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2020

Document Version:
Publisher's PDF, also known as Version of record

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Anson Ku

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DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Building 302 Lecture hall, Medicon Village, Lund
Date 2020-02-28 and time 0900.

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Faculty of Medicine
Translational Cancer Research
Medicon Village, Lund
Author: Anson Ku

Document name
Doctoral Dissertation

Date of issue

Sponsoring organization

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In all, this work contributes to PCa research by paving a new way to isolate and sequence the miRNA content from urinary EVs. This will provide a method for the routine use of EVs via liquid biopsy to better stratify patients in the hopes of reducing unnecessary tissue-biopsies and over-treatment of PCa.

Key words

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language
English

ISSN and key title

ISBN

Recipient's notes

Number of pages

Price

Security classification

Harnessing urinary extracellular vesicles microRNA by acoustic trapping as potential biomarkers for prostate cancer

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Cover photo by Anson Ku

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Department of Translational Medicine, Lund

ISBN 978-91-7619-882-7
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2020

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MADE IN SWEDEN

To my family and to Adam,

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I ***Ku A**, *Lim HC, Evander M, Lilja H, Laurell T, Scheding S, Ceder Y. Acoustic Enrichment of Extracellular Vesicles from Biological Fluids. Anal Chem. 2018;90(13):8011-9.
*Equal contributors

II **Ku A**, Ravi N, Yang M, Evander M, Laurell T, Lilja H, Ceder Y. A urinary extracellular vesicle microRNA biomarker discovery pipeline; from automated extracellular vesicle enrichment by acoustic trapping to microRNA sequencing. PLoS One. 2019;14(5):e0217507.

III **Ku A**, Fredsøe J.H, Sørensen K.D, Evander M, Laurell T, Lilja H, Ceder Y, High-throughput and automated acoustic trapping of extracellular vesicles from clinical prostate cancer urine samples. Manuscript

IV **Ku A**, Voss G, Lilja H, Ceder Y. The interplay between the androgen receptor and Hypoxia-inducible factor 2A on extracellular vesicle secretion from prostate cancer cells. Manuscript

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The four papers below are the focus of this dissertation:

I Pillarsetty N, Jhaveri K, Taldone T, Caldas-Lopes E, Punzalan B, Joshi S, Bolaender, A., Uddin, M. M., Rodina, A., Yan, P., **Ku, A.**, Ku T, Shah SK, Lyashchenko S, Burnazi E, Wang T, Lecomte N, Janjigian Y, Younes A, Batlevi CW, Guzman ML, Roboz GJ, Koziorowski J, Zanzonico P, Alpaugh ML, Corben A, Modi S, Norton L, Larson SM, Lewis JS, Chiosis G, Gerecitano JF, Dunphy MPS. Paradigms for Precision Medicine in Epichaperome Cancer Therapy. Cancer Cell. 2019;36(5):559-73.e7

II Thorek DLJ, **Ku A**, Mitsiades N, Veach D, Watson PA, Metha D, Strand SE, Sharma SK, Lewis JS, Abou DS, Lilja HG, Larson SM, McDevitt MR, Ulmert D. Harnessing Androgen Receptor Pathway Activation for Targeted Alpha Particle Radioimmunotherapy of Breast Cancer. Clinical Cancer Research. 2019;25(2):881-91

III McKnight BN, Kuda-Wedagedara ANW, Sevak KK, Abdel-Atti D, Wiesend WN, **Ku A**, Selvakumar D, Carlin SD, Lewis JS, Viola-Villegas NT. Imaging EGFR and HER3 through (89)Zr-labeled MEHD7945A (Duligotuzumab). Scientific Reports. 2018;8(1):9043

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Abbreviations

3'-UTR – 3'-untranslated region
4K-score – four kallikreins score
α-SMA - α-smooth muscle actin
A2M – α2-Macroglobulin
A4F – asymmetric-flow field-flow fractionation
aa – amino acid
ACT – α1-antichymotrypsin
ADT – androgen deprivation therapy
AFM – anterior fibromuscular
APC – antigen presenting cell
AR – androgen receptor
ARE – androgen response element
ARNT - aryl hydrocarbon receptor nuclear translocator
AS – active surveillance
AUA – American Urological Association
BCR – biochemical recurrence
bHLH – basic helix-loop-helix
BPH – benign prostate hyperplasia
BOO – bladder outlet obstruction
Bx – biopsy
CAF – cancer-associated fibroblast
CAP – Cluster Randomized Trial of PSA Testing for Prostate Cancer
CSS – charcoal-stripped serum
CM – conditioned media
CNA – copy-number alteration
cPSA – complexed prostate-specific antigen
CT – computed tomography
d-box – distal box
DAMP – damage-associated molecular pattern
DBD – DNA binding domain
DC – dendritic cell
DE – differential expression
DHT or 5α-DHT – (5α)-Dihydrotestosterone
DLS – dynamic light scattering
DRE – digital rectal examination
ECM – extracellular matrix
EGFR – epidermal growth factor receptor
ERSPC – European Randomized Study of Screening for Prostate Cancer
ESCRT - endosomal sorting complex required for transport
ETS – E26 transformation-specific

EVs – extracellular vesicles
FBS – fetal bovine serum
FC – flow cytometry
fPSA – free prostate-specific antigen
GS – Gleason score
GWAS – genome-wide association study
HGPIN – high grade prostatic intraepithelial neoplasia
HRS – hepatocyte growth factor-regulated tyrosine kinase substrate
IPSS – international prostate symptom score
ISEV – International society of extracellular vesicles
KLK2 – Kallikrein Related Peptidase 2
LBD – ligand binding domain
LBP – ligand binding pocket
mCRPCa – metastatic castration-resistant prostate cancer
miRISC – miRNA-induced silencing complex
miRNA – microRNA
MRI – magnetic resonance imaging
MVs –microvesicles
NGS – next-generation sequencing
NNS – number needed to screen
NNT – number needed to treat
NTA – nanoparticle tracking analysis
NTD – N-terminal domain
NTS – nanoparticle tracking analysis
ODD – oxygen-dependent degradation domain
OS – overall survival
p-box – proximal box
PAP – prostatic acid phosphatase
PAS – PER-ARNT-SIM
PCa – prostate cancer
PCA3 – prostate cancer antigen 3
PEG – polyethylene glycol
PHI – Prostate Health Index
PLD2 – phospholipase D2
PIN – prostatic intraepithelial neoplasia
PLCOS - U.S. Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial
polyQ – poly-glutamine
pre-miRNA – precursor microRNA
proPSA – precursor prostate-specific antigen
PRR – pattern recognition receptor
PS - polystyrene
PSA – prostate-specific antigen
RCT – randomized clinical trial

RT – room temperature
rt-qPCR – real time quantitative polymerase chain reaction
SEC – size exclusion chromatography
SHBG – sex hormone-binding globulin
SNP – Single nucleotide polymorphism
siRNA – small interfering RNA
sEVs – small extracellular vesicles
T – testosterone
TAD – transactivation domain
tau – transactivation unit
TEM – transmission electron microscopy
TGF-β - transforming growth factor-β
tPSA – total prostate-specific antigen
Tregs – regulatory T-cells
tRPS – tunable resistive pulse sensing
TRUS – transrectal ultrasonography
TSG101 – tumor susceptibility gene 101
UC – ultracentrifuge/ultracentrifugation
uEVs – urinary extracellular vesicles
UGS – urogenital sinus
UGM – urogenital mesenchyme
USPSTF – United States Preventive Service Task Force
VAMP – vesicle-associated membrane protein
VEGF – vascular endothelial growth factor
VHL – Von Hippel-Lindau

Abstract

Prostate cancer (PCa) is an interesting study case of clinical management of uncertainty. PCa is the most diagnosed but only the second-leading cause of non-cutaneous cancer-related mortalities among men in developed countries. To put simply, the life-time risk of PCa diagnosis and mortality is approximately 11% and 3% respectively; a ~3.6-fold difference. The large number of PCa incidence is attributed to the routine use of a serum biomarker known as prostate-specific antigen (PSA) produced by the prostatic lumen epithelium. PSA is a highly sensitive biomarker for detecting general prostate perturbations such as prostatitis, benign prostate hyperplasia (BPH) and PCa but it cannot easily distinguish them at low serum concentration. To confirm PCa diagnosis, prostate tissue biopsy is still required today. Thus, the screening strategy today creates a large pool of men that will go through PSA testing, prostate biopsy and, possibly, treatments that may not die as a result of the disease. In-addition to the unnecessary interventions, the economic and psychological toll of PCa diagnosis and treatment is burdensome to individuals. There is no doubt that PSA testing can save lives but PCa screening today can benefit from additional biomarkers in multiple areas. First would be to distinguish men likely to harbor clinically significant PCa and therefore require biopsies versus those that do not. Second, those that will likely progress to high grade or malignant disease thus requiring clinical intervention versus those that do not.

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Prostate

Overview

The prostate is the largest accessory gland of the male reproductive system. The main function of the prostate is to produce prostatic fluid that, in conjunction with seminal secretions form the major constituent of semen during ejaculation which protects, nourishes, and releases sperms from gel-matrix for fertilization. In human, the prostate gland is situated inferior to the bladder and anterior to the rectum. It envelops the descending urethra where prostatic fluid is discharged into via the prostatic ducts. Nourishment of the prostate is supplied by the prostatic arteries branched from the internal iliac arteries and drained by the prostatic venous plexus and periprostatic lymph vessels to the internal iliac nodes and the obturator.

Development

The prostate development commences at around 9 - 10 weeks of gestation and slows after birth (1). Prostate morphogenesis originates from the urogenital sinus (UGS) as a result of circulating androgen produced by the fetal testes. Androgen binds and activates the androgen receptor (AR) expressed by the urogenital mesenchyme (UGM) which triggers the budding of epithelial cell cords from the ventral, lateral and dorsal aspect of the UGS via paracrine signaling.(2, 3) In addition, the local conversion of testosterone (T) to 5α-dihydrotestosterone (5α-DHT or DHT), a potent agonist for AR, by 5α-reductase plays an essential role as *null*-mutation of 5α-reductase can significantly or completely abrogate the development of the prostate.(4) As the epithelial cords elongate, concurrent branching and canalization of the epithelial cord occurs at around 11 weeks and after. Canalization begins from the UGS and proceed towards the proliferating epithelial tip, thus transforming the solid epithelial cord into luminal ducts. It is during canalization that epithelial cells begin to differentiate into luminal and basal subtype remarkable by their expression of keratins 8/18, AR and NKX3.1 for luminal cells and keratins 5/14 and TP63 for basal subtype while the adjacent mesenchyme differentiates into smooth muscles. Separately, a third population of cells, known as neuroendocrine, which are predominantly of neural crest origin, expressing serotonin and chromogranin A can

be found beginning at 13 weeks of gestation.(5) At 19 weeks, a continuous layer of α-actin-positive smooth muscle layer can be found to encompass the nascent prostate that eventually becomes the anterior fibromuscular layer (6).

Prostate architecture and cellular makeup

A physiologically normal prostate is comprised of functionally distinct zones known as the central, transition and peripheral zones as defined by McNeal.(7) The peripheral zone comprises 70% of the prostate with loose stromal components, whereas the central and transition zones account for 25% and 5% of the prostate respectively and has dense stromal component (Table 1**Error! Reference source not found.**).(8) The prostate is encapsulated by the tough anterior fibromuscular layer comprised of fibrous and smooth muscular elements.

Table 1
Origin and characteristics of a normal adult prostate

Zones	Proportion of normal adult prostate	Stroma	Embryonic Origin
Peripheral	70%	Loose	Urogenital Sinus
Central	25%	Dense	Wolffian Duct
Transition	5%	Dense	Urogenital Sinus

At the basic level, a normal adult prostate is organized into tubuloalevolar structure that terminates at the acinus in the distal end from the prostatic urethra. The acinus cavities are lined by tall columnar secretory luminal cells that produce prostatic fluids. Histologically, luminal cells express cytokeratin-8 (CK8), the androgen receptor (AR) and are dependent on androgen for proliferation. They secrete proteins such as PSA, Kallikrein Related Peptidase 2 (KLK2) and prostatic acid phosphatase (PAP) with putative role of liquifying semen. Luminal cells are supported by a layer of basal cells that maintain the basal lamina which separates the epithelial compartment from the stromal compartment. Basal cells are characterized by the expression of CK5, CK15 and the transcription factor p63. In-addition, rare cells such as neuroendocrine cells, luminal progenitor and basal progenitor cells with capacity for luminal and basal cells renewal are present in small numbers.(9, 10)

Beyond the epithelial compartment lies the stromal compartment. The function of the prostate stroma is thought to maintain the architecture through the extracellular matrix (ECM). The cellular composition of the stroma is predominantly fibroblast and smooth muscle cells depending on location. They are supported by other cell types such as endothelial cells, neuronal cells and leukocytes.(11) In the stroma, AR is expressed in a subset of smooth muscle cells and fibroblast. Though the role of

AR in these cells remains unclear, tissue recombination studies suggest and that they have anti-proliferative effect on secretory luminal cells.(12, 13) Other studies have also observed that AR expression is significantly diminished in stroma of PCa samples and portend worst prognosis.(14) Therefore, the prostate stroma is likely to play a key role in maintaining the delicate balance of growth and cell death through paracrine signaling as evident during prostate development.

Androgen receptor and signaling

The androgen receptor (AR) is a hormone nuclear receptor located in the long arm of the X chromosome (Xq11:Xq12). The AR locus contains eight exons that code for a nominal protein size of 919 amino acids or 119 kDa. In the unbound state, AR is found in the cytoplasm associated with HSP70 and HSP90 chaperone proteins. Binding of free circulating T or DHT leads to the disassociation of co/chaperones proteins and translocation into the nucleus where they recruit additional coactivators, homodimerizes and binds to DNA response elements and activates the androgen-specific targets by binding to the androgen response element (ARE) : 5′-GGTTCT-3′, **Figure 1**). In the prostate, downstream target genes of AR activation include TMPRSS2, and the glandular kallikrein family of serine proteases such as KLK2 and PSA.

