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MicroRNAs as predictive and therapeutic tools in Prostate Cancer

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ABSTRACT

Prostate cancer (PCa) is one of the leading causes of cancer-related death in men. Despite significant advances in prostate cancer diagnosis and management, validation of new biomarkers able to distinguish among early, androgen-insensitive and metastatic tumors are still necessary to guide therapeutic decisions and to improve patient prognosis. Since c-Met is considered to have a role in PCa progression towards metastatic stage, we investigated the effect of this receptor in microRNA (miR) expression profiles of several prostate cancer cell lines and a cancer-stem cell population (PCSC-1) isolated from fresh surgery specimen. We focused on miR-130b which is considered an oncomiR able to confer tumorigenic and stemness properties to the cells. Expression of miR-130b in prostate cancer cell lines is directly correlated with c-Met and its forced expression in LnCaP cells led to a significant downregulation of AR and an increase of srivival genes, such as Bcl-2 and Mcl-1, and metastasis-associated markers such as IL-11 and CXCR-4. As a consequence, these cells were able to overcome *in vitro* inhibitory effects of Casodex and to grow orthotopically and form distant metastasis *in vivo* in immune-compromised mice, as compared with their control. Finally, we analyzed expression of miR-130b in a large dataset of patients and we found a strong correlation between miR-130b levels and tumor recurrence and metastasis. Taken together, our observations provide insights to the importance of miR-130b, that could be a novel biomarker to predict the prognosis and progression of patients with prostate cancer.

1- RATIONALE

In developed Western Countries, prostate cancer (PCa) is the most common malignant tumour in men, and is the second highest cause of cancer mortality after lung cancer (Damber JE et al, 2008). Curative options for patients with localized disease include radical prostatectomy (Bill-Axelson A et al, 2005) or radiotherapy (Morris DE et al, 2005), meanwhile for advanced stages androgen ablation and chemotherapy have significantly improved prognosis. Notwithstanding, most tumours relapse within 2 years to an incurable hormone-independent and chemotherapy-resistant state, which is ultimately responsible for prostate cancer mortality (Crawford ED et al, 2010). Bone metastasis, which occur in approximately 70% of these patients (Hall CL et al, 2005), are associated with impairment in quality of life due to the onset of skeletal-related events (SREs) such as pathologic fractures, spinal cord compression, need of surgery or radiotherapy on bone, hypercalcemia and bone pain (fig.1).

In the majority of cases, PC is diagnosed by *prostate-specific antigen* (PSA) screening and/or digital rectal examination followed by local biopsies. However, serum PSA level may be a consequence of different variable events such as benign prostatic hyperplasia or pharmacological therapy. For these reasons, despite PSA screening has led to a decrease in men diagnosed as having metastatic disease, prostate-specific antigen has been associated with no reduction in prostate cancer mortality, thus producing substantial overtreatment and treatment-related morbidity (Tosoian J et al, 2010). Therefore, the identification of predictive biomarkers able to discriminate between indolent and metastatic tumors would be helpful in guiding therapeutic decisions, by distinguishing individuals with potentially life-threatening disease for whom treatment is actually necessary. At the same time, new insight into castration-resistance and metastasis molecular mechanisms are required to develop new, more effective and more specific pharmacological tools that could improve clinical outcome.

2 – BACKGROUND

2.1 – Castration-resistant Prostate Cancer (CRPC)

The concept of androgen deprivation for the treatment of advanced prostate cancer was developed by Huggins and Hodges in 1941 (Huggins et al, 1941), and today, newer forms of androgen-deprivation therapies remain the first line treatment for this disease. The principle behind reducing the levels of circulating androgens for therapeutic purposes is based on the central role these hormones have on the development, differentiation, and maturation of male reproductive organs including the prostate (Yadav N et al, 2012). Almost all patients with advanced prostate cancer initially respond to androgen deprivation therapy, but virtually every patient will relapse due to the growth of castration-resistant cancer cells. Systemic therapies have also been an option in the CRPC management. However, chemotherapy is not well tolerated by all patients, who were often elderly men with limited bone marrow reserve and concurrent medical conditions. Treatment of patients with CRPC remains a significant clinical challenge. The key for the development of new drugs and to optimize androgenic suppression in advanced stages of CRPC is the identification and characterization of molecular targets and mechanisms that lead to tumor growth. There have been many theories proposed on the causes of prostate cancer recurrence. Postulated and documented mechanisms include androgen receptor (*AR*) gene amplification, *AR* gene mutations, bypass pathways and the involvement of tumor stem cells (Sharifi N, 2013; Attar et al, 2009; Pienta KJ et al, 2006).

2.1.1 - AR signaling in CRPC

Increasing evidences demonstrate the role of AR in the development of castration-resistant prostate cancer. One way in which prostate cancer cells circumvent the effects of androgen blockade is by developing the ability to use very low levels of androgen for growth. Increased sensitivity could result from AR gene amplification which is reported in approximately 30% of patients with CRPC, or from stabilization of the AR mRNA or protein.

Several investigators identified different mutations in the AR that confer to it the ability to be activated by weak androgen precursors (Mao HL et al, 2009), progesterone, estradiol, cortisol, or even antiandrogens. Those include point mutations in the AR ligand binding domain that can result in activation by nonandrogenic ligands, or mutations in other regions such as the amino terminus or the DNA binding domain that confer oncogenic properties to the AR. Although suspected to be low, the real frequency of AR mutations in CRPC is not known, mainly due to the difficult access to metastatic samples. However, it is well documented that some antiandrogens can lead to the selection of tumors expressing AR mutants that can be activated by the therapeutic agent. Some of those tumors can still be sensitive to a second-line antiandrogen.

2.1.2 - Bypass pathways

Alternatively, the androgen receptor pathway can be bypassed completely and prostate cancer cells develop the ability to survive independent of ligand-mediated or non-ligand-mediated androgen receptor activation. The best known bypass mechanism involves modulation of apoptosis. In androgen-dependent prostate cancer cells, androgen receptor activation stimulates cell proliferation and depletion of androgen leads to apoptosis of these cells. Androgen-independent prostate cancer cells have been shown to frequently up-regulate antiapoptotic molecules. Activation of Akt is one way in which androgen-independent prostate cancer cells escape apoptosis in an androgen-depleted environment (Debes JD et al, 2004; Thomas C et al, 2013). Another postulated bypass mechanism is the neuroendocrine differentiation of prostate cancer cells (Debes JD et al, 2004).

Prostatic NE cells are typically post-mitotic cells that resist androgen deprivation therapy due to their lack of AR expression. NE cells secrete many factors that induce mitogenic effects on prostate cancer tumor cells. IL-6 is among the most well-documented factor induced by castration. IL-6 can further enhance the NE phenotypes (Deeble PD et al 2001), thereby amplifying the wave of survival factor release. Moreover, these cells can increase the proliferation of neighboring cancer cells, allowing progression in a low-androgen environment.

2.1.3 - Cancer Stem Cells (CSCs)

CSCs are defined as a subset of tumor cells that has the ability to self-renew and generate the diverse cells that comprise the parental tumor. First evidence of CSCs existence and functionality has been provided in hematological tumor (Lapidot et al, 1994; Bonnet et al, 1997) but mounting data over recent years have indicated the presence of CSCs in multiple solid tumors, including breast (Al-Hajj et al, 2003), lung (Eramo et al, 2008), brain (Singh et al, 2004) and colon (O'Brien et al 2007; Ricci-Vitiani et al, 2007).
CSCs share important properties with normal tissue stem cells, including self-renewal (by symmetric and asymmetric division) and embryonic and survival genes expression. They may be distinct from the cell of origin which specifically refers to the cell type that receives the first oncogenic hit. Our knowledge on molecular mechanisms that govern CSCs behavior are limited, but several studies reported an overlap with the same pathways orchestrating the properties of adult tissue stem cells, such as Wingless-β Catenin, Notch-Jagged, Hedgehog cascade and Bone Morphogenetic Proteins (Valent P et al, 2012). However this scenario is further complicated by the evidence that CSCs do not necessarily originate from the transformation of normal stem cells but they can arise from restricted progenitors or more differentiated cells that have acquired self-renewing capacity.
CSCs hypothesis suggests several possible explanations for the main unsolved questions of treating patients with cancer: i) local recurrence after treatment of solid tumor by radiation or chemotherapy and ii) development of metastases that can appear many years after curative surgical treatment of primary tumor. Several groups have reported an increase of DNA-repair mechanisms, ABC-transporters functionality and survival

properties (Signore et al, 2013; Maugeri-Saccà et al, 2011) in CSCs, thus enabling them to survive the therapeutics regimens and to promote tumor re-growth. Moreover, resistant and quiescent CSCs that have drifted to distant sites may be responsible for cancer metastasis.

2.2 – C-Met receptor and Prostate Cancer

C-Met is a transmembrane tyrosine kinase receptor which was reported to be expressed in a variety of cell types such as endothelial, epithelial, neuronal end hematopoietic cells (Trusolino et al, 2010). This receptor was shown to be involved in several physiological processes such as embryogenesis (Bryant et al, 2008), wound repair (Gurtner et al, 2008) and organ homeostasis (Zaret et al, 2008). De-regulation of c-Met is highly implicated in cancer development, particularly in metastatic signaling promoting proliferation, survival and migration cues. *Hepatocyte Growth Factor* (HGF) is an ubiquitous protein and it is the unique known ligand of c-Met receptor (Bottaro et al, 1991). Once activated, the receptor dimerize and modulate a complex signal which involves the canonical pathways necessary to build and construct the malignant phenotype of a cancer cell. In particular, PI3K and MAPK pathways have a significant role in c-Met-induced anti-apoptotic and proliferative mechanisms respectively (Lai et al, 2009). In addiction Met activation results in cytoskeleton changes through interaction with RAC1 and PAK proteins (Joffre et al, 2011; Shrestha et al, 2012), conferring to the cells motility and invasiveness capabilities.
Molecular mechanisms by which HGF/c-Met axis regulates prostate cancer progression are still confounding. A large amount of *in vitro* studies demonstrated that HGF derived from prostate stroma significantly increases the proliferation, motility, and invasion of malignant epithelial cells through the c-Met protein. It has been observed that c-Met is highly expressed in the AR-negative prostate cancer cell lines, such as DU145 and PC-3, but slightly expressed in the AR-positive prostate cancer cell lines, such as LNCaP, LAPC-4, CWR22, and LuCaP (Pisters LL et al, 1995, Van Lendeers G et al, 2002). Moreover, it has been observed that c-Met is highly expressed in basal and intermediate cells in the prostate epithelium but not in AR-positive luminal cells (Gmyrek A et al, 2001). HGF receptor seems to be also involved in castration-resistant prostate cancer, based on several studies showing its activation after androgen blockade (Verras M et al,

2007; Maeda A et al, 2006) and its capabilities to induce a stem-like phenotype in prostate cancer cells (van Leenders GJ et al 2011). On the contrary, conflicting report are common for clinical studies involving c-Met analysis. Met expression does appear to increase during prostate disease progression, but the correlation of Met expression with Gleason grade has been tenuous. Approximately 50% of localized cancers (and even more metastatic cancers) express Met. Moreover, no correlation between Met expression and disease progression, in a 5-year follow up period indicating that HGF receptor doesn't represent a useful prognostic marker for stratifying patients (Jacobsen et al, 2013; Knudsen BS et al, 2002).

2.3 – MicroRNAs

MicroRNAs (miRNAs or miRs) are a family of small non-coding RNAs (21-25 nucleotides) which are able to recognize specific sequence of transcripts and regulate target genes expression mostly at post-transcriptional level (Winter et al, 2009). In the majority of cases, miRNA-target sequences have been identified in 3'-untraslated region of mRNA, but recently several reports demonstrated significant interactions with regions in the 5'-UTR (Andersson Ørom *et al.,* 2008) and in the coding sequence (CDS) (Tay *et al.,* 2008).

2.3.1 - MicroRNA biogenesis

MicroRNAs are synthesized from a long primary transcript (≥ 1000 nucleotides), which is defined "pri-miR". Pri-miRs may have independent transcription units or may originate from an intron of a specific gene. This transcript is recognized in the nucleus by the complex composed by the DGCR8 protein, which recognize and bind the RNA, and Drosha a ribonuclease able to cut the pri-miR and to produce a precursor (pre-miR) about 70nt long. The resulting pre-miRs are exported into the cytoplasm by the Exportin-5 complex involving RanGTP hydrolysis. When microRNAs are in the cytoplasm, the pre-microRNA processing complex, containing the endonuclease Dicer and TRBP, cuts the pre-miRs to produce a double strand mature miR about 22-25 nt long. Only one strand, actually representing the correct mature miR, remains stable on the RISC (RNA-induced

silencing complex) playing its role by completely or partially pairing to the 3' untranslated region (3'UTR) of target mRNAs , thus determining their degradation or translation inhibition (Inui *et al.,* 2010).

2.3.2 - MicroRNA and Cancer

MiRNAs offer a fast fine-tuning and energy-saving mechanism for post-transcriptional control of protein expression. Growing evidence indicates that miRNA family controls basic cell functions (Stefani G et al, 2008), ranging from stemness maintenance to apoptosis, and different miRNAs are deregulated in many diseases including cancer (Calin GA et al, 2006-2009; Garzon R et al, 2009). It has been published that increased levels of some miRNAs promote tumorigenesis and that other alterations occur as secondary events, leading to the acquisition of metastatic ability. On the other hand, some miRs act as tumor-suppressors by repressing master oncogenic signaling.

