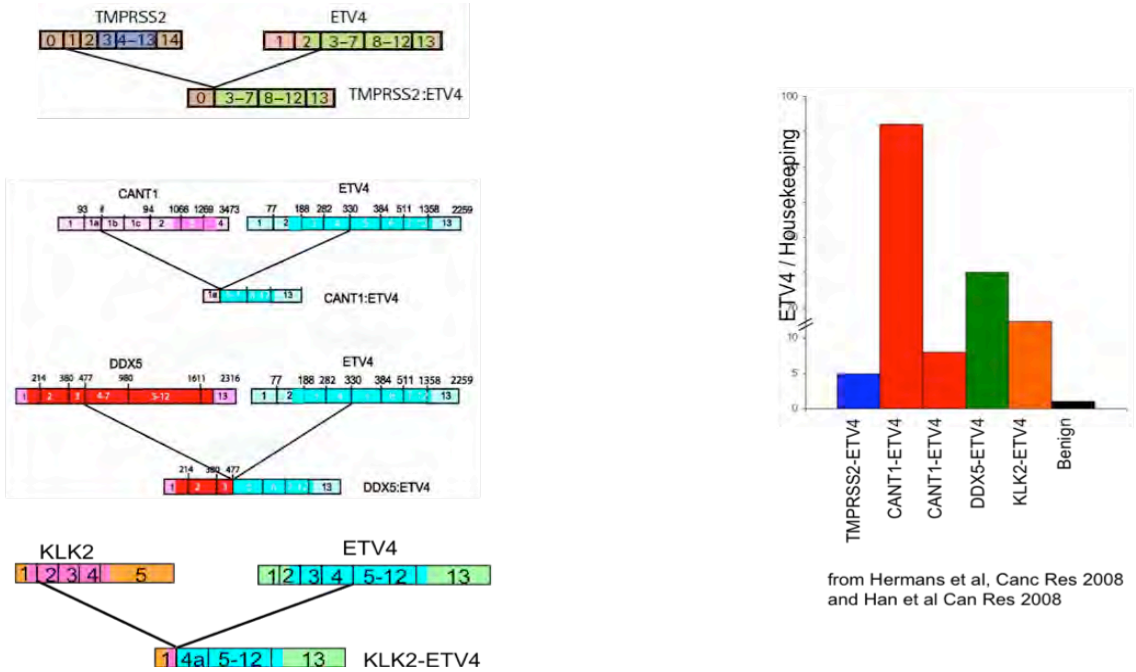

FIGURE 9: *(Left panel) Schematic diagram of prostate cancer translocations involving the ETV4: on the left are shown the different 5′ partners.*
(Right panel) Levels of ETV4 expression in tumors from prostate cancer patients carrying the indicated translocations.

1.10 Role of the EMT in tumor progression

Evidence has accumulated over the last years that a major mechanism underlying tumor invasion is that epithelial tumor cells may acquire a mesenchymal phenotype (Hugo et al., 2007; Thiery et al., 2009). This is similar to a process, crucial for embryonic development, called epithelial to mesenchymal transition (EMT). EMT plays a main role during gastrulation, neural crest formation, heart development and angiogenesis. During gastrulation, an early phase of embryogenesis, certain cells dissolve from the ectoderm and move towards the inside of the embryo to form the mesoderm, the precursor of mesenchymal tissues, fibroblasts and hematopoietic cells (Weinberg et al., 2007; Kalluri et al., 2009). During EMT, epithelial cells lose their epithelial characteristics such as cell-to-cell contacts as well as cell polarity, and downregulate epithelial-associated genes (Figure 10). In addition, EMT leads to upregulation of mesenchymal-associated genes as well as initiates major cytoskeletal changes in the cells in order to enable acquisition of fibroblast-like migratory phenotype (Stockinger et al., 2001)

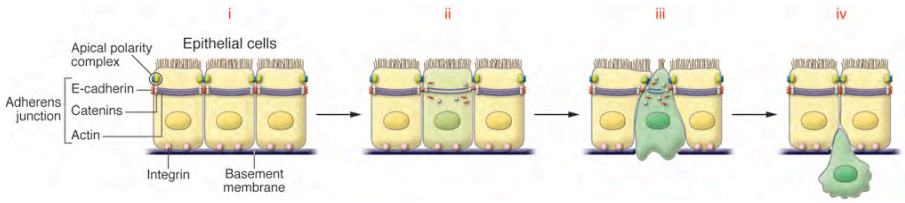

***FIGURE 10**. In epithelial cells, E-cadherin and integrins provide cell-cell and cell-basement adhesion, respectively (i). During EMT, the transcription of E-cadherin and integrins in epithelial cells is repressed, inducing the loss of polarity in these cells (ii). To invade the underlying tissue, the basement membrane is disrupted and the cytoskeleton remodeled to induce a migratory morphology (iii). This allows the cells to detach from the epithelial sheet and move into the surrounding stroma (iv) (from Acloque et al., 2009).*

In literature three different subtypes of EMT are recognized. The earliest EMT (type 1 EMT) in mammals occurs even before implantation during embryonic development. Then after implantation, EMT is required to start gastrulation, which results in the formation of the three germ layers and proper positioning of the cells in relation to the body plan (Wolpert, 2002). During type 1 EMT, primary epithelial cells of the embryo, derived from the ectodermal germ layer, give rise to primary mesenchymal cells that subsequently detach from each other and the basement membrane to ingress through the primitive streak (Figure 11) (Thiery et al., 2009). Type 1 EMT generates cellular plasticity, in order to form new structures and

tissues in the developing embryo. Besides playing a role in developmental processes, EMT is also observed in mature organisms.

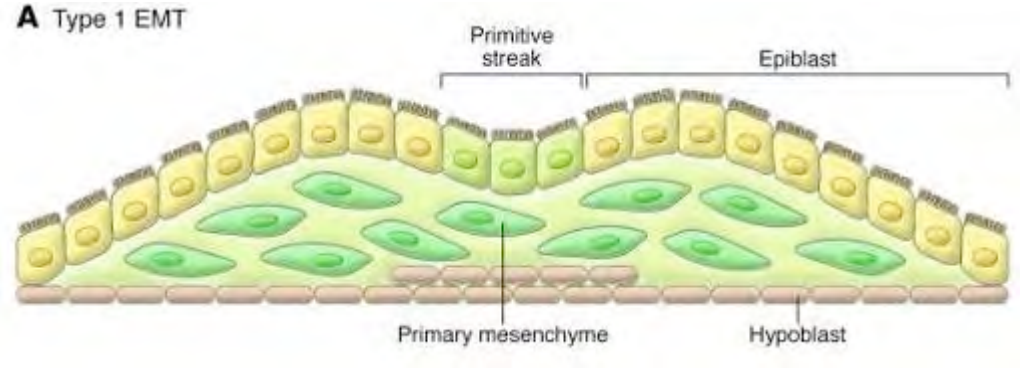

***FIGURE 11**: Type 1 -EMT (from Kalluri and Weinberg, 2009).*

The type 2 EMT is involved in tissue regeneration (Figure 12). During liver injury, for example, EMT is one of the mechanisms promoting repair. Liver cells that have undergone EMT and repopulate the tissue subsequently revert back to hepatocytes by MET (Choi and Diehl, 2009). Type 2 EMT involves the transition of an epithelial cell to a fibroblast, generated to reconstitute the damaged tissue and it is closely linked to inflammation. However, unlike type 1 EMT, fibrosis can also be an outcome of type 2 EMT, and so a physiological response to injury can result in a pathological event. (Thiery et al., 2009).

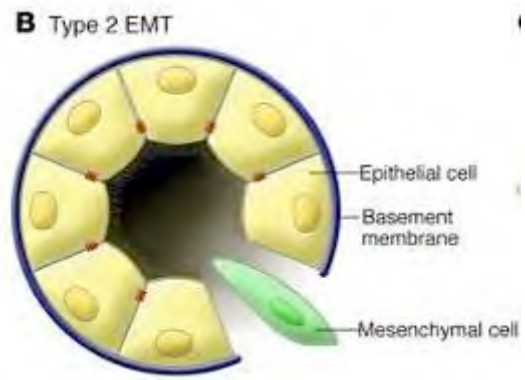

***FIGURE 12:** Type2- EMT (from Kalluri and Weinberg, 2009).*

The type 3 EMT is associated with cancer progression and occurs in epithelial tumors, which account for 90% of the human tumors (Kalluri and Weinberg, 2009;

Klymkowsky and Savagner, 2009). As a result of the genetic and epigenetic alterations that occur in a progressing tumor, cancer cells acquire certain capabilities that distinguish them from regular epithelial cells. These capabilities include increased replicative potential, reduced apoptosis and induction of angiogenesis (Hanahan and Weinberg, 2000). However, the capability of cancer cells to detach from the tumor, invade the tissue, enter the circulation and start a distant colony is what makes cancer difficult to treat. Cancer cells that obtain this invasive phenotype are, according to many reports, generated by activated type 3 EMT pathways (Figure 13) (Thiery, 2002).

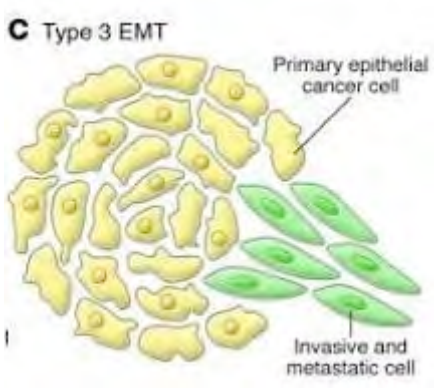

FIGURE 13: Type-3 EMT (from Kalluri and Weinberg, 2009).

The most important molecular event of EMT is the loss of E-cadherin, a transmembrane protein, which is important for cell-cell adhesion and tight cell binding in normal tissue, thus suppressing tumor growth and metastasis (Cano et al., 2000; Angst et al., 2001). In concomitance with loss of E-Cadherin, there is the increased expression of some mesenchimal markers: N-Cadherin, Vimentin. N-Cadherin is a glycoprotein of the cadherin family expressed in different cells: for example: neurons, hepatocytes (Angst et al., 2001). Vimentin belongs to the family of intermediate filaments and it contributes to the formation of cytoskeleton and to the maintenance of cell morphology; it has been also shown its role in cell motility (Helfand et al., 2011).

The activation of EMT follows the induction of a network of transcriptional factors involved in its regulation. These transcriptional factors are divided in two groups: direct regulators and indirect regulators of EMT. Among transcriptional factors

that bind and repress directly the promoter of E-Cadherin there are Snail, Slug e Zeb (Peinado, 2007; Thiery et al., 2009); Twist, instead, represses indirectly E-Cadherin promoter (Thiery et al., 2009). It is interesting that some transcriptional factors (for example Snail and Zeb1) regulate also other EMT's features. They repress some genes involved in cell polarity (Thiery et al., 2009) and stimulate the invasiveness, inducing the expression of some metalloproteinases that are able to degrade basal membrane (Thiery et al., 2009).

Evidence has accumulated over the last years that a major mechanism underlying tumor invasion is that epithelial tumor cells may acquire a mesenchymal phenotype. Thus, processes similar to EMT may play a central role in cancer progression (Klymkowsky and Savagner, 2009; Thompson et al., 2005). Accordingly, the acquisition of an EMT-like phenotype has been associated with the ability of cancer cells to migrate, to invade and to metastasize (Peinado et al., 2004; Thiery and Sleeman, 2006). EMT has been reported in different cancers including prostate (Thompson et al., 2005; Armstrong et al., 2011). It has been found that in patients with different types of neoplasia, the reduction of the expression of E-Cadherin is associated with various indices of tumor progression (Loric et al., 2001; Moody et al., 2005). In addition, it has also been shown an association between the increased expression of N-Cadherin and the tumor (Nieman, 1999; Tomita, 2000). Recent evidences suggest that EMT could be also related with ability of self-renewal of tumor cells (Polyak and Weinberg, 2009).

Thus, EMT is a crucial event not only in development but also in tumorigenesis and it may be associated with invasiveness, metastatic spread of cancer cells and tumor progression.

2. AIM OF THE STUDY

The aim of our study is the understanding the pathogenic role of the ETV4 over-expression in prostate cancer. Specifically, this study has focused on the characterization of the phenotypic and molecular features induced by ETV4 over-expression in prostate cells both *in vitro* and *in vivo*.

We have investigated the effect of ETV4 expression on proliferation and invasiveness of prostate cells by using 2 complementary experimental approaches: (i) Inducible shRNA-mediated silencing of ETV4 in human prostate cell lines over-expressing ETV4; (ii) ETV4 over-expression in non-transformed RWPE human prostate cell line.

Next, in order to define the role of ETV4 in the transformation of normal prostate cells and in cancer progression we have generated and characterized a mouse model of prostate-specific ETV4 over-expression. Furthermore, we have investigated the cooperation of ETV4 over-expression with the loss of the tumor suppressor PTEN, a genetic alteration frequently found in Prostate cancer, to define the contribution of this interaction on the clinical and biological variability of prostate cancers.

3. MATHERIAL AND METHODS

3.1 Cell lines

The prostatic cell line PC3 (IST Cell Bank and Cell Factory, Genoa, Italy) was grown in Ham's F12, supplemented with 10% bovine fetal serum, 2% L-glutamine and 1% penicillin/streptomycin. The non tumorigenic cell line RWPE (American Type Culture Collection, Manassas, VA, USA) was cultured with serum free KER (Keratinocyte SFM, Gibco), supplemented with epidermal growth factor and Bovine Pituitary Extract (Gibco, Carlsband, CA, USA). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

3.2 Vectors and transfection

ShRNAs against ETV4 (NM_001986) were designed with the 'siRNA selection program' (Whitehead Institute: http ://jura.wi.mit.edu/siRNAext) : GGCGCTTCCCAACTTCATA (sh65) and CCCTGTGTACATATAAATGAA (sh3). These shRNAs and an irrelevant shRNA (GCCTATTTACGCCTGACAA) were cloned in the pLVTHM plasmid: a vector whose shRNA expression is doxycycline-inducible in cells expressing the modified tetracycline repressor tTR-Krab. The fragment containing the tTR-Krab under the control of the human EF1a promoter was cloned into pcDNA3 to generate the pcEF-Krab vector. ShRNA vectors were used to transduce prostate cells transfected (doxycycline-inducible ETV4 silencing) or not transfected (constitutive ETV4 silencing) with pcEF-Krab.

PC3 cells were electroporated with pEF1-Krab plasmid and selected with G418 (Sigma, St Louis, MO, USA). The clones expressing higher Krab levels were stably transduced with pLVTHM vectors. shRNAs expression was induced by doxycycline (0.5 mg/ml, Sigma) for at least 5 days.

The FL-ETV4 and the Luc vectors were obtained cloning the human ETV4 cDNA, amplified from PC3 cells and the luciferase cDNA, in a pLG4.2 vector (Promega, Madison, WI, USA) derivative containing the EF1a promoter. RWPE cells were stably transfected with FL-ETV4 and luciferase-containing vector using

Lipofectamine (Invitrogen) and selected with 0.5 ug/ml puromycin (Sigma).

3.3 Cell proliferation assay

We tested the cell proliferation efficiency counting in log phase expansion culture systems and measuring of the H3 thymide uptake levels. Briefly, 5 x 104 PC3 cells were plated in triplicate in 75 cm2 flasks and left in the incubator. At different time points they were trypsinized and measured by direct counting of trypan-blue negative cells. For the H3 thymide uptake assay, 500 PC3 or RWPE cells were plated in quadruplicate in a 96 well dish. After the culture for different time and the following incubation time for 12 hours with 10 uC of H3 tymidine, the cells have been lysated with 3 cycles of freezing and throwing, transferred to a MultiScreen harvest plate FB (Millipore Corporation) containing individual filters of glass fibres at the bottom of each microplate well using Filtermate (Packard). The plate have been washed with water for 4 times, and allowed to dry. After the addition of 50µl of scintillation cocktail to the filters the material have been counted using a Topcount β counter (Packard).

3.4 Western blot analysis

The lysates were obtained from 70% confluent cells. The protein concentration was measured by Bradford Protein Assay (BioRad, Hercules, CA). Samples were loaded at 25 µg/lane, separated by either on 10 or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) (Millipore) membranes and probed with specific primary antibodies. Primary antibody incubation was performed overnight at 4° C. The primary antibodies we used were the following: ETV4 monoclonal antibody (Abnova, Atlanta, GA, USA); actin (Sigma-Aldrich, St Louis, MO, USA); vimentin, twist, E-cadherin, N-cadherin and Snail1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Zeb1 Zo-1 and Slug (Cell Signaling, Danvers, MA, USA). The detection was performed with horseradish peroxidase conjugated secondary antibody(anti-rabbit or anti-mouse) (Cell Signaling, Danvers, MA, USA) and with ECL (Superfemto Amersham, Piscataway, NJ) and ImageQuant 350 apparatus (GE Healthcare, Pittsburg, PA, USA).

3.5 RT-PCR and Quantitative real-time PCR

Total RNA was isolated with RNeasy Mini Plus Kit (Qiagen) and reverse transcribed using the Moloney Murine leukaemia virus (Applied Biosystem). In each reaction we used in the 1x reaction buffer: 0,5-1 µg of RNA, 1 µl of random primers (50µM), 25 mM MgCl2, 10 mM dNTPs, 20 U of RNasi Inhibitor, 50 U of Moloney Murine Leukemia Virus Reverse Transcriptase. The protocol we used is: 40' at 42°C e 5' at 99°C in a Thermal Cycler (Biorad). We used the following protocol: activation of polymerase at 98°C for 30''; 35 cycles of denaturation at 98°C for 5'' and of annealing/extension at 60°C for 10''.

The expression level of each gene was measured by relative quantification respect to the control sample with the method of 2ΔΔC(T) (Livak and Schmittgen , 2001). The expression of the genes have been normalized with 2 "housekeeping " genes: GAPDH and TBP. Each experiment has been performed three times in triplicate and the results shown are the average of the three experiments.

Table: primers used for quantitative PCR. m= murine; h=human

Primer name	**Forward primer**	**Reverse primer**
hmETV4	5'-GCTCGCTGAAGCTCAGGT-3'	5'-TCCTTCTTGATCCTGGTGGT-3'
hETV4	5'-CCACCAGGATCAAGAAGGAG-3'	5'-CTCAGGAAATTCCGTTGCTC-3'
hGAPDH	5'-AACGGATTTGGTCGTATTGGGC-3'	5'-TTGATTTTGGAGGGATCTCG-3
mGAPDH	5'-ACCCAGAAGACTGTGGATGG-3'	5'-GATGACCTTGCCCACAGC-3'
MMP1	5'-CGCTTAGGCTGGAGTGTAGG-3'	5'-CTTGAGGCCAGGAGTTTGAG -3'
MMP2	5'-CCCCTGTTCAAGATGGAGTC -3'	5'-CCCAGGTTGCTTCCTTACCT -3'
VIM	5'-CGCCATCAACACCGAGTTC-3'	5'-ATCTTATTCTGCTGCTCCAGGAA-3'
E-cadherin	5'-GAACGCATTGCCACATACAC-3'	5'-ATTCGGGCTTGTTGTCATTC-3'
N-cadherin	5'-TCAGTGGCGGAGATCCTACT-3'	5'-GTGCTGAATTCCCTTGGCTA-3'
SNAIL1	5'-GCGAGCTGCAGGACTCTAAT-3'	5'-GGACAGAGTCCCAGATGAGC-3'
SLUG	5'-GCCTCCAAAAAGCCAAACTA-3'	5'-CACAGTGATGGGGCTGTATG-3'
TWIST1	5'-GCCGGAGACCTAGATGTCATT-3'	5'-CACGCCCTGTTTCTTTGAAT-3'
ZEB1	5'-GCCAATAAGCAAACGATTCTG-3'	5'-TTTGGCTGGATCACTTTCAAG-3'
ZEB2	5'-AAGCCAGGGACAGATCAGC-3'	5'-CCACACTCTGTGCATTTGAACT-3'

3.6 Soft agar assays

1x104 and 1x105 cells of each cell line were suspended in 4 ml of 0.3% Noble agar (BD Biosciences; Bedford, MA, USA) in the appropriate medium (0.5 mg/ml of doxycycline was added for shRNA induction). The 4 ml agar–cell mixture was layered on 4 ml bottom layer of 0.5% agar medium. Weekly, 300 ml of medium (or medium with 0.8 mg/ml doxycycline) has been added to each dish. After 21 days at 37° C colonies were stained and counted by microscope. Experiments have been performed three times in triplicate.