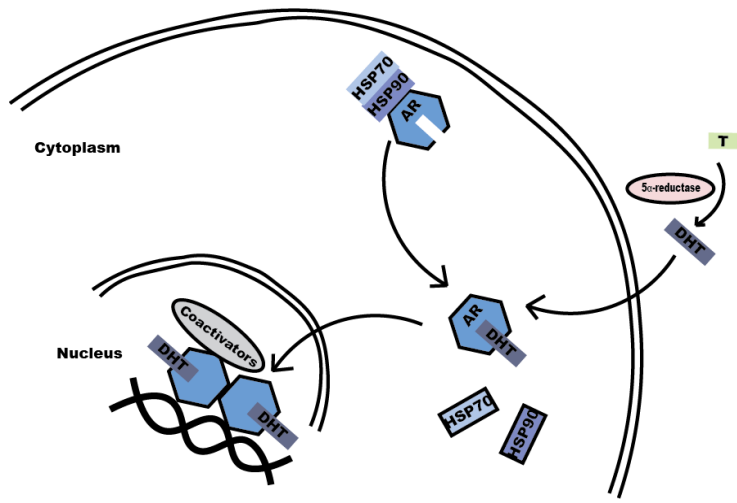

Figure 1. Illustration of testosterone (T) conversion into 5α-dihydrotestosterone by 5□-reductase followed by intracellular binding to androgen receptor (AR). Following AR activation, it is translocated into the nucleus and activate the androgen response element after homodimerization.

The AR protein has multiple functional domains including the N-terminal domain (NTD), DNA binding domain (DBD), hinge region and c-terminal ligand binding domain (LBD) (**Figure 2**). The NTD comprises 60% of the amino acids (aa) sequence of AR. The NTD executes the transactivation function in AR via the constitutively active, activation function 1 (AF1) that interacts with coactivators and the polymorphic region consisting of varying length of polyglycine or notably polyglutamine (polyQ) repeats that imparts functional diversity across human population.(15) Within AF1, the transactivation unit 1 (Tau-1) and Tau-5 contain the FQNLF nuclear receptor box and WHTLF motif that mediate the ligand-dependent interaction between the NTD and LBD as well as the ligand-independent p160 coactivators recruitment respectively that ultimately leads to the stabilization of the AR homodimer and transcriptional function.(16) The normal polyQ repeat contains anywhere from 8 – 31 repeats of glutamine. Longer repeats, up to 40 or more, can lead to spinal and bulbar muscular atrophy while shorter repeats increases susceptibility to prostatic neoplastic transformation.(17, 18) Mechanistic studies revealed that the deletion of the polyQ repeat increases AR activation by up to four-fold through its interaction with the LBD and coactivators, SRC-1e leading to an increased transactivation activity.(19, 20) Interestingly, deletion studies have shown that the NTD is constitutively active in the absence of LBD and retains full transcriptional activity within Tau-1 and Tau-5.(21)

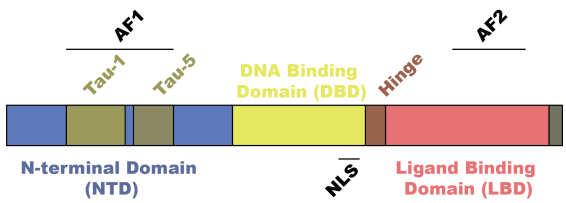

Figure 2. Illustration of androgen receptor protein functional domains. N-terminal domain (NTD) in blue harboring the activation function-1 (AF-1) in olive green, domain essential for interaction with coactivators. Next to the NTD is the DNA binding domain (DBD) that interacts with the DNA after activation. The DBD also contains the nuclear localization signal that is exposed after androgen binding. Lastly, the c-terminal sits after hinge region. It contains the ligand binding domain, the site where androgen binds to.

The DBD is a highly conserved region among the steroid receptor family that includes estrogen and progesterone receptor. The DBD of AR harbors two zinc fingers, four cysteine residues in each that coordinate a zinc ion to stabilize the folding of the protein structure. The N-terminal zinc finger contains a highly conserved proximal-box (p-box) that directly binds to the major groove of the DNA response element (two, hexameric half-site 5'-AGAACA-3' separated by a 3-nucleotides spacer: IR3) whereas the second zinc finger contains the distal-box (d-box) that interacts with the zinc finger on the second AR dimer. Upon, androgen binding, the AR changes conformation exposing a nuclear localization signal (NLS) located between the DBD and hinge region. Nuclear translocation is initiated by the

association of importin-α with the NLS that traffic the ligand-bound AR to the nucleus.

The LBD harbors the activation function 2 (AF-2) consisting of eleven α-helices and two anti-parallel β-sheets. Three helices (H5, H10 and H11) comes together to form the major part of the hydrophobic ligand binding pocket (LBP) and a fourth (H12) that closes the pocket after ligand binding. After nuclear translocation, the LBD is essential for stabilization of the AR homodimerization through interaction of the helix loop (H5) on each of the LBD. The ligand-induce conformation enables the NTD/CTD interaction and recruitment of AR co-regulators. Mutation in the H5 of the LBD region has been shown to cause androgen insensitivity disorder.(22) There are two natural agonists for AR, T and DHT with differing affinities (Table 2**Error! Reference source not found.**). In circulation of healthy male, the concentration of T is usually much higher than DHT and are bounded to sex hormone-binding globulin (SHBG) and albumin. The bioavailability of androgen is dependent on free androgen, *i.*e unbound to carrier proteins. T is the precursor for DHT synthesis by 5α-reductase, thus there is a strong correlation between serum of T and DHT levels in eugonadal men.(23)

Table 2
Agonists of AR and their serum concentration in eugonadal men

Agonists	Affinity (nM)	Unbound Serum Concentration (nM)
Testosterone (T)	0.29 – 0.49 (24)	2.9 – 33.9 (25)
5α-Dihydrotestosterone (DHT)	0.24 – 0.34 (24)	0.23 – 7.3 (25)

Pathologies of the Prostate

There are three common male urological afflictions that account for the majority of urological visits. They are prostatitis, benign prostate hyperplasia (BPH) and PCa but our discussion will only be focused on BPH and PCa. For an in-depth review of prostatitis, refer to (26).

Pathologies of the Prostate – Benign prostate hyperplasia

Benign prostate hyperplasia (BPH) is a common, non-lethal, lower urinary tract condition affecting 15% - 60% of male greater than 40 years of age.(27) BPH is characterized by the hyperproliferation of the cells in the stromal and epithelial compartment of the transition zone surrounding the urethra; as a result, it can cause bladder outlet obstruction (BOO) with symptoms presented as either irritative

(frequent and urgency of urination and nocturia) or obstructive (weak urine stream, straining, and urinary retention). Currently, clinical diagnostics of BPH relies on a combination patient self-reporting, validated questionnaires to clinical workup. Urodynamics and the International Prostate Symptom Score (IPSS), a set of eight questions can provide insight into the level of urine flow obstruction. Digital rectal examination (DRE) performed by physician can give an indication of prostate volume while PSA testing, or imaging modality such as transrectal ultrasonography (TRUS), computed tomography (CT) or magnetic resonance imaging (MRI) to measure the prostate volume. In multiple studies, PSA has been shown to have excellent discriminatory power for prostate volume. In one study, men with serum PSA level between 2.1 – 2.5 ng/mL has 72% chance of having prostate volume >30 mL while PSA level between 4.1 – 7.0 ng/mL has 69% chance of having prostate volume >40 mL.(28)

Currently, the etiology of BPH remains unclear though modifiable and non-modifiable risk factors such as age, metabolic syndrome, obesity, sex hormone levels and diets have been identified. The incidence of BPH increases with age.(29) The prostate volume is also increased significantly in men diagnosed with metabolic syndrome such as type 2 diabetes mellitus, hypertension, dyslipidemia.(30)

Pathologies of the Prostate – Epidemiology of prostate cancer and risk factors

PCa is the most commonly diagnosed and second leading cause of cancer-related deaths among men in the United States in 2019.(31) Historically, prostate cancer incidence exploded with the introduction of PSA as a screening tool in the early 1990s followed by an equally rapid reduction of incidence (**Figure 3**). The causes were likely multi-factorial, attributable to the depletion of the sampling pool, revision of the initial screening guidelines or potentially the use to Lovastatin.(32) As expected, screening of PCa and improvement in therapies led to a decrease in overall age-adjusted mortality with five year survival increased from 88.7% in 1990 to 99.3% in 2011 in the United States.(32) The current estimate of life-time risk of PCa diagnosis and mortality is 12% and 3% respectively.

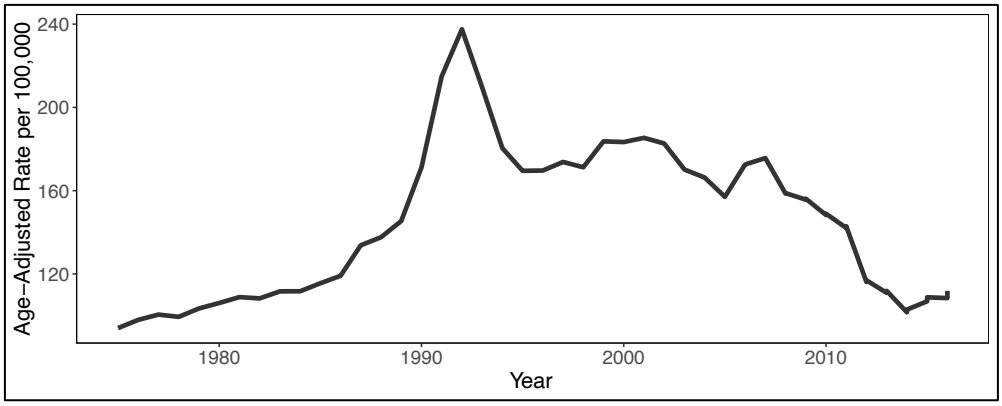

Figure 3. Age-adjusted prostate cancer incidence over time.

Recent data from the United States suggest that cancer mortality is on the rise again due, possibly, to reduction in routine PSA testing (see section "Pathologies of the Prostate – PSA screening").

There are well-established prostate cancer risks such as age, ethnicity and familial history being the most studied. The incidence of PCa increases significantly beginning at 45 – 50 years of age and peak at 70-74 years of age (**Figure 4**). Race is also a significant risk factor of PCa mortality with African American men having nearly two-fold the mortality rate compared to their white counterpart even after adjusting for socioeconomic status(33). Familial history also modifies PCa risk with diagnosis of one or more immediate family member, *e.g* brother or father significantly elevating the relative risk to greater than 2-fold.(34-36) The combination of race and familial history suggest a large component of PCa risks is due to genetics. Indeed, a recent epidemiological study of monozygotic and dizygotic Nordic twins with an median follow-up of 32 years suggested that prostate cancer has a strong inheritability component accounting for 57% of prostate cancer incidence at 100 years of age.(37) Single nucleotide polymorphism (SNP) identified by genome-wide association studies (GWAS) also pointed to overwhelming contribution of SNPs towards PCa development.(38) Furthermore, mutations in DNA repair proteins such as *BRCA1*, *BRCA2*, *CHEK2* and *ATM* can lead to the development of aggressive PCa at an early age.(39-41)

Figure 4. Figure 2. Prostate cancer incidence at different age.

In addition to genetic component, epidemiology studies have also demonstrated that extrinsic factors play an important role. Men of African descent or Asian descent living in different parts of the world display varying incidence compared to their country of origin, suggesting that diets, screening or other extrinsic factors do contribute to PCa incidence.(42) Indeed, epidemiological studies of diets high in saturated fat or β-carotene have been shown to increase risk for PCa.(43) Consumption of soy proteins or lycopene, abundant in tomatoes, have been associated with the reduction of PCa risks.(44) Separately, meta-analysis investigating the relationship between alcohol consumption with PCa and smoking with PCa revealed a dose-response association of the two habits and PCa mortality.(45, 46)

Pathologies of the Prostate – Prostate cancer, diagnosis and grading

There are many subtypes of PCa. They can be broadly categorized into adenocarcinoma (originates from the epithelium of the acini or ducts) and non-adenocarcinomas (sarcomas, neuroendocrine, small cell, transition cell carcinoma and squamous cell carcinoma) based on cell origin. Each of those subtypes is defined by different characteristics such as aggressiveness or presence of AR but owning to the large proportion of diagnosed PCa being adenocarcinomas (>99%), we will focus our discussion of PCa exclusively on prostate adenocarcinoma and use the term PCa and prostate adenocarcinoma interchangeably unless otherwise

stated. For additional insights into the rare subtypes of PCa, refer to the following reviews.(47, 48)

The development of PCa is a slow process often requiring decades whereby genetic and epigenetic alterations accumulate. Approximately 75% of detected PCa arises from the peripheral zone while 20% stems from the transition zone.(49) They are usually suspected as a result of routine DRE or PSA testing (see section: Pathologies of the Prostate – PCa Screening) and confirmed by extended sextant (10 – 12 cores) Bx. In the early stages, PCa are presented as atypical lesions known as prostatic intraepithelial neoplasia (PIN) before possibly progressing to prostatic carcinoma. PIN usually occurs as multifocal lesions in 60% - 90% (50) of the case and is thought to arise independently (51, 52). They can be sub-divided into three-grades, grade 1 (mild), grade 2 (moderate) and grade 3 (severe), the latter two are considered "high grade" PIN or (HGPIN) with high likelihood of progressing into adenocarcinoma. The transition from HGPIN to PCa occurs when lesion breach the basal cell layer and basal membrane into the stromal component of the prostate (**Figure 5**). When the tumors are locally confined within the prostate, *i.e.* no identifiable tumors in lymph nodes or distant organs, the survival rate is ~100% at 5 years however once tumors disseminate into distant organ, it is considered incurable with survival rate of 30% at 5 years.(32) Therefore, the clinical diagnosis and management of PCa can benefit from biomarkers in two ways. First, screening in order to identify the presence of high-risk PCa and therefore requiring prostate Bx for confirmation. Second, the use of biomarkers post-Bx to assess the likelihood of PCa progression and therefore requiring clinical intervention. Third, monitoring PCa treatment response.

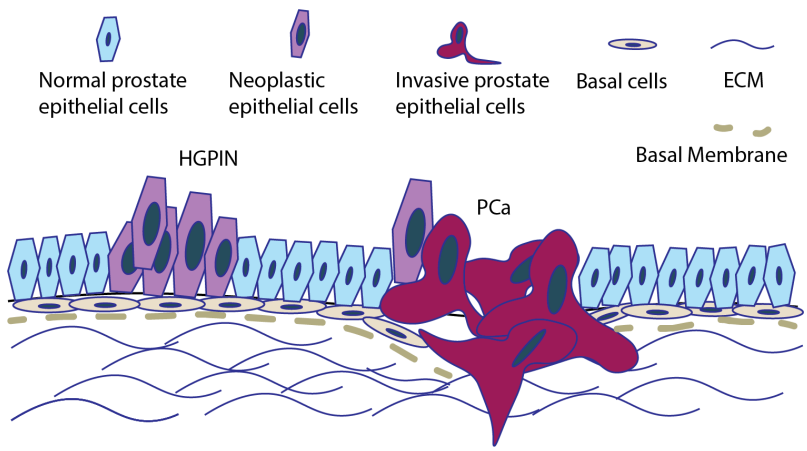

Figure 5. Illustration of normal prostate epithelial cells (blue) on top of basal cells (beigh) adjacent to the basal membrane (dashed line). Proliferating neoplastic epithelial cells (purple) form high grade prostate intraepithelial neoplasia on the left (HGPIN) and invasive epithelial cells (red) forming prostate cancer to the right.