Several studies have linked miRNAs to controlling self-renewal of embryonic stem cells (ESCs) (Wang et al, 2007; Marson et al, 2008), while others are involved in differentiation (Tay et al, 2008). Interestingly, the miRNA expression patterns in tumor cells often bear resemblance to those in ESCs. Let-7, for instance, is excluded in ESCs and often lost in cancer, including breast, lung and ovarian cancer. More recent evidence suggests that miRNAs may be also involved in tumor development by critically regulating CSCs. MiR-30 and let-7 family control the cell cycle and differentiation properties of breast cancer stem cells (BCSCs) while miR-200c regulates self-renewal by targeting the stemness marker BMI-1 (Yu et al, 2007-2010; Shimono et al, 2009).

Recently, it was discovered that extracellular miRNAs circulate in the blood of both healty and diseased patients (Sita-Lumsden et al, 2013). Most of these circulating RNAs are included in lipid vescicles and are, therefore, highly stable. The existence of circulating microRNAs has revealed a new world of putative diagnostic and prognostic biomarkers easy-detectable and highly specific for the different stages of cancer progression. It turns out to be evident that cancer-specific miRNA expression signatures may become very informative for diagnostic and prognostic purpose.

2.4 – MicroRNA and Prostate Cancer

Similar to other solid tumors, microRNAs regulate prostate cancer development and progression (Coppola et al, 2010). Experimental models have demonstrated a potential role for miRs as predictive biomarkers or therapeutic tools for patients treatment. In PCa, several miRNAs have been reported to be differentially regulated and act as both tumor-suppressor and oncomiRs. For instance, miR-221 and miR-222 have been shown to be up-regulated in castration-resistant prostate cancer cells compared to androgen-dependent prostate cancer cell line and significantly affect the response to DHT (Galardi et al, 2007; Mercatelli et al, 2008). Tumorigenic properties of miR-221 and miR-222 are attributed to their control of the cyclin-dependent kinase inhibitors $p27^{KIP1}$ and $p57^{KIP2}$, and thus control of G_1 to S phase transition, PI3K and PTEN signaling and other targets implicated in malignant transformation. Similarly, miR-21 has been identified to be up-regulated in prostate cancer and it seems implicated with drug-resistance acquisition. Overexpression of miR-21 was also observed in docetaxel resistant variant of PC3 cells, additionally, ectopic expression of this microRNA in wild type PC3 cells increased the resistance to docetaxel (Shi et al, 2010). The oncogenic and drug resistance properties of miR-21 were attributed to its control of downstream target, programmed cell death 4 (*PDCD4*) (Asangani et al, 2008; Frankel et al, 2008) . On the other hand, several miRNAs were found down-regulated in diseased patients. MiR-15 and miR-16 are among the most extensively studied tumor-suppressor miRs (Calin et al, 2002; Bonci et al, 2008). A stable lentivirus-mediated sequestering of miR-15a/miR-16in RWPE-1cells induced an increase in proliferation and migration in vitro and allowed these cells to form small tumor masses in non-obese diabetes/severe combined immunodeficiency (NOD/SCID) mice. On the contrary, lentivirus-mediated miR-15a/miR-16reconstitution in LNCaP cells resulted in a dramatic apoptotic effect *in vitro* and in considerable regression of tumor xenografts *in vivo* (Bonci et al, 2008). These observations were, at least in part, explained by the evidence that miR-15a/miR-16are able to repress the expression of BCL2, Cyclin-D1 and WNT3A genes, thus interfering with multiple oncogenic activities. Similarly, miR-145 (Zaman et al, 2010), miR-205 (Wang et al, 2013) and miR-200 family (Kong et al, 2009)) have been shown to be part of tumor-suppressor class. Down-regulation of these

microRNAs affect proliferation and EMT processes in PCa cells, increasing activation of EGFR/RAS/MAPK pathway and expression of ZEB-1 and ZEB-2 genes.

Further analysis in microRNA biology in prostate cancer are necessary due to the fact that, in spite of results obtained in experimental models, an unequivocal expression signature representative of diseased-patients is still lacking and variability among clinical studies gave back a more complex scenario. However, high-throughput technology applied to miRs offers an appealing tool to set up patient-tailored treatments. Identification of miRNA profiles associated with poor clinical outcomes may represent an innovative parameter for optimizing both the individual risk assessment and the evaluation of innovative pathway-targeted inhibitors coupled with predictive biomarkers discovery.

3 – MATERIAL AND METHODS

3.1 - Cell culture and treatments

RWPE-1, RWPE-2, 22Rv1, LnCaP, PC3 and DU145 were provided from ATCC and cultivated in the recommended medium. PCSC-1 cells were provided from University of Palermo after dissociation of a patient-derived tumor tissue and selection in EGF-FGF supplemented medium (Eramo et al, 2008).
PCSC-1 line was treated with 25nM of HGF (Peprotech) and cells were collected after 8 hours and 24 hours for RNA and protein extraction. PCSC-1 line was treated with Crizotinib inhibitor (Sigma-Aldrich) at 3µM final concentration for 48 hours, while PC3 cells were treated with SU11274 inhibitor (Sigma-Aldrich) at 5µM final concentration for 48 hours. Cells were collected for RNA extraction and analysis. LnCaP/TW and LnCaP/miR-130b were treated with Casodex (Sigma-Aldrich) or Docetaxel (Sigma-Aldrich) at final concentration of 5µM and 30nM respectively.

3.2 - Proliferation assay

$3x10^3$ LnCaP/TW and LnCaP/miR-130b cells were plated in a 96-well plate and treated with Casodex or Docetaxel, as described above. Proliferation rate were measured using Cell-Titer Glo reagents (Promega) according to manufacturer instructions.

3.3 - Soft agar assay

For the soft agar assay, $1x10^2$ PCSC-1 cells or $1x10^4$ LnCaP/TW and LnCaP/miR-130b were suspended in culture medium supplemented with 0.4% agar and plated in a 24-well plate previously coated with a 3-mm layer of the same modified medium. Cells were cultured for 3 weeks before Crystal Violet staining.

3.4 -Transfection

pBABE control vector and TPR-MET vector were provided from Addgene company. Transfection was performed at 1µg/10^5 LnCaP cells concentration using XtremGene HP reagent (Roche) according to the manufacturer's instructions. Puromycin was used at 1µg/ml concentration for 48h and cells were collected for further analysis. MiR-130b-mimic, miR-130b-inhibitor and negative control (Qiagen) were transfected at 25nM/ 10^5 LnCaP cells concentration using Dharmafect 2 reagent (Thermo Scientific), according to the manufacturer's instructions. After 48h from trasnfection, cells were collected for further analsysis.

3.5 - Generation of lentiviral vectors and gene transfer

For TW-EGFP/miR130b generation, miR-130b was PCR amplified from human genomic DNA with the following primers:

- miR-130b FW - TCTGTGCAAGTGCCCAAGAT
- miR-130b REV - GCAAAGTCTGAAGGTGCCA

The 299bp fragment was subcloned in the lentiviral vector TW under the control of the CMV promoter. An empty vector was used control. Cells were plated in a 6-well plate 24 hours prior to viral infection and incubated overnight with 2ml of complete optimal medium (with serum and antibiotics). The day after the medium was removed and cells were infected adding 2 ml of complete medium with Polybrene (5µg/ml) and lentiviral particles described previously. Transduction efficiency was measured through EGFP expression analysis while microRNA overexpression was confirmed by qRT-PCR.

3.6 - Protein extraction and Western Blot analysis

Total proteins from NSCLC were extracted with radioimmunoprecipitation assay (RIPA) buffer (0.15mM NaCl, 0.05mM Tris-HCl, pH 7.5, 1% Triton, 0.1% SDS, 0.1% sodium deoxycholate and 1% Nonidet P40). Sample extract (20µg) was resolved on 4–12% precast polyacrylamide gels (NuPAGE) using a mini-gel apparatus (Invitrogen–Life Technologies) and transferred to Hybond-C extra nitrocellulose (GE HealthCare). Membranes were blocked for 1h with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20, incubated overnight with primary antibody, washed and incubated for 1h with secondary antibody, and visualized by chemiluminescence using . The following primary antibodies were used:

- Phospho-Met (Tyr 1234/1235) (Cell Signaling Technology)
- AR (SantaCruz Biotechnology)
- Bcl-XL (SantaCruz Biotechnology)
- Mcl-1 (SantaCruz Biotechnology)
- Actin
- Tubulin
- Nucleolin (SantaCruz Biotechnology)

A secondary anti-rabbit or anti-mouse immunoglobulin G (IgG) antibody peroxidase conjugate (GE HealthCare) was used. Actin, Tubulin and Nucleolin monoclonal antibodies were used as loading controls, compatible to the relevant weight of the proteins.

3.7 - Immunofluorescence

- For immunofluorescence analysis, 2×10^4 cells were seeded in culture medium on 15-mm polylysine-coated coverslip placed in 24-well plates. Cells were fixed with 2% paraformaldehyde for 20' and permeabilized with PBS 0.1 % Triton X-100 for 3' at room temperature (RT). Primary antibodies were diluted in PBS 3% BSA, 0.05% Triton X-100 and incubated overnight at 4° C. Antibodies used were:
- Phospho-Met (Tyr 1234/1235) (Cell Signaling Technology)
- CK 8/18 (SantaCruz Biotechnology)
- CK14
- P63

For immunofluorescence analysis of xenograft tissues, cryostatic sections of 5 µm, obtained using a cryomicrotome (Kriostat 1720 MGW Leitz), were fixed with paraformaldehyde and then blocked with 5% serum in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% Triton X-100. After 1h of incubation at room temperature, sections were stained overnight at 4°C with CK8/18 (SantaCruz Technology). For primary antibody detection, cells or tissues were incubated for 45' with Alexa Fluor 488 or 647-conjugated secondary antibodies (Gibco). After extensive washing, the nuclei were stained with 4′, 6′-diamidino-2-phenylindole (DAPI) for 20'. Coverslips were mounted on microscope slides with the storage solution (glycerol/PBS) and sealed with Prolong Gold Antifade reagent (Life Technologies–Invitrogen). Fluorescent signals from a single optical section were acquired by a three-laser confocal microscope (Olympus FV1000).

3.8 - Immunohistochemistry analysis

PCSC-1 derived OCT-embedded tumor tissues were dissected in a 5µm sections using Kriostat 1720 MGW Leitz. Tissue sections were fixed with 2% paraformaldehyde for 20 min and permeabilized with phosphate-buffered saline 0.1% Triton X-100 for 5 min at room temperature. Immunohistochemical stainings for CK8/18, CK5, p63 and PSA proteins were performed using avidin-biotin-peroxidase complex (Envision Plus HRP; Dako, Glostrup, Denmark). Slides were counterstained with hematoxylin and permanently mounted.

3.9 - Flow Cytometry analysis

For Flow Cytometry staining, cells were fixed with 2% paraformaldehyde for 20 min and if necessary permeabilized with phosphate-buffered saline 0.1% Triton X-100 for 3 min at room temperature. Cells were then incubated for 30 min on ice with primary antibody diluted in phosphate-buffered saline 1% bovine serum albumin. The antibodies used include: CD44-FITC (1:10; 555478- BD Pharmigen), c-MET-FITC (1:10; FAB3582F- R&D Systems), CD44v6 (1:10; FAB3660F- R&D Systems), CD133-PE (1:10; #130-080-801- Milteny Biotec.) and CD24-FITC (1:5; 555428- BD Pharmigen , c-Kit (1:200; SC19983- SantaCruz), ABCG2 (1:5; FAB995P- R&D Systems), EGFR-PE (1:5; 555997- BD Pharmigen).
Cells incubated with non-conjugated antibodies were washed twice and then incubated for 30 min on ice with appropriate secondary antibodies. Stainings were analyzed with BD FACSCanto flow cytometer (Becton, Dickinson and Company, San Jose, CA, USA).

3.10 - RNA extraction and quantitative qRT–PCR

Total RNA was extracted with TRIzol (Life Technologies–Invitrogen) following protocol indications. For evaluation of microRNA levels, 50 ng of RNA were reverse transcribed with M-MLV reverse transcriptase (Life Technologies–Invitrogen) and cDNA was diluted 1:10 in the PCR reactions. A microRNA specific looped-primer was used for miR-130b retrotranscription and a TaqMan microRNA assay (Life Technologies–Applied

Biosystem) for miR-130b was used for PCR amplification. Normalization was performed using snRNA U6 as reference (RNU6B TaqMan microRNA assay, Life Technologies). Values are expressed in terms of $2^{-\Delta\Delta CT}$ where $\Delta\Delta C_T = \Delta C_{Tsample} - \Delta C_{Tcalibrator.}$; ΔC_T is the difference in threshold cycles between the miR and U6 amplicons, and C_T is a parameter given by ABI PRISM 7700 Sequence Detector software by negative correlation with an internal reference dye (ROX). For Sybr Green analysis 1 µg of RNA was reverse-transcribed with random primers and the following oligos were used at 300 nM for quantitative PCR:

- AR FW - CACAGTTGGAGACTGCCAGG
- AR REV - TGCTGGCGCACAGGTACTTCT

For c-MET, IL-6, IL-11 and CXCR4 mRNA evaluation a specific TaqMan assay was used (Life Technologies–Applied Biosystem). Normalization was performed with GAPDH amplification using the following primers:

- GAPDH FW - TTCCAGGAGCGAGATCCCT
- GAPDH REV - GGCTGTTGTCATACTTCTCATGG

3.11 - TaqMan Array MicroRNA Cards

The TaqMan Array Human MicroRNA Card (Life Technologies–Applied Biosystems) Set v3.0, is a two-card set containing a total of 384 TaqMan MicroRNA Assays per card that enables accurate quantification of 754 human miRNAs. Included on each array are three TaqMan MicroRNA Assays as endogenous controls to aid in data normalization and one TaqMan MicroRNA Assay not related to human as a negative control. An additional pre-amplification step was enabled by using Megaplex PreAmp Primers, Human Pool Set v3.0 for situations where sensitivity is of the utmost importance or where the sample is limiting.