3.7 Cell cycle analysis

Apoptosis was analyzed using the AnnexinV/PI apoptosis kit (BD Biosciences) on FACScan flow cytometer (BD Bioscience). Cell cycle analysis was performed on synchronized cells (using 10mM hydroxyurea overnight) by flow cytometry. At different time points from hydroxyurea removal, cells were washed, fixed with 70% ethanol, centrifuged and suspended in a lysis buffer and propidium iodide (50 mg/ml) staining buffer. Cell cycle distribution was analyzed using the ModFit LT software (BD biosciences).

3.8 Migration assay

A wound was created by a yellow tip on cells grown to confluence on 6-well plates in cell lines PC3 and RWPE; fresh medium (with 0.5 mM Mitomycin C to prevent cell proliferation) was added and migration was measured on photographs taken at 0 and 48 h.

3.9 Invasion assay

Transwell filters of 8-mm pore size (Corning, Lowell, MA, USA) were coated with 50 ml of 5% matrigel (BD Biosciences); 500 ml of the appropriate medium with 10% fetal bovine serum was loaded in the lower chambers. Single-cell suspensions (1 x105 PC3 or 1.5x105 RWPE cells/well) in medium without serum (and without supplements for RWPE cells) were loaded in the upper chambers.

Filters were incubated 24 h at 37°C in CO2 incubator and, after removal of cells from the upper surface with a cotton swab, were stained with DIFF-QUICK (Medicult, Firenze, Italy). Invading cells, attached to the lower surface, were counted by microscope.

3.10 Mouse xenograft

Athymic nude immunodeficient mice (Harlan, Udine, Italy) were maintained at the CeSAL (University of Florence, Firenze, Italy) and treated according to the European Union guidelines and to protocols approved by the local Animal Ethical Committee. Xenografts were generated by subcutaneous injection of 200 ml of phosphate-buffered saline containing 4x106 PC3 cells. Half of the animals were randomly chosen to receive doxycycline (1 mg/ml in drinking water together with 5% saccarose) to induce shRNA. After 8 weeks tumor weight and volume were measured.

3.11 Immunofluorescence

Cells, after overnight growth on sterile cover slips, were fixed with 2% formaldehyde, permeabilized with 0.1% Triton X-100 and blocked in 5% horse serum. Cells were incubated with primary antibodies against E-cadherin and N-cadherin (Santa Cruz biotechnology) followed by incubation with AlexaFluor488-conjugated goat-anti-rabbit secondary antibodies (Invitrogen) and nuclei counterstained with propidium iodide (Sigma). Immunofluorescence was examined using a Leica SP2-AOBS confocal microscope (Leica Microsystems, Nussloch, Germany).

3.12 Vectors construction and generation of ETV4 transgenic mice

In order to construct the vector pARR2PB-dETV4, we first constructed the TME-ETV4 replacing the 5' end of ETV4 into the FL-ETV4 vector with a PCR amplicon, in which we have introduced the 5' portion of TMPRSS2–ETV4 fusion gene. Then, the TMPRSS2-ETV4 cDNA was excised from TME-ETV4 vector by the VspI-NheI restriction enzymes, both ends were blunted using Klenow enzyme; this fragment

was ligated within the ARR2PBCAT plasmid (Zhang et al., 2000), that has been previously digested with ClaI-SacII restriction enzymes to eliminate the CAT gene and whose ends have been blunted. Thus the plasmid contains the rat probasin PB promoter, with two tandem repeats of the androgen responsive region (*ARR2PB*), the TMPRSS2-ETV4 fusion cDNA and the SV40 polyadenylation sequence.

The fragment of 3.96 kb containing the above sequences was released by digestion with ApaLi-SspI, isolated by agarose gel, purified by a Qiagen gel extraction kit (Qiagen, Valencia, CA) according to the instructions from the manufacturers, and then used for transgenic injections. The pronuclear injection of the pARR2PB-dETV4 fragment into FVB mouse fertilized eggs and the placement of the embryos into pseudo-pregnant females were performed at the LiGeMA Facility of the University of Florence, Italy.

3.13 Genotyping of the transgene mice by PCR analysis

The mice carrying the hETV4 containing transgene and/or the heterozygosis for the Pten allele were screened by PCR on mouse tail DNAs. The primers specific for human ETV4 were: 5'-CCACCAGGATCAAGAAGGAG-3' in exon 6 and 5'-CTCAGGAAATTCCGTTGCTC-3' in exon 7. For the detection of the heterozygotes Pten mice we used the following forward primers: 5'-CGAGACTAGTGAGACGTGCTACTTCC-3', in neomycin gene and 5'-ACTCTACCAGCCCAAGGCCCGG-3' in Pten gene. As control for the amplification reaction we amplified the murine β-actin using the following primers 5'-ACCACAGCTGAGAGGGAAATC -3' and 5'- AGAGGTCTTTACGGATGTCAA -3'. In each reaction we used in the 1x reaction buffer: 100 ng of DNA, 1.25mM MgCl2, 0.2mM dNTPs, 300nM forward and reverse primers, 1.25U of DNA polimerase (Amplitaq DNA Polymerase 5U/µl) e sterile H2O in a final volume of 25µl. The protocol we used is: one cycle at 94° C for 5' followed by 35 cycles (30'' at 94°C, 30'' at 57°C, 45'' at 72°C) and finally 1 cycle at 72°C for 5'. We used the Thermal Cycler (Biorad).

3.14 Southern blot

Genomic DNA was digested overnight at 37°C with EcoRV restriction enzyme and separated by gel electrophoresis, using an 1% agarose gel. For determination of DNA size, we ran also 1 Kb DNA ladders. The DNA was denatured into single strands by incubation for 20 minutes with a solution containing 1,5 M NaCl e 0,5 M NaOH.

The DNA was transferred from the gel to a positive charged nylon membrane (Zeta Probe GT genomic, Biorad). We used a tank in which there was the same denaturing solution (1,5 M NaCl e 0,5 M NaOH) and a paper bridge with the two sides immersed in the liquid. We transferred the agarose gel on this bridge, and then on the gel we added the nylon filter and filter papers that help the DNA transfer by capillarity. The transfer was done overnight. DNA fragments retained on the nylon membrane the same pattern of separation they had on the gel. The nylon membrane was neutralized with two washes in a solution containing 1,5 M NaCl e 200 mM Tris-HCl ph 7,8. The membrane was then incubated with a nucleic acid probe with the ETV4 sequence labeled with P32. The hybridization was done overnight at 65°C, using a solution containing Dextran sulphate 10% (GE Healthcare), SDS 1%, NaCl 1M. After the hybridization, the unhybridized probe was removed from the nylon membrane by several washing with SSC (SSC 20X: NaCl 3 M, Na citrato 0,3 M) and SDS. The washing steps were:

2 washes with SSC 1X, SDS 0,1% for 10 minutes at 65°C

1 wash with SSC 0.5X, SDS 0,1% for 10 minutes at 65°C

The bound labeled probe was revealed using phosphoroimaging instrument (Typhoon, GE Healthcare)

3.15 Paraffin inclusion and HE coloration

Prostate tissues were fixed in formalin 4% for 24 h, dehydrated using solution with increasing concentration of ethanol (70%, 80%, 95%, 100%) followed by toluene and then paraffin. The tissues were embedded in paraffin and then sectioned at 5 μm of thickness. The tissues sections were mounted on an appropriate slide and let them dry. For the HE coloration, the sections were deparaffinized with two washing in toluene of 10 minutes and hydrated with

washing of 5 minutes at decreasing concentration of ethanol: absolute ethanol, ethanol 95%, ethanol 70% and ethanol 50%: After a step in distillated water, the sections have been stained with hematoxylin, specific dye of the nucleus. Then the sections are stained with eosin, a specific dye of the proteins. The sections are dehydrated with increasing concentration of ethanol until to 100% and after a washing in toluene mounted on slides.

3.16 Immunohistochemistry

For immunohistochemical detection deparaffinized slides were subjected to microwave-citrate antigen retrieval and blocking of endogenous peroxidase with 3% of H2O2. The slides were then incubated over-night at 4°C with Mouse anti Ki67(550609 BD Pharmigen), mouse anti–p63 antibody (MS-1081; NeoMarkers, Fremont, CA) and mouse monoclonal anti– smooth muscle actin antibody (M0851; DakoAb, Carpinteria, CA), followed by the incubation with the appropriate secondary antibodies. The visualization was performed using a standard biotin–streptavidin complex (Ultra-vision) and the diaminobenzidin (Dako); the nucleus were counter stained with hematoxylin, followed by dehydration and mounting of the slides.

3.17 Statistical analysis

All data are expressed as mean ± s.d. Statistical analysis was performed using t-test. Statistical significance was accepted for $P < 0.05$.

4. RESULTS

4.1 ETV4 is expressed at different levels in human prostate cell lines

We measured the ETV4 expression levels in normal prostatic tissue, in one immortalized but not-malignant RWPE human prostate cell line and in four human prostate cancer cell lines: DU145, PC3, LnCap and V-Cap. ETV4 expression was not detectable in LnCap and V-Cap cell lines. The ETV4 mRNA levels were increased in PC3 cell lines by about 7 fold; in RWPE and in Du145 human prostate cell lines ETV4 mRNA levels were similar to that found in the normal prostate tissue (Figure 14 (a) and (b)). However, the level of ETV4 protein in prostate cancer DU145 cell line was about 5 fold higher than in non-malignant RWPE cell line. Thus, in DU145 and in PC3 cell lines ETV4 is over-expressed and may be casual to their cancer phenotype.

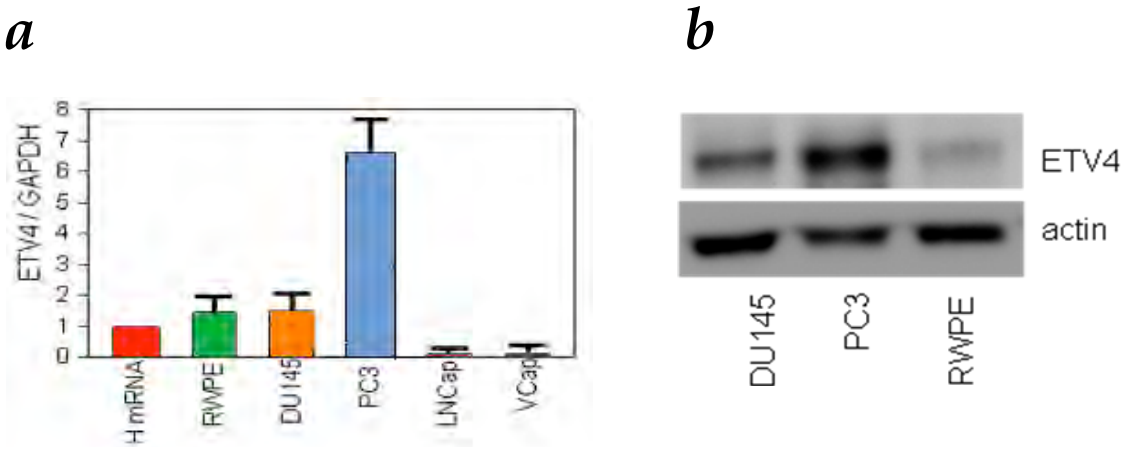

FIGURE 14: *ETV4 expression levels in human prostate cell lines.* ***(a)*** *ETV4 expression level (normalized to the housekeeping gene GAPDH) in comparison with normal human prostate was assessed by qRT-PCR in RWPE, Du145, PC3, LNCap and VCap .* ***(b)*** *Expression of ETV4 assessed by western blot analysis.*

4.2 Downregulation of ETV4 inhibits proliferation, anchorage-independent growth and migration in PC3 cells

Before I started to work on this project, some preliminary experiments obtained in my laboratory showed that the silencing of ETV4 by RNA interference technology in DU145 cells, reduces the proliferation rate and the anchorage-independent growth, suggesting that *ETV4* expression is important for both these features typical of the tumorigenesis.

However, in the DU145 cells the ETV4 protein levels are just as high to that of the non-malignant prostate cell line RWPE; for this reason I started to use another cellular model, the human PC3 prostate cancer cell line that expresses high levels of ETV4. In order to study the role of ETV4 expression I used either a constitutive or a doxycycline inducible vector (Wiznerowicz and Trono, 2003), expressing specific short hairpin (sh) RNA against ETV4. We transfected the PC3 cells stably with 2 different shRNA (sh3 and sh65) to down regulate the expression of ETV4. The expression of both shRNAs, by either the constitutive or the inducible system, was able to produce a stable and efficient reduction of ETV4 levels in PC3 cell line. In PC3 transduced with these silencing vectors the expression levels of ETV4 mRNA was about 10 fold less of those transduced with the irrelevant shRNA vector and this reduction was seen both at mRNA by qRT-PCR (Figure 15a) and at protein levels by western blot analysis (Figure 15b). We used all the stable transfectant PC3 cell lines we have generated to investigate the phenotypic effects of ETV4 silencing.

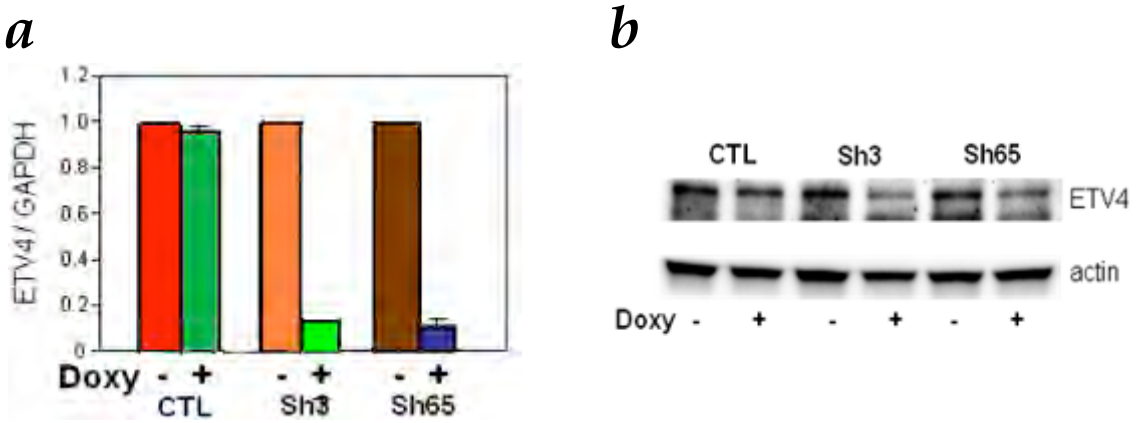

FIGURE* 15: (a)** *ETV4 expression level in PC3 cell line (normalized to the housekeeping gene GAPDH) assessed by qRT-PCR before and after the doxycycline (Doxy).* ***(b) *Expression of ETV4 assessed by western blot analysis before and after doxycycline (Doxy).*

Cell proliferation. The usage of our doxycycline-inducible stably transduced PC3 cell lines, has reduced the inherent experimental variability of growth assays, because it was possible to test in parallel exactly the same cells with or without ETV4 silencing. Using this silencing system we have studied if the reduction of ETV4 expression affects the proliferation rate by counting the cell number after tripan blu exclusion and by H3 thymidine uptake in the PC3 cell line. First, we verified the effect on cell growth of the concentration of doxycycline we have used in our experiments: we found that at this concentration doxycycline affected only slightly the growth of PC3 cell lines expressing an inducible irrelevant shRNA: after 14 days 84.8±12.0% reduction of cell number. In contrast, doxycycline induction reduced the growth of this cell line and after 14 days we had the reduction of 20.7±5.6%. Similar results were obtained by H3 thymidine uptake experiments (Figure 16). After doxycicline induction of either one of the two shRNA against ETV4, the cells showed reduced uptake of H3 in comparison with the same cells without doxycicline induction, whereas there was no difference in the H3 uptake with and without induction of the control shRNA.

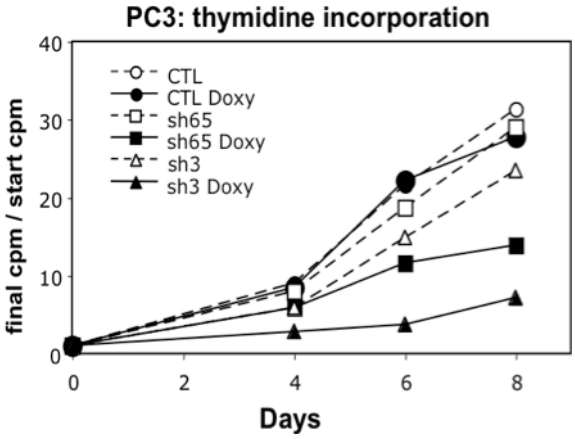

FIGURE 16: *Analysis of cell proliferation by uptake with H^3 in PC3 transduced with two different shRNA against ETV4 (sh65 e sh3) and a control shRNA (CTL) before and after induction with Doxycycline.*

Anchorage-independent cell growth. The anchorage-independent growth is another typical characteristic of cancer phenotype. We have used a classical soft-agar assay to evaluate the ability ETV4–silenced PC3 cells to grow independently from the substrate. We found that the reduction of ETV4 expression (figure 17a and b), with either sh3 and sh65, strongly impaired the ability to form colonies in soft agar and the reduction of the PC3 colonies was about 55% (sh65) and 68% (sh3). These effects of ETV4 inhibition suggest that in PC3 cell lines the expression of ETV4 is important in determining their proliferation rate and their ability to grow independently from anchorage. These results, obtained with two different shRNA molecules, support the notion that their effect is mediated by specific reduction of ETV4 mRNA and not to off-target modulation.

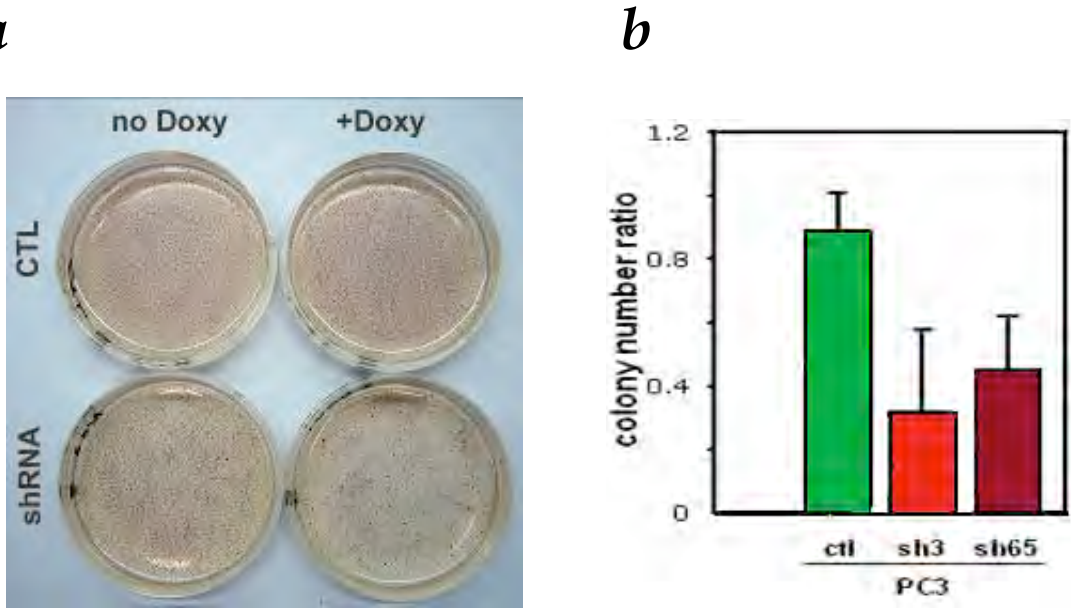

FIGURE 17: *Doxycycline-induced ETV4 silencing reduces the substrate-independent growth of PC3 cell line. A representative experiment of PC3 transduced either with the shRNA against ETV4 (shRNA: lower row) or with a control irrelevant shRNA (CTL: upper row) without (no Doxy, on the left) and with Doxycycline (+Doxy, on the rigth).* ***(b)*** *In the bar diagram each bar represents the ratio between the number of colonies from plates with doxycycline and the number of colonies from plates without doxycycline (mean of three experiments in triplicates).*

Migration and invasion. ETS proteins such as ERG and ETV1 play an important role in cell motility and invasiviness of prostate cell lines (Klezovitch et al., 2008; Tomlins et al., 2008; 2007), and ETV4 is important for motility of prostate, breast, oral squamous, colon cancer cells etc (Hollenhorst et al., 2011; Kaya et al., 1996;

Shindoh et al., 1996; Horiuchi et al., 2003). Furthermore, I tested if PC3 cells are able to efficiently migrate in the wound-healing assay and to invade matrigel. The wound healing assay evaluates the ability of the cells to migrate in a wound made by a yellow tip on a monolayer of cells grown to confluence and where the proliferation is inhibited by mitomycin C. After doxycycline induction, the migration of PC3 containing an inducible ETV4 shRNA was 3.5±0.8 fold less of PC3 containing an irrelevant shRNA (P<0.01) (Figure 18).