Pathologies of the Prostate – PCa genomic alterations

The extensive integrated-omics characterization of primary PCa in recent years had revealed distinct genomic features of PCa. In all, epigenetic alteration, copy-number alterations (CNA), complex chromosome rearrangement with low rate of point mutations are defining characteristics of PCa. One of the hallmark of PCa is the fusion of downstream AR-regulated genes with transcription factors of the E26 transformation-specific (ETS) family *i.e. TMPRSS2*:*ERG* fusion being the most common via the chromosomal deletion of 21q22.2-3.(53-55) Other pathways commonly dysregulated in PCa include DNA repair, *PI3K*, *P53* and *MYC*. The PI3K pathway includes the tumor suppressor, *PTEN* and the *PIK3CA* kinase that are found to be mutated at an estimated 25% - 70% of primary PCa and 74% - 100% of metastatic PCa.(55, 56) The large incidence of PI3K pathway alteration suggested that it could be actionable. P53, a well-established tumor suppressor gene, is commonly inactivated in PCa at 5 – 40% of the localized tumors with deletion being the dominant mode of inactivation. Other rare mutation events such as SPOP (11%), FOXA1 (3%), IDH1 (1%) and others (26%) are observed in local PCa that is associated with distinct genomic alterations.(55) Separately, miRNA expression can be distinguished between ETS altered and wildtype primary PCa tumors.

The androgen axis is a central therapeutic target of PCa. Interestingly, studies showed that treatment naïve PCa harbor low level of AR alteration in comparison to metastatic castration-resistance PCa (mCRPCa).(55, 57, 58) The high level of AR alteration observed in mCRPCa is induced by the androgen deprivation or anti-androgen treatments. The selection pressure drives AR amplification and missense mutation that renders it ligand promiscuous or ligand independent. Other genes related to androgen signaling axis, such as AR coactivators, *NCOA2* and AR-associated transcription factor *FOXA1* are also found to be mutated in mCRPCa.(59) Frequent CNA of P53 and Rb1 tumor suppressors and activating mutations of PIK3CB, PIK3R1 is also associated with mCRPCa.(60)

Pathologies of the Prostate – PSA screening

PSA testing has been performed for nearly three decades beginning in the 1990s. The routine screening of PCa by PSA assay has been controversial. Systematic review of RCT of population-based total PSA screening in the USA (U.S. Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; PLCOS) and the UK (Cluster Randomized Trial of PSA Testing for Prostate Cancer; CAP) have concluded no benefit in overall survival (OS) (61, 62) or 27% reduction in PCa mortality at 13 years-median follow-up in the European Randomized Study of Screening for Prostate Cancer (ERSPC) trial (63) or 42% risk reduction in the Göteborg Trial (64).

Part of the confusion stems from the fact that the participants of the PLCOS and CAP trials had undergone prior PSA testing before enrollment which reduced the incidence of high grade PCa in those cohorts. In addition, early efforts to estimate the number needed to screen (NNS) and the number needed to treat (NNT) to save one life yielded 1254 and 43 respectively at 9 years.(65) The disappointing results led the United States Preventive Service Task Force (USPSTF) to recommend against the use of PSA as a general screening tool in 2012. Since then, PSA screening have been reduced by 9% in the USA.(66, 67) The consequences of the refrained use of PSA screening led to a moderate increase in distant stage PCa mortality (68) while other studies concluded that the clinical stage of PCa is higher on initial Bx (cT > 2b) in the post-USPSTF recommendation era (69). Consistent with the previous observation, the incidence of metastatic events observed in the active surveillance (AS) arm compared to the treatment arm (radiotherapy or radical prostatectomy) increased at 10 years.(70)

There is no doubt that PSA screening save lives in the long run.(71) The ERSPC trial, now with greater than 13 years of follow-up time had revised the NNS and NNT for PSA screening (Table 3)**Error! Reference source not found.**. With the longer follow-up time from 9 to 13 years, the NNS and NNT both decreased by nearly 50% and the benefit of screening

Table 3
Number needed to screen (NNS) and number needed to treat (NNT) to save one life data from the European Randomized Study of Screening for Prostate Cancer Study.(65)

Years of follow-up	Number needed to screen	Number needed to treat
9	1410	48
11	979	35
13	781	27

becomes more apparent. Thus, the net benefit of PSA screening will increase with longer follow-up time. It is now understood that the use of PSA screening should be a joint decision between the patient and their physician after informing the former of the benefits and risks given their susceptibility factors and life-expectancy (see section: Pathologies of the Prostate – Epidemiology of prostate cancer and risk factors).

Pathologies of the Prostate – PSA

The majority of serum PSA (80% – 90%) (72) exists covalently complexed to the α1-antichymotrypsin protease inhibitor (ACT; together known as cPSA) or as free PSA (fPSA; 10% - 20%) in an precursor (also known as proPSA), active or inactive

form.(73) PSA can also be complexed with α2-Macroglobulin (A2M) but because there is no accessible epitope to PSA, this form of complexed PSA is not detected in the clinic.(74) The combined fPSA and cPSA is known as total PSA (tPSA). Different permutations of PSA are used for the risk assessment and treatment monitoring of PCa (Table 4).

Table 4
Additional PSA-based assays for PCA risk stratification

PSA assays	Measurements
%fPSA	fPSA/tPSA
PSA-specific reference ranges	Age-specific PSA range reference
PSA velocity	Rate of tPSA increase
PSA doubling time	Doubling time of PSA (months)
PSA density	PSA level/prostate volume
PSA-based nomograms	Multiparametric risk score: age, family history, DRE, prostate volume and tPSA
PSA glycoforms	PSA glycosylation form

tPSA display remarkable sensitivity (0.78 – 1.00) for PCa however, it is limited by low specificity (0.06 – 0.66) as conditions such as prostatitis, BPH, prostate Bx and PCa have been shown to affect PSA level.(75) Studies have shown clear concordance between PCa risks and PSA level.(76) However, there is a PSA range between 1-10 ng/mL, the "grey zone" where PSA level cannot predict PCa on Bx accurately (76) though clinically significant PCa (Gleason ≥7) have found been found.(77) In or near this PSA range, fPSA/tPSA or other FDA-approved assays such as the four kallikrein score (4Kscore), Prostate cancer antigen 3 (PCA3) and Prostate Health Index (PHI) can aid in the PCa risk assessment and therefore reduce the need for unnecessary Bx (Table 5**Error! Reference source not found.**).(78)

Table 5
US FDA approved clinical assays for PCa risk assessment.

US FDA approved Commercial Assays	Assays	Materials
PHI	proPSA, fPSA	Serum
4Kscore	Intact fPSA, tPSA, klk2 and	Serum
PCA3	PCA3 RNA, PSA RNA	Post-DRE urine sediments

PCa – Diagnosis and Risk Stratification

Abnormal DRE and high PSA levels are triggers for prostate Bx. Though variations exist, 10 – 12 cores, TRUS-guided Bx with 18G needle in an extended sextant pattern parallel to the sagittal plane is the standard of care. The estimated sensitivity of TRUS-Bx are between 31% - 38% at finding clinically significant PCa.(79-81) Like all invasive procedures, prostate Bx is associated with risks of pain, bleeding

(10% – 84%), urinary retention (0.2% - 1.7%), erectile dysfunction and infections (0% – 6.3%) with rare cases of sepsis.(82) In addition, prostate Bx can exact psychological tolls and economic burdens on individuals and societies.(83) Thus, prostate Bx should only be used when clinically significant PCa is suspected.

Prostate Bx allows for histological evaluation of tissue samples to assess risk of mortality from PCa. One of the best prognostic indicator for PCa is the Gleason score (GS) system developed in 1974 by the pathologist Dr. Ronald Gleason.(84) GS began as a 5-tier grade system (grade 1-5) that reflects the cellular architecture and differentiation state of the biopsied material but have since gone through significant changes. In its current usage, the GS is derived from summation of the two most prominent histological patterns. Therefore, GS can range from 6 – 10 (3 + 3 or 5 + 5). Since multiple cores are taken from the prostate, each core could be presented with different Gleason grade, in such case, the two dominant pattern is taken. The most updated guideline from the assigns GS into 5 different prognostic groups with different risks for biochemical recurrence (Table 6). The major feature is the differentiation of GS 3+4 and 4+3 which portents different risk. In clinical practice, risk assessment is often not performed with GS alone but in combination with other

Table 6
Gleason scores (GS) prognostic groups based on Gleason grades assignment.

Gleason Score (GS)	Prognostic Group (GS group)
GS ≤ 6 = 6	Prognostic group I
GS 3+4 = 7	Prognostic group II
GS 4+3 = 7	Prognostic group III
GS 4+4=8, 3+5=8, 5+3 = 8	Prognostic group IV
GS 4+5=9, 5+4=9, 5+5=10	Prognostic group V

parameters. There are currently a number of risk assessment models developed over the years that will provide a risk score for PCa but it is beyond the scope of this thesis; for an in-depth analysis, see (85). In clinical practice, the AUA and EAU have summarized risk into different levels based on PSA, clinical grade and GS or GS prognostic groups (Table 7).

Table 7
Risk categories produced by American Urological Association (AUA) and European Association of Urology (EAU) using PSA, clinical grades and Gleason score or gleason prognostic group as parameters.

Risk Category	AUA localized disease (86)	EAU (87)
Very Low Risk	PSA <10 ng/mL & GS Group 1 & cT1-T2a & <34% of Bx cores positive & no core with >50% involved, & PSA density <0.15 ng/mL/cc	PSA < 10 ng/mL & GS < 7 & cT1-2a, Localized
Low Risk	PSA 10-20 ng/mL & GS Group 1 & cT1-2a	

Intermediate Risk	PSA 10-20 ng/mL | Grade Group 2-3 | cT2b-c Favorable: GS Group 1 (PSA 10-<20) | GS Group 2 (PSA<10) Unfavorable: GS Group 2 (PSA 10-20 | cT2b-c) | GS Group 3 (PSA < 20)	Favorable: PSA 10-20 ng/mL | GS 7 | cT2b Localized Unfavorable: PSA >20 ng/mL | GS>7 | cT2c
High Risk	PSA >20 ng/ml or GS group 4-5 or clinical stage >T3*	Any PSA Any GS cT3-4 or cN+ Locally advanced

MicroRNA

MicroRNA (miRNA) is a class of small, regulatory noncoding RNAs of 18 – 24 nucleotides (nt) in length that are highly conserved across plants and animals.(88) It was first discovered in 1993 by Lee *et al.* while studying the timing of larval development in *C. elegans*.*(89)* Today, there are over 2000 annotated human miRNAs with essential functions related to development and homeostasis. Given their role in development and tissue maintenance, it is unsurprising that tissues have different miRNA expressions (90, 91) and their importance can be appreciated as knockout of conserved miRNA can lead to embryonic or post-natal lethality in mice.(92)

Biogenesis

In eukaryotes, nascent miRNAs are canonically produced from primary miRNAs (pri-miRNA) which are primarily transcribed by RNA polymerase II, but also RNA polymerase III, in the nucleus and are several hundreds to over a thousand kilobase in length. The pri-miRNAs contain sequence motifs, double-stranded RNA/single-stranded RNA junctions and hairpin structures that govern their processing by the DGCR8/Drosha protein complex into an approximately 70 nt stem-loop structure, termed precursor miRNA (pre-miRNA), with a characteristic 2 nt overhang at the 3'-end.(93) An alternative pathway is the production of mirtrons from introns (*i.e.* miR-1003/1006/1008) through splicing. The product, like pre-miRNAs, is exported into the cytoplasm by Exportin-5/Ran-GTP, which recognizes the 3'-overhang, where it matures into miRNA after the cleavage of the stem-loop structure by the DICER complex. The cleavage of pre-miRNA results in a ~22 nt duplex, one strand of which is loaded onto an Argonaute (Ago) protein. Asymmetric retention of either the 5' or 3' strand (guide strand) in the Ago protein (guide strand) forms the miRNA-induced silencing complex (miRISC) while the expelled strand (passenger strand) is rapidly degraded. Evidence are now emerging that the strand specificity of Ago loading is miRNA, cell and cell-state dependent. In some cases, both the 5' and 3' strand has been observed to be active. In animals, there are four known Ago proteins, Ago1 – Ago4, with Ago2 having the highest tissue expression and the only one endowed with ribonuclease activity which enables it to cleave target mRNA upon full complementary binding.(94) The miRISC complex exerts post-

transcriptional gene regulation by full or partial complementary base-pairing to the mRNA at either the coding region(95) or the 3'-untranslated region (3'-UTR) of the mRNA.(96). Silencing is mediated either by direct degradation (miR-196:HOXB8) (97), inhibition of translation initiation via eukaryotic initiation factors (eIFs)(98, 99) or in majority of the time by mRNA decay (100, 101) resulting from poly(A) degradation at the 3'-end that ultimately affect the protein level. The later requires three additional interacting proteins, TNRC6, TNRC6A (also known as GW182) and TNRC6C.(102)

miRNA expression, binding and target specificity

It has been predicted that 60% of the mammalian protein coding genes are targets of miRNA.(103) The abundance of binding sites allows miRNA to fine-tune the gene expression of cells in order to maintain homeostasis. This is achieved through either full or partial base-pairing of miRNA-mRNA, both of which exerts different mechanistic repression. Regardless, the binding specificity of miRNA to targets is achieved predominantly at the seed region (2 – 7/8th position) of the miRNA. In addition, crosslinking studies identified non-canonical binding regions (13 – 16th position) of the miRNA that can contribute to the stability of miRNA-target interactions while fine-tuning target diversity within a miRNA family.(104, 105) Usually, full complementarity is only found in the seed region, whereas overall partial complementarity is enough to exert an effect on the target. This binding permutation allows miRNAs to target tens to hundreds of different mRNAs simultaneously. Post-transcriptional modification of miRNA can further increase the diversity of binding targets. The most commonly observed miRNA modification includes the alterative cleavage by Drosha or Dicer leading to the formation of isomiR (106), A-I editing of pri-miRNA by ADAR enzyme (107, 108) and non-templated nucleotide addition at the 5'- or 3'-end of miRNA (109). It has been reported that A-I editing of pri-miRNA can completely block miR-151 maturation (110) or alters the binding target entirely in miR-376-5p (108) all of which has been shown to affect cancer progression (111).