3.12 - Tissues microdissection and microRNA expression analysis

Formalin fixed and paraffin embedded tissues (metastases and primary tumors) were cut in 8 µm slides and arranged on laser-microdissection RNAse free slides (Nikon). They were maintained at 70 °C for 20 minutes and then treated with xilene for 20 minutes. Tissues were re-hydrated with scalar percentage of ethanol and distilled water and finally stained with Harris hematoxilyn. Tumoral tissues were microdissected with a UV-laser microscope by Nikon, avoiding contamination of surrounding stroma. Laser cut samples were treated with TRIzol protocol for RNA extraction. Megaplex A retro-transcription and amplification system and TaqMan^{R} Human Array microRNA Card A (Life Technologies–Applied Biosystems) were used for miRNA profiling evaluation following recommended protocols.

3.13 - Animal models

Male 6- to 8-week old *NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ* (NSG) mice (The Jackson Laboratory) were used for *in vivo* experiments. For the subrenal capsule (SRC) model, injection of 3 x 10^5 cells in 15 µl of Matrigel was administered with a 29G needle in the subcapsular space of the left kidney under a stereomicroscope. For the orthotopic prostate model, an injection of 3,5 x 10^5 cells in 15 µl of Matrigel was made in the left anterior prostate using a 29G needle under a stereomicroscope. For the intracardiac model, a percutaneous injection (1 x 10^5 cells in 50 µl of DPBS) was made in the left ventricle using a 29G needle. For the intratibial model, a small cavity was created in the tibial shaft of the right hind limb through the drilling of a 23G needle, and then 2,5 x 10^5 cells in 10 µl of Matrigel were inoculated using a 29G needle.

For bioluminescence imaging, mice were injected intraperitoneally with D-Luciferin (150 mg/kg) approximately 10 minutes before imaging procedure and then were anesthetized with Isoflurane (XGI-8 anesthesia system, Caliper Life Sciences). A cryogenically cooled imaging system (IVIS 100 Imaging System, Caliper Life Sciences) was used for data acquisition. Whole animal imaging was used to monitor tumor localization and tumor growth. Signal intensities were quantified as the sum of detected photons in a specific ROI (Region of interest). For fluorescence imaging, mice were sacrificed at established timepoints and single organs were removed and imaged under a stereomicroscope

(Olympus SZX-10), coupled with a fluorescence unit equipped with GFP filter. Transmitted light images and fluorescence images of single organs were acquired using a digital colour camera (Olympus XC-50). For histological analysis of *in vivo* experiments, tumor masses were snap-frozen in OCT and stored at −80°C. Cryostatic sections were stained with hematoxylin and eosin, dehydrated and mounted with xylene. For microtomography analisys, mice were sacrificed at established timepoints and tibiae were dissected and analyzed with a microcomputed cone-beam X-ray system (Skyscan 1072 µ-CT) without addition of contrasting agent.

3.14 - Statistical analysis

Plotted data are presented as the mean+s.d. of at least two independent experiments. Statistical significance was calculated by the Student's *t*-test analysis.

4 – RESULTS

4.1 - AR and c-Met expression in prostate cancer cell lines

The role of AR and c-Met in prostate cancer has been investigated by a large number of studies. However, only few reports described a possible molecular interaction between the two receptors indicating their possible implication in castration resistance and metastasis development (Verras M et al, 2007; Maeda A et al, 2006). In order to provide new insights into a putative Met/AR regulation, we firstly analyzed the expression of both receptors in prostate cancer cell lines representative of tumor progression (tab.1). According to the literature (Tilley WD et al, 1990; Niu Y et al, 2008), c-Met receptor was found overexpressed in basal cells (fig.3a), while Western blot analysis revealed a strong expression of AR only in luminal cells, such as LnCaP and 22Rv1 (fig.3b). Same results were observed at the RNA level through qRT-PCR (fig. 3c-d). Interestingly, in our model c-Met expression seems to be independent of tumorigenic properties since no relevant variations were observed among low- and high-aggressive cell lines. This contradiction was explained by several studies reporting significant differences in the functional state of the receptor whose activation is enhanced in metastatic cell lines, such as PC3 and DU145 (Knudsen BS et al, 2004; Day Y et al, 2011). We obtained similar results analyzing c-Met activation through immunofluorescence. As shown in fig.3e, we found a strong increase in Met phosphorylation in PC3 metastatic cell line compared with RWPE-1 non-tumorigenic cells and RWPE-2 low-aggressive cells. Interestingly, the majority of activated receptor is dispersed in cytoplasm rather than membrane. Moreover, they seem to be included in distinct microvescicles which were previously reported as part of a crucial mechanism regulating receptor tyrosine kinase (RTK)-dependent tumorigenesis (Joffre et al., 2011).

4.2 - C-Met overexpression in LnCaP cells and microRNA profiling

The role of c-Met receptor in prostate cancer is still controversial. Experimental models showed its direct correlation with metastatic cascade, drug-resistance and stemness properties acquisition. On the other hand, clinical models failed to find a significant association among c-Met expression and the different stages of the disease, indicating that c-Met could not be used as diagnostic or prognostic biomarker to stratify patients. Based on these assumptions, we hypothesized that c-Met role in tumor progression is not due to its expression but mainly on its activation state which unfortunately is not easily detectable with standard clinical techniques such as immunohystochemistry. To avoid this bias, we decided to analyze the downstream microRNA effectors of c-Met signalling cascade. We transfected LnCaP cell line with an engineerized plasmid carrying a constitutive activated c-Met (TPR-MET) (Dean M et al, 1987) or its relative control (pBABE). Receptor overexpression was confirmed through qRT-PCR (fig.4a) and Western blot assay (fig. 4b). Significant reduction in AR expression ($p=0.02$) was observed in LnCaP/TPR-MET cells, providing evidences for a new putative regulation of both receptors (fig. 4c). Transduced cells were analyzed for microRNA expression profile using TaqMan Array Cards which enable accurate quantification of 754 human microRNAs. Only 2-fold changed miRs were considered for further analysis (fig. 4d). In particular, we focused on miR-130b which was previously reported as an oncomiR regulating EMT process, metastasis signalling and stem-phenotype acquisition. Moreover, miR-130b is considered as a putative regulator of AR expression based on *in silico* analysis of TargetScan (fig. 4d), which is one of the most used software for microRNA-targeting prediction.

4.3 - MiR-130b *in vitro* analysis

To investigate the potential role of miR-130b in prostate cancer cells, we analyzed initially miRNA expression in commercial prostate cancer cell lines. High levels of miR-130b were found only in basal and metastatic cells with the exception of LnCaP cells which have a luminal phenotype (fig5a). Interestingly, PC3 cells treated with c-Met receptor inhibitor SU11274 showed a significant reduction in miR-130b level, confirming its dependence on c-Met activation (fig.5a). These data corroborates our previous results showing a direct correlation with c-Met expression. Moreover, miR-130b seems to be strictly associated with tumor progression suggesting a possible application as a prognostic biomarkers in prostate cancer. To investigate *in vitro* the biological effects of miR-130b, we stably transduced negative LnCaP cells with an EGFP-lentiviral vector carrying miRNA genomic locus (LnCaP/miR-130b) and the empty vector (LnCaP/Tween) as relative control. qRT-PCR confirmed miR-130b overexpression in miR- versus empty vector-transduced cells (fig.5b). Proliferation rate *per se*, was not influenced by increased miR-130b levels (fig.6a). On the contrary, soft-agar assay revealed a strong induction of anchorage-independent growth abilities in LnCaP/miR-130b cells, compared with their relative controls (fig.6b). Moreover, we observed an increase in invasive capabilities due to the fact that miR-overexpressing cells were able to degrade the semi-solid medium, to migrate and to attach on plate surface (fig.6c). In order to translate these results into a molecular pattern, we analyzed in overexpressing cells changes in RNA and protein expression, which could be responsible for the observed phenotype. As shown in fig.7a, miR-130 is able to regulate activation of several genes implicated in survival, proliferation and metastatic spreading. Furthermore, increasing expression of genes such as IL-11 (Kang et al, 2003), CXCR-4 (Wang et al, 2006), RUNX-2 (Blyth et al, 2005) and RANKL (Jones et al, 2006), indicates that this microRNA could be a putative mediator of bone metastasis. Based on these assumptions, we hypothesized a sophisticate mechanism in which c-Met is able to regulate AR through miR-130b induction leading to a more aggressive phenotype which represents a prerequisite for acquisition of metastatic capabilities. To assess our hypothesis, we firstly analyzed AR protein level after miR-130 overexpression. As shown in fig.7b, LnCaP transfected with a synthetic miR-130b mimic displayed a reduction in AR expression, similar to results obtained after TPR-MET

ectopic expression. This effect was counteracted when LnCaP/TPR-MET cells were treated with a synthetic miR-130b inhibitor (fig.7c). At the same time, wild-type LnCaP cells depleted of endogen miR-130b, showed an increase in AR protein level. These data give further evidences of Met and miR-mediated AR regulation.

4.4 - MiR-130b controls Casodex sensitivity of LnCaP cells

Several studies reported the central role of androgen receptor in survival mechanisms (Debes JD et al, 2002; Shiota et al, 2012). On the contrary, our experiments demonstrated that miR-130b expression not only results in AR downregulation but also in increasing levels of anti-apoptotic factors, such as Bcl-2 and Mcl-1. Based on these results, we hypothesized a possible miR-130b implication in androgen-deprivation resistance and, consequently, in castration-resistant state progression. To corroborate our hypothesis, we treated LnCaP/miR-130b cells and their relative controls with 5 µM of Casodex (CDX), a non-steroideal antiandrogens, searching for proliferative capability impairments. We observed a 50% increase in proliferative index of miR-overexpressing cells compared with LnCaP/TW controls (fig.8a).

4.5 - MiR-130b *in vivo* analysis

To determine whether miR-130b forced expression could affect tumorigenic properties *in vivo*, we injected stably transduced LnCaP/miR-130b cells and their relative controls in NSG mice prostate, to reproduce a more reliable microenvironment for tumor growth. This protocol, defined orthotopic injection, represents the gold-standard technique model for prostate cancer studies. After 3 weeks post-injection, we observed a significant difference in tumor engraftment with a 50% (n=5/9) of miR-overexpressing-cells-inoculated mice, presenting a distinguishable luciferase signal at IVIS imaging system (fig.8b). On the contrary, a reduced engraftment rate (20%, n=2/10) was reported in control mice. These results, which contradict *in vitro* data where no proliferative differences were observed between LnCap/miR-130b and control cells, suggest that miR-130b is able to accelerate cell homing, survival and proliferative capabilities, probably empowering tumor–microenvironment stimuli. In order to investigate miR-130b role in metastatic properties acquisition, we inoculated LnCaP/miR-130b and control cells

directly into NSG mice myocardium through intracardiac injection. After 8 weeks, 50% (n=3/6) of mice inoculated with miR-overexpressing cells showed a distinguishable luciferase signal in thoracic region, suggesting lung metastasis formation (fig.8c). On the contrary, LnCaP/Tween cells were able to from distant metastasis only in 10% of inoculated mice (n=1/8). Thus, this experiment demonstrated the capability of miR-130b cells to survive in the blood flow and invade organs.

4.6 - Analysis of c-Met/AR circuitry in Prostate Cancer Stem Cells

Cancer stem cells are defined as those cells within a tumor which are able to self-renew and drive tumorigenesis. Moreover, increasing evidence indicate that these cells are responsible for drug resistance, tumor recurrence and metastasis formation. Based on these assumptions, we decided to analyzed c-Met/AR circuitry in a stem-like population (PCSC-1) isolated from patient-derived tumor tissue through mechanical/enzymatical dissociation and selection in cell culture medium containing EGF and FGF factors (fig.9a). *In vitro* characterization revealed a significant expression of stem-associated biomarkers, such as CD44 and EGFR, and basal markers, such as CK5, CK14 and p63 (fig.9c). Moreover, these cells were able to grow in soft-agar plate demonstrating clonogenic capabilities and anchor-independent proliferation properties (fig.9b). We characterized PCSC-1 cells also *in vivo* using three different experimental models: i) subcutaneous injection model, ii) ortothopic injection model and iii) intratibial injection model (fig.10a-c). These cells were able to grow in all conditions confirming their high tumorigenic potential. Moreover, when injected in NSG mouse prostate, PCSC-1 cells were able to reproduce primary tumor luminal phenotype, as indicated by *de novo* expression of CK-8/18 and aberrant glandular-like structure formation (fig.10b). Interestingly, when injected in a non-permissive microenvironment such as bone, PCSC-1 cells were able to proliferate and to cause bone fractures and osteolytic lesions (fig.10c) which represent one of the most common pathological features of prostate cancer metastatic patients. These data confirm that cancer stem cells represent an experimental model more reliable to patient for prostate cancer research.