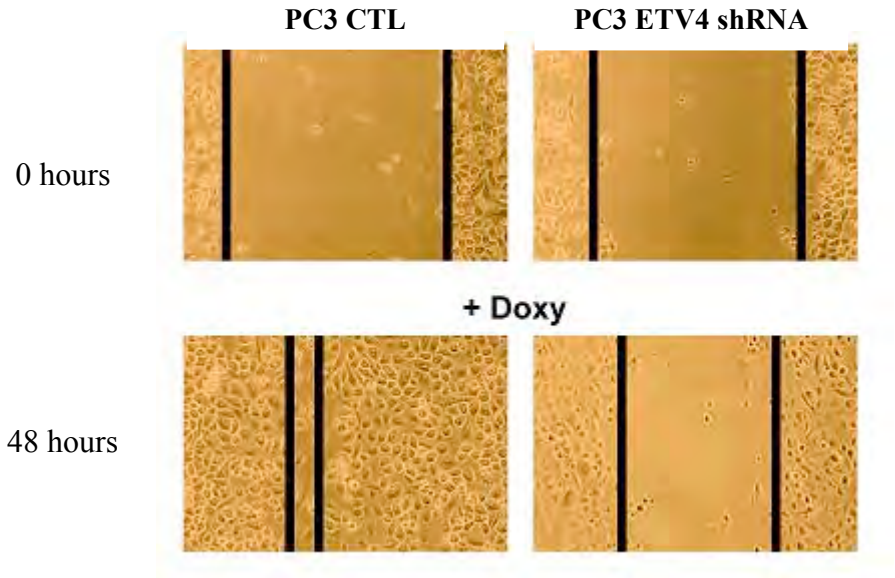

FIGURE 18: *Monolayer of stably transduced PC3 cells after culture for 5 days with doxycycline was scored and then cultured in medium for the time shown in the picture and in presence of mitomycin C. Cell migration into the wound was examined by phase contrast microscopy.*

Furthermore, I tested the ability of PC3 cells to invade a matrigel matrix, a material that reproduce the basal membranes of tissues. Single cell suspensions were loaded on filters coated with matrigel; only the cells able to degrade the matrigel moved through pores of the membrane. Doxycycline induction reduced of 2.9±0.2 fold the number of matrigel invading cells of PC3 containing an inducible ETV4 shRNA whereas slightly reduced (about 1.5 fold) the invading ability of PC3 containing the irrelevant shRNA. Matrix metalloproteinases (MMPs) play pivotal roles in invasion through the degradation of extracellular matrix of tissues surrounding tumors (Crawford et al., 1994) and ETV4 is known to regulate some of them (Higashino et al., 1995; Kaya et al., 1996; Horiuchi et al., 2003). We found that the reduction of the ETV4 levels in PC3 was associated with decreased levels

of MMP1 (0.28±0.2) and MMP3 (0.14±0.1) mRNA and only a slight decrease of MMP9 (0.7±0.2), whereas there was no variation in the expression level of MMP7, PLAU and ADAMs (ADAM9, ADAM10, ADAM17, ADAMTS1). In addition, there was an increased level of the metalloproteinase inhibitor TIMP1 (3.24±0.1) but not of TIMP2 (Bourboulia et al., 2010) (Figure 19). Thus, ETV4 expression is important for the migration and the invading ability of PC3 cells.

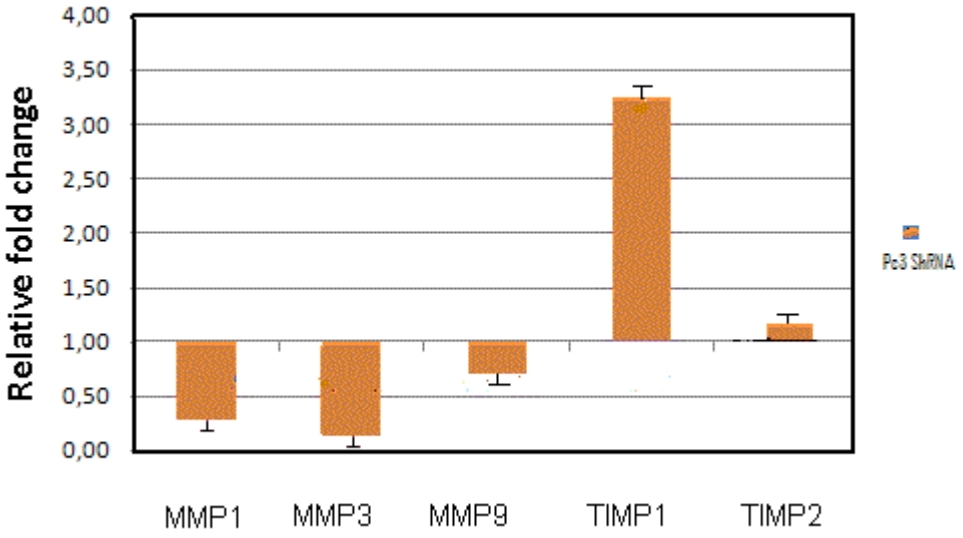

FIGURE 19: *Expression mRNA level of methalloproteinases (MMP) and their inhibitors (TIMP) in cell lines PC3 where ETV4 is silenced.*

4.3 Overexpression of ETV4 stimulates proliferation, anchorage-independent growth, migration and invasiviness in the non-malignant RWPE prostate cells

In order to study the effect of ETV4 over-expression I used as model the RWPE, a commercially available non tumoral prostatic cell line (Webber et al., 2001). The RWPE cell line was obtained immortalizing by infection with the human papilloma 18 (HPV-18) virus. The RWPE cells do not show any of the phenotypic features associated to the neoplastic transformation. In fact, the RWPE cells are not able to growth in soft-agar, to invade and to develop tumors in immunodeficient mice (Bello et al., 1997). For my experiments, I stably transfected the RWPE with either a vector encoding the human full length ETV4 cDNA (RWPE-ETV4) or a vector encoding the luciferase gene (control RWPE). Cells transfected with ETV4, showed an expression of ETV4 about 7.5 fold higher than untransfected or luciferase-vector transfected RWPE cells; at protein level the increase of ETV4 was about 5 fold (Figures 20a and 20b).

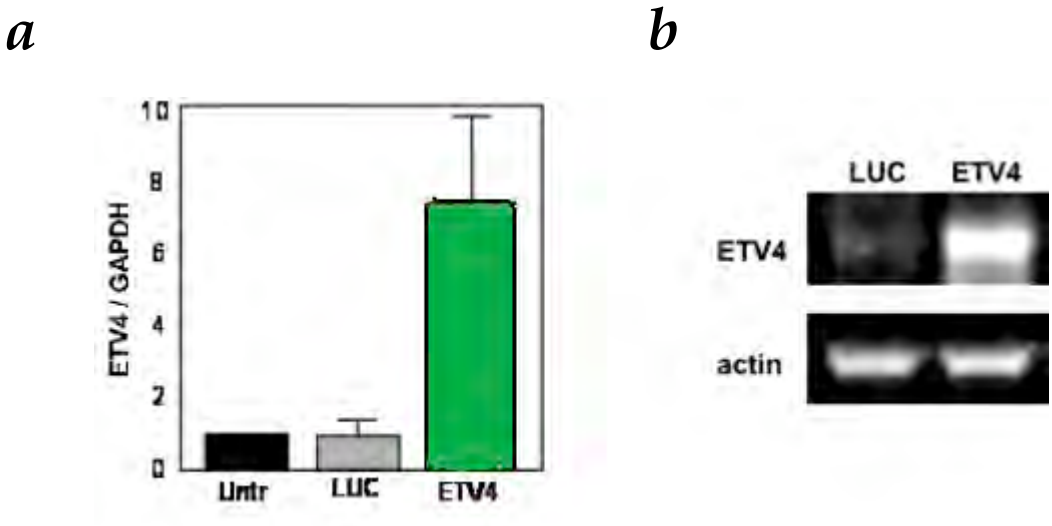

FIGURE 20: *ETV4 expression in RWPE cells untransfected (untr), mock-transfected (LUC) or with ETV4 expression vector.* ***(a)*** *ETV4 expression (normalized to the housekeeping gene GAPDH) assessed by quantitative real time PCR.* **(b)** *ETV4 expression level assessed by western blot.*

By H3 thymidine incorporation assay, I have tested the rate of proliferation in the RWPE cells, and I showed that the over-expression of ETV4 increased the rate the proliferation by about 2 fold compared to control cells (Figure 21).

Furthermore, we tested the ability of these cells to grow in soft agar. The RWPE cells over-expressing ETV4 were able to form a number of colonies three fold higher than control RWPE (Figure 22); in addition, the size of the RWPE-ETV4 colonies was much bigger than that of control RWPE colonies. These experiments, in triplicate, show that over-expression of ETV4 provides the RWPE with anchorage-independent growth, a typical characteristic of the tumor cells.

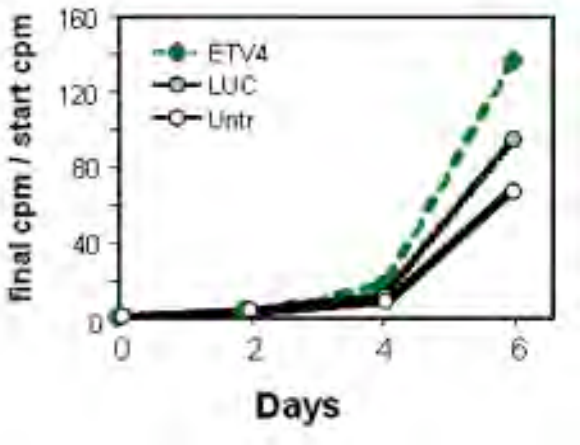

FIGURE 21: *ETV4 overexpression increases the rate of proliferation of RWPE cell line assessed by H3-thymidine uptake. It is represented the ratio between initial and the actual c.p.m.(average of 3 experiments done in quadruplicate).*

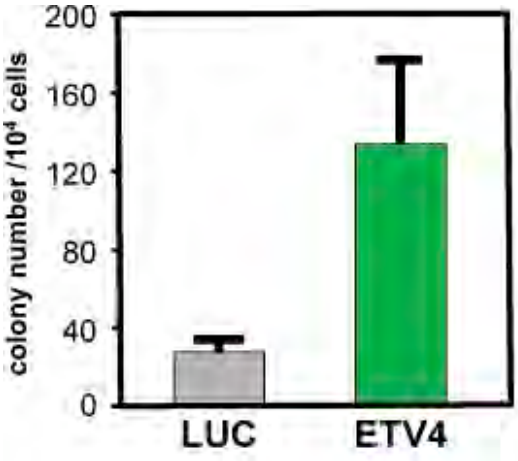

FIGURE 22: *ETV4 over-expression increases the substrate-independent growth of RWPE cell lines. Each bar represents the average number of colonies (3 experiments done in triplicate).*

Then, we studied the effect of the ETV4 over-expression on the migration in a wound-healing assay. After 48 hours, the RWPE over-expressing ETV4 heals almost totally the wound, while the control RWPE cells are not able to heal the

wound (Figure 23); showing that ETV4 over-expression increases the migratory capacities of the RWPE cells.

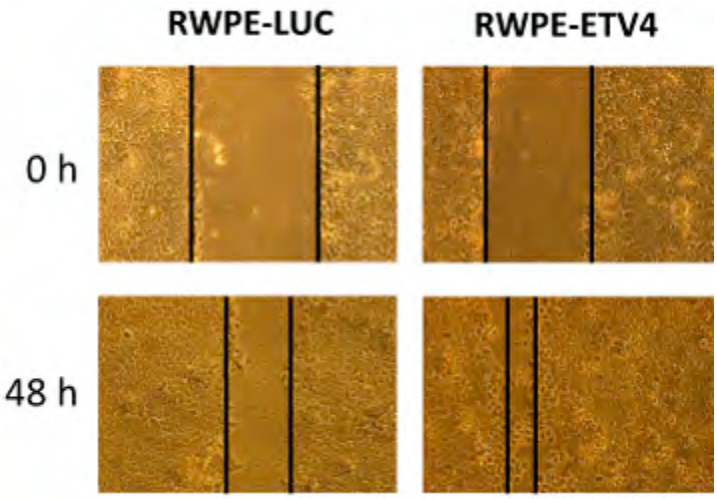

FIGURE 23: *Monolayer of mock transfected, ETV4 transfected RWPE cells was scored and then cultured in medium for 48 hours in the presence of mitomycin C. Cell migration into the wound was examined by phase contrast microscopy.*

Finally, we have analyzed the effect of ETV4 over-expression in invasion assay through matrigel matrix. In our experiments we found that the over-expression of ETV4 increased the invasion ability of about 3.5 fold when compared with control cells (Figures 24 and 25).

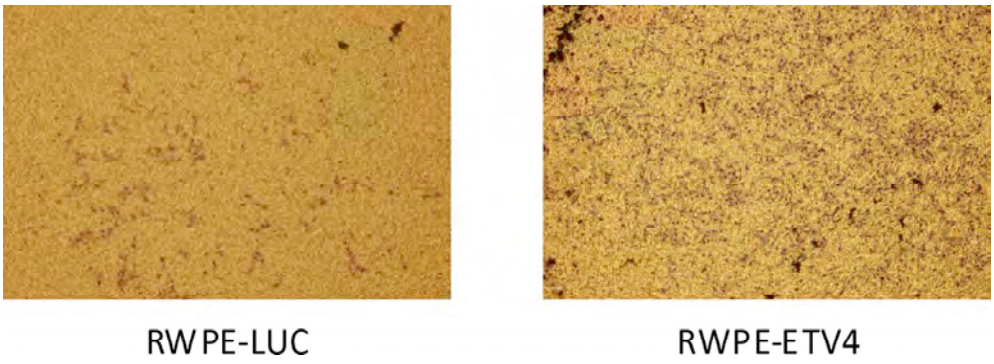

FIGURE 24: *ETV4 over-expression increases the migration through matrigel of RWPE cell lines: mock-transfected (LUC) vs. ETV4 transfected (ETV4-RWPE) RWPE.*

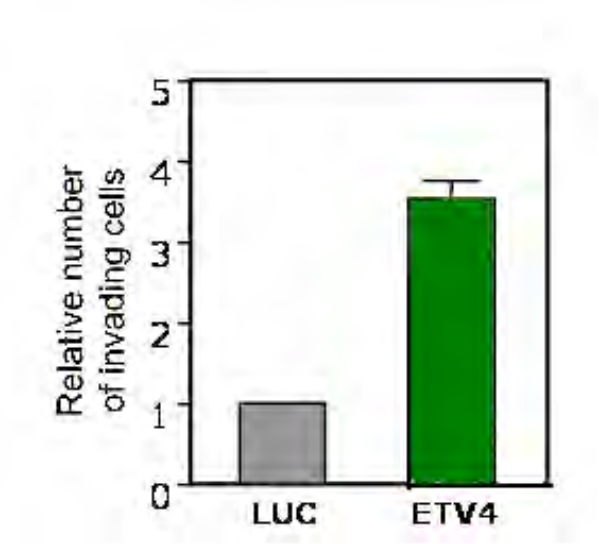

FIGURE 25: *Migration through matrigel of mock-transfected (LUC) and ETV4 transfected (ETV4-RWPE) RWPE cell lines. Cells that migrated out of the chamber were stained and counted. Each bar shows the number of migrating cells normalized to that of the mock-transfected cells. The mean + SD of three experiments done in triplicates is shown.*

These results confirmed that ETV4, also in RWPE cells, has a role in cell proliferation, anchorage-independent growth, migration and invasion ability.

4.4 Downregulation of ETV4 inhibits tumor growth in immune-deficient mice

Many types of cancer cells give tumors when injected in immune-deficient mice (xenograft model): this is true for also for PC3 (Ware et al., 1985). To reduce experimental variability of such experiments we have used PC3 transfected with the doxycycline-inducible shRNA against *ETV4*. Thus, we have injected in each mouse cells derived from exactly the same culture and, after two months, we have compared the size of the xenograft of mice assigned randomly to drink water with or without doxycycline.

We found that the average weight of PC3 xenografts from mice treated with doxycycline was about half of control mice ($P<0.04$) (Figure 26 and 27). It is interesting that in PC3 xenografts harvested from doxycycline-treated mice the ratio ETV4-silencing vector/GAPDH at DNA level was 4% of the ratio in xenografts from control mice, suggesting that the tumors in doxycycline-treated mice are mainly derived from the few untransfected cells included in the initial injection. Thus, ETV4 downregulation in PC3 cells reduces their ability to form tumors in mice supporting the notion that ETV4 expression is necessary to maintain the cancer status of these human prostate cancer cell lines.

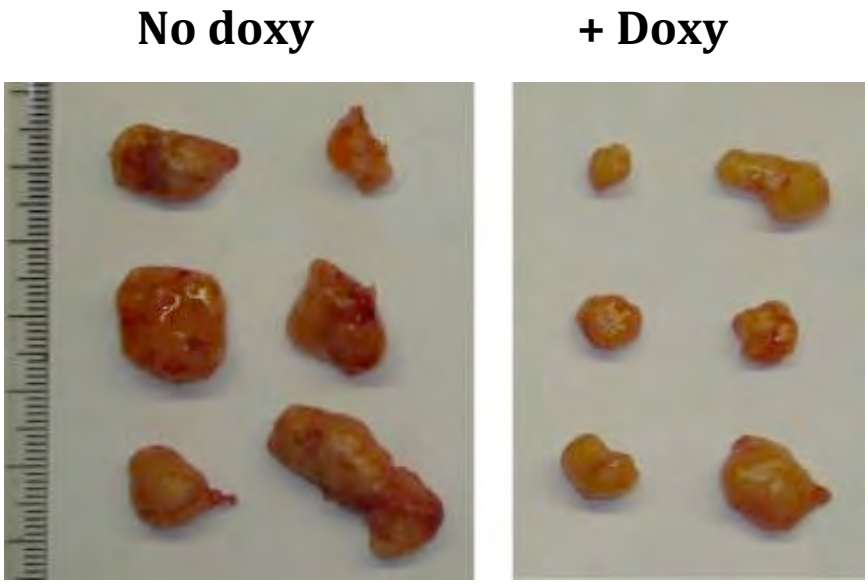

FIGURE 26: *Tumors derived from PC3 cells stably transduced with doxycycline-inducible vectors expressing the anti-ETV4 shRNA 65 and injected in athymic nude mice (representative experiment). Tumors have been excised from the immunodeficient mice treated with doxycycline after 8 weeks from the injection.*

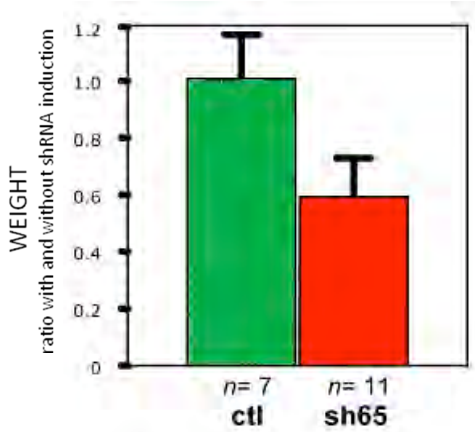

FIGURE 27: *In the histogram is indicated the ratio between the average weight of the tumors recovered from doxycycline treated mice and the average weight of tumor recovered from untreated mice. The bar of errors represents the Standard Deviation.*

4.5 The role of ETV4 in the modulation of proliferation through the cell cycle regulation

In principle cell growth could be increased either by an increased rate of cell proliferation or by a decreased rate of apoptosis. To identify the mechanism involved in growth alterations caused by ETV4 silencing or over-expression in prostate cell lines, we have analyzed apoptosis and cell cycle.