Regulation and Turnover

In order to understand the role of miRNA in human diseases, the factors that affect its production and degradation must be understood. Under steady-state conditions, the turnover of mature miRNAs can range from minutes to days with turnover specific to each miRNA.(112) Recent metabolic pulse-labelling studies in *Drosophila melanogaster* have shown that the 42 most abundant mature miRNAs

can be transcribed at 700 molecules per minute and their intracellular abundance level is correlated to their transcription rate with the exception of mirtrons that are transcribed by alterative processing without DROSHA.(113) Importantly, duplex miRNAs are rapidly degraded unless they are loaded onto Ago. The finding is in agreement with previous reports that Ago proteins hold the largest reservoir of miRNAs and that systemic depletion of Ago can lead to global reduction of miRNAs.(114-116) Thus, Ago abundance can directly affect the intracellular miRNA abundance. Indeed, phosphorylation of Ago2 at Tyr393 by epidermal growth factor receptor (EGFR) or Tyr529 potentially reduces miRNA loading.(117, 118) Interestingly, the converse is also true where miRNA biogenesis can stabilize Ago protein levels.(116) During target repression, Ago-bounded miRNA can be edited by nucleotidyl transferase that increases its instability if the complementarity base pairing is high. Another proposed mechanism such as sequestration of miRNA into exosomes has been implicated as a potential mechanism for regulating miRNA activity.(119)

miRNA detection

There are currently several quantitation methods for miRNA that differ based on sensitivity selectivity and cost. They include, but are not limited to, reverse transcription quantitative polymerase chain reaction (rt-qPCR), microarray and next-generation sequencing (NGS) each with their advantages and disadvantages. We will focus our discussion on NGS due to its excellent sensitivity, specificity, and ability to detect different isoforms, and due to its relevance to our work. For an in-depth review of other methods, refer to (120).

NGS is based on massively parallel sequencing by synthesis. The advantage of the approach is the ability to interrogate different miRNA isoforms and modifications simultaneously with high sensitivity. However, NGS has a high initial cost and requires extensive bioinformatic knowledge and computational resources for analysis which may not be available to everyone. The initial consideration when applying NGS methods is the objective of the experiment which will dictate the depth of sequencing, number of cycles and downstream bioinformatic analysis.(121) Sequencing depth refers to the average number of unique reads covering the span of the target assembly. For miRNA, sequence with 2 – 10 million reads should be sufficient for differential analysis and maybe identification of different isoforms and miRNA editing events at high sequencing depth. The discovery power will depend on the abundance level of each isoform and editing events. As noted earlier, cost is a barrier for miRNA detection. In that regard, multiplexing, or pooling multiple samples with unique indexes for the analysis could significantly reduce cost and is a standard today.

For sequencing to be effective, biases must be reduced at every point beginning with library preparation. A sequencing library is the collection of RNAs *i.e.* inserts with 3' and 5' adapters compatible with the flow cell. This could be accomplished by polyadenylation of the insert at the 3'-end or by ligation. Polyadenylation *i.e.* poly(A)-tailing and ligation based method is affected by secondary structure of RNA but denaturation can effectively prevent that problem.(122) Different enzymes have different substrate specificity, like-wise, the commonly used *E. coli* poly(A) polymerase does not favor uridine at the 3'-end of the insert. For ligation, the truncated bacteriophage T4 RNA ligase 2 is used. Ligation-based methods do harbor biases caused by the secondary structure of the inserts and insert circularization that lead to their dropout.(123, 124) The addition of at least three random nucleotides at the ligation end of the adapter can overcome the secondary structure bias that occurs during the ligation step. Polymerase chain reaction (PCR) of the library is necessary to amplify their content. During this step, biases can arise as a result of the high G/C content and the length of miRNAs that leads to dropout. An effective remedy is extending the denaturation time to allow for better strand separation.(125) For miRNA, post-PCR clean-up and size-selection is highly recommended in order to remove excess primers, reagents and more importantly primer-dimers that arise during initial library preparation. Another consideration should be the complexity of miRNA sequencing that could impact the quality of the reads. When in doubt, spiking in Phi-X could help increase the complexity of the libraries.

Sequencing results in stacks of color images that needs to be reconstructed into nucleotide information along with sorting of samples if they were multiplexed. The workflow usually entails removing low quality reads, and length followed by adapters trimming at the 3'-end. The reads can then be mapped to genomes of interest which can be accomplished by different software today. Once the mapping is completed, it can be tabulated for follow-up analysis *e.g.* differential expression analysis. For additional information of the different approaches, refer to (126-128)

miRNA in PCa

Like many cancers, miRNAs have been implicated in many steps of PCa progression. Macroscopic analysis by microarray can differentiate cancer types based on miRNA expression profile *i.e.* PCa/breast cancer can be identified by let-7i/ miR-27b/181b, suggesting that it could be used as disease-specific biomarkers.(129) On a microscopic level, functional analysis of miRNAs dysregulation often converges on four classical pathways of cancer such as apoptosis, differentiation, invasion/migration and androgen signaling. One of the first discovered oncological miRNA (oncomiR) miR-21 is common enriched in many cancers.(130) In PCa, it has been shown to target PTEN, RECK and BTG2 which affects proliferation, androgen signaling, invasions and

transdifferentiation.(131-134) Many other miRNAs have since been implicated in PCa progression (Table 8). Notable oncogenic miRNAs include miR-32/221/222 that impinges on cell cycle regulator such as Skp2 and $p27^{Kip1}$.(135) The miR17-92 cluster has been implicated in PCa progression by inhibiting apoptosis via STK4 by miR-18a.(136)

Table 8
Non-exhausive summary of PCa-related miRNAs.

miRNA	Function	Refs
Proliferation & Apoptosis		
Let-7a	Proliferation, cell cycle and differentiation	(137)
miR-1	Tumor suspressive, cell cycle control	(138, 139)
miR-21	Promotes tumor progression	(131, 133)
miR-24	Inhibits apoptosis	(140)
miR-32	Inhibits apoptosis	(141)
miR-96	Autophagy, growth and metastasis	(142)
miR-182	Proliferation, inhibit apoptosis	(143)
miR-205	Inhibits apoptosis	(144)
miR-221/222	Enhance cell proliferation and survival	(135)
Invasion & Migration		
miR-21	Represses inhibitor of MMP	(145)
miR-96	Represses cell-cell adhesion	(146)
miR-143	Inhibits MAPK pathways	(147)
miR-18a	Promotes cancer progression	(136)
miR-210	Hypoxia-related	(148, 149)
Androgen Signaling		
miR-21	Promotes tumor progression	(134)
miR-34c	Androgen receptor repression	(150)
miR-125b	Androgen signaling and castration-resistance	(151)
miR-135b	Target ER, AR HIF1AN	(152)
miR-145	Negatively regulates AR	(153)
miR-183	Positively regulates PSA	(154)

Extracellular vesicles

Overview

Extracellular vesicles (EVs) are anucleate cellular products with a phospholipid bilayer that harbor a combination of protein, carbohydrates, nuclei acids and lipids. EVs biogenesis is evolutionarily conserved across the three branches of life, their putative function is related to adaptation and communication with the environment (155). EVs were first observed using electron microscopy during reticulocytes differentiation and was later extended to the study of B-lymphocytes and dendritic cells.(156) Initially, EVs were thought to be cellular debris of insignificant value, however, research from the past decade has proved that they are anything but insignificant. Today, EVs are broadly defined by two classes known as exosomes and microvesicles (MVs, aka ectosome or microparticles) based on physical properties and specific route of biogenesis (**Figure 6**). In-addition, apoptotic bodies are often considered the third class of EVs but will not be discussed further. Though they have different origins, exosomes and microvesicles share many physical properties such as size and density that challenge many of the isolation and characterization techniques today. Furthermore, exosomes and MVs can employ similar molecular processing machineries that further masquerade the two classes of vesicles (discussed below). In the sections below, we will discuss the biogenesis of EVs, its relevance in oncology and lastly, isolation and characterizations techniques.

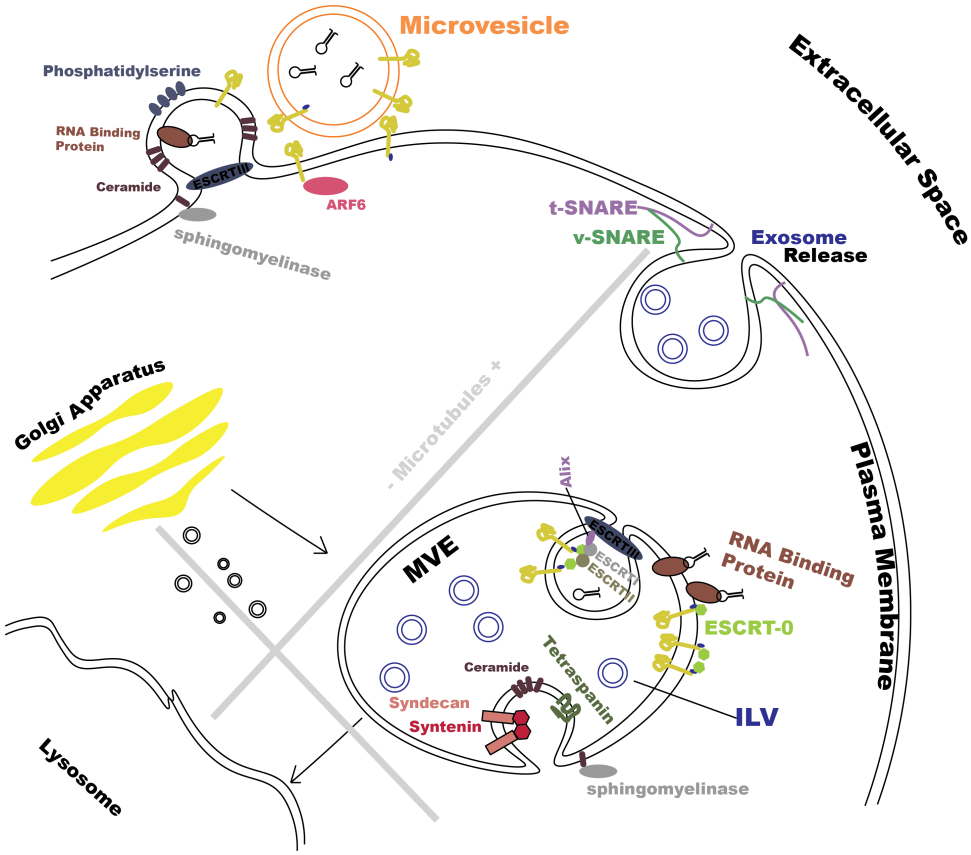

Figure 6. Simple illustration of exosome and microvesicle biogenesis. Microvesicle biogenesis (top) is initiated by RNA sorting and lipid such as ceramide aggregation. Exosome biogenesis begn with invagination of endosome. Vesicles inside the multivesicular body (MVE) *i.e.* intraluminal vesicle (ILV) is destined to either lysosomal degradation or fusion with the plasma membrane.

Exosomes

Exosomes are between 50 – 150 nm in diameter and 1.12 – 1.19 g/mL (157) in density that originating from the endosome which is an organelle that sorts and trafficks molecular cargoes derived from multiple sources such as the plasma membrane and Golgi apparatus. Exosomes begin as inward budding of the early endosomal membrane to become intraluminal vesicles (ILVs) during endosome maturation. The ILVs within the endosome, collectively known as the multivesicular endosomes (MVEs) are destined either towards the lysosome for degradation or exocytosis via fusion with the plasma membrane to becomes exosomes. The production of exosomes is a dynamic process driven by cargoes and therefore highly enriched in specific proteins, lipids, RNA and DNA. As such,

exosomes content reflects the cell origin and state of the cells. The molecular machineries that create exosomes have only been described in detail recently but still not completely understood.

Biogenesis

For protein cargoes, the endosomal sorting complex required for transport (ESCRT) pathway has been the most studied. The ESCRT pathway is consisted of 30 proteins assembled into four complexes termed ESCRT-0 to ESCRT-III to carry out ILV formation during endosome maturation (**Figure 6**). Mono-ubiquitinylated transmembrane proteins on the endosomal membrane are recognized by ESCRT-0 and aggregate with other ubiquitinylated protein-ESCRT-0 complexes. ESCRT-I and ESCRT-II are recruited to destabilize the local membrane structure followed by scission of the bud by the recruitment of ESCRT-III. The recognition of ubiquitinylated transmembrane proteins by ESCRT-0 is achieved by the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS).

HRS recruits tumor susceptibility gene 101 (TSG101), a subunit of ESCRT-I which leads to the association of ESCRT-II. ESCRT-III contains the protein, Snf7 that recruits another protein, Alix to stabilize and promote the vesicular scission. The assembled ESCRT proteins complex is released after scission of the bud by VPS4 where it can then be recycled.(157) Other proteins have also been implicated in exosome biogenesis such as syndecan and syntenin however, whether the proteins are part of the essential exosome biogenesis pathways or mere cargoes that initiates ILVs remains unclear.(158) It is important to note that, the mechanisms of exosome biogenesis is highly cell dependent as *in-vitro* knockdown studies of the components in the ESCRT pathway often resulted in disparate outcomes in different cell lines.(157, 159-161) Other ESCRT-independent exosome biogenesis pathways have been observed to induce ILV formation. They include the HSC70 chaperone and tetraspanin family of proteins CD9, CD63, CD81 and TSPAN8.(162, 163) Due to their role in exosome biogenesis, the aforementioned proteins are widely used as markers for vesicles.

Exosomes are highly enriched in cholesterol, sphingomyelin, ceramide and phosphatidylserine rendering it “rigid” in comparison to other vesicles. Indeed, lipids-induced exosomes biogenesis have been traced to both neutral sphingomyelinase 2 (nSMase) enzyme which mediates the hydrolyse of sphingomyelin into phosphocholine (164) and ceramide and phospholipase D2 (PLD2) which hydrolyze phosphatidylcholine into phosphatidic acid (165). Studies have also revealed that ceramide can induce negative curvature at the inner leaflet of the endosomal membrane to initiate ILVs formation.(165)

Like other biomolecules, RNAs are sorted into exosomes. The most studied of the RNA species is a class of small regulatory RNAs known as microRNAs (miRNAs,

see section “MicroRNA”). miRNAs can contains sequence motifs such as GGAG located in the 3’-end of miR-198 and miR-601 (166) or GGCU in miR-194-2-3p and miR-365-2-5p (167) that are selective for exosomal sorting. In addition, 3’uridylation also distinguish the exosomal miRNA from cellular miRNA.(168) Other proposed sorting mechanisms include Y-Box-binding protein 1 with miR-223 (169) or major vault protein with miR-193a(170). Separately, McKenzie et al. have shown that miRISC complex can be loaded onto exosome and that phosphorylation of Ago2 at ser387 of the miRISC complex can inhibit Ago2-MVE interaction.(119)

The MVE trafficking to either lysosome or plasma membrane is facilitated by the RAB family of small GTPase proteins and SNAREs/SNAP complexes. The former consists of near 70 proteins with the task of vesicular trafficking while the latter includes vesicle-associated membrane proteins (VAMP) for vesicular fusion. Their expression is organelle-specific and can be found in compartments such as the endoplasmic reticulum, Golgi apparatus and endosomes. For exosomes, RAB5, RAB7, RAB11, RAB27 and RAB35 have been reported to promote exosome secretion at different stages of endosome maturation.(171) To target vesicles towards the plasma membrane, proteins such as SNARE forms complex with SNAP on the opposing membrane to mediate membrane fusion. VAMP7 or VAMP8 with SNAP-23 has been implicated in the fusion of MVE with the plasma membrane or lysosomal compartment. Many additional Rabs, SNAREs and SNAPs proteins have been shown to facilitate membrane fusion and trafficking, for in-depth review see (172-174).