Based on previous data, we analyzed c-Met and AR expression in PCSC-1 cell line. These cells showed an high level of c-Met receptor, even though it was not evenly expressed in

the entire population, suggesting the presence of several sub-populations which could have a different role in cancer progression (fig.11a). Surprisingly, we found a significant expression of AR both at RNA and protein levels (fig.11b-c), in spite of PCSC-1 basal phenotype. To validate the possible regulation of AR expression by c-Met, we treated PCSC-1 cells with HGF and we observed a reduction in AR protein and RNA expression (fig.12a-b). At the same time, when we treated our cells with Crizotinib, an inhibitor of c-Met phosphorylation, we found an increase in AR RNA levels (fig.12c-d). We obtained similar results when we separated PCSC-1 line in two sub-populations, MET^{high} and MET^{low}, and we treated these cells with crizotinib. No differences in AR expression was found before treatment (fig. 12c). As shown in fig.12d, only MET^{high} population was affected by c-Met inhibitor increasing RNA level of AR. Finally, we analyzed miR-130b expression in our prostate cancer stem cell model. As shown in fig.12e, significant levels of miR-130b were detected in PCSC-1 cells validating its involvement in stemness phenotype (Ma et al., 2010). Moreover, we extended our analysis to PCSC-1 sub-populations and we observed an increase of miR-130b relative expression in MET^{high} cells as compared to their negative counterpart, confirming its direct correlation with c-Met receptor (fig.12f).

4.7 - MicroRNA expression profiles in patient-derived tumors and metastasis

In order to determine whether miR-130b correlates with advanced stages in prostate cancer patients, we decided to move into a clinical setting analyzing patient-derive tumor tissues. We microdissected 9 formalin-fixed and paraffin embedded prostate cancer tissues from 6 patients with clinical evidence of recurrent disease, 2 patients with negative follow-up after prostatectomy and 3 prostate tumor metastases (2 bone and 1 linfonodal metastases). We analyzed microRNA signatures using “TaqMan Array Human MicroRNA A Card v2.0” (Applied Biosystem–Life Technologies), which include a panel of 754 miRs commonly expressed in cells (fig.13a). As a reference and internal control, we used microdissected epithelial cells from a pool of three formalin fixed normal prostate tissues. We observed that cancer-associated microRNAs, such as miR-103 (Martello et al, 2010), miR-494 (Romano et al, 2012) and miR-135a (Chen et al, 2012), were upregulated in all samples, while miR-130b was expressed only in recurrent and

metastatic samples (fig.13a). We performed a similar analysis using a free online database (GEO record GSE21032) comprehensive of 218 prostate tumors genomic and transcriptomic profiles, described by Taylor and colleagues in 2010 (Taylor et al, 2010). This database includes microRNA expression data of 28 normal prostate tissues and 113 prostate tumor tissues consisting in 21 recurrent patient-derived primary tumors, 78 disease-free patient-derived primary tumors and 14 metastatic tissues. MiR-130b expression levels were higher in metastatic samples compared with primary tumors and normal tissues (p=0.0001, fig.13b). In order to evaluate miR-130b association with clinicopathologic features, we subdivided primary tumors in two groups, disease-free and recurrent patients, based on follow-up assessment after prostatectomy. As shown in fig.13c, miR-130b is significantly higher in patients who experienced recurrence compared with patients with a negative follow-up (p=0.0009). Finally, to assess the correlation between miR-130b and disease recurrence rate, we divided patients in two groups according to the miR levels (high and low). A Kaplan-Meier survival analysis revealed that miR-130b up-regulation is associated with an increased likelihood of disease recurrence (p=0.0004, fig.13d).

Taken together, these results indicate a prognostic role of miR-130b as a marker which could be used to distinguish between indolent and recurrent tumors. Moreover, it could be useful to predict patient-responsiveness to androgen-blockade therapies uncovering a new scenario for personalized-clinical protocol based on c-Met targeting.

5 – FIGURES AND TABLES

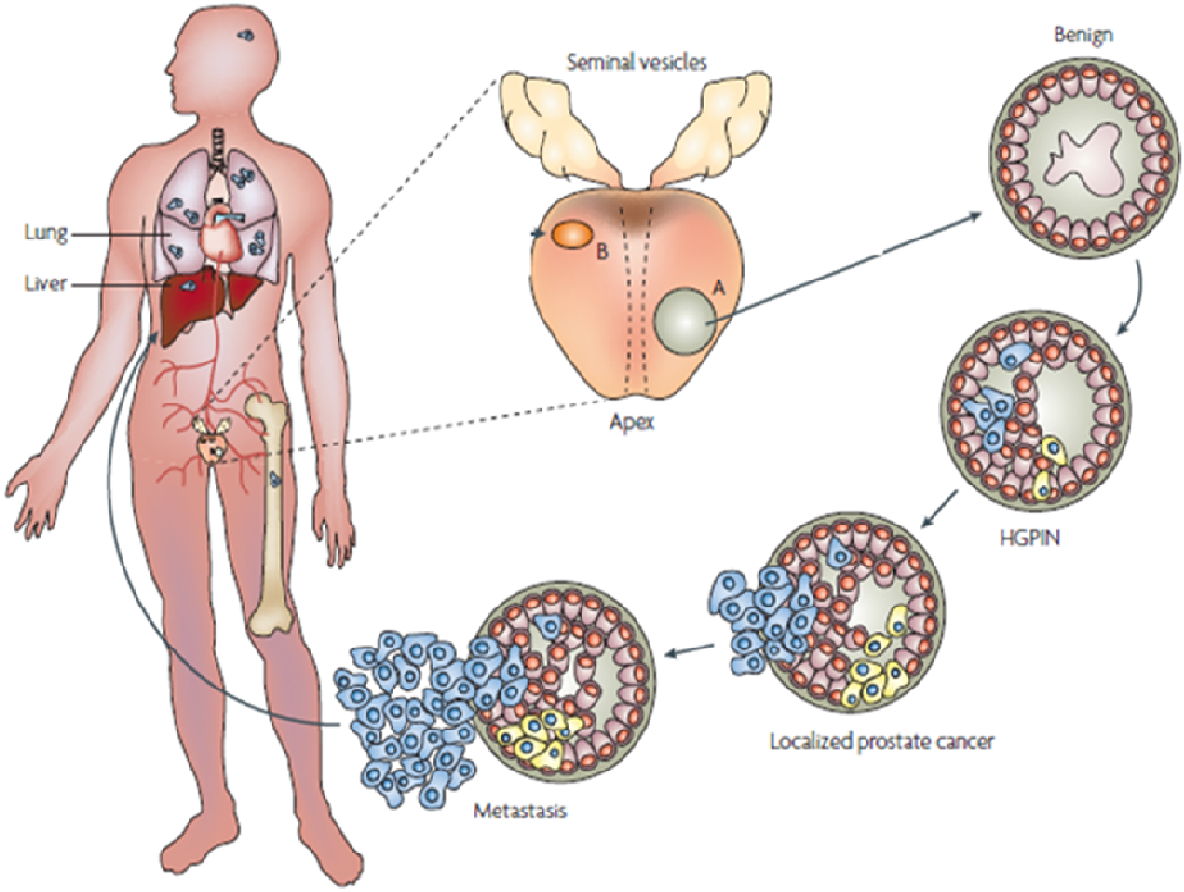


Fig.1: representative scheme of prostate cancer tumor progression (Kumar-Sinha et al, 2008)

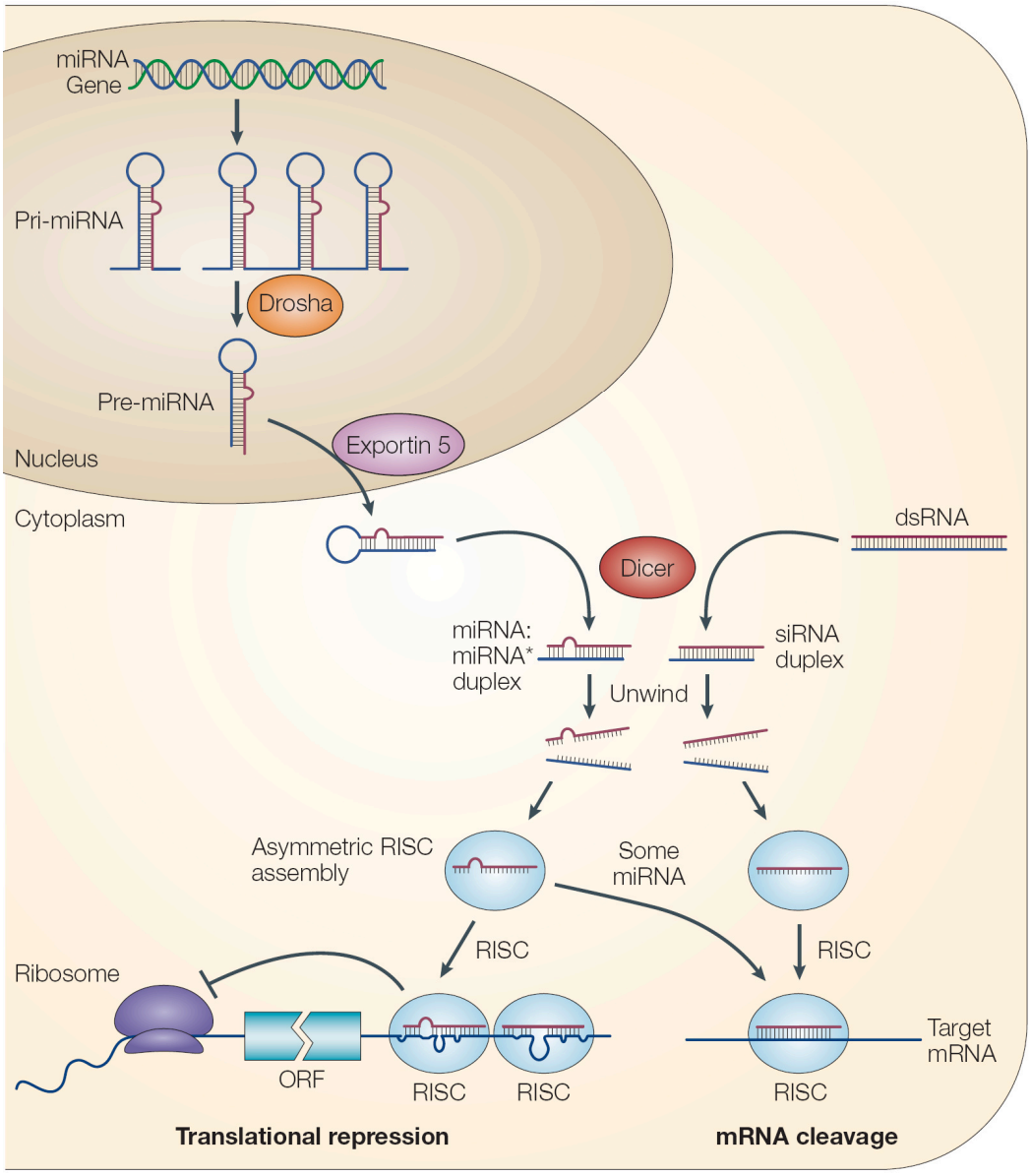


Fig.2: miRNAs biogenesis and mechanism of action (He L and Hannon GJ, 2004)