The apoptosis was studied by flow cytometry using Annessin V/7ADD staining; the Annessin V is a specific staining for apoptotic cells, while the 7AAD is specific for the necrotic cells. We did not observe any significant variation of apoptotic cells percentage after ETV4 silencing in PC3 nor after ETV4 over-expression in RWPE cells (data not shown).

Next, we analyzed cell cycle progression. We synchronized the cells in G1 phase with hydroxyurea and then we removed the hydroxyurea at different time points and we analyzed the cells by flow cytometry. Four hours after synchronization the fraction of PC3 cells expressing the shRNA against ETV4 in S phase was reduced in comparison with controls (Figure 28a). This result indicates that silencing of ETV4 causes a slower cell cycle progression. The analysis of the cell cycle in RWPE cells overexpressing ETV4 were a mirror image of the above: there was an increase in the fraction of cells in S phase after 4 h after synchronization compared with controls (Figure 28 b).

In order to investigate the molecular basis of the observed changes, we have tested some of the proteins known to be involved in cell cycle regulation (Herold et al.,

2009; Malumbers et al., 2009). In RWPE-overexpressing ETV4, we have found reduced expression of P21 (WAF1/CIP1) and P27, together with a slight increase of MYC, CHECK1 and MCM7 expression. In the ETV4- silenced PC3, we have found increased expression of P21 (Figures 28c and d) and reduced expression of MYC, CHECK1 and MCM7 (Figure 28c). These results suggest that ETV4 play a direct or an indirect role in cell cycle regulation.

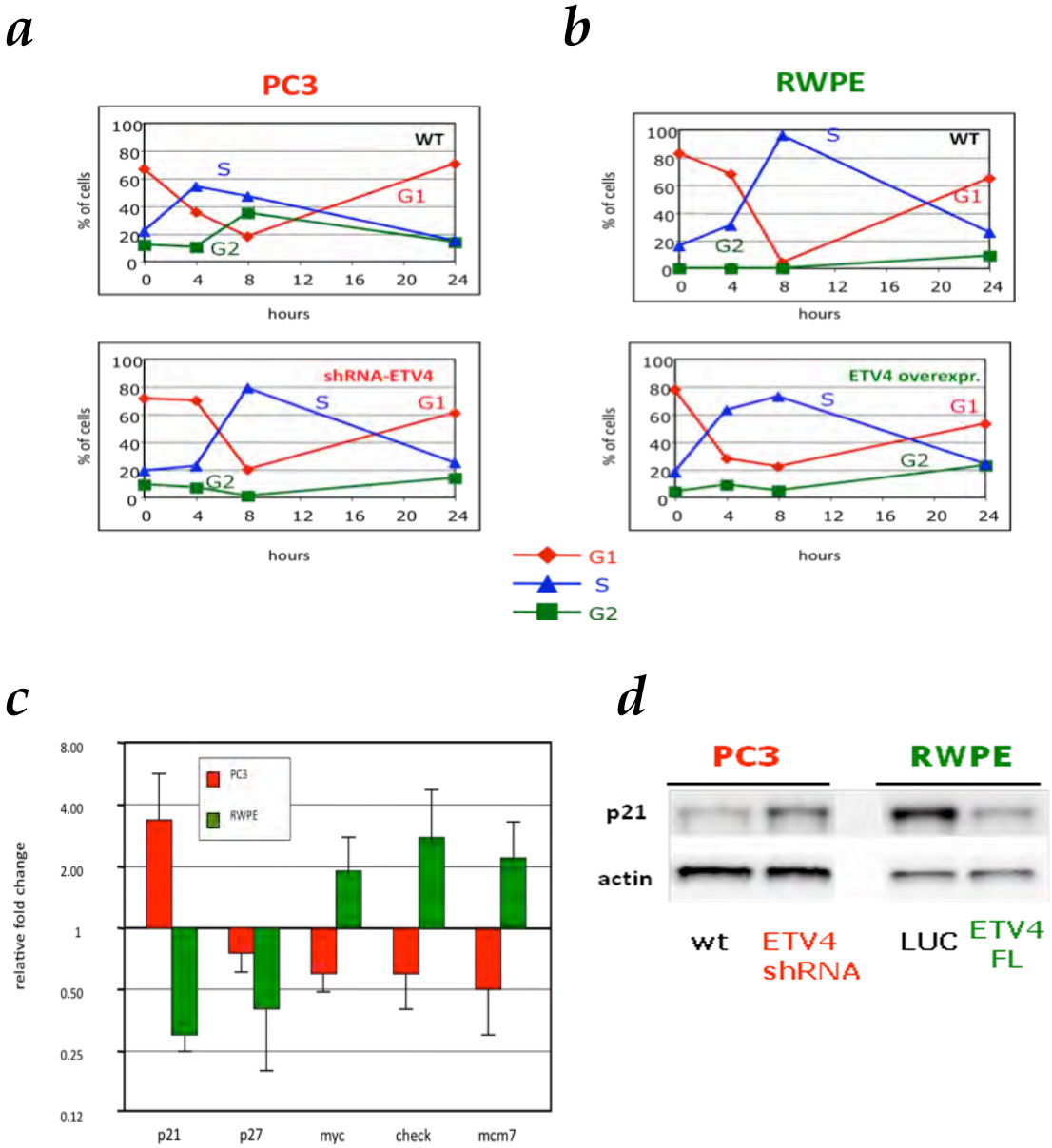

FIGURE 28: *Cell cycle analysis carried out in PC3* **(a)** *and RWPE* **(b)** *cells after the treatment with 10 mM hydroxyurea for 14 hours followed by culture in normal medium. The analysis of the cell cycle phases was performed at 0, 4, 8 and 24 hours.* **(c)** *Fold changes of expression levels (normalized to the housekeeping gene GAPDH) of genes known to be involved in cell cycle in PC3 sh3 compared with PC3 CTL and RWPE cells transfected with ETV4 compared with LUC-transfected cells.* **(d)** *Expression by western blot analysis of the protein level of p21(Waf1/Cip1) in PC3 and RWPE cell lines.*

4.6 ETV4 overexpression induces EMT in non-malignant RWPE prostate cells

ETV4 over-expression in the non-malignant RWPE cells results in an increase of their ability to invade and to migrate and it is paralleled by the modulation of the expression of genes involved in this function such as metalloproteinase and their inhibitors. In fact, we found the increased expression of MMP2 where the FL-RWPE results increased about 41±5.5 fold and the expression of MMP3 is increased about 16±1.2 fold; while we found a slight reduction of TIMP1 about 0.8±0.1 fold (Figure 29).

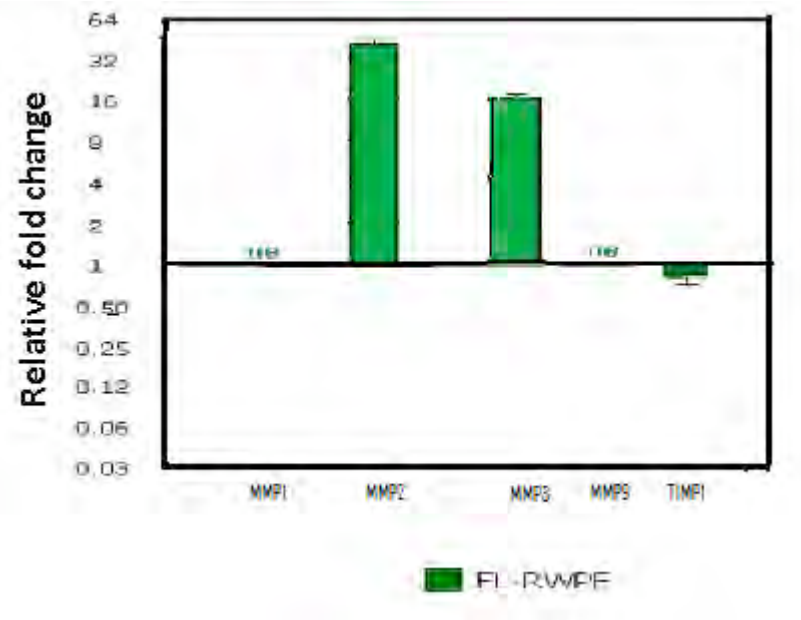

FIGURE 29: *Expression mRNA level of metalloproteinases(MMP) and of an MMP inhibitor (TIMP) in cell lines RWPE where ETV4 is over-expressed.*

Moreover, we observed that RWPE cells transfected with *ETV4* expression vectors underwent shape changes. The RWPE cells show a polygonal morphology typical of the epithelial cells and only rarely spindle-shaped (mesenchymal-like) cells are present in control RWPE cells (about 1%); in RWPE over-expressing ETV4 the frequency of spindle-shaped cells raised to 53% (Figure 30). Thus, the over-expression of ETV4 in RWPE cells does not only increase the invasiveness and migratory abilities, but induces RWPE cells to acquire a mesenchymal-like shape.

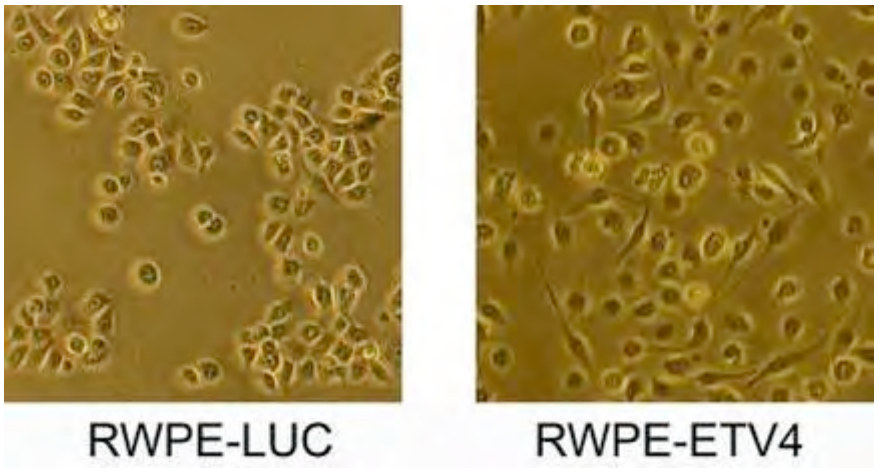

FIGURE 30: *Representative microphotographs of RWPE cells. The cells transfected with a control vector (RWPE-LUC) displayed a rounded epithelial cell shape with rare spindle-shaped cell. A large fraction of cells RWPE transfected with ETV4 displayed a spindle-like shape*

In the last years evidence has accumulated that a major mechanism underlying tumor invasion is the activation of a transcriptional program of the Epithelial Mesenchymal Transition (EMT). In order to verify if the cell morphology changes we have observed in RWPE cells upon ETV4 expression are due to the activation of the transcriptional program EMT, we studied the expression of EMT molecular markers. We analyzed by Real Time–PCR and Western Blot the expression levels of E-Cadherin, a glycoprotein expressed on the membrane of epithelial cells in adherent junctions, a marker of the epithelial phenotype and of N-Cadherin and Vimentin, two mesenchymal markers.

Over-expression of ETV4 was associated with decreased expression of E-cadherin (epithelial markers) and increased expression of Vimentin and N-cadherin (mesenchymal markers) both at RNA and protein levels (Figures 31a and 31b).

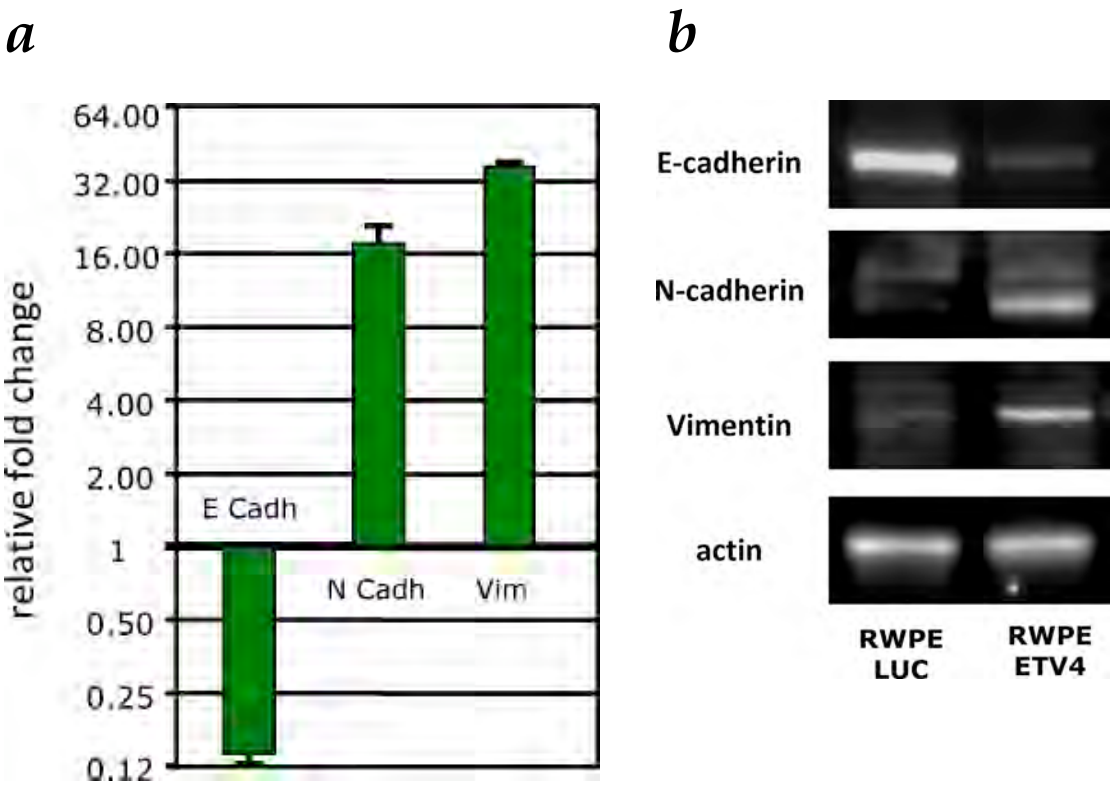

***FIGURE 31* (a):** *Fold changes of expression levels (normalized to the housekeeping gene GAPDH) of epithelial (E-Cadherin) and mesenchymal (N-Cadherin,Vimentin) markers in RWPE cells transfected with ETV4 vector compared with the RWPE control cells.* **(b)** *Expression of these Epithelial-mesenchymal markers assessed by western blot analysis.*

In addition, with analysis by confocal microscopy, we have found that the luciferase transfected RWPE cells do not express N-cadherin whereas E-cadherin is expressed and localized on the plasma membrane. In keeping with the acquisition of mesenchymal morphology we found that in RWPE cells over-expressing ETV4, the epithelial marker protein E-cadherin is reduced and migrates from the plasma membrane to the cytoplasm (Figure 32). In parallel, there is up-regulation of the mesenchymal protein N-cadherin and it is now seen in both cytoplasm and plasma membrane (Figure 32). We can therefore presume that the morphological changes of RWPE cells induced by ETV4 expression are mediated by changes in the levels and distribution of E-cadherin and N-cadherin typical of mesenchymal cells.

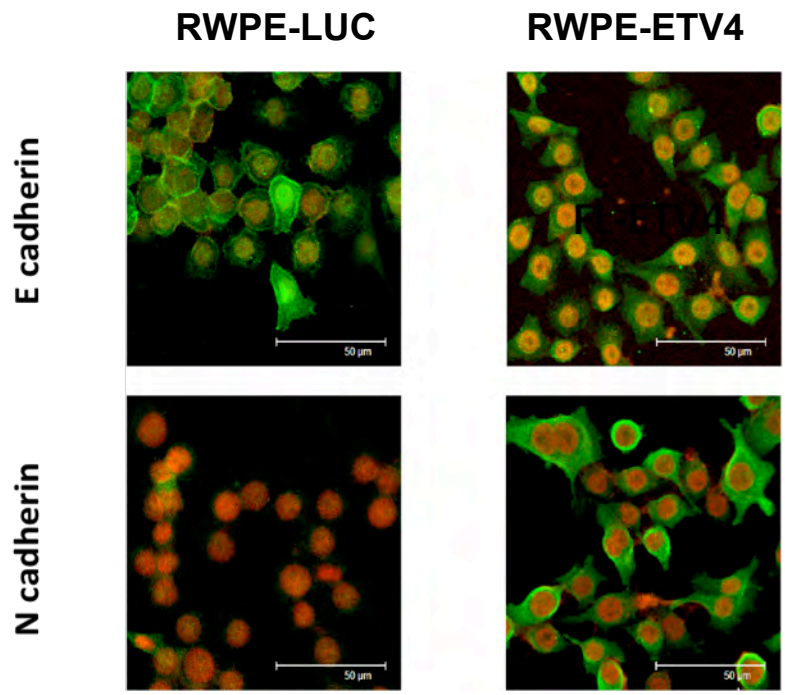

FIGURE 32 : *Analysis by confocal microscopy of E-Cadherin and N-Cadherin in RWPE cells transfected with mock and ETV4 vector. The control cells do not express the mesenchymal marker protein N-Cadherin and the epithelial marker protein E-Cadherin is expressed and localized on the plasma membrane. In RWPE cells overexpressing ETV4, the E-Cadherin is reduced and has migrated from the plasma membrane to the cytoplasm; the N-Cadherin is upregulated and appeared either in the cytoplasm and plasma membrane.*

Next we have tested for proteins that are known to be direct repressors of the E-Cadherin (SLUG, ZEB1, ZEB2, SNAIL), and others are indirect repressors (TWIST1). The expression levels of these transcriptional factors were measured by Real Time-PCR. The RWPE cells over-expressing ETV4 have increased expression levels of TWIST (10 fold), ZEB (40 fold) and ZEB2 (4 fold), while there is a slight increase of SLUG (2 fold) (Figure 33a); we did not observe variation of SNAIL1 expression level. These results were confirmed also at protein level by western blot analysis (figure 33b).

These data indicate that ETV4 is a powerful inducer of EMT: the increase of several transcription factors relevant to EMT is in keeping with this notion.