Microvesicles

MVs are produced by the shedding of plasma membrane which results in vesicles with sizes ranging from 100 nm to greater than 1 μm in diameter. Like exosomes, MVs biogenesis is initiated by cargoes utilizing some of the same molecular machineries of exosomes biogenesis. Enrichment of lipids, proteins and RNAs have been observed in MVs. Lipids species such as ceramide, In MVs, local aggregation of ceramide can destabilize membrane leaflet.

The mechanisms responsible include Ca^{2+}dependent for proteins sorting include ARRDC1 which interacts with TSG101 (175) and ARF6 which regulate proteins sorting (176). RNA sorting mechanism by has also been elucidated in glioblastoma derived MVs.(177) Similar to exosomes, sequence motifs CUGCC forming a hairpin structure at the 3’UTR of the mRNA dictates its sorting into MVs.

EVs in diseases

The explosion of EV research has refined our understanding of its role under normal and pathophysiological condition. It is now understood that EVs can act as messengers to the surrounding or distant tissues. For instance, EVs released by B-cells express MHC can stimulate T-cell activation.(178) In addition, EVs loaded with FAS-ligand, and TRAIL are secreted into the placenta as immune-repellent in order to protect the fetus during pregnancy.(179) In oncology, the horizontal transfer of EV cargoes by tumors to surrounding tissues have been implicated in nearly all facets of tumor progression such as altering microenvironment, initiating angiogenesis, immune suppression, ECM degradation, treatment resistance and preparing metastatic milieu in distant tissues.

During early tumor development, the increasing metabolic demands of proliferative cells will, at some point, outgrow the supply of oxygen and nutrients provided by the vasculature. At that point, tumors such as pancreatic cancer can adapt by secreting EVs expressing Tspan8 that leads to endothelial cells activation and angiogenesis (163) or in breast cancer where MVs expressing a truncated VEGF that have low affinity to Bevacizumab (a VEGF inhibitor) can potently activate endothelial cells (180). In addition, EVs expressing transforming growth factor-β (TGF-β) on the surface can convert fibroblast into myofibroblast phenotype or cancer-associated fibroblast (CAF) in the context of cancer.(181) Myofibroblast is usually found during wound healing, their presence (usually determined by vimentin, tenascin and/or α-smooth muscle actin: αSMA staining) in cancer leads to angiogenesis, ECM remodeling and inflammatory response, generally known as a reactive stroma.(182) The transfer of miRNA such as miR-125b, to tumor stroma can induce CAF transition.(183) The reactive stroma observed in many cancer led Dvorak to famously assert that tumors are “wounds that never heal”.(184) In PCa, reactive stroma with collagen I staining has been observed and it is correlated with GS.(185) The activated stroma in turn sustain tumor progression through reciprocal secretions of growth factors and EVs in positive feedback cycles.(186) Recently, Nabet et al. have identified a novel mechanism whereby excess unshielded RN7SL1 which serve as a damage-associated molecular pattern (DAMP) are exported into exosomes of breast cancer.(187) The RN7SL1 is detected by pattern recognition receptor (PRR) with RIG-1 after exosomal uptake by immune cell that led to the induction of STAT1 and NOTCH signaling that promoted metastasis.

The role of EVs as a mode of communication between immune cells have been intensively studied. It is found that antigen presenting cells (APCs) secrete EVs as an antigen presentation intermediate that stimulate the activation of T-cells albeit at lower efficiency than with interaction of APC.(188-190) Thus it is not surprising that tumor derived EVs can also impact the function of immune cells. Studies from EVs derived from tumors exhibit immune-suppressive, and occasionally stimulatory functions. In the former, proteins such as Fas ligand, TRAIL and/or galectin-9 were identified on EVs that promoted T-cell apoptosis.(191, 192) In-addition, clinical studies have shown that high level of circulating EVs expressing PD-L1 from

metastatic melanoma and glioma patients can also suppress cytotoxicity mediated CD8+ T-cells and could potentially be used as biomarker for immunotherapy.(193, 194) Alternatively, immune-suppression has been shown to be a result of shifting balance to myeloid derived suppressor cells and regulatory T-cells (Tregs) that inhibit the activation of CD8+ T-cells.(195, 196)

The acquisition of a motile phenotype and invasive properties is a hallmark of cancer. As part of the metastatic cascade, tumor needs to degrade ECM, intravasate into and extravasate out of blood circulation and finally expand in the new niche. Tumor utilizes EVs or invadopodium as part of their escape mechanism to achieve local and distant invasion. EVs secreted by cancer have shown to contain exosome-associated markers such as TSG101, RAB27a/b and ARF6 and metalloproteinases (MMP) such as MMP-2/9/14 which can degrade collagen-I.(197-199) The production of MMPs can be targeted directly by miRNA such as miR-183(200) or indirectly by via secondary effect such as targeting the NOTCH or EGFR pathway by miR-139/34a (201, 202). In-addition, secreted EVs are enriched in fibronectin which is necessary for directional movement of tumors.(203) Invadopodium is actin-filament extension that mediates motility of tumor cells induced by specific external stimuli such as growth factor signaling, hypoxia and ECM.(204) Interestingly, invadopodium employs the same exosomes-biogenesis machineries to deposit expressing MMPs at the tip to promote ECM degradation.(205) Activated invadopodium signature is a negative prognostic indicator for breast cancer.(206) As part of the preparation for distant migration, EVs have been documented to prime the future metastatic site by educating the local sites such as the bone marrow via MET (207) or Kupffer cells in the liver via TGF-β to facilitate their seeding (208). Metastatic site could also be hospital due to the local effects such PTEN suppression due to the horizontal transfer of EVs containing miR-17-92 cluster of miRNAs secreted by astrocytes in the brain.(209)

EVs are present in all biological fluids that are readily accessible with minimally invasive procedure. The unique expression and function of biomolecules harbored by EVs make it a potential source of biomarkers. Thus, the term “liquid Bx” is given to EV isolation and analysis intend for obtaining prognostic and diagnostic information.

EVs liquid Biopsy of blood and urine

EVs are found in all biological fluids at different concentration and associated with different proteins. Studies have found miRNA profile is sufficient to discriminate the composition of different bodily fluids.(210, 211) The two most common biological fluids studied for PCa EV research are blood and urine.

Urine

Urine from healthy individuals is composed of water, electrolytes and small quantities of proteins. Proteins in urine are mainly derived from plasma (< 40 kDa), cell shedding (48%), Tamm-Horsfall proteins (THPs, 49%), and urinary EVs (uEVs, 3%).(212) uEVs are specific to the urinary tract as evident by the expression of specific proteins such as aquaporin-2 and sodium transporters (213, 214). The concentration of uEVs is highly dependent on collection time of the day and the void fraction. uEVs concentration is usually the highest in the morning but could be affected by hydration, diet and medication. Thus, researchers often collect morning, first-void urine as they should provide the highest levels of uEVs. If morning first void urine is not possible, prostate massage could enhance prostatic EVs secretion into urine but creates additional barrier to its routine use.

Unlike other biological fluids, uEVs are mostly associated with THPs in solution. THPs is a glycoprotein produced at the thick ascending limb of loop of Henle. The function of THP is diverse, it includes immunomodulation and activation.(215) The significance of THP function can be observed as mutation can lead to uromodulin-associated kidney disease.(216) In urine, THP form a mesh-like network that traps bacteria such as *E. coli* and thereby preventing it from traveling upstream of the urinary tract. THP can also enmesh uEVs within their fiber network which effectively pellet them together at low speed.(217) Therefore, investigators interested in enrichment of uEVs by centrifugation should consider the effect of THP. The addition of DTT have been shown to liberate uEV thereby increasing the yield and purity of the vesicles.(218)

uEVs have been shown to contain a wealth of biomarkers (219). Detectable levels of ERG-TMPRSS gene fusion, a prostate lesion marker have already been demonstrated from urine samples.(220) Other protein markers of prostate such as PSA, PSMA and TMPRSS can be detected in uEVs.(221) In addition, miRNA from urinary EVs has been shown to predict the presence of PCa (Table 9).(222-224)

Table 9
Non-exhaustive list of miRNA from urine reported to have prognostic or diagnostic value for prostate cancer management.

miRNAs	Isolation method	Relevance	Ref
miR-1290/375	Exoquick	PCa OS, AUC = 0.66	(225)
miR-196a/143	UC	PCa +/- AUC: 0.92, 0.72	(222)
miR222/24/30c	NA (whole urine)	PCa +/- AUC: 0.89 - 0.95	(224)
miR-107/574	Urine cell pellet	PCa +/- AUC 0.74, 0.66	(226)
miR-205/214	NA (whole urine)	PCa +/- AUC: 0.83, 0.92	(227)
miR-196a/501	UC	PC +/- AUC 0.69 – 0.73	(222)
miR-151a/204/222/23b	miRCURY Exosome Isolation	OS: HR 1.88	(228)

EVs enrichment and characterization

Overview

It is currently not possible to identify the origin and mode of biogenesis of EVs of similar sizes once they are released into the extracellular space, they can only be inferred by their biophysical properties such as size, density and biomolecular cargoes as described in the previous sections.(229) But because EVs are heterogeneous, the choice of isolation technique will likely bias one population over another. Therefore, a well-established characterization and isolation technique is necessary to investigate the different populations of EVs, however, due to the pace of EV research, the discovery of novel markers and isolation techniques can out-pace their validation.(230) This led many studies to misuse markers and nomenclature (231) that cumulated to a proposed set of requirements for EVs studies by the International society of extracellular vesicles (ISEV) (232). We will therefore denote the use of small EVs (sEVs) to be < 200 nm and m/l EVs (m/lEVs) to be > 200 nm.

Enrichment

The choice of enrichment method can greatly affect the purity, yield and population of final EVs product. This is complicated by the fact that different biological fluids (*i.e* serum/plasma, urine, seminal fluids, cerebral spinal fluids, etc) have different properties that renders one type of enrichment more suitable than other (discussed by methods). Researchers interested in EVs should consider the type biological fluids, purity, yield requirement and downstream applications when choosing the appropriate isolation method. Today, the enrichment of EVs can be broadly categorized into sedimentation, size-exclusion chromatography (SEC), chemical precipitation, immune-affinity, membrane filtration and microfluidics. Each type of method will impact the aforementioned purity and yield to different degree.

In order to discuss the different isolation methods, we must first discuss the non-exhaustive source of contaminations (biological fluids dependent) during EV isolation, that is, particles in solution with similar size, density or biomolecules to EVs. EV enrichment are mainly based on size and density. Non-vesicular

contaminants that can overlap in those size and density range include protein aggregates and lipoproteins (**Figure 7**).(233)

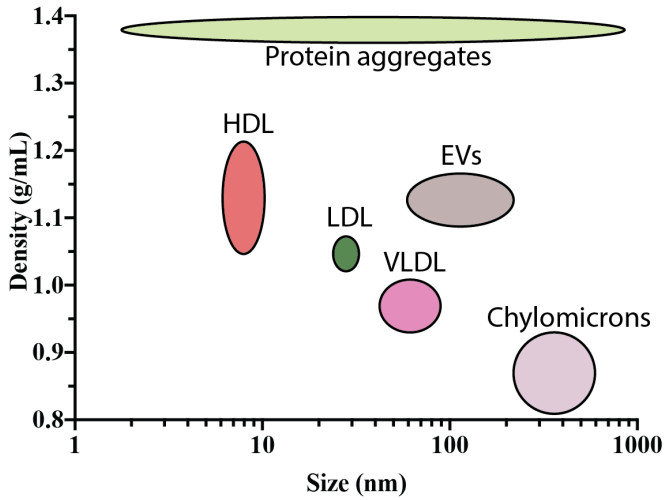

Figure 7. Illustration of the size and density overlap between extracellular vesicles, lipoproteins and protein aggregates.

Sedimentation

Sedimentation in UC is based on centrifugal force, buoyance of the particles and viscous drag force on the particles with larger and denser particles sediments faster. Therefore, the choice of rotors for UC, the density of suspension medium and centrifugation duration can greatly influence the precipitants, for an excellent review see (234). To harmonize the different rotor types, investigator should consider the use of *k*-factor to normalize their UC time to achieve reproducibility. Differential ultracentrifugation (UC) and its variant with or without density gradients proposed by Thery *et al.* in 2006 remains the most widely used EVs isolation method.(235) In the protocol, unwanted particles *i.e.* cells, cellular debris large vesicles are sequentially removed at 300xg to 10,000xg before the small EVs are precipitated at >100,000xg for 1 – 2 h. Washing and microfiltration with 0.22 μm can be used on the final pellet with the benefit of increasing purity (defined as the number of particles/protein content) but decreasing yield, due in part to the formation of EV aggregates.(235, 236) This can negatively impact the downstream application intended for the EVs such as functional studies. Density gradient can be added in this setup to further resolve the subpopulation of vesicles. As noted earlier, UC performance is affected by viscosity, biological fluids such as serum or plasma

when used as whole will decrease the sedimentation efficiency of EVs due to higher viscosity.(237) Urine, due to the large quantities of Tamm-Horsfall proteins (THPs) traps small EVs will precipitate at much lower speed, thus, the standard protocol will lead to significantly reduction of EVs yields as well as the large level of protein contaminations.(218)

The advantage of using UC for EVs isolation is yield, cost and intra-laboratory reproducibility. It has been well-documented that UC can yield EVs with diameter of 30 – 250 nm and expression of proteins such as CD9, CD63, TSG101, Alix, depending on source.(238) The disadvantage of UC however, includes EVs aggregation/integrity, low-purity, throughput and inter-laboratory reproducibility.(239) The final products of UC without density gradient often contains significant amount of proteins aggregates, nucleosomes and lipoproteins which will negatively impact the characterization and functional studies.(233) The complexity of UC also led to the use of different protocols in different laboratories resulting in low reproducibility.(240) Lastly, UC is labor intensive and time consuming, with each round of processing requiring ~4 h not including handling time. This reduces the translational aspect of UC where throughput and reproducibility are paramount.