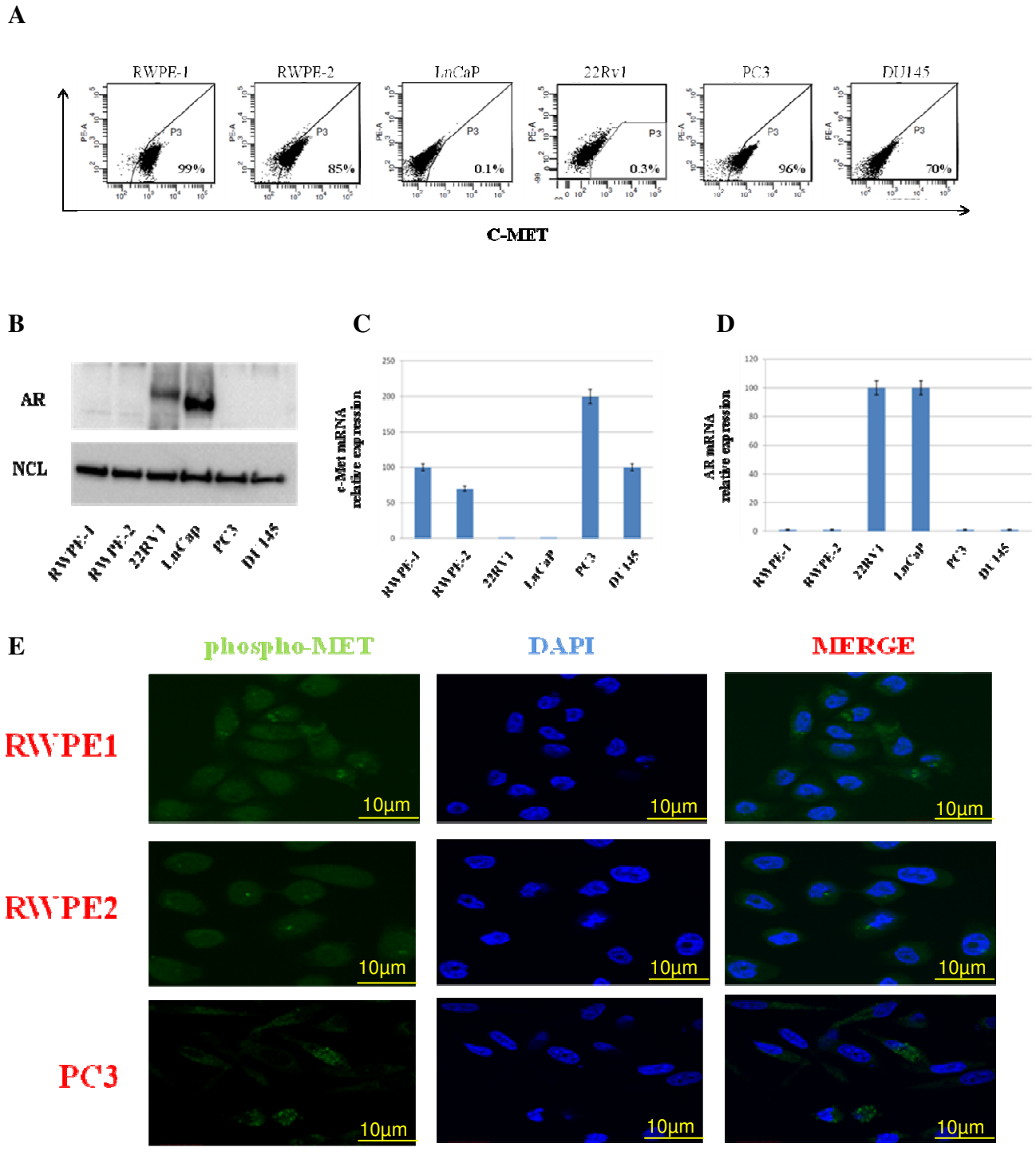
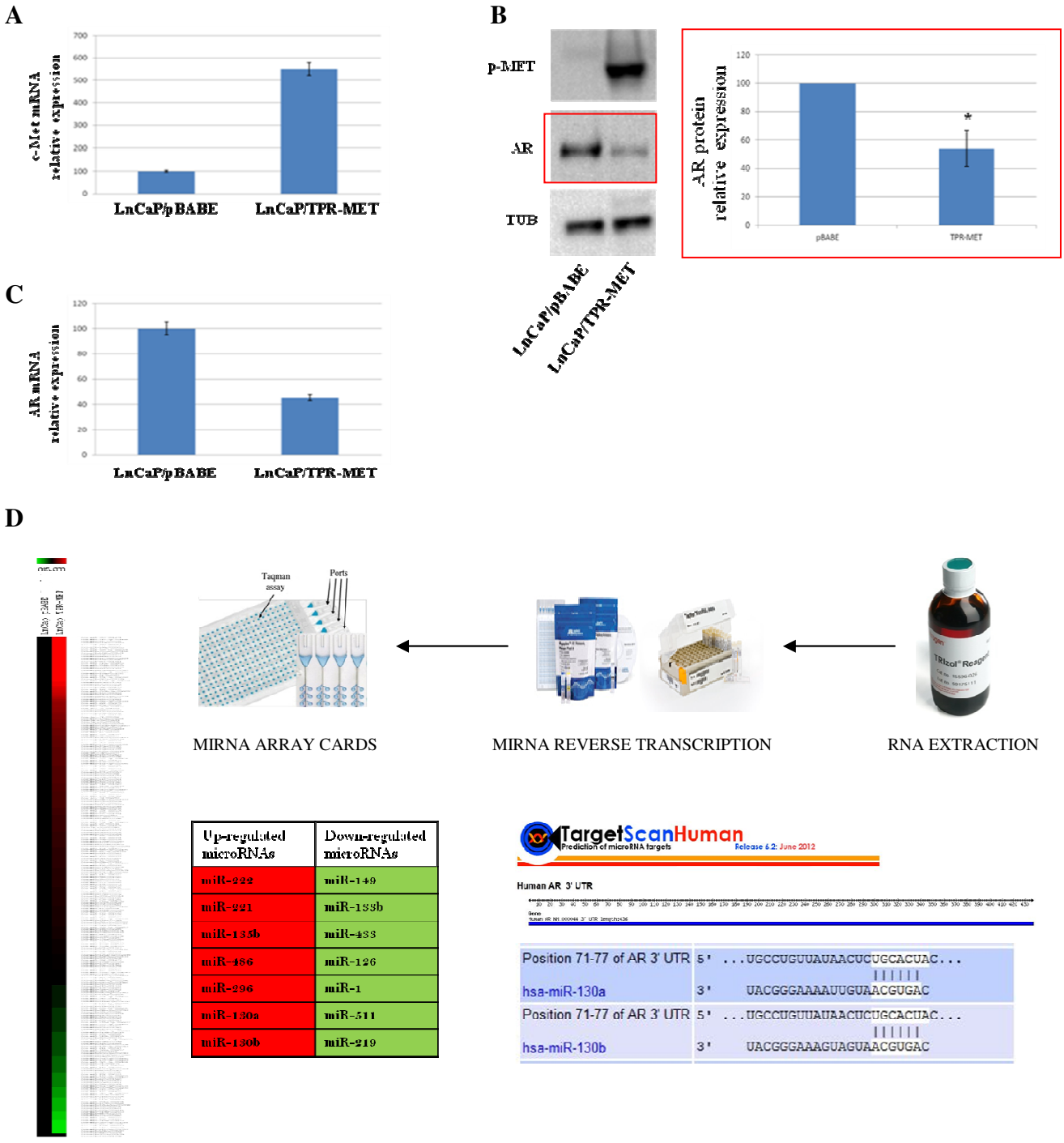


Fig.3: a) cytofluorimetric [fluorescence-activated cell sorting (FACS)] analysis of c-Met expression in prostate cell lines; **b)** Western blot analysis of AR in prostate cancer cell lines. Nucleolin (NCL) was used as loading controls; **c-d)** qRT-PCR analysis of c-Met and AR mRNA expression.; **e)** IF analysis of phospho-Met expression in RWPE-1, RWPE-2 and PC3 cells, alone or merged with DAPI (4′, 6′-diamidino-2-phenylindole).



Up-regulated microRNAs	Down-regulated microRNAs
miR-222	miR-149
miR-221	miR-133b
miR-135b	miR-433
miR-486	miR-126
miR-296	miR-1
miR-130a	miR-511
miR-130b	miR-219

Fig.4: a) qRT-PCR of c-Met overexpression in LnCaP cells transduced with TPR-MET plasmid and relative control; **b)** Western blot analysis of phospho-Met and AR expression in LnCaP cells transduced with TPR-MET plasmid and relative control. Tubulin (TUB) was used as loading controls; Columns, mean±SD of three independent experiments; **P*<0.02; **c)** qRT-PCR of AR expression in LnCaP cells transduced with TPR-MET plasmid and relative control; **d)** schematic representation of TaqMan microarray Cards protocol and final heatmap analysis. (bottom) significantly miRNA de-regulated after MET expression (right) and Schematic presentation of predicted miR-130b and miR-130a target sites identified in the AR 3′UTR (left).

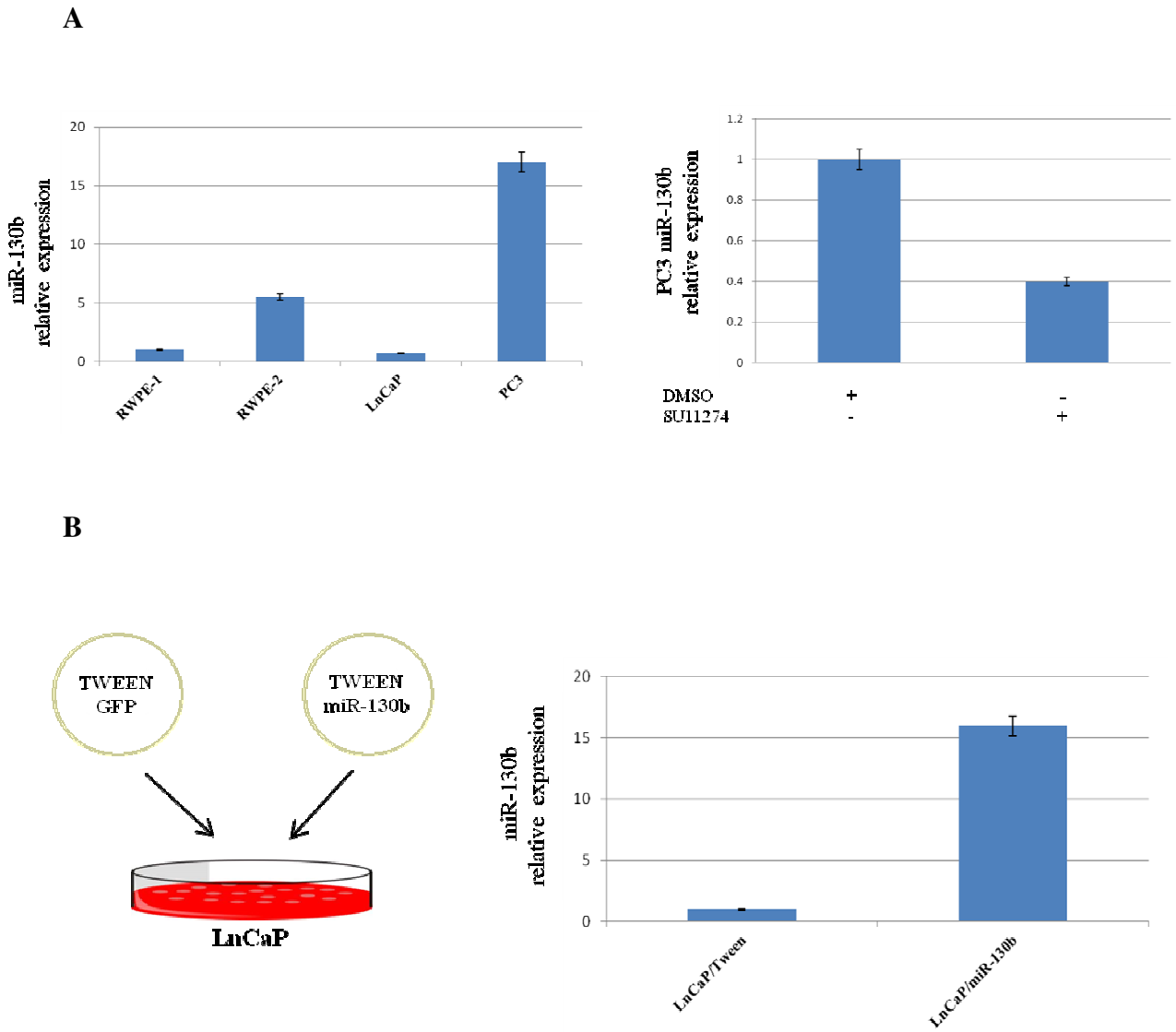


Fig.5: a) qRT-PCR analysis of miR-130b expression in prostate cancer cell lines representatives of tumor progression (left) and miR-130b expression in PC3 cell line after treatment with c-Met activation inhibitor SU11274 (right); **b)** schematic representation of LnCaP lentiviral transduction with miR-130b genomic coding region and relative control (left) and qRT-PCR analysis of miR-130b overexpression after cell transduction (right).

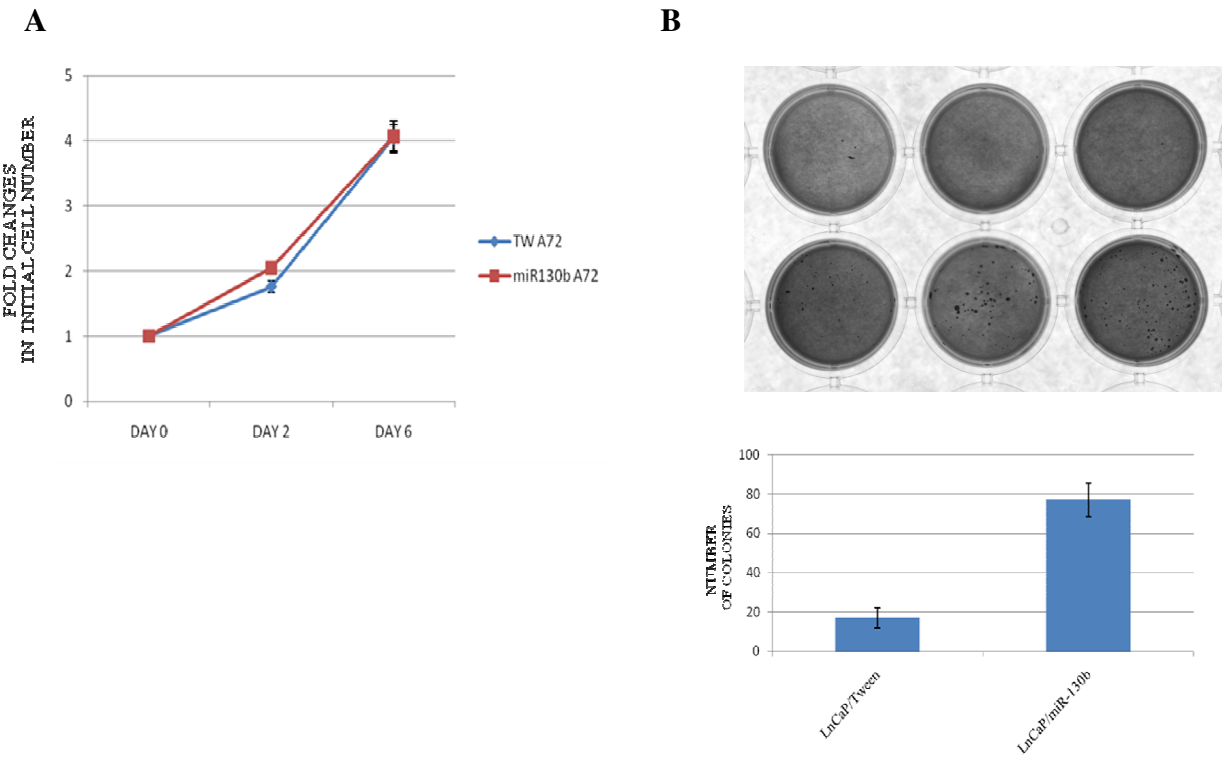


Fig.6: a) cell proliferation of LnCaP overexpressing miR-130b and their relative control as determined with Cell Titer Glo assay; **b)** analysis of soft-agar assay in which LnCaP/miR-130b and their relative controls were seeded at $1x10^3$ cells per 35-mm dish and cultured in 0.4% soft agar in RPMI plus 10% FBS at 37°C for 14 days; **c)** representative images of Tween- and miR-130b-derived colonies (upper) and migrated cells (bottom). EGFP expression indicate transduced cell derivation.