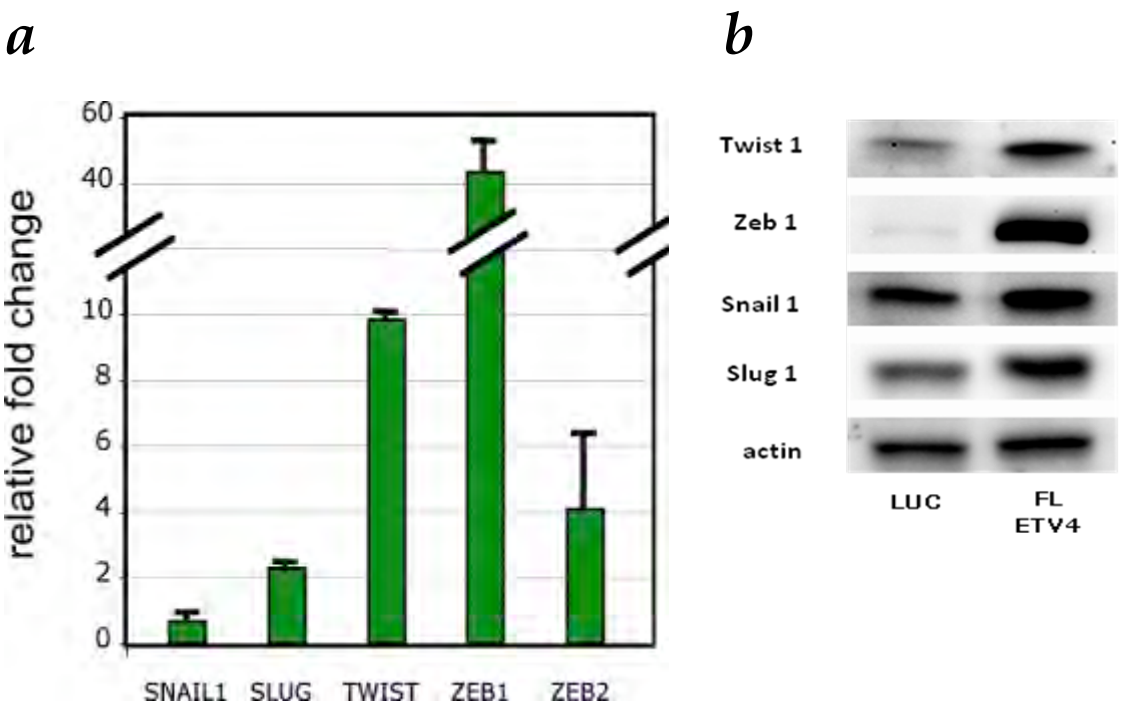

FIGURE 33: *The effect of ETV4 over-expression on the expression of genes involved in EMT in the RWPE.* **(a)** *Expression level (normalized to the housekeeping gene GAPDH) of transcription factors associated with EMT in RWPE cells transfected with mock and ETV4 plasmid.* **(b)** *ETV4 expression level assessed by western blot analysis of some transcription factors (TWIST1, ZEB1, SNAIL1 and SLUG1) associated with EMT in RWPE cells transfected with either mock (Luc) or ETV4 expressing plasmids.*

4.7 Generation of mice with prostate-specific ETV4 expression

The *in vitro* studies we have performed during this research project have generated a very interesting amount of the data about the role of ETV4 expression in prostate cells. These studies have shown that in prostate cells ETV4 expression may induce most of neoplastic features, such as increased proliferation and cell motility. In addition, they have provided some information about the down-stream pathways through which ETV4 expression induce such neoplastic features.

Although suggestive, these *in vitro* experiments alone are not sufficient to determine whether ETV4 may play a causal role in the development and progression of human prostate cancer. The study of the human diseases has taken advantage of the presence of animal model that have been useful to verify hypothesis on the molecular mechanisms involved in the disease derived from *in vitro* study or from the analysis of the clinical phenotype of the patients. In order to verify the pathogenetic role of the ETV4 over-expression in prostate carcinoma initiation or progression in a *in vivo* model we have resorted in developing a mouse model over-expressing ETV4 in prostate epithelial cells. We have generated a transgenic mice in which we have inserted the *TMPRSS2-ETV4* fusion cDNA found in prostate cancer patients. First, we have modified the vector used for the *in vitro* study, replacing the 5' end of ETV4 with a PCR amplicon, in which we have engineered the 5' portion of TMPRSS2–ETV4 fusion gene (see "Material and Methods for details"). Second, the *TMPRSS2-ETV4* fusion cDNA was inserted in a vector containing : (1) the androgen inducible and prostate specific modified rat probasin (ARR2PB) promoter that includes two tandem repeats of the androgen responsive region, and (2) the poliA signal from the SV40 virus (Zhang et al., 2000). A schematic representation of the ARR2PB-ETV4 construct is shown in Figure 34.

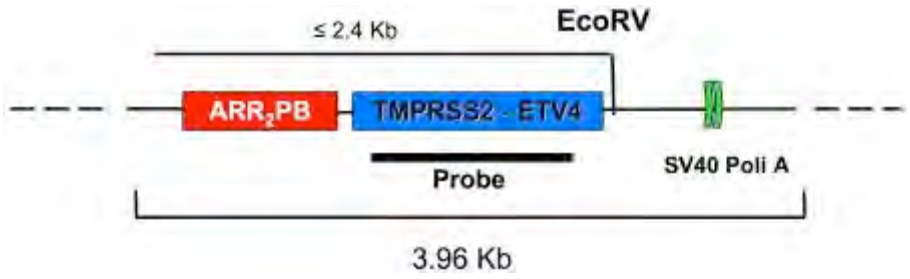

FIGURE 34: *Schematic representation of the ARR2PB-ETV4 construct. The promoter is in red, the coding sequence of the fusion TMPRSS2-ERT4 gene is in blue and the SV Poly A in green. The position of the unique Eco RV site is shown.*

The fragment containing the ARR2PB promoter, the TMPRSS2-ETV4 and the poly A sequences (ARR2PB-ETV4) was released from the vector and then used for the pronuclear injection into FVB mouse fertilized eggs at the LiGeMA Facility of the University of Florence, Italy. We obtained 3 ARR2PB-dETV4 founders that, after PCR analysis, resulted to be positive for the presence of the ARR2PB-ETV4 transgene. These founders were crossed with wild type FVB mice and each of them generated viable offspring: thus, we have obtained 3 mouse lines that we named A, B and C. The litters of each of the 3 lines were screened for the presence of the ARR2PB-ETV4 transgene by PCR on mouse tail DNA, using primers specific for human ETV4 and primers from murine β-actin as control (see Material and Methods). All the mice from the litter A were negative for the presence of the ARR2PB-ETV4 transgene thus the founder of the line A was not able to transmit the transgene. At variance, mice carrying the ARR2PB-ETV4 transgene were present in the offspring of both B and C mouse lines (Figure 35).

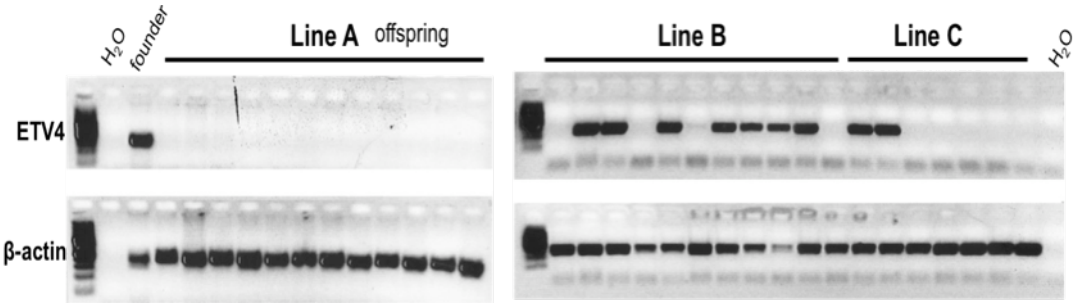

FIGURE 35: *Analysis by PCR of the offspring of the 3 mouse lines. In the offspring of the founders A there were no mice carrying the ARR2PB-ETV4 transgene.*

The founders of line B and C and their F1 offspring were characterized for construct integration by Southern blot analyses (Figure 36). The genomic DNA was

digested with a restriction enzyme Eco RV that cuts once in the ARR2PB-ETV4 transgene: specifically, the Eco RV is at 3' end of the ETV4. The hybridization with the human ETV4 cDNA labeled probe (see figure 34) showed the presence of a band of about 4 kb in all the mice positive for the presence of the ARR2PB-ETV4 transgene by PCR, an additional band was present only in the mice from the line B, no bands were present in the mice negative by PCR.

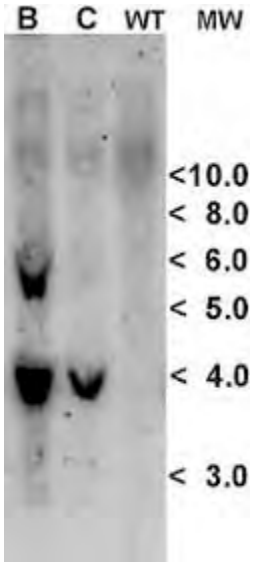

FIGURE 36: *Southern blot analysis of B and C ARR2PB-ETV4 transgenenic mouse lines.*

Because the length of the transgene used for the injection was of 3.96 kb, the presence of a band of the same length suggests that in both mouse lines there were multiple integrations in tandem. The presence of an additional band is expected because it derives from the combination of the Eco RV site within the transgene and an Eco RV present in the genomic DNA flanking the 5' of the integration site of the transgene (see Figure 37) we found an additional band in the mice from line B, but not in mice from the line C. The absence of such additional band in the line C can be explained by the fact that the Eco RV site in genomic DNA flanking the integration site of the transgene was either too far or too close to generate a fragment with a size detectable by Southern blotting. In conclusion, mice from both lines had only one transgene integration site but with multiple copies of the transgene.

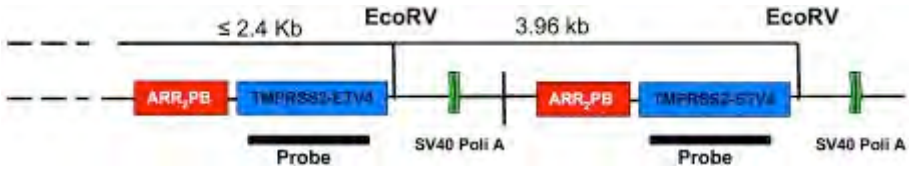

Figure 37: *Schematic representation of a tandem insertion of the ARR2PB-ETV4 transgene.*

Finally, we evaluated the expression of the transgene in the prostate by quantitative RT-PCR using a pair of primers localized in 2 different exons of ETV4 in a region identical in both human and murine ETV4 sequence (see the table of primers in Material and Methods).

The gene expression has been normalized using as reference the GAPDH expression level. This analysis showed that the ETV4 expression was increased ten fold in line C compared with the control wild type mouse, whereas the line B showed similar levels of ETV4-espression than the wild type mouse (Figure 38). This result suggested that the mice from line B were not able to express the transgene.

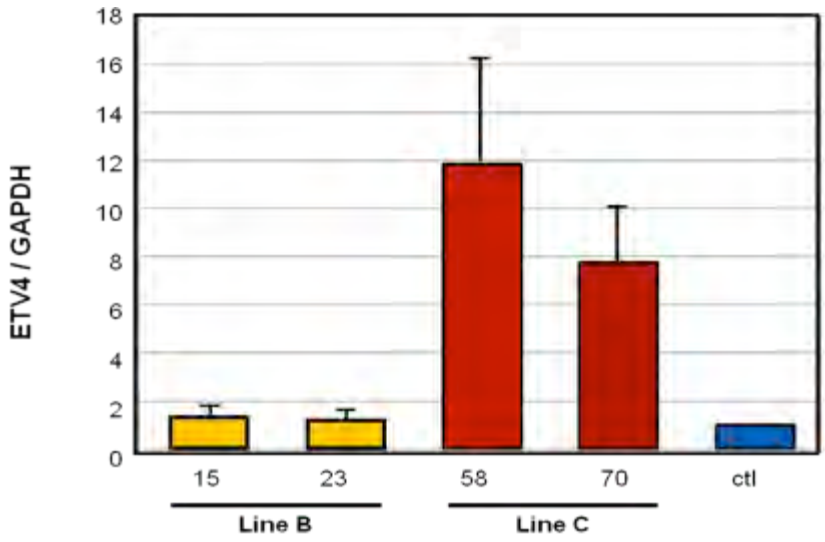

Figure 38: *Analysis by real time PCR of the ETV4 expression in the mice of lines B and C. The mean of three experiments is shown.*

4.8 Transgenic Expression of ETV4 in the Mouse Prostate Induces Prostatic Intraepithelial Neoplasia (PIN)

We expanded the line C by crossing the mice carrying the ARR2PB-ETV4 transgene with wild type mice; the mice resulting from this crossings have been genotyped for the presence of the ARR2Pb-ETV4 transgene and male mice have been followed up. We analyzed the prostate of both wild type and ARR2Pb-ETV4 transgenic male mice at 6 and at 10 months: after careful dissection of prostate from each mouse, the prostate was embedded in paraffin for classical hematoxylin and eosin staining studies and for further studies.

We found neither gross nor microscopic prostate lesions in any of the wild type mice (6 months: *n*=4; 10 months: *n*=10) (Figure 39a and 40a). We also did not find prostate lesions in 5 ARR2Pb-ETV4 transgenic mice at 6 months of age (Figure 39b).

a ***b***

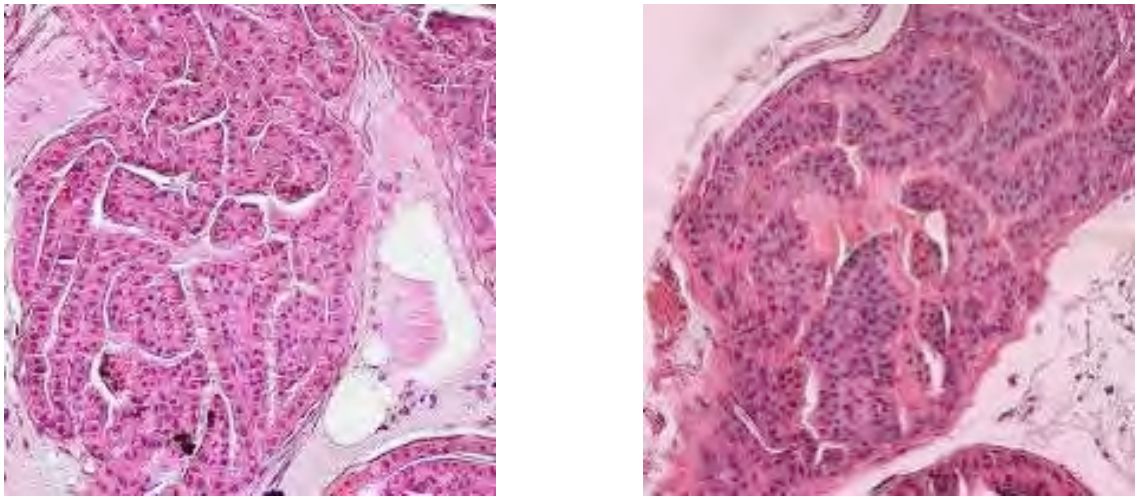

FIGURE 39: *Hematoxylin and eosin stained sections of anterior prostates from 6 months old WT FVB* **(a)** *and ARR2Pb- ETV4 FVB* **(b)** *mice. There are no evidence of lesions in both mice. The magnification is 4x.*

At variance, in some of older ARR2Pb-ETV4 mice we observed that in the context of prostate with normal architecture were present focal lesions characterized by nuclear atypia and altered cellular stratification, consistent with the definition of

mPIN (Figure 40b). Specifically we found that by 10 months of age, mPIN were present in about 55% of 18 ARR2Pb-ETV4 mice (10 out 18 mice).

These results show that over-expression of ETV4 is sufficient to induce pre-neoplastic lesions in a fraction of our ARR2Pb-ETV4 mice.

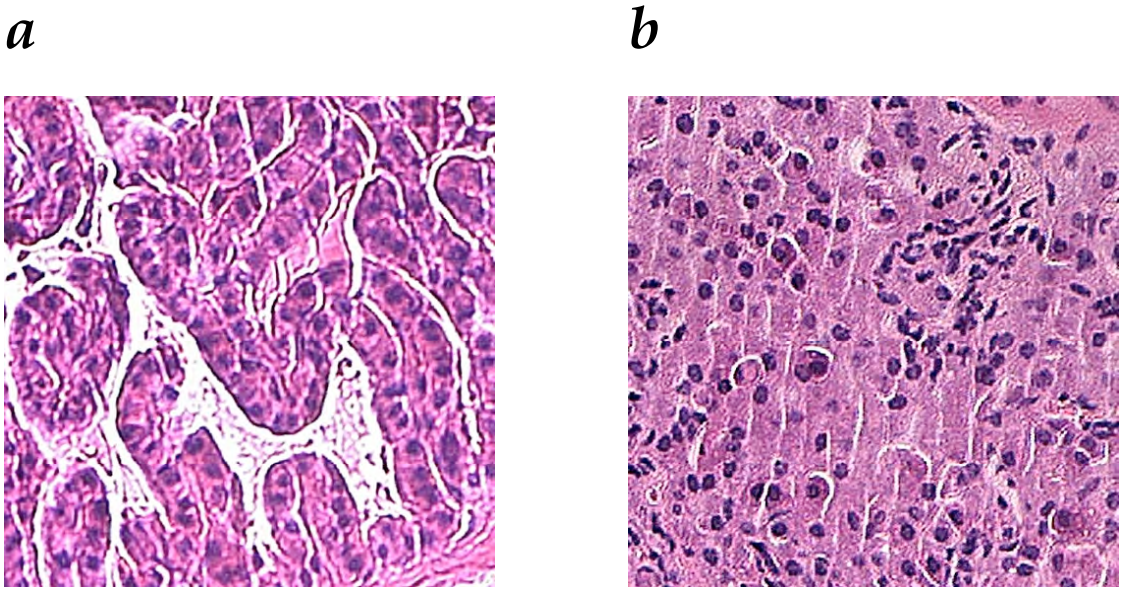

FIGURE 40: *Hematoxylin and eosin stained sections of anterior prostates from 10 months old* **(a)** *WT FVB and* **(b)** *ARR2Pb-ETV4 FVB mice. Histological characterization of prostate in ARR2Pb-ETV4 FVB mouse shows a morphological alterations similar to PIN lesion. The magnification is 10X.*

Currently, we are analyzing the specimens from these mice by immune-staining. One of the critical hallmarks distinguishing the normal human prostate gland from prostate cancer is the loss of basal cells and we will test the loss of basal cells by using the anti–p63 antibody a specific marker of those type of cells. The results of the immune-staining with anti–p63 and with anti–smooth muscle actin (marker for the stromal cell compartment) antibodies will show the presence of the displacement of the basal cells. In addition we will measure the proliferation rate in the wild type and ETV4 mice by the immune-staining with anti Ki67 and BrdU, both markers of proliferation.

4.9 Interaction of ETV4 prostate expression with Pten deficiency

Next, we decided to test whether the interaction of the aberrant prostatic expression of ETV4 with another gene would cooperate and promote carcinoma and eventually, metastasize. One of the most common genetic alterations found in prostate carcinoma is the loss of the tumor suppressor Pten. Pten heterozygous (Pten+/–) mice develop HGPIN with an increased proliferative rate after a long latency with incomplete penetrance (Di Cristofano et al. 2001). For our study we have obtained this mouse model of heterozygous Pten loss (Pten+/– mice) from Pier Paolo Pandolfi (Boston). We crossed our ARR2Pb-ETV4 mice with Pten+/– mice: this cross generates offspring with a mixed genetic background because the strain of ARR2Pb-ETV4 mice is FVB and that of Pten+/– mice is C57/Bl6. We have systematically analyzed only the first generation (F1) of mouse derived from this crossing.

The F1 mice deriving from this ARR2Pb-ETV4;Pten+/- cross were screened by PCR on mouse tail DNA for the presence of the ETV4 transgene (see above) and for the inactivated Pten allele by a specific PCR amplification of the neomycin gene, present only in the inactivated Pten allele (see "Material and Methods"). This crossing may result in four different genotypes in the respect of the ARR2Pb-ETV4 and the inactivated Pten: (i) wild type; (ii) ARR2Pb-ETV4; (iii) Pten +/-; (iv) ARR2Pb-ETV4;Pten+/- (Figure 41). All these F1 male mice derived from this cross were followed up, and sacrificed at 7 (n=18) and at 10 (n=11) months of age.