Size exclusion chromatography

Purification by SEC is separation of particles based on differences in hydrodynamic diameter with larger (hydrodynamic diameter) particles traversing faster than smaller particles through the resin. Thus, with increasing elution volume, particles of decreasing sizes can be enriched into different fractions. There is a lower and an upper limit to the sizes of the particles that can be resolved depending on the resin. Elution time decreases linearly to the hydrodynamic volume of the particles of interest.(241) The advantages of using SEC include scalability (volumetric requirement can be tailored by changing the diameter of the column), reproducibility and purity.(242) The system can further be automated via fraction collector which reduces manual labor and operator errors. However, SEC for EVs isolation is gravity-driven to preserve the integrity of the vesicles thus the processing time for each sample could be significant. Furthermore, if columns are reused, cleaning and equilibrating the column will take additional time which reduces its throughput in clinical setting.(241)

Chemical precipitation

Chemical precipitation of EVs remains one of the simplest, high yield and cost-effective method of EV isolation. There are many precipitation methods based on

different reagents such as polyethylene glycol (PEG), protamine, sodium acetate and acetone, each yielding varying degree of purity. The main principle is to selectively reduce the solubility of EVs such that it can be collected by benchtop centrifuge. PEG is the most commonly used reagent for EVs research. It has been demonstrated on biological fluids such as serum/plasma and cell culture media.(243, 244) Isolation of EVs by PEG is based on polymeric dehydration of water molecules. PEG is non-specific and thus, proteins and lipoproteins can coprecipitate along with EVs, making it one of the least suitable isolation methods for downstream proteomic analysis. EVs isolation with PEG requires little labor, it is added to samples followed by incubation at 4ºC overnight and centrifugation. The combination of ease and cost makes it suitable for research purposes. However, the chemical precipitation requires long duration and operator involvement and not readily automatable, all of which makes it unlikely to be used in the clinical setting.

Immunoaffinity capture

Immuno-affinity capture of EVs is based on antibody fixture onto solid phase support follow by manipulation of the solid phase to achieve enrichment. The solid phase can be magnetic beads (commonly found in commercial EVs purification assays) or microfluidic devices. Antibody, usually CD9/63/81 are used to capture EVs onto magnetic or agarose beads that can be readily enriched by an external magnetic field or centrifugation. The critical parameter is the choice of antibody where affinity and non-specificity binding needs to be established. In-addition, because EVs display a range of different antigens at different concentration by different cells, the choice of antibody will greatly affect the selected EV population. Therefore, an understanding of the EVs subtype should be known prior to antibody selection and characterization.

Immuno-affinity capture method has high cost, low yield and not readily scalable but generate vesicles with high purity and integrity. Thus, the method is excellent for vesicle characterization but ideal for *in-vivo* functional studies as large quantities of vesicles are often needed. The isolation protocol is simple but could be automated especially when designed as a microfluidic system.(245) Therefore, immuno-affinity capture technique could be translated for clinical use for routine diagnosis.

Membrane filtration

Membrane filtration has a long history in biomedicine due to its cost and accessibility. The use of different pore-size membrane enables researcher to selectively retain particles of interest. For EV isolation, hydrostatic dialysis or ultrafiltration are two common methods used. Hydrostatic dialysis is excellent for enriching diluted EVs samples such as urine or conditioned media (CM). On the other hand, membrane filtration is relatively simple to use, successively smaller

pore-size membranes are deployed to filter out large particles until the population of interest is achieved. The method has been applied to uEV isolation (246) and EVs from FBS (247) successfully. It can be used as standalone (248) or in combination with other techniques such as UC (249). The disadvantage of the membrane filtration is the adhesion of EVs to the membrane, clogging and deformation of the vesicles.(238) Another membrane based method called asymmetrical-flow field-flow fractionation (A4F) have been successfully used. A4F is based on cross flow patterns that selectively retain particles with different hydrodynamic diameter. It is considered to be milder in comparison to membrane filtration alone or UC. The method has been applied to studying urine samples from prostate cancer patients (250) and analysis of circulating miRNA carriers (251). A4F can resolve hydrodynamic diameter of different particles very efficiently. In one study, Zheng *et al.* could resolve large (90 – 120 nm), small (60 – 80 nm) EVs and the non-vesicular exomeres (35 nm) population which contained different cargoes using A4F.(252) A4F requires significant optimization and cannot be easily applied elsewhere.(253)

Today there are a large variety of sizes and type of membrane filter used for EV isolation which makes the technique accessible. However, it also complicates the effort to standardize EV isolation and reproducibility of the experiments.

Microfluidics EV isolation

Currently, the microfluidic system for EV isolation include miniaturization of the techniques described above *i.e.* immunoaffinity or filtration (sieving).(254, 255) Other novel techniques include nanowire microfluidic system that has been used for urinary EV capture and *in-situ* miRNA quantitation.(256) Other method achieve EV size separation by passive lateral displacement (257). For additional information on microfluidic systems for EV isolation, refer to (258, 259)

Acoustic Trapping

Manipulation of particles by sound was first demonstrated using an experimental contraption known as the Kundt's tube in 1866. Since then application of acoustic waves in biological study have been quiescent until 1971 when ultrasound was found to induce erythrocyte separation in chick embryos.(260) Since then, the application of acoustics in biomedicine has exploded.

Acoustic trapping is a label-free, microfluidic technique that capitalizes on the advantages of small scale, namely the compatibility with low-volume samples,

laminar flow profile and sensitivity.(261) Acoustic trapping is operated at resonance with the dimension of the acoustic resonator chosen at multiples of quarter-wavelength (λ/4) of the actuating sound wave (λ). There are three forces exerted on particles situated in a standing sound wave, primary radiation forces, lateral radiation force and inter-particle forces that play a key role in acoustic trapping (**Figure 8**).(262) The primary radiation force pushes the particles to the node or anti-node (for lower density particles relative to medium) of the acoustic waves while the lateral forces overcome the viscous drag forces of the moving fluid. Inter-particle forces (aka secondary force) generated from the scattering sound field retains EVs (discussed later). The primary radiation force exerted on spherical particles much smaller than the acoustic wavelength, λ can be approximated by:

$$F_{PR} = -V_P E_{AC} k \emptyset \sin(2kx) \qquad \text{(Eq) 1}$$

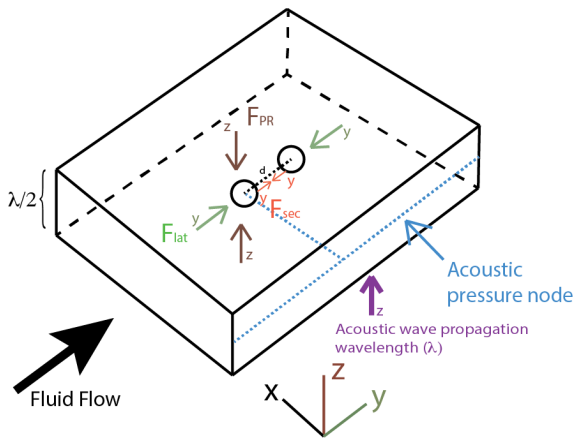

Figure 8. Illustration of the three forces acting on the particles in an acoustic field in an acoustic resonantor with height (Z-axis) equals λ/2. Acoustic pressure wave (purple) with wave length λ is propagating from the bottom to top creating an acoustic pressure node (dashed blue line). Primary radiation force (brown) acting on the particles in the z direction while lateral force (green) is again to push particles against flow. Lastly, secondary forces (orange) creates an attractive force pulling the two particles separated by distance *d* together in the y direction.

Where V_p denotes the volume of the particle, E_{ac} denotes acoustic energy density, k denotes the wave number, x denotes the position of the particle relative to the node and ϕ denotes the acoustic contrast factor that can be expanded as:

$$\emptyset = \frac{3(\rho_p - \rho_m)}{2\rho_p + \rho_m} + \frac{\beta_m - \beta_p}{\beta_m} \qquad \text{(Eq) 2}$$

The acoustic contrast factor describes the aggregate difference in particle density ρ_p and compressibility β_p in relation to the surround medium density ρ_m and compressibility β_m. From equations 1 and 2, it can be observed that the physical

properties of the particles can directly affect the primary radiation force, *i.e.* with greater forces exerted on larger, denser and more compressible particles relative to the medium (positive contrast factor). As a result, the particles will move toward the node to minimize the primary radiation force. Conversely, if the particles have lower density or compressibility compared to medium then (negative contrast factor), the particles will move towards the pressure node. In practice, EVs with densities ~1.11 – 1.19 g/mL and compressibility of ~ $3.5x10^{10}$ Pa^{-1} (263) will have a positive acoustic contrast and therefore move to the node while most lipoproteins will move towards the anti-node.

Lateral forces arise due to the difference in acoustic energy as it propagates through the medium. It can be described by:

$$F_{lat} = V_p \nabla E_{AC} \left[\frac{3(\rho_p - \rho_m)\cos^2(kx)}{2\rho_p + \rho_m} - \frac{(\beta_m - \beta_p)\sin^2(kx)}{\beta_m} \right] \quad \text{(Eq) 3}$$

where ∇E_{AC} denotes the differences acoustic energy density across space. Note that the lateral force operating at the anti-node will depend on the density and little on the compressibility of the particles. Note that when the particles are at the node, the contribution of the second term, compressibility term, becomes zero and does not contribute to the lateral force.

Lastly, the inter-particle forces will keep the particles clustered together. Assuming particles of the same size, inter-particle force is described by:

$$F_{sec} = 4\pi\, a^6 \left[\frac{v^2(x)(\rho_p - \rho_m)^2(3\cos^2(\theta) - 1)}{6\rho_m d^4} - \frac{\omega^2\, p^2(x)\rho_m(\beta_p - \beta_m)^2}{9d^2} \right] \quad \text{(Eq) 4}$$

where a is the diameter of the particles, *v(x)* is the velocity field, *p(x)* is the pressure field, *d* is the center-center inter-particle distance and θ is the angle formed by the imaginary line linking the two particles and the direction of the propagating wave and ω is the angular frequency.

The actuation of standing wave in a medium can produce bulk fluid movements known as acoustic streaming caused by, among many things, the dissipation of acoustic energy into the fluid. Acoustic streaming produces a fluid drag force on particles that reduces the trapping efficiency. As the particle becomes smaller (less than one micron), the primary, lateral and inter-particle forces diminish while drag force persists leading to the overall reduced trapping efficiency. To overcome this hurdle, Hammarström *et al*. exploited the use of "seed particles" to enhance the isolation of EVs-sized by the inter-particle forces.(261) They demonstrated that the 100 nm particles and rod-shaped *E. coli* (~2μm in the long dimension) can be trapped with the technology. The acoustic trapping technique has since been developed into a commercially available system by AcouSort AB (Lund, Sweden).

In the current iteration, the system can be completely automated via scripting of commands into the software. The system is compatible with multiple plate formats from 24 – 96 well plates which allows user to tailor the assay for individual purpose.

Extracellular Vesicles Quantitation

Different isolation method above can yield different quantities, purity and populations of EVs. There are few methods for quantifying the concentration and purity of the vesicles. Like the method of isolation, they range in cost, complexity and detection limits. Below details some of the most commonly used systems for characterizing isolated EVs.

Nanoparticle tracking analysis

One of the first semi-quantitative method for EVs sizing and quantitation is based on nanoparticle tracking analysis (NTA). NTA captures the two dimensional Brownian motion or random diffusion of the particles illuminated by incident laser via a highly sensitive camera. Since Brownian motion of the particles is related to its hydrodynamic diameter, temperature and viscosity of the fluid, tracking the particles over time can reveal the diffusion pattern which then can be modelled by the Stokes-Einstein equation.(264) Currently, the NanoSight system manufactured by Malvern (UK) is the most widely used. The system can measure particles from $10^7 – 10^9$ per milliliter in concentration and a size range of 30 nm – 1 μm. In practice, the NTA system has been validated for particles between 30 – 200 nm (265) and polydisperse samples are shown to be accurately represented (266). There are several major limitations of NTA. The first is that it cannot distinguish vesicles from non-vesicular particles such as protein aggregates or lipoproteins. The second is that large contaminants can potentially skew the measurements as the high scattering intensity could overwhelm the smaller vesicles. Fluorescent labelling of the sample by antibody or other means could help identify the nature of the particles but because the unbound fluorophores remain in the medium during measurement, it can generate high background level.

Transmission electron microscopy

Transmission electron microscopy (TEM) is an indispensable method that enables researchers to verify the particle size distribution obtained by other instrumentations such as NTA. TEM is a semi-quantitative imaging technique that can visualize EVs with nanometer resolution. As such, the size, morphology and purity of the vesicles can be ascertained in great details. On its own, biological samples which are

composed mainly of carbon, hydrogen, oxygen and nitrogen do not readily interact with the electron beams from TEM. For visualization, the biological samples need to be fixed for structural preservation and stained such that electrons will be scattered by the contrast agents. Fixation does introduce artefacts such as protein aggregates thus, addition of immunogold labelling can be applied to increase specificity. One of the drawbacks of using TEM is that it is done under dehydrated state which means that the morphology of the vesicles may not reflect the "true" morphology in solution. This is evident by the "cup-shaped" vesicles that have been observed in the past.(156) The staining procedure can also induce artefacts into the images.(267) Cryo-EM can overcome the aforementioned problem, but the technique is more time consuming and is not wide available.

Hypoxia

Overview

Hypoxia is a result of reduced oxygen availability to below normal level (normoxia) in order to maintain homeostasis. The oxygen reduction can be external, such as changing altitude, that leads to systemic oxygen reduction or internal such as changes to local metabolic demand or profusion *i.e.* ischemia. Under normal atmospheric condition at sea level, the oxygen level is approximately 21% O_2 (160 mmHg) however, the peripheral tissues of human do not experience the atmospheric oxygen level. Instead, the highest measured oxygen level was recorded at the alveolar compartment, at 14.5% O_2, while peripheral tissues is measured between 3% - 7% O_2 with metabolically active tissues such as the brain, prostate and liver having the lowest measured oxygen level near 3% O_2.(268)

During tumor development, the increasing metabolic demand of the proliferating cells and the increasing separation from capillaries engender hypoxic region. The limited oxygen availability limits the growth of the tumor to 5-10 cell layers or 100 – 200 μm from the capillaries.(269) At that point, tumor becomes quiescent until it can initiate the angiogenic switch that promotes angiogenesis by secreting vascular endothelial growth factors (VEGF).(270) Interestingly, the angiogenic switch can also lead to the deregulated secretion of pro-angiogenic factors that impede proper neovascularization to support oxygen delivery.(271-273) Thus hypoxia is a common feature of cancer, its presence often correlates to worst clinical prognosis.(274) In prostate cancer, hypoxia have been documented clinically by direct oxygen measurements (275, 276), immunohistochemistry (277, 278) or imaging (279, 280). Its presence is correlated to earlier BCR.(281-283)

Hypoxia sensing

Eukaryotic organisms have developed an elegant system of sensors and effectors to adapt to changing oxygen conditions. In animals, the family of proteins known as hypoxia-inducible factors (HIFs) are the mediators of cellular oxygen sensing. The HIF family consists of three oxygen sensitive α subunit, HIF1α, HIF2α and HIF3α located in the cytoplasm and a constitutively expressed oxygen-insensitive β

subunits, HIF1β or aryl hydrocarbon receptor nuclear translocator (ARNT), located in the nucleus. HIF1α and HIF2α are the two most studied of the oxygen sensitive subunit and therefore we will focus our attention to them. For additional information about HIF3α please refer to the following review (284).