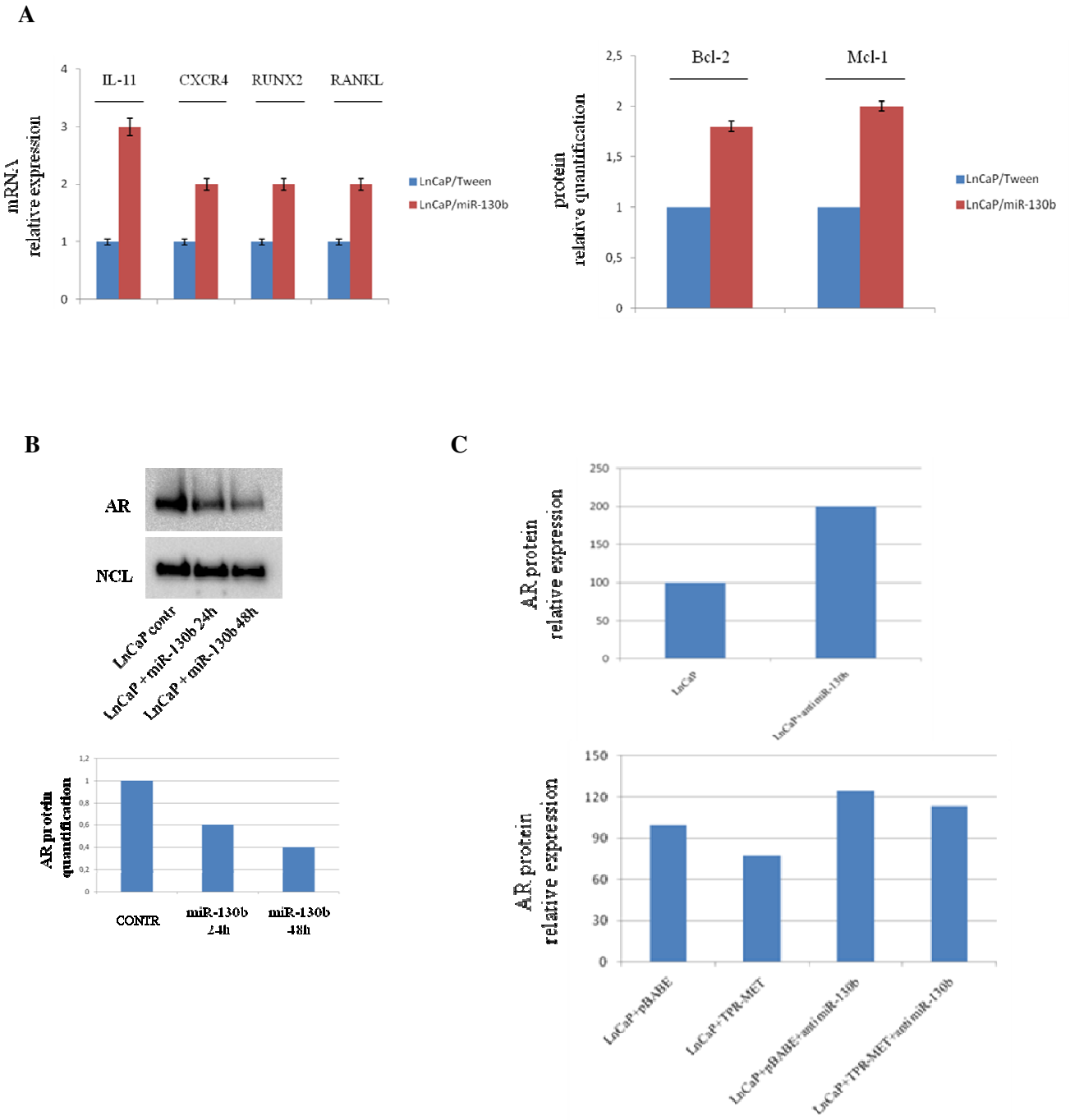


Fig.7: a) qRT-PCR (left) and Western Blot (right) analysis of miR-130b-mediated increase of genes involved in survival and migration pathways; **b)** Western Blot image of miR-130b-mediated AR protein reduction after transiently transfection with synthetic miR-mimic of LnCaP cells (upper) and relative quantification analysis (bottom); **c)** Western blot relative quantification analysis of AR expression in LnCaP treated with anti-miR-130b (upper) and phenotype rescue after co-trasfection with TPR-MET vector and miR-130b inhibitor (bottom). Nucleolin (NCL) was used as loading controls.

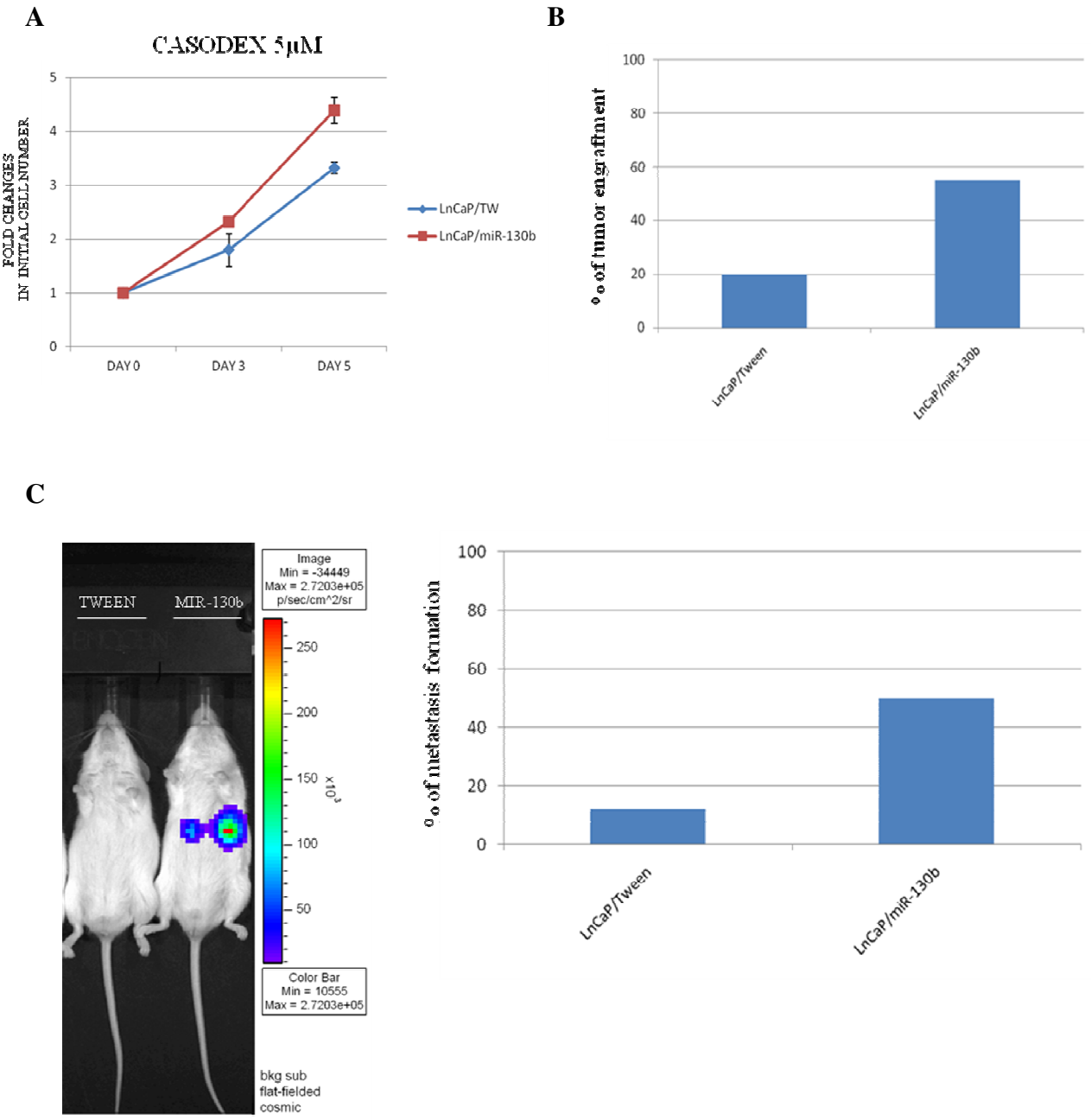
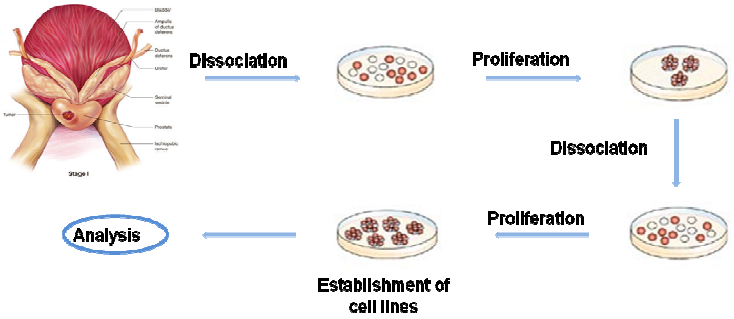


Fig.8: a) proliferation assay analysis of LnCaP/miR-130b and relative controls after Casodex (5µM) treatment; **b)** engraftment rate of LnCap/miR-130b and control cells after 3 weeks post-injection in NSG prostate; **c)** (left) IVIS representative image of mice injected intracardially with LnCaP/miR-130b and relative controls and (right) histogram showing metastasis formation rate of described cells.

A



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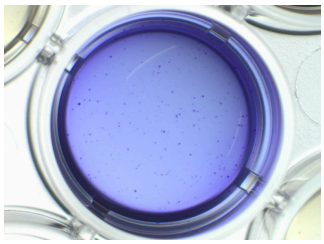

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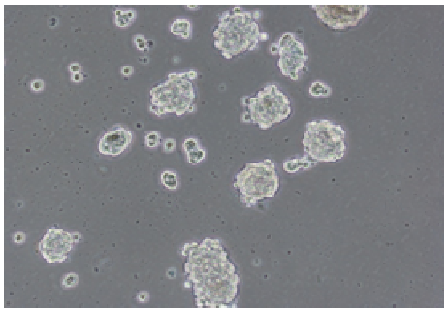

ANTIBODY	PCSC-1
CD 44	94.8%
CD 24	0.5%
CD 133	6.1%
CD 44v6	96.9%
c-Met	46.9%
c-Kit receptor	25.3%
CXCR4	22%
EGFR	59.9%
ABCG2	2.6%

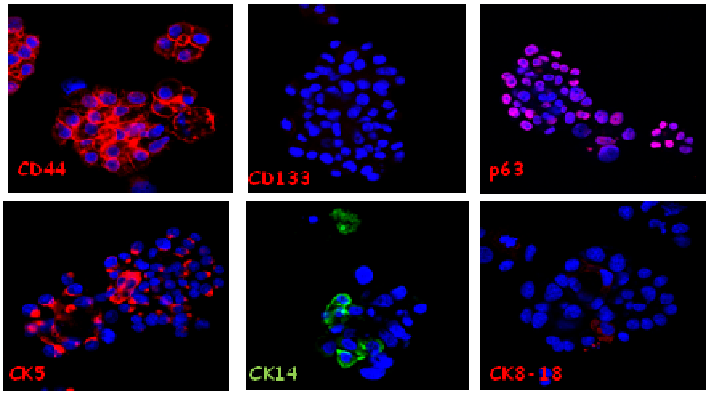

Fig9: **a**) cancer stem cells isolation: from tumor dissociation to cell line expansion and phenotyping; **b)** soft-agar assay of PCSC-1 cells; **c**) FACS (upper) and immunofluorescence (bottom) analysis show stem-associated and prostate cancer-associated marker expression profiles in PCSC-1 spheres.