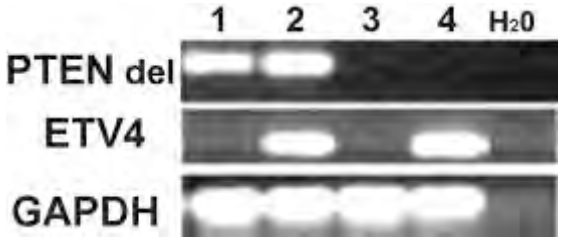

FIGURE 41*: PCR genotyping of Pten deletion and of ETV4 transgene in the mice. In lane 1 there is a mouse positive for pten deletion (PTen+/-), in lane 2 a mouse positive for both pten deletion and etv4 transgene, in lane 3 a wild type mouse, and in lane 4 a mouse positive for etv4 transgene, in the last lane there is H20 as negative control of PCR.*

The analysis of the hematoxylin-eosin stained specimens showed neither prostate hyperplasia nor PIN in both 7 months wild type (n=3) and ARR2Pb-ETV4 (n=3) mice, and we observed, as expected, PIN only in one third of the age-matched Pten+/– mice (n=3) (Figure 42, 43 and 44).

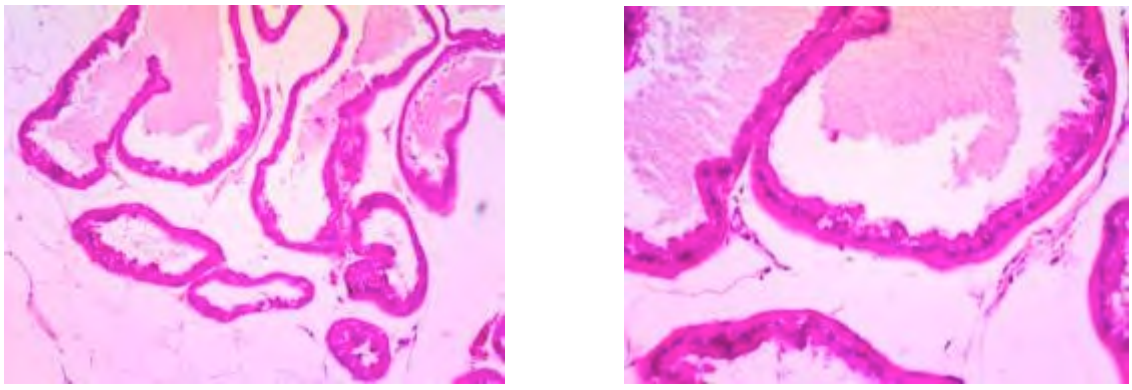

FIGURE 42: : *Hematoxylin and eosin stained sections of ventral prostates from 7 months old WT mouse (4x on the left, 10 x on the right). Histological characterization of prostate shows a normal tissue.*

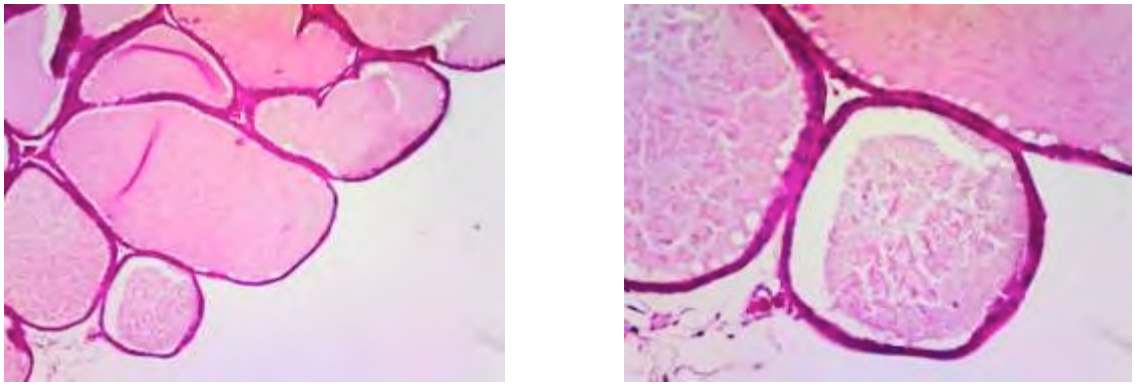

FIGURE 43: *Hematoxylin and eosin stained sections of ventral prostates from 7 months old ARR2Pb-ETV4 mouse (4x on the left, 10 x on the right). Histological characterization of prostate shows normal tissue.*

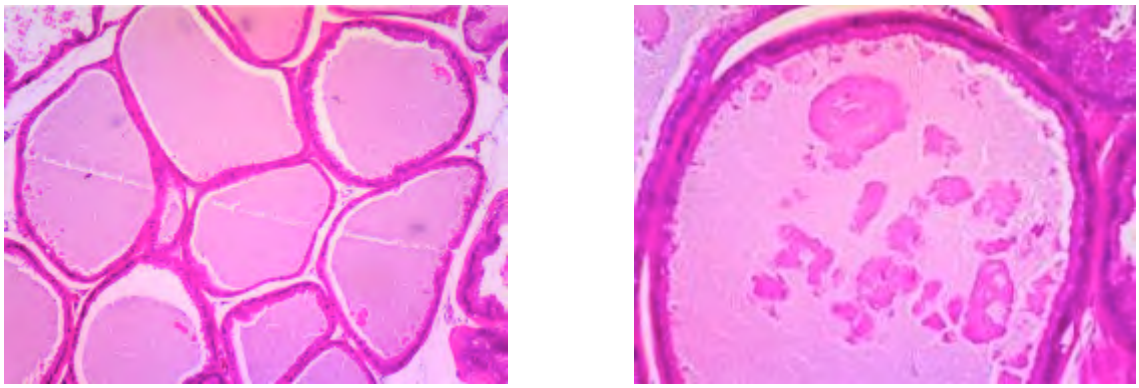

FIGURE 44: *Hematoxylin and eosin stained sections of ventral prostates from 7 months old Pten+/– mouse (4x on the left, 10 x on the right). Histological characterization of prostate shows normal tissue*

The number of control mice and of those with a single genetic alteration we have so far analyzed is still small, nevertheless the phenotype we have observed in ARR2Pb-ETV4; Pten+/- mice is strikingly different. In fact, we have found that all our ARR2Pb-ETV4; Pten+/- mice(n=9) mice showed PIN lesions and that PIN were multifocal in most of the case. These findings indicate that ETV4 expression combined with Pten loss reduces cancer latency, increases cancer penetrance and produce a more severe phenotype in the respect of parental mice (Figure45).

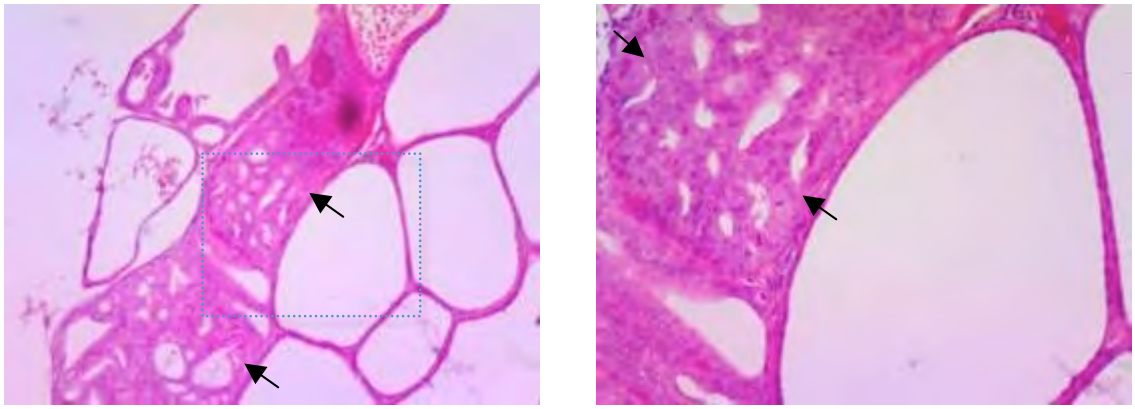

FIGURE 45: *Hematoxylin and eosin stained sections of ventral prostates from 7 months old ARR2Pb-ETV4; Pten+/- mouse (4x on the left, 10x on the right). Histological characterization of prostate shows multifocal PIN lesions. The arrows indicate the PIN.*

Furthermore, we have analyzed few ARR2Pb-ETV4;Pten+/- mice at 10 months of age: in all ARR2Pb-ETV4;Pten+/- mice (n=3) we found the presence of multifocal PIN with architectural and cytological modifications more florid than those observed in the same mice at younger age (7 months) (Figure 46).

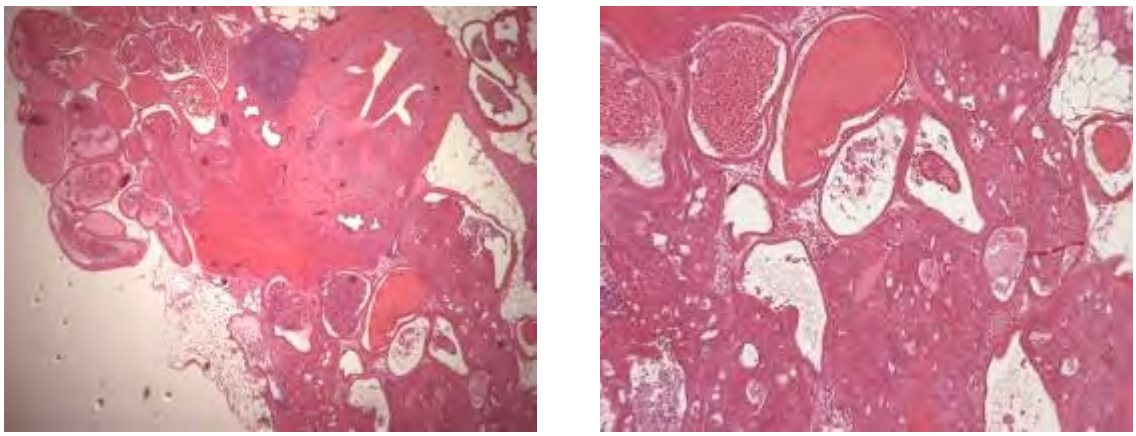

FIGURE 46: *Hematoxylin and eosin stained sections of ventral prostates from 10 months old ARR2Pb-ETV4; Pten+/- mouse (4x on the left, 10x on the right). Histological characterization of prostate shows multifocal PIN lesions more atypical evident than in 7 months old mice.*

We are currently analyzing the specimens from these mice by immune-staining using the anti–p63 antibody, anti–smooth muscle actin and anti Ki67 and BrdU.

These transgenic mice models suggest that ETV4 over-expression is sufficient to initiate neoplastic transformation and cooperates with Pten loss in promoting prostate cancer progression.

5. DISCUSSION

The discovery of translocations involving ETS proteins is a major advance in understanding the molecular basis of Prostate cancer. Among the ETS proteins, ERG, ETV5 and ETV1 have emerged as major factors in invasiveness and cell migration, but not in fostering cell proliferation; (Tomlins et al., 2008; Klezovitch et al., 2008; Tomlins et al., 2007). Indeed, in a mouse model the prostate-specific overexpression of ERG has proven important in cancer progression, but its ability to initiate cancer is still controversial (Tomlins et al., 2008; Klezovitch et al., 2008; Carver et al., 2009; King et al., 2009). In contrast, we know that ETV4, an ETS protein over-expressed in several types of carcinomas as breast, lung and colon (Hiroumi et al., 2001; Horiuchi et al., 2003; Bieche et al., 2004), and a partner of TMPRSS2 in chromosomal translocations found in Prostate cancer, exerts a powerful function not only in invasiveness and in migration, but also in cell proliferation. During my PhD I have investigated the effect of ETV4 expression by using 2 complementary experimental approaches: (i) Inducible shRNA-mediated silencing of ETV4 in human prostate cell line PC3 over-expressing ETV4; (ii) ETV4 over-expression in non-transformed RWPE human prostate cell line. In addition, we are currently investigating a mouse model of prostate-specific ETV4 we have generated during my PhD.

5.1 ETV4 confers migration and invading ability to prostate cancer cells

The PC3 cells are able to efficiently migrate in the wound-healing assay and to invade in the matrigel assay. Our experiments confirmed that the ETV4 silencing reduces the migration and invasion of PC3 cells (Hollenhorst et al., 2011). In a variety of tissues ETV4 is a transcriptional activator of several MMPs (Higashino et al., 1995; Habelhah et al., 1999; Crawford et al., 2001), endopeptidases capable of degrading the extracellular matrix and thus promoting tumor invasion (Kessenbrock et al., 2010). Indeed, when we silenced ETV4 in PC3 cells the

expression of MMP1, MMP3 and MMP9 decreased. In complementary experiments, ETV4 overexpression confers migration and invading ability to the nonmalignant RWPE cells, and this is probably mediated by increased expression of MMP2 and MMP3.

5.2 ETV4 is involved in cell proliferation and in cell cycle regulation

We have confirmed previous evidence that ETV4 silencing reduces the anchorage-independent growth of PC3 cells (Hollenhorst et al., 2011). In addition, we have shown that ETV4 expression increases anchorage-independent growth of RWPE cells. Whereas these data already suggest that ETV4 over-expression is oncogene-like, we have also uncovered an effect of ETV4 directed onto cell proliferation. Indeed, the progression of PC3 cells through the cell cycle is slowed down by ETV4 silencing; whereas the progression of RWPE cells through the cell cycle is accelerated by ETV4 expression. This effect on the rate of cycling appears to be mediated by or associated with the modulation of the cell cycle genes P21 (WAF1/CIP1), MYC, MCM7 and CHECK1; in RWPE cells also P27 (KIP1) is down regulated.

Thus, ETV4 over-expression promotes cell cycle progression of the nonmalignant RWPE prostate cells probably by causing a reduction in P21 expression: accordingly, ETV4 silencing increases the levels of P21 in PC3 cells. These data are strengthened by the fact that for most genes the down regulation in PC3 is the mirror image of the upregulation in RWPE cells. The mechanism by which ETV4 hinders P21 transcription remains to be investigated. As P53 is a major activator of P21 transcription, one might have presumed that the ETV4 effect is mediated by P53 (el-Deiry et al., 1993; Abbas et al., 2009). However, this effect is seen even in PC3 that do not have P53 cells (Isaacs WB et al., 1991). It is possible that ETV4 down regulates directly P21 gene, resembling the down regulation of the ErbB2 (HER-2/neu) gene reported in breast and ovarian cancer cells as a result of ETV4 binding to the ErbB2 promoter (Xing et al., 2000; Yu et al., 2006). However, it is also possible that part of the effect of ETV4 on P21 is mediated by a slightly

increase of the expression of MYC and of its targets (Herold et al., 2009; Coller et al., 2000), which we have observed in prostate cell lines over-expressing ETV4.

5.3 ETV4 overexpression activates EMT

The acquisition of invasiveness and metastatic capabilities by epithelial tumor cells is associated with a set of morphological and functional changes that are similar to EMT (Thiery et al., 2009; Klymkowsky et al., 2009). Various studies have reported about EMT-like process in prostate cancer (reviewed in Nauseef and Henry, 2001) and in prostate cell lines after different stimuli (transforming growth factor-b),(Ao et al., 2006; Slabakova et al., 2011) DAB2IP(Min et al., 2010; Xie et al., 2010). Recently, it has been reported that the TMPRSS2-ERG translocation is associated with EMT (Leshem et al., 2011; Gupta et al., 2011). Here, we have shown that overexpression of ETV4 in RWPE cells induces EMT changes: in fact, not only the cells did acquire a fibroblast-like shape, but this was also associated with an increased expression of mesenchymal markers (vimentin, N-cadherin and cadherin-11) and a decreased expression of epithelial markers (E-cadherin, zonula occludens 1). Specifically, the reduction of the epithelial marker protein E-cadherin paralleled quantitatively an increase in the mesenchymal marker protein N-cadherin on the cell membrane. Thus, just as in lung cancer cells (Upadhyay et al., 2009) and in ovarian cancer cells (Cowden et al., 2009) ETV4 expression is able to induce EMT transition also in prostate cancer cells.

As for the mechanism whereby ETV4 can induce EMT, this is probably mediated by several transcription factors, including members of the Snail family (SNAIL1, SNAIL2/SLUG), of the zinc finger E-box-binding homeobox family (ZEB1 and ZEB2), that directly repress E-cadherin transcription, and others that are indirect E-cadherin repressors (such as the basic helix loophelix factor TWIST1). (Yang et al., 2004; Thiery et al., 2009) Recently, it has been found that ETV4 upregulates TWIST1 in a murine breast cancer cell line (Qin et al., 2009) and SNAIL1 in MCF10a (an immortalized non tumorigenic human breast cell line) (Yuen et al., 2011); in addition, a direct correlation between the mRNA levels of ETV4 and SNAIL1 has been observed in breast cancer patients (Yuen et al., 2011). We show here that ETV4 expression in the nonmalignant RWPE prostate cells results in a

strong increase of TWIST1, ZEB1 and ZEB2 expression; but, unlike in breast cell lines, not that of SNAIL1: this suggests, that the mechanism whereby ETV4 induces EMT is at least in part tissue specific. ETV4 resembles ERG in some of the mechanisms leading to EMT, such as activation of the WNT pathway (Gupta et al., 2011) and induction of ZEB1 and ZEB2 (Leshem et al., 2011). However, ETV4 differs from ERG because it does not affect the expression of ZEB2 regulators. At any rate, although the mechanism through which ETV4 and ERG induce EMT may be somewhat different, it appears that EMT is a final common pathway through which these ETS proteins exert their oncogenic potential in prostate cells.

5.4 ETV4 induces early neoplastic lesions in transgenic mice

Despite the high frequency of cases of human prostate cancer that are associated with the presence of ETS gene fusions, the role of these genes in prostate oncogenesis is not yet completely defined. The expression of ETS genes, specifically ERG and ETV1, confer various neoplastic characteristics to prostate cells *in vitro*; however, the investigations of their role by using transgenic mouse model has produced conflicting results. For this reason, although the suggestive results of our *in vitro* experiments, we have decided to generate a transgenic model with prostate-specific expression of ETV4 protein under the control of the modified *probasin* (ARR2PB) promoter.

We have succeeded in generating a transgenic line that effectively express ETV4 in the prostate tissue. The pathological analysis of our ARR2PB-ETV4 mice has shown that focal lesions with cytological and architectural alterations are present only in a fraction of mice with advanced age (10 months). The alterated cytological and histological organization of the epithelial cells observed in ARR2PB-ETV4 prostates resemble the early modifications observed in human Pin; however, the specific nature of these lesions has to be confirmed by additional immuno-histochemical stainings that are in progress. These observations indicate that ETV4 expression is able to induce focal PIN with a long latency and a partial penetrance. It is intriguing, that only similar early lesions have been observed in most of mouse models of prostate cancer carrying ETS fusion genes (ERG, ETV1)

[REF] or other single genetic lesion (i.e. NKX3-1+/− and PTEN+/− mice): this suggest that for prostate cancer progression and invasion are required multiple genetics events. Having this in mind, we decided to investigate the outcome of the combined expression of ETV4 with a genetic lesion frequent in human prostate cancer, namely the heterozygote loss of PTEN. Thus, we have crossed our mice with mice carrying the deletion of one Pten allele. The preliminary pathological analysis of few mice deriving from this cross has shown that the combination of ETV4 expression with the heterozygote PTEN loss results in a full penetrance and a shorter latency in the development of focal PIN. In addition, these lesions appear to be more aggressive of those observed in ARR2PB-ETV4 mice because are multifocal and show more severe morphological abnormalities.

Overall, this very preliminary analysis of the transgenic ARR2PB-ETV4 mice and of their cross with mice carrying the deletion of the tumor suppressor PTEN indicate that ETV4 expression contribute to the neoplastic transformation of prostate cells *in vivo*. The ongoing analysis of these mouse models we have generated will clarify the role of ETV4. In addition, by using these mouse models we will have the possibility to verify whether the pathways we have identified *in vitro* are also responsible of the ETV4 oncogenic activity *in vivo*.