The HIF proteins are part of the highly conserved basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) family of transcription factors. The HIFα subunits consist of bHLH DNA binding domain and the PAS domain necessary for heterodimerization and binding to the hypoxia-response element. The oxygen-dependent degradation (ODD) domain is the critical region that modulates the stability of HIFα subunit. The N-terminal transactivation (N-TAD) domain and the C-terminal transactivation (C-TAD) domain are crucial for interacting with coactivators and confer target specific (**Figure 9**).(285)

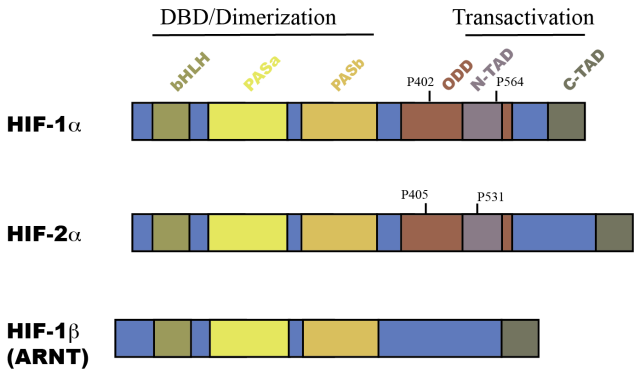

Figure 9. Schematic illustration of the protein domains of HIF1α, HIF2α and HIF1β. HIF1α and HIF2α contain the basic helix-loop-helix (bHLH), PER-ARNT-SIM (PAS) domain for DNA binding and heterodimerization, oxygen dependent degradation (ODD) domain for oxygen sensing and the N/C-terminal transactivation domain for interacting with coactivators and target specificity. HIF1β is similar to both HIF1α and HIF2α without ODD.

Under normoxic condition, the HIFs propyl-hydroxylase proteins (PHD), PHD1, PHD2 and PHD3 work together to promote the degradation of the α subunit to prevent HIF activation. Specifically, PHDs catalyze the hydroxylation HIFs at the P402 and P564 proline residues on HIF1α or P405 and P531 proline residues on HIF2α. The process is dependent on the availability of oxygen, α-ketoglutarate and requires iron as a cofactor. The hydroxylation increases the binding specificity of von Hippel-Lindau (pVHL) protein to HIFα by 1000-fold thereby targeting it for subsequent proteasomal degradation.(286) During low oxygen availability or absence of iron cofactor, the activity of PHD is diminished and the protein level of HIFα is stabilized. HIFα then translocate to the nucleus where it can heterodimerize with ARNT to activate hypoxia response.

Hypoxia miRNA

The expression of tumor miRNA are frequently altered during hypoxia.(287-289) HIF activation have been shown to affect the transcription of miRNA and miRNA clusters.(290) One of the miRNA that is the consistently up-regulated during hypoxia is miR-210. miR-210 has been implicated in tumorigenesis (291), angiogenesis (292), cell survival (293), DNA repair (294) and susceptibility to cytotoxic activity of T-cells (295). It is also found in EVs secreted by metastatic breast cancer cells where it was shown to enhance angiogenesis by suppressing specific genes in endothelial cells.(296) Therefore, hypoxia miRNA can potentially contribute to the progression of cancer.

The present investigation

Paper I : Acoustic enrichment of extracellular vesicles from biological fluids

Overall Aims: The general aim of this paper was to assess the performance of the acoustic trapping technology for extracellular vesicle (EVs) enrichment.

Summary

In this paper, we investigated the applicability of the acoustic trapping technology for EV enrichment in comparison to ultracentrifugation (UC). First, we determined the efficiency of the acoustic trapping system with polystyrene (PS) particles across a range of sizes that recapitulate EVs. It ranged from ~1% to 82% for 0.1 – 1 μm particles and demonstrated invariance to a log-fold dilution at concentrations that recapitulates biological fluids. Next, we investigated the use of acoustic trap and UC on three biological fluids, CM, urine and plasma. We found that the sufficient quantity of EVs could be enriched from all three biological fluids as determined by nanoparticle tracking analysis (NTS). Interesting, we found that overall, the size-distribution of particle obtained from UC is larger than acoustic trapping. We then determine the quality of the EVs by TEM and found that they are morphologically round, intact and harbor EVs with sizes ranging from exosome to large vesicles similar to UC. We also confirmed the NTS finding that UC-derived vesicles are generally larger than those obtained from acoustic trapping. Interestingly, we also observed that the urine sample from UC contained significant Tamm-Horsfall proteins (THP) whereas the same was not observed in acoustic trapped sample. To ascertain the identity of the EVs observed by NTS and TEM, we performed CD9/63/81 ELISA assays on the enriched samples from CM, urine and plasma by acoustic trapping and UC. We found that the expression of CD9/63/81 varies depending on biological fluid. In CM, CD81 was the highest expressing marker while CD63 and CD9 was the highest in urine and plasma respectively. The concentration of CD9/63/81 were lower in acoustic trapped samples compared to UC by 2 – 12-folds but show high concordance. Lastly, we showed that microRNAs

(miRNAs) can be detect in the enriched samples from acoustic trapping and UC and protected from degradation suggesting that they are encapsulated by membrane.

Discussion

Acoustic trapping is a novel technology that could potentially revolutionize the isolation of EVs from biological fluids. We have discussed in the section "Enrichment" the current state of EV isolation and the dearth of a low-volume compatible and robust isolation technique today. Given the large number of isolation methods today, it is often difficult to find a suitable one for that functions well across the different biological fluids while compatible with downstream applications. To that end, acoustic trapping provides a robust, low-volume compatible and potentially automated method for the isolation of EVs in different biological fluids. Given the operating theory of acoustic trapping it is expected that different size particles will be captured with different efficiencies.(261) We experimentally validated this dependence with PS beads from 0.1 μm to 1 μm and show that the isolation of EVs in or near this size range should be feasible. At the 0.1 μm range, we found the trapping efficiency to approximately 1%. It is important to note that because PS beads have lower density than sEVs, 1.05 g/mL compared to 1.11 – 1.19 g/mL (297), it is expected that sEVs will have higher trapping efficiencies compared to PS beads of the same size.

Our finding from NTA and TEM confirmed that acoustic trapping can enrich EVs that are morphologically round and intact. Interestingly, NTA and UC of the EVs enriched by acoustic trapping are smaller across all three biological fluids tested compared to UC. This is unsurprising as UC is known to induce aggregation during centrifugation that could impact its utility for function studies.(236) Furthermore, abundant level of THP was observed in the urine samples from UC but not acoustic trapping preparation which suggesting that the latter may overcome of the limitations of UC. An important criteria for EVs is the display of tetraspanin, CD9/63/81 on the surface of vesicles. Examination of the tetraspanins from acoustic trapping revealed that they are present at different concentration across the different biological fluids and are generally lower compared to UC. As UC is known to induce EVs aggregation and co-isolate protein complexes, it is possible that the combination can lead to higher ELISA signal. In addition to protein markers, EVs is also known to encapsulate small RNAs such as miRNA. Our investigation showed that miRNAs are present and are protected by intact EVs similar to UC. Interestingly, unlikely the ELISA results the level of miRNAs are similar to UC.

Together, the data demonstrated that the EVs obtained from acoustic trapping meets the standard of EVs ranging from exosomes to MVs by size, integrity, surface markers and RNA content. Compare to UC, acoustic trapping could offer a less laborious and potentially higher purity preparations compared to UC.

Paper II : A urinary extracellular vesicle microRNA biomarker discovery pipeline; from automated extracellular vesicle enrichment by acoustic trapping to microRNA sequencing

Overall Aims: The overall aim of this paper was to establish an optimized pipeline for RNA isolation and miRNA sequencing that is compatible with the acoustic trap technique. To verify the pipeline, we assessed the concordance of the miRNA expression profiles between acoustic trapping and UC method as a mean of validation. In addition, we assessed the repeatability of the library construction from the acoustic trapped samples.

Summary

In this paper, we obtained 100 mL of spot urine from healthy male donor in sterile Falcon tube. We cleared the sample by centrifugation followed by EVs isolation using acoustic trapping (9.75 mL) and UC method. The isolated EVs were then treated with RNase to remove free circulating RNA followed by RNA isolation and library preparation using two, commercially available, small RNA library preparation methods, NEXTFlex and CATS. Using a column-based RNA extraction method, we estimated 0.79 ng and 138 ng of RNAs were recovered (80% and 56% yield) from samples derived from acoustic trapping and UC respectively. Libraries can be generated from an estimated 130 pg of total RNA. Sequencing results revealed that NEXTFlex library preparation resulted in larger number of reads ($2.3x10^5$ – $9.5x10^6$) and higher miRNA content (35% - 60%) for both acoustic trap and UC samples compared to CATS ($7.7x10^3$ – $1.7x10^5$ and <3% miRNA). Comparing the results from NEXTFlex prepared libraries, we found that the miRNA profile of the acoustic trap sample is highly correlated with the UC (Spearman rho = 0.70). Technical replicate of the libraries prepared from acoustic trapped sample also showed high correlation (Spearman rho = 0.81). A large number of unique miRNAs were found common between the two acoustic trap replicates (111/200 and 111/190). Similarly, a set of unique miRNAs were found to be common between UC and acoustic trap (200/507 and 200/279 respectively).

Discussion

In paper I, we have shown that EVs with characteristics of exosomes and MVs can be isolated by acoustic trapping. In this paper, we build on our previous work to develop an RNA isolation and small RNA library preparation pipeline that is compatible with acoustic trapping. There are currently three obstacles that hinder

the routine use uEVs miRNA as biomarkers. The first is the lack of an efficient, low-volume compatible and robust method for EVs isolation. The second is the low abundance of RNA contained within EVs that challenge our current isolation and quantitation methods.(298) For example, the use of phenol/chloroform is incompatible with RNA isolation from minute quantities EVs.(299) The third are the significant biases that can arise during miRNA sequencing.(300) For example, biases could arise during RNA-adapter ligation (301) or during amplification (125) that leads to miRNA dropouts. Our pipeline overcame those technical challenges which enabled 80% of the total RNA to be recovered from the acoustic trapped samples while our library preparation method resulted in libraries with only 130 pg of input. Next, we assessed two library preparation methods based on either polyadenylation (CATS) or 4N random nucleotides ligation (NEXTFlex) and found that the latter produced robust reads when compared to the polyadenylation method. Our finding is consistent with other studies that had demonstrated the superiority of random nucleotides for ligation in comparison to traditional methods in reducing ligation biases.(302)

The power of an experiment depends on the technical variability of the methods to be much less than the biological variability. Therefore, we assessed the repeatability of library preparation with NEXTFlex using 130 pg of RNA as input and found that it generated two libraries with nearly identical species. Importantly, the miRNA profile of the two samples showed excellent concordance. However, when we investigated the number of unique miRNAs, we found that the two replicates share only 55% – 60% of miRNA in common. The low percentages of common miRNAs are a result of sequencing depth. We observed that the exclusive set of miRNAs have lower expression compared to the common set and therefore, likely to be variable. As UC is the best documented EV isolation method, we compared the miRNA profile obtained from acoustic trapping to UC. The two methods showed high correlation suggesting that the miRNAs are derived from similar pool of EVs. However, analysis of the common miRNAs between UC and acoustic trapping showed that the latter could only identify ~50% of the miRNAs found in UC. We speculate that the result arose due to the error in quantitation as well as the lack of size-selection of the cDNA libraries which led to a 10-fold difference in sequencing depth. It is expected that with size-selection, the libraries size and concentration can be better estimated and therefore will overcome the challenge observed in this experiment.

In this paper, we sought to overcome the limitations of EV research by developing a pipeline that can enrich uEVs from ~1.7 mL of urine and isolate sufficient RNA for small RNA sequencing. With further development of our EV isolation system and increasing the miRNA sequencing depth by size-selection, we expect that the uEVs miRNA expression could be investigated in a robust, low-volume compatible and automated manner in the future.

Paper III : High-throughput and automated acoustic trapping of extracellular vesicles from clinical prostate cancer urine samples

Overall Aim: The overall aim of this paper is to investigate the role of urinary EVs miRNA (uEVs-miRNA) as predictive biomarker for patients with high (GS≥8) or low (GS ≤7) Gleason grade which includes non-detectable PCa using the pipeline established in paper II.

Summary

In this paper, we obtained a total of 207, 1 mL first-void urine samples without prostate massage from Aarhus University Hospital. The PCa status of the patients were confirmed by 10-core Bx and includes relevant clinical parameters such as PSA and Gleason score but were blinded to researchers. The order of the samples was randomized prior to processing as described in paper II with minor modification. Briefly, the samples were retrieved from -80^{o}C and equilibrated at RT. To begin the samples were cleared by centrifugation at 2000xg for 10 mins at RT follow by EVs isolation using 900 μL for acoustic trapping. The isolated EVs were treated with RNase A followed by RNA isolation, sequencing library preparation and finally sequencing with five, high-throughput flow cell. In all, the application of our pipeline resulted in high success rate from isolating uEVs and library preparation.