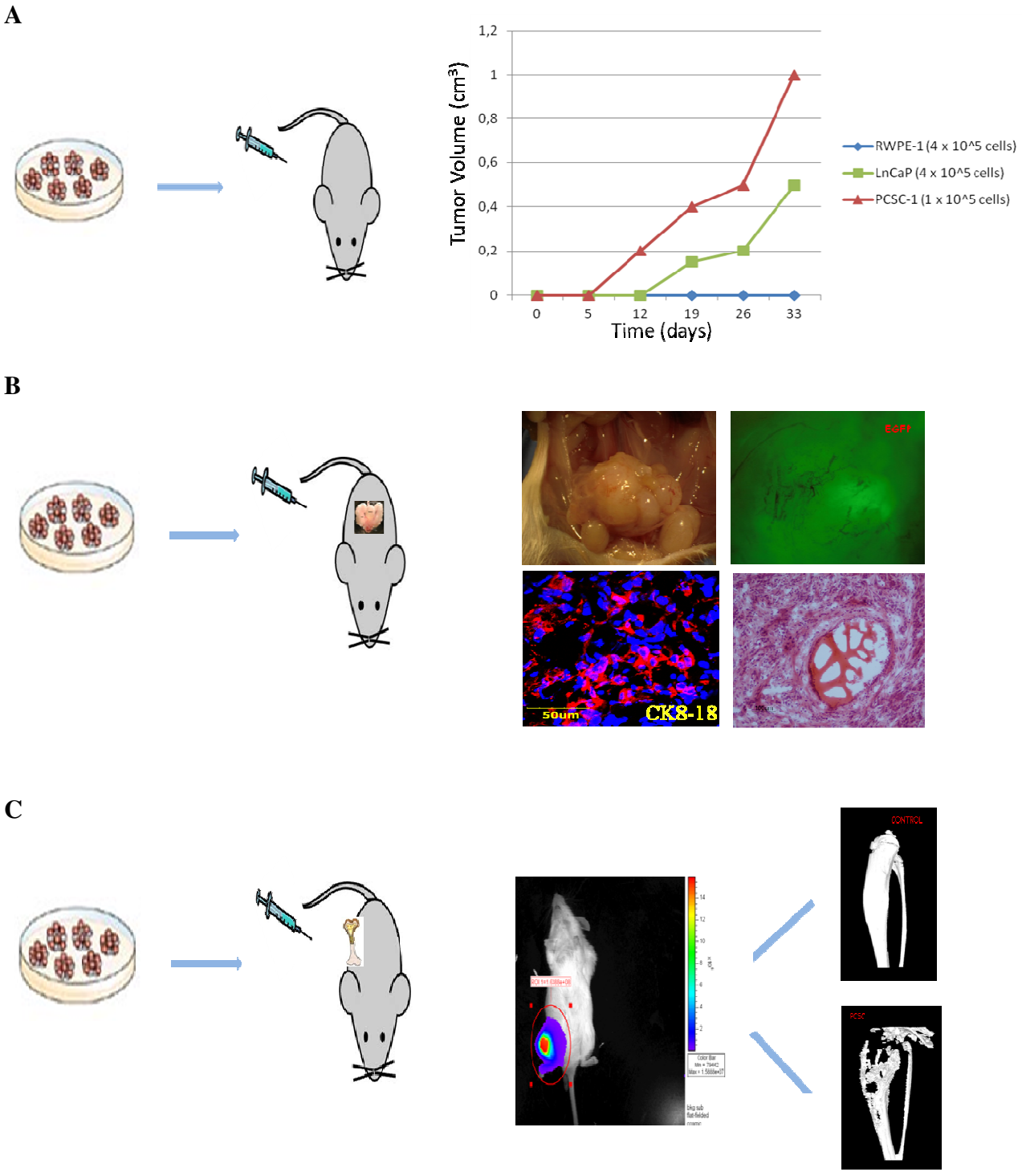

Fig.10: **a)** schematic representation of PCSC-1 subcutaneous injection (left); tumor growth rate (right) of PCSC-1 cells compared with lymph node metastasis-derived, LnCaP, and immortalized epithelial, RWPE-1 cell lines; **b)** schematic representation of PCSC-1 orthotopical injection (left); stereo microscope image showing PCSC-1 tumor mass, IF analysis and hematoxilin/eosin staining showing differentiation of PCSC-1 cell line (right); **c)** schematic representation of PCSC-1 intratibial injection (left); microCT analysis (right) showing skeletal lesions caused by PCSC-1 cells.

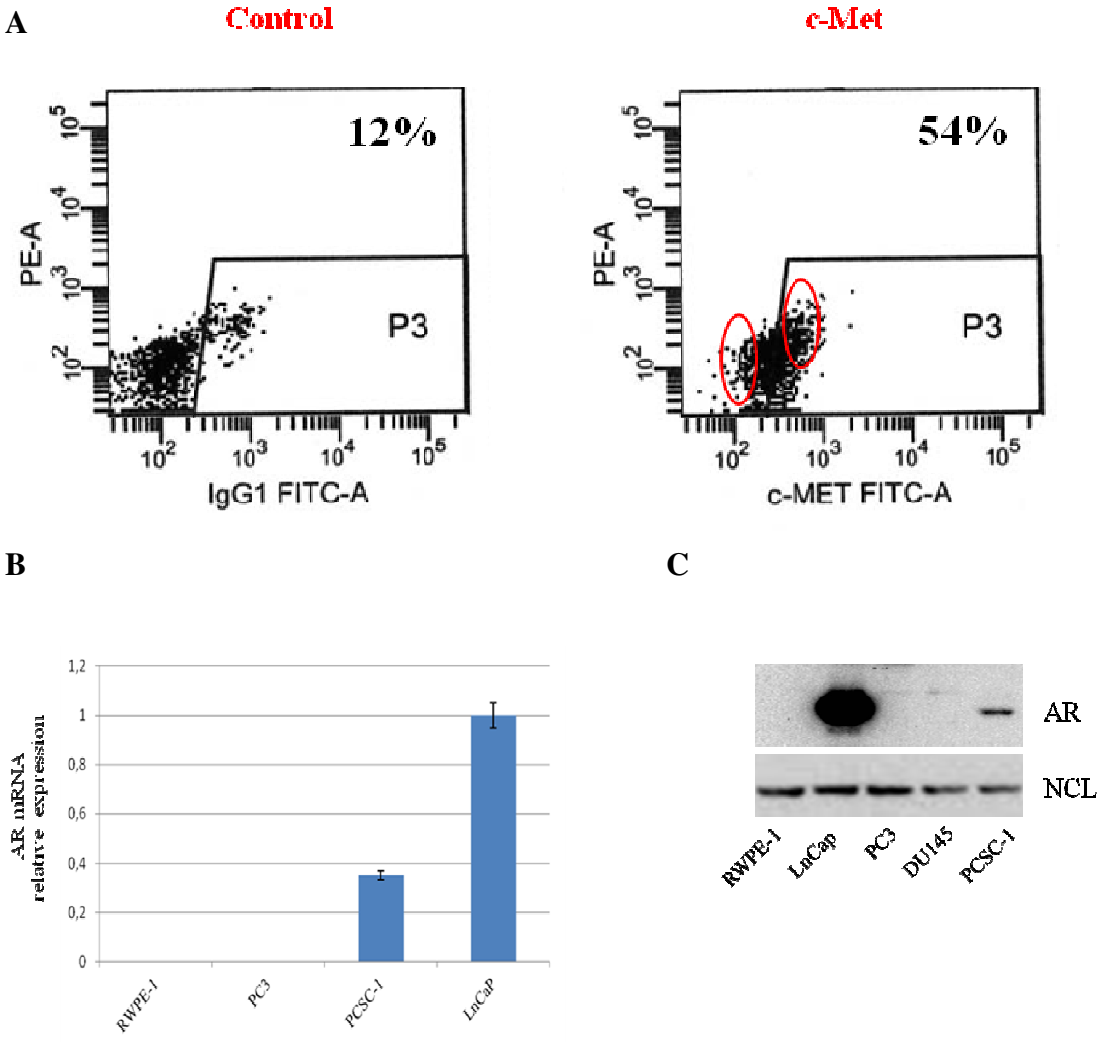

Fig.11: a) flow cytometry analysis of c-Met expression in PCSC-1 population. **b)** representative Real-Time and **c)** Western Blot analysis showing AR relative expression in PCSC-1 cells and prostate cancer cell lines.

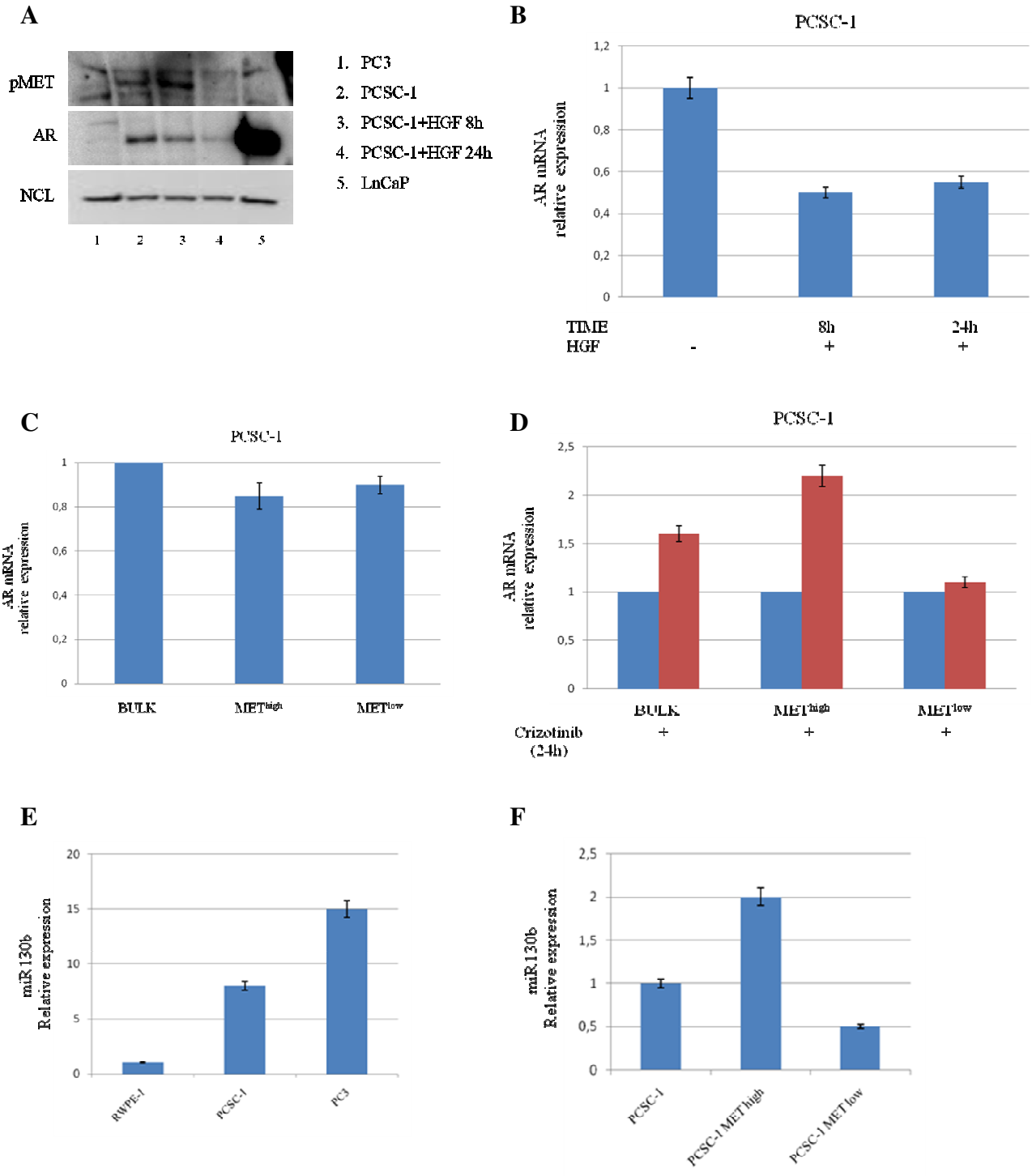

Fig.12: a) Western Blot and **b)** qRT-PCR analysis showing AR relative expression in PCSC-1 cells after HGF treatment; **c)** AR RNA levels in PCSC-1 and PCSC-1 sub-population MET high and MET low; **d)** AR RNA levels in PCSC-1 cells and PCSC-1 MET^{high} (+) and MET^{low} (-) sub-population after treatment with Crizotinib; **e)** qRT-PCR analysis of miR-130b in PCSC-1 cells compared with RWPE-1 and PC3 cell lines; **f)** qRT-PCR analysis of miR-130b in PCSC-1 cells compared with MET^{high} and MET^{low} sub-populations.