5.5 Perspectives

The studies performed during my thesis have shown by using two complementary models (silencing and over-expression) that the over-expression of ETV4 is likely to be responsible of several characteristic in the transformation of prostate epithelial cells: down regulation of ETV4 inhibits proliferation, anchorage-independent growth and migration in prostate cells. Furthermore ETV4 can induce EMT regulating the expression of several transcription factors that are direct or indirect repressors of E-cadherin. These findings strongly support the notion that a certain level of expression of ETV4, by affecting positively both proliferation and invasiveness, is capable of inducing most and possibly all neoplastic features of prostate cancer. Most important, the oncongenic ability of ETV4 has been confirmed *in vivo* by the results of the xenograft experiments and by the preliminary observations in the transgenic mice we have generated. Thus, it is

possible that ETV4 over-expression, whether driven by the TMPRSS2 promoter or otherwise, may turn out to be a powerful oncogene. This is of considerable importance, since the correlation between translocations involving ETS genes and the clinical course of prostate cancer is still controversial. From this point of view, it is tempting to regard ETV4 and its down-stream pathways as a potential target for new therapeutic approaches. It is also possible, that some of the ETV4 down-stream genes are deregulated in the same way, but by different mechanisms, also in prostate cancers that do not express ETV4. This will extend the relevance of our results to a larger number of patients. The identification of these genes directly or indirectly regulated by ETV4 in prostate cancer will take advantage from the genetic tools, both cell lines and transgenic mouse, that has been generated during the work of my thesis. Finally, the transgenic mice will be also useful models for the *in vivo* preclinical testing of drugs and of other therapeutic approaches.

REFERENCES

Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer, 2009; 9: 400–414.

Abdulkadir SA, Magee JA, Peters TJ, Kaleem Z, Naughton CK, Humphrey PA, Milbrandt J. Conditional loss of Nkx3.1 in adult mice induces prostatic intraepithelial neoplasia. Mol Cell Biol, 2002; 22:1495–1503.

Acloque H, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA. Epithelial mesenchymal transitions: the importance of changing cell state in development and disease. J Clin Invest , 2009;119:1438-1449.

Ao M, Williams K, Bhowmick NA, Hayward SW. Transforming growth factor-beta promotes invasion in tumorigenic but not in nontumorigenic human prostatic epithelial cells. Cancer Res 2006; 66: 8007–8016.

Angst B, Marcozzi C and Magee A. The cadherin superfamily: diversity in form and function. J. Cell. Sci., 2001; 114(4): 629-41.

Armstrong AJ, Marengo MS, Oltean S, Kemeny G, Bitting RL, Turnbull JD et al.. Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. Mol. Cancer Res., 2011 ; 9:997–1007.

Attard G, Rizzo S, Ledaki I, Clark J, Reid AH, Thompson A, Khoo V, de Bono JS, Cooper CS, Hudson DL. A novel, spontaneously immortalized, human prostate cancer cell line, Bob, offers a unique model for pre-clinical prostate cancer studies. Prostate. 2009 Oct 1;69(14):1507-20.

Baert JL, Beaudoin C, Coutte L and de Launoit Y. ERM transactivation is upregulated by the repression of DNA binding after the PKA phosphorylation of a consensus site at the edge of the ETS domain, J. Biol. Chem., 2002; 277(2):1002-12.

Baker DA, Mille-Baker, Wainwright SM, Ish-Horowicz D and Dibb NJ. Mae mediates MAPK phosphorylation of ETS transription factor in Drosophila . Nature, 2001; 411, 330–334.

Barrios R, Lebovitz RM, Wiseman AL, Weisoly DL, Matusik RJ, DeMayo F, Lieberman MW. RasT24 driven by a probasin promoter induces prostatic hyperplasia in transgenic mice. Transgenics 1996; 2:23–28.

Bello D, Webber MM, Kleinman HK, Wartinger DD and Rhim JS. Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis, 1997; 18(6):1215-23.

Benz CC, O'Hagan RC, Richter B, Scott GK, Chang CH, Xiong X et al. HER2/Neu and the Ets transcription activator PEA3 are coordinately upregulated in human breast cancer. Oncogene 1997; 15: 1513–1525.

Bieche I, Tozlu S, Girault I, Onody P, Driouch K, Vidaud M and Liderau R. Expression of PEA3/E1AF/ETV4, an Ets-related transcription factor, in breast tumors: positive links to MMP2, NRG1 and CGB expression. Carcinogenesis, 2004; 25(3) :405-411.

Blasi F and Sidenius N. The urokinase receptor: focused cell surface proteolysis, cell adhesion and signaling. FEBS Lett., 2010; 584(9):1923-1930.

Bojovic BB and Hassell JA. The PEA3 Ets transcription factor comprises multiple domains that regulate transactivation and DNA binding. J. Biol. Chem., 2001; 276(6):4509-21.

Bonneau D and Longy M. Mutations of the human PTEN gene. Hum Mutat, 2000;16:109–22.

Bourboulia D and Stetler-Stevenson WG. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion. Semin. Cancer Biol., 2010; 20:161–168.

Brothman AR, Maxwell TM, Cui J, Deubler DA, Zhu XL. Chromosomal clues to the development of prostate tumors. Prostate 1999; 38: 303–12.

Brown LA, Amores A, Schilling TF, Jowett T, Baert JL, de Launoit Y and Sharrocks AD. Molecular characterization of the zebrafish PEA3 ETS-domain transcription factor. Oncogene, 1998; 17(1):93-104.

Cairns P, Okami K, Halachmi S, et al. Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. Cancer Res,1997;57:4997–5000.

Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Bianco MJ, del Barrio MG, Portillo F and Nieto MA. The transcription factor Snail controls epithelial-mesenchymal transition by repressing E-cadherin expression. Nat. Cell. Biol; 2000. 2(2):76-83.

Carver BS, Tran J, Gopalan A, Chen Z, Shaikh S, Carracedo A, Alimonti A, Nardella C, Varmeh S, Scardino PT, Cordon-Cardo C, Gerald W and Pandolfi PP. Aberrant ERG expression cooperates with loss of PTEN to promote cancer progression in the prostate. Nat. Genet., 2009; 41:619–624.

Casey M, L Fang, Hynes P G, Wassim G. Abou-Kheir, Philip L. Martin, Heather S. Tillman, Gyorgy Petrovics, Hibah O. Awwad, Yvona Ward, Ross Lake, Luhua Zhang, Kathleen Kelly. TMPRSS2- Driven ERG Expression *In vivo* Increases Self- Renewal and Maintains Expression in a Castration Resistant Subpopulation. Plos one, 2012 ; 7: e41668.

Cerveira N, Ribeiro FR, Peixoto A, et al. TMPRSS2-ERG gene fusion causing ERG overexpression precedes chromosome copy number changes in prostate carcinomas and paired HGPIN lesions. Neoplasia, 2006, 8:826-832.

Choi, S. S, and Diehl, A.M. Epithelial-to-mesenchymal transitions in the liver. Hepatology 50, 2007-2013, 2009.

Chotteau-Lelievre A., R. Montesano, J. Soriano, P. Soulie, X. Desbiens, Y. De Launoit. PEA3 transcription factors are expressed in tissues undergoing branching morphogenesis and promote formation of duct-like structures by mammary epithelial cells *in vitro*. Dev. Biol, 2003; 259: 241–257.

Clark J.P. , Cooper C.S. ETS gene fusions in prostate cancer, Nat. Rev. Urol. 2009; 6:429–439.

Coller HA, Grandori C, Tamayo P, Colbert T, Lander ES, Eisenman RN et al. Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. Proc Natl Acad Sci USA, 2000; 97:3260–3265.

Colombel M, Blanc E, Lerolland B, Gasnen D, Chopin D, Abbon C. Comparative study of treatment of prostatic adenoma: laser photocoagulation versus endoscopic resection. Prog Urol., 1995 Dec; 5(6):974-9.

Cowden Dahl KD, Dahl R, Kruichak JN and Hudson LG. The epidermal growth factor receptor responsive miR-125a represses mesenchymal morphology in ovarian cancer cells. Neoplasia, 2009; 11:1208–1215.

Crawford HC, Fingleton B, Gustavson MD, Kurpios N, Wagenaar RA, Hassell JA et al. The PEA3 subfamily of Ets transcription factors synergizes with beta-catenin-LEF-1 to activate matrilysin transcription in intestinal tumors. Mol Cell Biol, 2001; 21:1370–1383,.

Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, Bartsch G, Klocker H. Androgen receptor activation in prostatic tumor cell lines by insulinlike growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res, 1994; 54:5474–5478.

Cunha G R, Donjacour A A , Cooke P S et al. "The endocrinology and developmental biology of the prostate," Endocrine Reviews, 1987; 8:338–362.

De Jong M and Maina T. "Of mice and humans: are they the same?—implications in cancer translational research," Journal of Nuclear Medicine, 2010; 51 (4): 501–504.

de Launoit Y, Chotteau-Lelievre A, Beaudoin C, Coutte L, Netzer S, Brenner C et al. The PEA3 group of ETS-related transcription factors. Role in breast cancer metastasis. Adv Exp Med Biol, 2000; 480: 107–116.

De Marzo AM, Platz EA, Epstein JI, et al: A working group classification of focal prostate atrophy lesions. Am J Surg Pathol, 2006; 30:1281-1291.

Demichelis F, Setlur SR, Beroukhim R, Perner S, Korbel JO, Lafargue CJ, Pflueger D, Pina C, Hofer MD, Sboner A, Svensson MA, Rickman DS, Urban A, Snyder M, Meyerson M, Lee C, Gerstein MB, Kuefer R, Rubin MA. Distinct genomic aberrations associated with ERG rearranged prostate cancer. Genes Chromosomes Cancer, 2009 Apr; 48(4):366-80.

Dehm SM, Schmidt LJ, Heemers HV, Vessella RL, Tindall DJ. Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. Cancer Res, 2008 Jul 1;68(13):5469-77.

Di Cristofano A, De Acetis M, Koff A, Cordon-Cardo C, Pandolfi PP. Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse. Nat Genet, 2001; 27:222–224.

Discenza MT, Vaz D, Hassell JA, Pelletier J. Activation of the WT1 tumor suppressor gene promoter by Pea3. FEBS Lett, 2004; 560: 183–191.

Dong JT. Prevalent Mutations in Prostate Cancer. J. Cell. Biochem, 2006; 97:433- 447.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K and Tuschi T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature, 2001; 411: 494-498.

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM et al. WAF1, a potential mediator of p53 tumor suppression. Cell, 1993; 75: 817–825.

Elgavish A, Wood PA, Pinkert CA, Eltoum IE, Cartee T, Wilbanks J, Mentor-Marcel R, Tian L, Scroggins SE. Transgenic mouse with human mutant p53 expression in the prostate epithelium. Prostate, 2004; 61:26–34.

Ellwood-Yen K, Graeber TG, Wongvipat J, Iruela-Arispe ML, Zhang J-F, Matusik RJ, Thomas GV, Sawyers CL. 2003. Myc-driven murine prostate cancer shares molecular features with human prostate tumors. Cancer Cell 4:223–238.

Epstein JI, Allsbrook WC Jr, Amin MB, Egevad LL. Update on the Gleason grading system for prostate cancer: results of an international consensus conference of urologic pathologists. Adv Anat Pathol., 2006; 13(1):57-9.

Gleason DF. Classification of prostatic carcinomas. Cancer Chemother Rep., 1966; 50(3):125-8.

Gopalan A, Leversha MA, Satagopan JM, et al. TMPRSS2-ERG gene fusion is not associated with outcome in patients treated by prostatectomy. Cancer Res, 2009; 69:1400–6.

Gotoh A, Kao C, Ko SC, Hamada K, Liu TJ, Chung LW. Cytotoxic effects of recombinant adenovirus p53 and cell cycle regulator genes (p21waf1/cip1 and p16 cdkn4) in human prostate cancers. J Urol, 1997; 158:636-641.

Greenall A, Willingham N, Cheung E, Boam DS and Sharrocks AD. DNA binding by the ETS-domain transcription factor PEA3 is regulated by intramolecular and intermolecular protein-protein interactions. J. Biol. Chem., 2001; 276(19):16207-15.

Greenberg NM, DeMayo FJ, Finegold MJ, Medina D, Tilley WD, Aspinall JO, Cunha GR, Donjacour AA, Matusik RJ, Rosen JM. Prostate cancer in a transgenic mouse. Proc Natl Acad Sci, 1995; 92:3439–3443.

Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G et al. The miR-200 ZEB1 and SIP1. Nat Cell Biol, 2008; 10:593–601.

Gronberg H, Isaacs SD, Smith JR et al. Characteristics of prostate cancer in families potentially linked to the hereditary prostate cancer 1 (HPC1) locus. JAMA 1997; 278: 1251–55.

Gupta S, Iljin K, Sara H, Mpindi JP, Mirtti T, Vainio P, Rantala J, Alanen K, Nees M and Kallioniemi O. FDZ4 as a mediator of ERG Oncogene-Induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. Cancer reserch, 2011; 70(17): 6735-45.

Habelhah H, Okada F, Kobayashi M, Nakai K, Choi S, Hamada J et al. Increased E1AF expression in mouse fibrosarcoma promotes metastasis through induction of MT1-MMP expression. Oncogene 1999; 18:1771–1776.

Han B, Mehra R, Dhanasekaran SM, Yu J, Menon A, Lonigro, Wang X, Gong,1,3 Lei Wang Y, Shankar S, Laxman B, Shah RB, Varambally S, Palanisamy N, Tomlins SA, Kumar-Sinha C and Chinnaiyan AM. A fluorescence in situ hybridization screen for E26 transformation-specific aberrations: identification of DDX5-ETV4 fusion protein in prostate cancer. Cancer Res., 2008 Sep 15; 68(18):7629-37.

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell., 2000; 100(1):57-70.

Hazan R, Qiao R, Keren R, et al. Cadherin Switch in Tumor Progression. Annals of the New York Academy of Science, 2004; 1014:155-163.

Helfand BT, Mendez MG, Murthy SN, Shumaker DK, Grin B, Mahammad S, Aebi U, Wedig T, Wu YI, Hahn KM, Inagaki M, Herrmann H, Goldman RD. Vimentin organization modulates the formation of lamellipodia. Mol. Biol. Cell., 2011; 22(8):1274-89.

Helgeson BE, Tomlins SA, Shah N, Laxman B, Cao Q, Prensner JR, Cao X, Singla N, Montie JE, Varambally S, Mehra R and Chinnaiyan AM. Characterization of TMPRSS2:ETV5 and SLC45A3:ETV5 gene fusions in prostate cancer. Cancer Res., 2008 Jan 1; 68(1):73-80.

Herold S, Herkert B, Eilers M. Facilitating replication under stress: an oncogenic function of MYC? Nat Rev Cancer, 2009; 9:441–444.

Hermans KG, Bressers AA, van der Korput HA, Dits NF, Jenster G and Trapman J. Two unique novel prostate-specific and androgen-regulated fusion partners of ETV4 in prostate cancer. Cancer Res., 2008; 68: 3094–3098.

Hida K, Shindoh M, Yoshida K, Kudoh A, Furaoka K, Kohgo T et al. Expression of E1AF, an ets-family transcription factor, is correlated with the invasive phenotype of oral squamous cell carcinoma. Oral Oncol 1997; 33: 426–430.

Higashino F, Yoshida K, Noumi T, Seiki M, Fujinaga K. Ets-related protein E1A-F can activate three different matrix metalloproteinase gene promoters. Oncogene, 1995; 10:1461–1463.

Hiroumi H, Dosaka-Akita H, Yoshida K, Shindoh M, Ohbuchi T, Fujinaga K and Nishimura M. Expression of E1AF/PEA3, an Ets-related transcription factor in human non-small-cell lung cancers: its relevance in cell motility and invasion. Int J Cancer, 2001; 93:786–791.

Hollenhorst PC, Shah AA, Hopkins C and Graves BJ. Genome-wide analyses reveal properties of redundant and specific promoter occupancy within the ETS gene family. Genes Dev 2007 Aug; 21(15):1882-94.

Hollenhorst PC, Chandler KJ, Poulsen RL, Johnson WE, Speck NA, Graves BJ. DNA specifity determinants associate with distinct transcription factor functions. PLoS Genet. 2009 Dec; 5(12): e1000778.

Hollenhorst PC, Paul L, Ferris MW and Graves B. The ETS gene is required for anchorage-independent growth and a cell proliferation gene expression program in PC3 prostate cells. Genes and Cancer, 2010; 1(10):1044–1052.

Hollenhorst PC, McIntosh LP and Graves BJ. Genomic and biochemical insights into the specificity of ETS transcription factors. Annu. Rev. Biochem., 2011; 1:1044–1052.

Horiuchi S, Yamamoto H, Min Y, Adachi Y, Itoh F and Imai K. Association of ets-related transcriptional factor E1AF expression with tumour progression and overexpression of MMP-1 and matrilysin in human colorectal cancer. J. Pathol., 2003; 200: 568–576.

Huggins C and Hodges CV. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. J Urol., 2002 Jul; 168(1):9-12.

Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED et al. Epithelial–mesenchymal and mesenchymal–epithelial transitions in carcinoma progression. J Cell Physiol, 2007; 213:374–383.

Iljin K, Wolf M, Edgren H, Gupta S, Kilpinen S, Skotheim RI, Peltola M, Smit F, Verhaegh G, Schalken J, et al. TMPRSS2 fusions with oncogenic ETS factors in prostate cancer involve unbalanced genomic rearrangements and are associated with HDAC1 and epigenetic reprogramming. Cancer Res, 2006; 66:10242–10246.

Isaacs JT. Molecular markers for prostate cancer metastasis. Am J. Pathol, 1997; 150:1511-1521.

Isaacs JT. New strategies for the medical treatment of prostate cancer. BJU Int., 2005; 96(2): 35- 40.

Isaacs WB, Carter BS, Ewing CM. Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. Cancer Res, 1991; 51:4716–4720.

Isaacs WB, Bova GS, Morton RA, Bussemakers MJ, Brooks JD, Ewing CM. Molecular biology of prostate cancer. Semin Oncol Review, 1994; 21(5):514-21.

Janknecht R. Analysis of the ERK-stimulated ETS transcription factor ER81, Mol. Cell. Biol., 1996; 16(4): 1550–1556.

Jemal A, Siegel R, Ward E, Murray T, Xu J; Smigal C, Thun MJ. Cancer statistics. CA Cancer J Clin., 2006; 56:106–130.

Jiang J, Wei Y, Liu D, Zhou J, Shen J, Chen X et al. E1AF promotes breast cancer cell cycle rogression via upregulation of Cyclin D3 transcription. Biochem Biophys Res Commun, 2007; 358:53–58.

Johnson D. G. and Walker C. L.. Cyclins and cell cycle checkpoints. Annu. Rev. Pharmacol. Toxicol, 1999; 39:295–312.

Joshua AM, Evans A, Van der Kwast T, Zielenska M, Meeker AK, Chinnaiyan A, Squire JA. Prostatic preneoplasia and beyond. Biochim. Biophys. Acta, 2008; 1785:156- 181.

Kalluri R and Weinberg RA. The basics of epithelial-mesenchymal transition. J. Clin. Invest., 2009; 119(6):1420–1428.

Kawasaki T, Tomita Y, Bilim V, Takeda M, Takahashi K, Kumanishi T. Abrogation of apoptosis induced by DNA-damaging agents in human bladder-cancer cell lines with p21/waf1/cip1 and/or p53 gene alterations. Int J Cancer, 1996; 68:501-505.

Kaya M, Yoshida K, Higashino F, Mitaka T, Ishii S, Fujinaga K. A single ets-related transcription factor, E1AF, confers invasive phenotype on human cancer cells. Oncogene, 1996; 12:221–227.

Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell, 2010; 141:52-67.

Kim MJ, Cardiff RD, Desai N, Banach-Petrosky WA, Parsons R, ShenMM, Abate-Shen C. Cooperativity of Nkx3.1 and Pten loss of function in a mouse model of prostate carcinogenesis. Proc Natl Acad Sci USA, 2002; 99:2884–2889.

King JC, Xu J, Wongvipat J, Hieronymus H, Carver BS, Leung DH, Taylor BS, Sander C, Cardiff RD, Couto SS, et al. Cooperativity of TMPRSS2-ERG with PI3-kinase pathway activation in prostate oncogenesis. Nat Genet, 2009; 41: 524–526.

Klezovitch O, Risk M, Coleman I, Lucas JM, Null M, True LD, Nelson PS and Vasioukhin V. A causal role for ERG in neoplastic transformation of prostate epithelium. PNAS, 2008; 105:2105–2110.