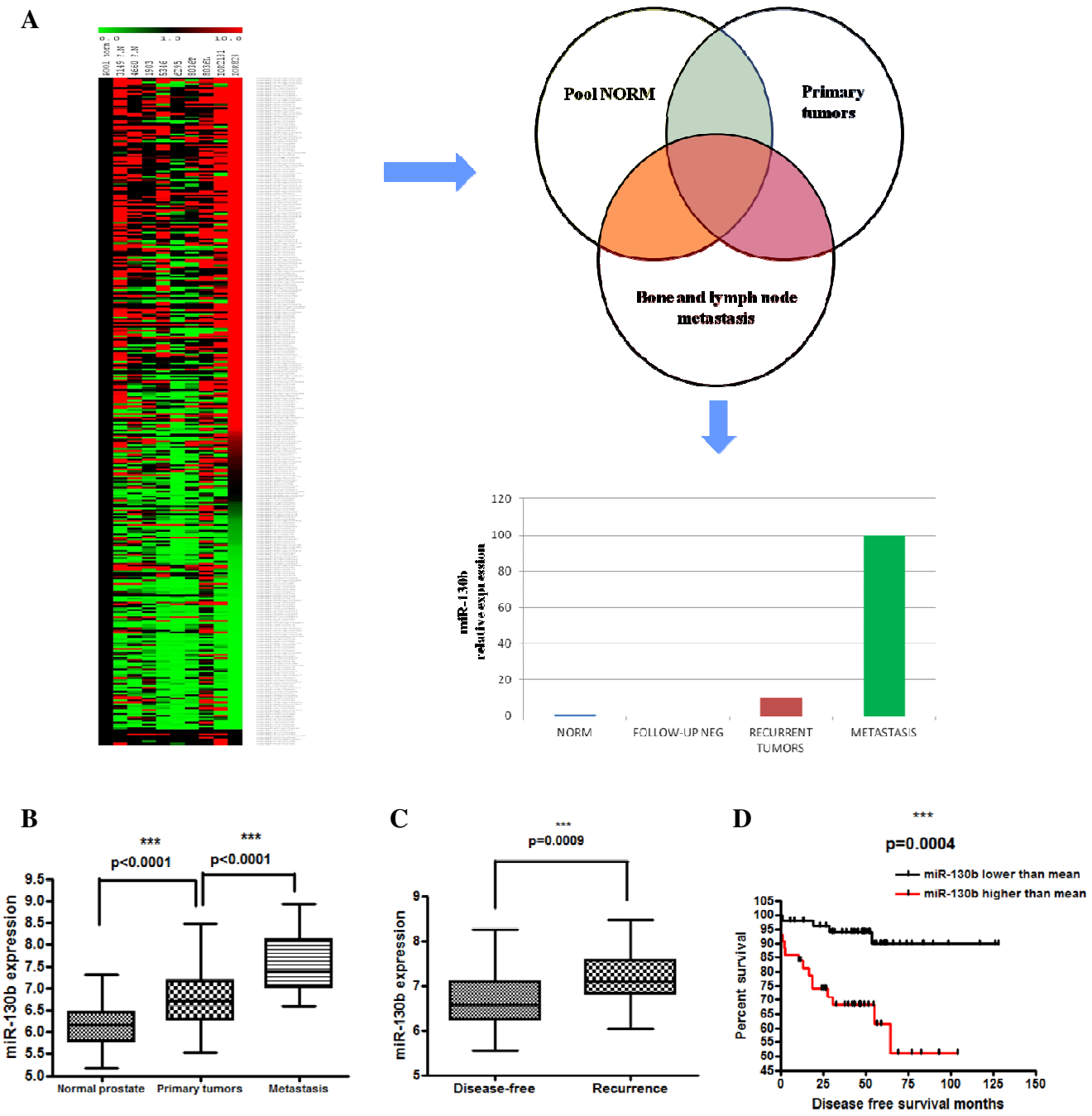

Fig.13: a) (left) MicroRNA expression profiles of microdissected-patient-derived tumor tissues of different prognosis. Normal epithelial tissue was used as control. (right) Histogram showing miR-130b direct correlation with disease progression; **b)** Box-Plot analysis showing correlation between miR-130b expression and different stages of prostate cancer progression; **c)** Box-Plot analysis showing direct correlation between miR-130b expression and recurrent patients compared with negative follow-up patients; **d)** PCa patient samples were subdivided into two groups according to low and high expression of miR-130b with mean split of all samples. A Kaplan-Meier plot representing the disease-free survival of patients was stratified.

CELL LINE	DERIVATION	PHENOTYPE
RWPE-1	Human prostate normal epithelial cell line	BASAL
RWPE-2	RWPE-1 derivation through Ki-Ras transformation	BASAL
22RV1	Human prostate carcinoma epithelial cell line	LUMINAL
LnCaP	Human prostate metastatic cell line – Lymph node metastasis derivation	LUMINAL
PC3	Human prostate metastatic cell line – Bone metastasis derivation	BASAL
DU145	Human prostate metastatic cell line – Brain metastasis derivation	BASAL
PCSC-1	Patient-derived stem cell-like line	BASAL

Table 1: prostate cancer cell lines

6 – DISCUSSION

Prostate Cancer is the second most frequently diagnosed malignancy and one of the main cause of cancer-related death in men worldwide. Early detection and localized tumors treatment have increased patients prognosis, however a significant percentage of them experience biochemical recurrence. Androgen ablation represents the first-line therapy option for recurrent and advanced disease, but most patients progress into a castration-resistant state within two years after treatment initiation, which is ultimately responsible for PCa mortality (Kirby et al, 2011). Androgens are primary regulators of normal prostate as well as cancer cell growth and proliferation. During androgen-dependent progression, prostate cancer cells depend on the androgen receptor as the primary mediator of growth and survival (Heinlein et al, 2004). On the contrary, during castration-resistant state prostate cancer develop a variety of cellular pathways to survive and grow in an androgen-depleted environment. Documented mechanisms include i) AR gene amplification or mutations (Taplin et al, 1995; Koochekpour et al, 2010), ii) ligand-independent activation (Yuan et al, 2009), iii) alternative molecular pathways (Dutt et al, 2009) and iiii) the involvement of tumor stem cells (Sharifi et al, 2006). In particular, the last two topics are becoming more attractive subjects to study, supplying new strategies for target-therapy and biomarker validation. Among alternative de-regulated pathways in prostate cancer, we focused on HGF/c-Met signaling which regulates multiple cellular processes that stimulate cell survival, proliferation and invasion (Benvenuti et al, 2007). Moreover, c-Met receptor has been implicated in CRPC, raising during androgen-ablation and conferring to the cells a stem-like phenotype (van Leenders et al, 2011). Unfortunately, in spite of experimental and clinical data demonstrating an improvement in prognosis of a sub-group of patients after c-Met targeting, the role of this receptor in PCa remains unclear with conflicting reports over its function in tumor development and progression. In fact, Met expression seems to increase during prostate disease progression, but the correlation of this receptor with Gleason grade has been tenuous. Approximately 50% of localized cancers (and even more metastatic cancers) express c-Met. Moreover, no correlation between Met expression and disease progression, in a 5-year follow up period indicating that HGF receptor doesn't represent a useful prognostic marker for stratifying patients (Jacobsen et al, 2013; Knudsen BS et al, 2002). In this study, we propose a sophisticate model of CRPC in which c-Met receptor is able to negatively regulate AR expression through a miRNA-mediate mechanisms, leading to an increase of tumorigenic

properties and androgen-deprivation resistance. In order to prove our assumptions, we firstly evaluated c-Met effects in our prostate cancer experimental models, 6 cancer cell lines representative of tumor progression and 1 patient-derived stem-like population, focusing in particular in the possible c-Met/AR circuitry responsible for disease progression. According to the literature, we found a strong expression of c-Met in basal cells while luminal cells, such as LnCap and 22Rv1, resulted completely negative after protein and RNA analysis. The opposite results were obtained when we estimated AR levels confirming the negative correlation between the two receptors. In our model c-Met expression seems to be independent of tumorigenic properties since no relevant variations were reported among low-aggressive and high-aggressive cell lines. On the contrary, when we evaluated the activation of the receptor through immunofluorescence analysis, we found a strong increase in Met phosphorylation in metastatic cell lines compared with non-tumorigenic cells. Interestingly, the majority of activated receptor appeared to be dispersed in cytoplasm rather than membrane. Moreover, they seem to be included in distinct microvescicles which were previously reported as part of a crucial mechanism regulating RTK-dependent tumorigenesis (Joffre et al, 2011). Based on these data, we hypothesized that c-Met role in tumor progression is not due to its expression but mainly on its activation state. In order to verify the effects of functional receptor, we transduced negative LnCaP cells with an engineerized vector carrying a constitutive active c-Met and its relative control. Surprisingly, we observed a strong reduction of AR protein concomitant to c-Met activation. Since there are no evidence of AR regulators induced by HGF receptor, we hypothesized an epigenetic-mediated regulation, focusing in particular on microRNA pattern. MicroRNAs are small 20-25 nucleotide-long RNA which are able to govern almost all molecular mechanisms of the cell, spacing from metabolism to apoptosis. MiRs involvement in disease development and progression is a well-proven assumption, in particular for cancer pathology where they are divided in oncomiRs and tumor-suppressor miRs (Lee et al, 2009). Moreover, these small RNA can act as biomarkers for identifying tumor progression since they are easy detectable in body fluid such as blood and urine (Sita-Lumsden et al, 2013). In order to verify our assumptions, we analyzed changing in microRNA expression profiles in Met-transduced-LncaP cells and their relative controls. Among positively de-regulated miRs we found tumorigenic-associated RNAs, such as miR-221 and miR-222, and stemness-associated miRs, such as miR-17-92 cluster and miR-181 family members. We focused on miR-130b which was previously reported to act as an oncomiR, conferring to the cells stemness properties

and tumorigenic capabilities. Moreover, this microRNA was reported as a putative regulator of AR expression based on TargetScan prediction software. Firstly, we analyzed miR-130b expression in prostate cancer lines. Accordingly with previous results, significant microRNA level was found in basal-phenotype Met-expressing cells while luminal cells, such as LnCaP and 22Rv1, did not present detectable values of it. Since miR-130b expression is higher in both metastatic cell lines PC3 and DU145 than in low-aggressive cells, it seems to be directly correlated with c-Met activation state and tumor progression. Interestingly, when we treated PC3 cells with SU11274, which is a class I c-Met inhibitor that competes with ATP to bind to the activation loop of the receptor, we observed a strong decrease in miRNA expression confirming its dependence on receptor activation. The close relationship between c-Met and miR-130b was also evaluated in PCSC-1 stem-like population, which represents a more reliable experimental models. MiRNA expression in bulk population and sorted MET^{high} and MET^{low} sub-populations were compared and, as expected, MET^{low} cells showed lower level of miR-130b than higher receptor counterpart and bulk population. In order to verify the biological effects of miR-130b, we stably transduced LnCaP cells, which we have previously reported as a low-expressing miRNA cell line, with a lentiviral vector carrying the genomic miR-130b locus. qRT-PCR analysis showed an increase of genes correlated with invasiveness, such as IL-11 and CXCR-4, and metastatic spread to the bone, such as RUNX2 and RANKL, which still represent an unresolved question for prostate cancer management (El-Amm et al, 2013). Moreover, miR-130b overexpressing cells presented increased levels of genes involved in survival signaling, such as Bcl-XL and Mcl-1, whose genomic amplification was shown to be correlated with CRPC (Krajewska et al, 1996; McDonnell et al, 1992). Interestingly, we observed in these cells a reduction of AR protein levels accordingly with our previous hypothesis. The same results were obtained in LnCaP cells transiently transfected with a synthetic miR-130b mimic. On the contrary, miR inhibitor treatment was able to counteract the effects of c-Met activation in LnCap cells, confirming Met-mediated targeting. Based on these results, we decided to evaluate the role of miR-130b in castration-resistant state acquisition. Active cell death, or apoptosis, plays a central role in maintaining tissue homeostasis and proper disposal of damaged cells, including the epithelial cells of the prostate after castration or administration of antiandrogens. Casodex (Bicalutamide) is a non-steroidal antiandrogen which competitively antagonizes the action of androgens of both testicular and adrenal origin by binding to cytosol androgen receptor in the target tissue. In addition, bicalutamide accelerates

the degradation of the androgen receptor (Waller et al, 2000) triggering tumor cells to undergo apoptosis (Floyd et al, 2009). We treated LnCaP/miR-130b with Casodex and verifying changes in the proliferation rate. Data obtained demonstrated that miR-overexpressing cells display an higher proliferative capacity than their counterpart, partially avoiding drug-induced apoptotic stimuli. The effects of miR-130b where further analyzed *in vivo*. Transduced LnCaP cells were ortothopically injected in immunocompromised mice. After 3 weeks post-injection, engrafment rate was higher in miR-130b overexpressing cells compared with relative controls, with 50% of mice presenting significant luciferase signal at IVIS system. Moreover, LnCaP/miR-130b were able to survive in blood flow and form metastasis to distant organs. Finally, we moved into a clinical setting analyzing miR expression in microdissected formalin-fixed paraffin-embedded primary tumor tissues, including recurrent and negative follow-up patients, and metastatic tissues. We observed a direct correlation between miR-130b levels and tumor recurrence and metastasis. Furthermore, we evaluated miR expression in an available online large dataset of prostate cancer patients carrying information about their RNA profiles. *In silico* analysis validated our experimental model, giving a statistical significance to data obtained.

PSA screening is widely used for prostate cancer diagnosis and its introduction in routinely performed clinical protocols contributed to increase early-stage cancer detection, as compared woth digital rectal examination (Hoffman, 2011). However, there are no evidence that testing reduces the risk of death from prostate cancer. Most abnormal PSA values represent false positive results that can be caused by benign prostatic hyperplasia, prostatitis or cystitis, perineal trauma, or the recent use of instruments for testing or surgery in the urinary tract. At the same time, normal PSA values cannot exclude neoplastic transformation of prostatic gland (Thompson et al, 2004). Therefore, the identification of new predictive and specific biomarkers for patient stratification and therapy-responsiveness prediction should represent some of the main goals for increasing prostate cancer management. In this study, we provided evidence of the role of Met-induced miR-130b as a new putative biomarker for prostate cancer screening, distinguishing patients who will develop recurrence after prostatectomy from patients carrying indolent tumors. Moreover, the effects of miR-130b in casodex resistance acquisition could be useful to discriminate patients who will benefit from androgen-blockade therapies and chemotherapy, avoiding over-treatment and quality-of-life impairment.

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