Klymkowsky MW and Savagner P. Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe. Am. J. Pathol., 2009; 174:1588–1593.

Kovar H. Dr. Jekyll and Mr. Hyde: The Two Faces of the FUS/EWS/TAF15 Protein Family. Seminars in Cancer Biology, 2005; 2011: 837474.

Kumar-Sinha C, Tomlins SA and Chinnaiyan AM. Evidence of recurrent gene fusions in common epithelial tumors. Trends Mol. Med., 2006; 12(11):529-36.

Kumar-Sinha C, Tomlins SA and Chinnaiyan AM. Recurrent gene fusions in prostate Cancer. Nat. Rev. Cancer, 2008; 8(7):497-511.

Lacronique V, Boureux A, Della Valle V, Poirel H, Tran Quang C, Mauchauffè M, Berthou C, Lessard M, Berger R, Ghysdael G and Bernard OA. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukaemia. Science, 1997; 278(5341):1309-12.

Laget MP, Defossez PA, Albagli O, Baert JL, Dewitte F, Stehelin D and de Launoit Y. Two functionally distinct domains responsible for transactivation by the Ets family member ERM. Oncogene, 1996; 12(6):1325-36.

Lapointe J, Kim YH, Miller MA, et al. A variant TMPRSS2 isoform and ERG fusion product in prostate cancer with implications for molecular diagnosis. Mod Pathol, 2007; 20:467–73.

Leshem O, Madar S, Kogan-Sakin I, Karner I, Goldstein I, Brosh R, Cohen Y, Hirsch JJ, Ehrlich M, Ben-Sasson S, Goldfinger N, Loewenthal R, Gazit E, Rotter V and Berger. TMPRSS2/ERG promotes epithelial to mesenchymal transition through the ZEB1/ZEB2 axis in a prostate cancer model. Plose One, 2011; 6: e21650.

Lin B, Ferguson C, White JT et al. "Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2". Cancer Res, 1999; 59(17):4180–4.

Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-46 Delta Delta C(T)). Methods, 2001; 25(4): 402-408.

Loric S, Paradis V, Gala JL, Berteau P, Bedossa P, Benoit G and Eschwège P. Abnormal E-cadherin expression and prostate cell blood dissemination as markers of biological recurrence in cancer. European Journal of Cancer, 2001; 74:111-122.

Maher CA, Palanisamy N, Brenner JC, Cao X, Kalyana-Sundaram S et al. Chimeric transcript discovery by paired-end transcriptome sequencing. Proc. Natl. Acad. Sci. USA, 2009.

Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer, 2009; 9:153–166.

Martínez-Piñeiro L. Personalised Patient Diagnosis and Prognosis in Prostate Cancer: What Are the Future Perspectives?. European Urology Supplements, 2010; 9(11): 794-799.

McNeal J E. "Origin and development of carcinoma in the prostate," Cancer, 1969; 23: 24–34.

Meeks JJ and Schaeffer EM. Genetic regulation of prostate development .J Androl., 2011; 32(3):210-7.

Mehra R, Tomlins SA, Yu J, et al. Characterization of TMPRSS2-ETS gene aberrations in androgen-independent metastatic prostate cancer. Cancer Res, 2008; 68:3584–90.

Mercado-Pimentel M, Runyan R. Multiple Transforming Growth Factor- ®Isoforms and Receptors Function during Epithelial-Mesenchymal Cell Transformation in the Embryonic Heart. Cells Tissues Organs, 2007; 185:146-15.

Miccalizi D, Farabaugh S, Ford H. Epithelial-Mesenchymal Transition in Cancer: Parallels Between Normal Development and Tumor Progression. Journal of Mammary Gland Biology and Neoplasia, 2010; 15(2): 117-134.

Mickey DD, Stone KR, Wunderli H, Mickey GH, Vollmer RT, Paulson DF. Heterotransplantation of a human prostatic adenocarcinoma cell line in nude mice. Cancer Res, 1977; 37:4049–4058.

Min J, Zaslavsky A, Fedele G, McLaughlin SK, Reczek EE, De Raedt T et al. An oncogene-tumor suppressor cascade drives metastatic prostate cancer by coordinately activating Ras and nuclear factor-kappaB. Nat Med, 2010; 16: 286–294.

Moody SE, Perez D, Pan TC, Sarkisian CJ, Portocarrero CP, Sterner CJ, Notorfrancesco KL, Cardiff RD and Chodosh LA. The transcriptional repressor Snail promotes mammary tumor recurrence. Cancer Cell, 2005; 8: 197–209.

Mosquera JM, Perner S, Genega EM, et al: Characterization of TMPRSS2-ERG fusion highgrade prostatic intraepithelial neoplasia and potential clinical implications. Clin Cancer Res, 2008; 14:3380-3385.

Nam RK, Sugar L, Yang W, et al. Expression of the TMPRSS2:ERG fusion gene predicts cancer recurrence after surgery for localised prostate cancer. Br J Cancer, 2007; 97:1690–5.

Nauseef JT, Henry MD. Epithelial-to-mesenchymal transition in prostate cancer: paradigm or puzzle? Nat Rev Urol, 2011; 8:428–439.

Nelson W, De Marzo A, Isaacs W. Prostate Cancer. N Engl J Med Rewiew article ; 2003; 349:366-381.

Nieman MT, Prudoff RS, Jhonson KR and Wheelock MJ. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J. Cell. Biol., 1999; 147(3):631-44.

Oh S, Shin S and Janknecht. ETV-1, 4 and 5: An oncogenic subfamily of ETS transcription factors. Biochim. Biophys. Acta., 2012; 1826(1):1-12.

Oikawa T and Yamada T. Molecular biology of the Ets family of transcription factors. Gene, 2003; 303:11-34.

Paulo P, Barros-Silva JD, Ribeiro FR, Ramalho-Carvalho J, Jeronimo C, Henrique R, et al. FLI1 is a novel ETS transcription factor involved in gene fusions in prostate cancer. Genes Chromosomes Cancer, 2011; 51:240–249.

Papavassiliou AG. Molecular medicine. Transcription factors. N. Engl. J. Med, 1995; 332:45-47.

Papsidero LD, Croghan GA, Asirwatham J, Gaeta J, Abenoza P, Englander L, Valenzuela L. Immunohistochemical demonstration of prostate-specific antigen in metastases with the use of monoclonal antibody F5. Am J Pathol., 1985; 121(3):451-4.

Patikoglou and Burley. Eukaryotic transcription factor-DNA complexes. Ann. Rev. Biophys. Biomol. Struct, 1997; 26:289- 325.

Peinado H, Marin F, Cubillo E, Stark HJ, Fusenig N, Nieto MA and Cano A. Snail and E47 repressors of E-cadherin induce distinct invasive and angiogenic properties *in vivo*. J. Cell Sci., 2004; 48: 365–375.

Peinado H, Olmeda H and Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nature Reviews Cancer, 2007; 7, 415-428.

Perner S, Demichelis F, Beroukhim R, et al. TMPRSS2:ERG fusion-associated deletions provide insight into the heterogeneity of prostate cancer. Cancer Res, 2006; 66:8337-8341.

Perner S, Mosqueta JM, Demichelis F, Hofer MD, Paris PL, Simko J, Collins C, Bismar TA, Chinnaiyan AM, De Marzo AM, et al.TMPRSS2-ERG fusion prostate cancer: an early molecular event associated with invasion. Am J Surg Pathol, 2007; 31:882-888.

Pflueger D, Rickman DS, Sboner A, Perner S, LaFargue CJ et al. N-myc downstream regulated gene 1 (NDRG1) is fused to ERG in prostate cancer. Neoplasia, 2009 Aug;11(8):804-11.

Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, Cordon-Cardo C, Catoretti G, Fisher PE, Parsons R, Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proc Natl Acad Sci USA, 1999; 96:1563–1568.

Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer, 2009; 9: 265–273.

Qin L, Liu Z, Chen H and Xu J. The steroid receptor coactivator-1 regulates twist expression and promotes breast cancer metastasis. Cancer Res., 2009; 69:3819–3827.

Rajput AB, Miller MA, De Luca A, et al. Frequency of the TMPRSS2:ERG gene fusion is increased in moderate to poorly differentiated prostate cancers. J Clin Pathol, 2007; 60:1238–43.

Rouzier C, Haudebourg J, Carpentier X, et al. Detection of the TMPRSS2-ETS fusion gene in prostate carcinomas: retrospective analysis of 55 formalin-fixed and paraffin-embedded samples with clinical data. Cancer Genet Cytogenet, 2008; 183:21–7.

Rubin M A., Maher C A., Chinnaiyan A M. Common Gene Rearrangements in Prostate Cancer. Journal of Clinical Oncology, 2011; 29:3659-3668.

Rubin M. ETS rearrangements in prostate cancer. Asian J. Androl., 2012.; 14(3):393-9.

Saramaki OR, Harjula AE, Martikainen PM, Vessella RL, Tammela TL, Visakorpi T. TMPRSS2:ERG fusion identifies a subgroup of prostate cancers with a favorable prognosis. Clin Cancer Res 2008; 14:3395– 400.

Schneider A, Brand T, Zweigerdt R, Arnold H. Targeted disruption of the nkx3.1 gene in mice results in morphogenetic defects of minor salivary glands: Parallels to glandular duct morphogenesis in prostate. Mech Dev, 2000; 95:163–174.

Seth A and Watson DK. ETS transcription factors and their emerging roles in human cancer. Eur. J. Cancer, 2005; 41(16):2462-78.

Shaikhibrahim, Z., Lindstrot, A., Langer, B., et al. Differential expression of ETS family members in prostate cancer tissues and androgen-sensitive and insensitive prostate cancer cell lines. Int. J. Mol. Med, 2011; 28:89-93.

Sharrocks AD, Brown AL, Ling Y and Yates PR. The ETS-domain transcription factor family. Int. J. Biochem. Cell. Biol., 1997; 29(12):1371-87.

Sharrocks AD. The ETS domain transcription factor family. Nat. Rev. Mol. Cell. Biol., 2001; 2(11):827-37.

Shenn M, Abate-Shen C. Pten inactivation and the emergence of androgenindependent prostate cancer. Cancer Res, 2007; 67:6535- 6538.

Shin Sook, Tae-Dong Kim, Fang Jin, Jan M. van Deursen, Scott M. Dehm, Donald J. Tindall, Joseph P. Grande, Jan-Marie Munz, George Vasmatzis, and Ralf Janknecht. Induction of Prostatic Intraepithelial Neoplasia and Modulation of Androgen Receptor by ETS Variant 1/ETS-Related Protein 81. Cancer Res, 2009; 69:8102-8110.

Shindoh M, Higashino F, Kaya M, Yasuda M, Funaoka K, Hanzawa M, Hida K, Kohgo T, Amemiya A, Yoshida K and Fujinaga K. Correlated expression of matrix metalloproteinases and ets family transcription factor E1A-F in invasive oral squamous-cell-carcinoma-derived cell lines. Am. J. Pathol., 1996; 148(3):693-700.

Slabakova E, Pernicova Z, Slavickova E, Starsichova A, Kozubik A, Soucek K. TGFbeta1-induced EMT of non-transformed prostate hyperplasia cells is characterized by early induction of SNAI2/Slug. Prostate, 2011; 71: 1332–1343.

Sommers CL, Heckford SE, Skerker JM, Worland P, Torri JA, Thompson EW, Byers SW and Gelmann EP. Loss of epithelial markers and acquisition of vimentin expression in adriamycin- and vinblastine-resistant human breast cancer cell lines. Cancer Res., 1992; 49:4258-4263.

Sreenath TL, Dobi A, Petrovicts G and Srivastava S. Oncogenic Activation of ERG: Apredominant mechanism in prostate cancer. J. Carcinog., 2011. Rewiew.

Stanbrough M, Leav I, Kwan PW, Bubley GJ, Balk SP. Prostatic intraepithelial neoplasia in mice expressing an androgen receptor transgene in prostate epithelium. Proc Natl Acad Sci USA, 2001; 98:10823–10828.

Stockinger A, Eger A, Wolf J, Beung H and Foisner R. E-cadherin regulates cell growth by modulating proliferation-dependent beta-catenin transcriptional activity. J. Cell. Biol., 2001; 153(5):1049-60.

Thiery J. Epithelial-mesenchymal transitions in tumor porgession. Nature Reviews Cancer, 2002; 11:442-454.

Thiery JP and Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol, 2006; 7:131–142.

Thiery JP, Acloque H, Ruby Y, Huang and Nieto MA. Epithelial-Mesenchymal Transitions in Development and Disease. Cell, 2009 ; 139: 871–890.

Thompson EW, Newgreen DF and Tarin D. Carcinoma invasion and metastasis: a role for epithelial-mesenchymal transition?. Cancer Res., 2005; 65: 5991–5995.

Tomita K, Van Bokhoven A, Van Leenders GJ, Ruijter ET, Jansen CF, Bussemakers MJ and Schalken JA. Cadherin switching in human prostate cancer progression. Cancer Res., 2000; 85: 446-450.

Tomlins SA, Mehra R, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW et al.. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science, 2005 Oct 28; 310(5748):644-8.

Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA and Chinnaiyan AM. TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer. Cancer Res., 2006 Apr 1; 66(7):3396-400.

Tomlins SA, Laxman B, Dhanasekaran SM, Helgeson BE, Cao X, Morris DS, Menon A, Jing X, Cao Q, Han B, Yu J, Wang L, Montie LE, Rubin MA, Pienta KJ, Roulston D, Shah RB, Varambally S, Mehra R and Chinnaiyan AM. Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. Nature, 2007 Aug 2; 448(7153):595-9.

Tomlins SA, Laxman B, Varambally S, Cao X, Yu J, Helgeson BE, Cao Q, Prensner JR, Rubin MA, Shah RB, Mehra R and Chinnaiyan AM. Role of the TMPRSS2-ERG gene fusion in prostate cancer. Neoplasia, 2008; 10: 177–188.

Tomlins SA, Bjartell A, Chinnaiyan AM, Jenster G, Nam RK, Rubin MA and Schalken JA. ETS gene fusions in prostatic cancer: from discovery to daily clinical practice. Eur Urol, 2009 Aug; 56(2):275-86.

Turner DP, Findlay VJ, Moussa O, Watson DK. Defining ETS transcription regulatory networks and their contribution to Breast cancer progression. J. Cell. Biochem, 2007; 102:549- 559.

Upadhyay S, Liu C, Chatterjee A, Hoque MO, Kim MS, Engles J, et al. LKB1/STK11 suppresses cyclooxygenase-2 induction and cellular invasion through PEA3 in lung cancer. Cancer Res., 2006; 66:7870–7879.

Vaarala MH, Porvari KS, Kellokumpu S et al. "Expression of transmembrane serine protease TMPRSS2 in mouse and human tissues". J. Pathol, 2001; 193(1):134–40.

Valkenburg KC and Williams BO. “Mouse Models of Prostate Cancer,” Prostate Cancer, 2011; doi:10.1155/2011/895238.

Van Poppel H, Joniau S, Van Cleynenbreugel B, Mottaghy FM, Oyen R. Diagnostic evaluation of PSA recurrence and review of hormonal management after radical prostatectomy. Prostate Cancer Prostatic Dis., 2009; 12(2):116-23.

Van Weerden WM and Romijn JC. Use of nude mouse xenograft models in prostate cancer research. The Prostate, 2000; 43(4):263-71.

Vesalainen SI.B, Lipponen PK, Talja MT, Alhava EM, Syrjanen KJ. Proliferating cell nuclear antigen and p53 expression as prognostic factors in T1-2M0 prostatic adenocarcinoma. Int J. Cancer, 1994; 58:303-308.

Visakorpi T, Kallioniemi OP, Heikkinen A, Koivula T, Isola J. Small subgroup of aggressive higly proliferative prostatic carcinomas defined by p53 accumulation. J. Natl Cancer Inst, 1992; 84:883-887.

Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinänen R, Palmberg C, Palotie A, Tammela T, Isola J, Kallioniemi OP. *In vivo* amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet., 1995; 9(4):401-6.

Voelkel-Johnson C, Voeks DJ, Greenberg NM, Barrios R, Maggouta F, Kurtz DT, Schwartz DA, Keller GM, Papenbrock T, Clawson GA, Norris JS. Genomic instability-based transgenic models of prostate cancer. Carcinogenesis, 2000; 21:1623–1627.

Xie D, Gore C, Liu J, Pong RC, Mason R, Hao G et al. Role of DAB2IP in modulating epithelial-to-mesenchymal transition and prostate cancer metastasis. Proc Natl Acad Sci USA 2010; 107: 2485–2490.

Xing X, Wang SC, Xia W, Zou Y, Shao R, Kwong KY et al. The ets protein PEA3 suppresses HER-2/neu overexpression and inhibits tumorigenesis. Nat Med, 2000; 6:189–195.

Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell, 2004; 117: 927–939.

Yang J and Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev. Cell., 2008; 14:818–829.

Yoshimoto M, Joshua AM, Chilton-Macneill S, Bayani J, Selvarajah S, Evans AJ, Zielenska M and Squire JA. Three-color FISH analysis of TMPRSS2/ERG fusions in prostate cancer indicates that genomic microdeletion of chromosome 21 is associated with rearrangement. Neoplasia, 2006 Jun; 8(6):465-9.

Yu Z, Xia W, Wang HY, Wang SC, Pan Y, Kwong KY et al. Antitumor activity of an Ets protein, PEA3, in breast cancer cell lines MDA-MB-361DYT2 and BT474M1. Mol Carcinog, 2006; 45: 667–675.

Yuen HF, Chan YK, Grills C, McCrudden CM, Gunasekharan V, Shi Z, et al. Polyomavirus enhancer activator 3 protein promotes breast cancer metastatic progression through Snail-induced epithelial-mesenchymal transition. J. Pathol., 2011; 224:78–89.

Wang S, Gao J, Lei Q, Rozengurt N, Pritchard C, Jiao J, Thomas GV, Li G, Roy-Burman P, Nelson PS, Liu X, Wu H. Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. Cancer Cell, 2003; 4:209–221.

Ware JL, DeLong ER. Influence of tumour size on human prostate tumour metastasis in athymic nude mice. Br. J. Cancer., 1985; 51:419–423.

Warren AJ. Eukaryotic transcription factors. Curr. Op. Struct. Biol., 2002; 12:107- 114.

Webber MM, Quader STA, Kleinman HK, Bello-DeOcampo D, Storto PD, Bice G, DeMendonca-Calaca W and Williams DE. Human Cell Lines as an *In Vitro*/*In vivo* Model for Prostate Carcinogenesis and Progression. The prostate, 2001; 47(1):1-13.

Wei GH, Badis G, Berger MF, Kivioja T, Palin K, Enge M, Bonke M, Jolma A, Varjosalo, Gehrke AR, Yan J, Talukder S, Turunen M, Taipale M, Stunnenberg HG, Ukkonen E, Hughes TR, Bulyk ML and Taipale JL. Genome-wide analysis of ETS-family DNA-binding *in vitro* and *in vivo*. EMBO J., 2010; 29(13):2147-60.

Weinberg A. The biology of CANCER. Garland Science, Taylor & Francis Group, 2007; 597-621.

Wiznerowicz M and Trono D. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. J. Virol., 2003; 77: 8957–8961.

Wolpert, L. Principles of Development, 2 edn (Oxford University Press) ; 2002.

Wu J and Janknecht R. Regulation of the ETS transcription factor ER81 by the 90-kDa ribosomal S6 kinase 1 and protein kinase A. J. Biol. Chem., 2002; 276:41856-41861.

Zhang J, Thomas TZ, Kasper S, Matusik RJ. A small composite probasin promoter confers high levels of prostate-specific gene expression through regulation by androgens and glucocorticoids *in vitro* and *in vivo*. Endocrinology, 2000 Dec; 141(12):4698-710.

Zong Y, Xin L, Goldstein AS, et al. ETS family transcription factors collaborate with alternative signaling pathways to induce carcinoma from adult murine prostate cells. Proc Natl Acad Sci U S A , 2009; 106:12465-12470.