Acoustic trapping had successfully enriched a total of 199 samples (96%) with eight failed due to either bubble formation in the microfluidic channel or seeding cluster wash-out. Samples were treated with RNase A prior to RNA isolation and library construction which yielded a total of 173 libraries. The libraries were pooled and size-selected to target miRNA species. Quality assessment of the sequencing run revealed that the median sample reads per flow cell are $7.7x10^6$, $7.1x10^6$, $8.0x10^6$, $7.0x10^6$ and $8.4x10^6$. Demultiplexing and reads alignment to the human reference genome resulted in approximately $9x10^4$ – $2x10^7$ mappable reads with an average length of 21 nt. Of the mapped reads, 80% aligned to protein coding, retained intron, processed transcript, lincRNA, antisense and nonsense-mediated decay whereas miRNA contributed to less than 1% of the mapped reads. We performed hierarchical clustering to investigate the underlying structure of the miRNA expression but found that it does not group according to any clinical parameters, Bx outcome, level Gleason score or clinical grade. To address whether miRNA expression can discriminate high (>7) and low (≤7) Gleason grade, we performed differential expression analysis, adjusting for batch effect and found that a total of 14 miRNAs are dysregulated in urinary EVs. The topmost up-regulated miRNAs are miR-29a-3p, miR-4433a-3p and miR-27a-3p while the two down-regulated miRNAs are miR-1-3p and miR-10a-5p. In order to understand the effect of the up-regulated

miRNAs, we performed pathway analysis on the up and down-regulated miRNAs by DIANATool(303). The results revealed pathways such as ECM-receptor interactions, proteoglycans in cancer, pluripotency of stem cells being affected, ErbB signaling are affected by up-regulated miRNAs. In contrast, pathways such as fatty-acid biosynthesis, pyrimidine metabolism, and Hippo signaling are affected by down-regulated miRNAs.

Discussion

Prostate cancer remains the second most diagnosed cancer world-wide largely due to PSA screening. PSA screening does reduce PCa-specific mortality in the long run (304), however, it has also increased the morbidities associated with PCa diagnosis especially those that will not benefit from it. Thus, there is an urgent need to stratify high risk vs low risk patients to receive confirmatory Bx in order to spare the latter from unnecessary medical procedures as well as psychological and economic burden (83). uEVs has shown great promise as a source of biomarkers for PCa. The challenge thus far has been developing a high throughput and robust isolation method that is compatible with downstream analysis.(305) As a result, many investigators interested in genomics biomarkers has resorted to bulk fluid RNA isolation without prior EV isolation.(306-308)

We had previously developed a uEV isolation and miRNA sequencing method in paper II, but it has yet been tested on a large scale until now. In this work, we showed that uEVs can be isolated robustly from 900 µL of urine with a success rate of 96% using the automated acoustic trapping method. Including library preparation, 86% of all samples resulted in cDNA libraries. Thus, the failed samples mainly occur during library construction and not during trapping. At this time, we cannot compare of our method to other microfluidics-based EV isolation methods due to the dearth of processing statistics. In-addition, our analysis of the cDNA library concentrations and sequencing depth revealed that our methods are not biased towards Bx-negative or Bx-positive sample groups. To our knowledge, this is the first time a high throughput, automated microfluidic EV enrichment method has been applied to clinical samples on this scale.

Investigation into the RNA species revealed that our reads were derived predominantly from long coding and intronic sequences which points to their degradation. As a result, miRNAs were out competed by the abundance of degraded products during sequencing. Our previous work along with other publications have shown that uEV miRNA should compose greater than 10% of the sequencing reads.(222, 309, 310) Nevertheless, our analysis revealed 14 miRNAs that are differentially dysregulated however, we caution its interpretation as the disparate reads in combination with a sensitive analysis likely resulted in type 1 error. This could be observed from miRNAs such as miR-183/100/205/223/615/99b/4433a where the dotplot showed only one or two samples

contributing to the differential expression of the miRNA. The result did provide miRNAs such as miR-1 and miR-29a have been reported previously to be a tumor suppressor in PCa.(311-313)Overall, our work here demonstrated that the acoustic trap can robustly and automatically enrich uEVs from PCa patients and that potential miRNA biomarkers for high grade PCa could be identified but addition work will be needed to validate the finding.

Paper IV : The interplay between the androgen receptor and Hypoxia-inducible factor 2A on extracellular vesicle secretion from prostate cancer cells

Overall Aim: The overall aim of this paper is to explore the effect of androgen signalling and hypoxia on EV secretion in PCa cell lines.

Summary

In this paper, we investigated the role of hypoxia and AR signaling on EVs production using four PCa cell lines, two dependent and two independent of AR. We explored the kinetic of EVs production in PCa cells incubated at either 1% or 21% O_2 over 96 h by harvesting conditioned media (CM) at every 24 h. We found that in DU145 and PC3, AR null (AR-) PCa cells, there are remarkable increase in EVs secretion over the 96 h period at 1% O_2 while the EV secretion of 22Rv1 and LNCaP, AR positive (AR+) PCa cells, remain unaffected by hypoxia. Interestingly, we found that the cell-adjusted EVs level were much higher in AR+ when compared to AR- cells. We hypothesized that the differences in EVs secretion under hypoxia could be a result of androgen signaling. To investigate our hypothesis, we incubated LNCaP cells for 96 h at either normoxic (20% O_2) or hypoxic condition (1% O_2) with media containing androgen with fetal bovine serum (FBS) or androgen-depleted medium with charcoal-stripped serum (CSS) with or without synthetic androgen, R1881. The results revealed that androgen depletion significantly reduces EVs secretion compared to FBS medium and that addition of R1881 could rescue EVs secretion in both normoxic and hypoxic condition. The finding was confirmed by knockdown of AR via small-interfering RNA (siRNA) where siAR reduces the EV secretion compared to control under normoxic condition. Next, we examined the expression of hypoxia-inducible factors (HIFs), HIF-1α and HIF-2α as initiators of EVs secretion during hypoxic episode. In AR- cells, the protein expression of HIF-1α were not observed whereas HIF-2α expressions were elevated from 24 – 96 h of hypoxia exposure. In AR+ cells, HIF-1α was moderately increases at 24 – 48 h but HIF-2α expression was not observed in 22Rv1 and mildly induced at 24 – 48 h. As the induction of HIF-2α was consistent with EVs secretion in AR- cells, we investigated the role of HIF-2α as initiator of EVs secretion under hypoxic exposure. Using an VHL298, an inhibitor of Von Hippel-Lindau (VHL) tumor suppressor protein which prevents the degradation of HIF proteins, we observed that the EVs secretion is elevated in DU145 upon VHL298 treatment compared to vehicle control under normoxic condition. Furthermore, transduction of inducible mutant-HIF2A under the tetracycline promoter into LNCaP confirmed that induced

HIF2A expression increases EVs secretion. It has been documented that the hypoxia can alter the cellular miRNA and extracellular vesicles miRNA (EV-miRNA) content. We assess the effect of hypoxia on the cellular and EVs-miRNA expression of the four PCa cell lines at 96 h by next-generation sequencing. Interestingly, in AR- cells, we found that 15 and 39 cellular miRNAs are altered significantly in DU145 and PC3 cells but in AR+ cells, only 5 and 2 for 22Rv1 and LNCaP respectively. Overall, canonical hypoxia-associated miRNAs such as miR-210 and miR-30 family were consistently altered in most or all cell lines. KEGG pathway analysis revealed that the PI3, FoxO, TGF-β, Hippo and Wnt signaling pathways are affect in AR- cells. In AR+ cells, pathways associated with neurological pathologies such as morphine addiction, cGMP-PKG, gliomas, and prion diseases pathways can be observed.

EV miRNAs were also affected by hypoxic exposure, with 15 and 1 miRNAs expression significantly altered in DU145 and PC3 cells respectively and 2 and 0 miRNAs expression altered in 22Rv1 and LNCaP respectively.

Discussion

Hypoxia is generally associated with the onset of aggressive cancer. In PCa, hypoxia markers have been shown to be an independent prognostic marker for treatment resistance and survival.(281, 282, 314, 315) In our study, we sought to understand the impact of hypoxia on EV secretion and its cargoes using *in-vitro* model of PCa cells,. Two previous studies examining the topic had resulted in divergent results. In one conducted by Panigrahi *et al.*, 1% O_2 hypoxic exposure increased EV secretion in all tested PCa cell lines including 22Rv1, LNCaP and PC3 (316) while Ramteke *et al.* showed that 1% O_2 did not altered the secretion of EVs in LNCaP cells (317). Our results are consistent with the latter publication where the EV secretion of LNCaP or generally in our work, AR+, is invariant to 1% O_2. In contrast, AR- cells showed remarkable induction of HIF2A during the 96 h of hypoxia. Our data points to the transcriptional repression of HIF2A by AR or AR signaling as the likely explanation for the muted hypoxic response observed in both ours and Ramteke *et al* work. In line with this view, Geng *et al.* have recently demonstrated that androgen signaling can repress HIF1α induced GPI up-regulation during hypoxia.(318) In addition, analysis of over 19 tumor types by *Bhandari et al.* had found that PCa ranks the second lowest in hypoxia score.(287) Androgen deprivation therapy (ADT) can effective rescue the repression caused by androgen signaling during hypoxia. It is not known why HIF2A is repressed by AR in PCa. A possible explanation could be that HIF2A is a transcription factor capable of initiating dedifferentiation.(319, 320) Thus, repression of HIF2A could possibly prevent undesirable dedifferentiation of prostate epithelial cells.

Another striking finding from our results is that AR+ cells have significantly higher EV secretion when compared to AR- cells. Investigation into the role of androgen signaling and EV secretion revealed that androgen can positively modulate the secretion of EVs. ADT or knockdown of AR could reduce the EVs secretion in LNCaP. This finding supports the previously work by Mitchell *et al.* whom observed that ADT in patients reduces the levels of EVs in urine.(321)

miRNA deregulation could be observed in many cancers. In our study, we explored the impact of hypoxia on cellular and EV miRNA expression. Interestingly, the miRNA expression changed little in AR+ cells whereas AR- cells resulted in significantly number of differentially expressed miRNA. The result is consistent with our earlier finding that AR+ cells may have a muted response towards hypoxia when compared to AR- cells. Regardless, miR-210, a hypoxia associated miRNA is enriched in three of the four cell lines, the two AR+ and one AR-, which suggest that, all of the cells do experience hypoxia. The finding also points to a possible alternative to detecting hypoxia in PCa through miR-210 instead of traditional immunohistochemistry for HIF proteins or hypoxia related proteins.

As part of our investigation into the impact of hypoxia on miRNA expression, we explored the possible sorting of miRNA into EVs. Interestingly, our analysis showed that miR-210-5p and miR-210-3p are down-regulated in the EVs compared to cells in 22Rv1 and PC3 respectively. The result points to the possibility that miR-210 might be sequestered into EVs during normoxia however, during hypoxia, sorting of miR-210 is inhibited which leads to accumulation inside the cells. Interestingly, miR-210-3p has a GGCU exosome sorting motif at the 3'-end that interacts with SYNCRIP.(322) Whether or not miR-210-3p is sorted via SYNCRIP or that SYNCRIP is prevented from sorting miR-210 during hypoxia remains unknown.

This work has demonstrated that AR and HIF pathways do interact. In our model, AR constitutively repress HIF2A on a transcriptional level while promoting EV secretion under normoxic condition. During hypoxic condition, since AR signaling is still active, EV secretion remains unaffected. In contrast, hypoxia can potently initiate hypoxic response of AR- cells leading to elevated EV secretion and miRNA changes.

Conclusion and Future Perspective

With the number of PCa cases on the rise world-wide, a personalized screening strategy will reduce over treatment while preventing PCa mortality. This necessitate the transition from identifying PCa to identifying clinically significant PCa with high risk of death within the life-expectancy of an individual. In that regard, PSA alone will not solve the problem. To mitigate the problem of overdiagnosis and overtreatment, additional biomarkers could help identify patients harboring high-grade PCa and therefore requiring confirmatory Bx from those who will not benefit from it. The recent demonstration that EVs along with its miRNA cargoes contributes to disease progression suggest that it could be a potential source of biomarkers for PCa. However, since the hurdles for EVs isolation have yet been completely resolved, the translation of EVs as a clinical diagnostic tool remains fulfilled.

The overall aim of this work is to demonstrate that a novel, microfluidic technique termed "acoustic trapping" can overcome the limitations of current EV isolation methods. Towards that end, we began by documenting the performance and applicability of acoustic trapping for EV isolation in paper I. We showed that EVs with sizes ranging from exosome to microvesicles could be isolated from conditioned media, urine and plasma samples by NTS and TEM. The acoustic trap enriched EVs are smaller compared to those enriched by UC. EV protein markers and encapsulated miRNAs could also be detected from the enriched samples. Having established that acoustic trapping can isolate EVs of all sizes, we then optimized a pipeline tailored towards miRNA biomarker discovery from urine in paper II. We showed that sufficient RNA can be isolated from small volume of urine for NGS of small RNA. Importantly, the miRNA profile is highly correlated to UC and that the libraries can be produced repeatably. In paper III, we investigated the applicability of the pipeline for high throughput biomarker discovery from urine samples of PCa patients. We demonstrated that acoustic trapping can process 207 urine samples with high success rate. Investigation into the RNA content revealed abundance of degraded RNAs species but 14 differentially expressed miRNAs can be observed that distinguished patients with high and low Gleason score. Although the current processing time for urinary EV is sufficient for biomarker discovery, it could benefit from additional improvements to the processing time of uEVs. Improvements to the microfluidic

channel or the use of higher harmonic frequencies or increasing the power could reduce the processing time below two hours for 900 μL samples.

As part of the investigation into the role of EVs in PCa, we explored the impact of hypoxia on EV secretion and miRNA expression in PCa cells, *in-vitro*. The study revealed that AR and AR signaling can fundamentally alter the response of PCa towards hypoxia. Cells with AR have a muted hypoxia response, that includes miRNA, while cells without it are sensitive to hypoxia. Regardless, we found that miR-210 is elevated in both cell lines expressing AR and therefore could be a better marker than HIF proteins for hypoxia.

Taken together, our work has demonstrated that the acoustic trap is a robust, automated and high-throughput EV isolation platform. We hope that the instrument and the RNA sequencing pipeline we developed will lower the barrier for EV research and hopefully clinical translation of EVs. In regard to PCa, we hope that the deregulated miRNAs from hypoxia or high grade tumors we found from our experiments could help fine-tune the stratification of patients in the future. Doing so will reduce the number of unnecessary biopsies, and with that, overdiagnosis and over-treatment of PCa without sacrificing the gains of the past three decades.

Popular science summary

Prostate cancer is the most common cancer among men in developed countries. That is, an estimated one out of every eleven men will be diagnosed with the disease within their lifetime. But prostate cancer is not a death sentence. In fact, many men above 50 are living with the disease without their knowledge of it nor are they likely to die as a result of it. The dilemma then becomes, what choice should one take when they are at risk of prostate cancer? Should they opt for a diagnostic procedure that entails invasive biopsy with possible side effect that includes urinary incontinence or should they opt for active surveillance, that is to wait but increase the frequency of check-ups? The choice is difficult, the first will subject an individual to procedures that might not benefit them within the next 5 to 10 years while the second leaves them living in uncertainty. However, there might be a silver-lining? That is, if there is a way to tell patients that they are likely to have the high risk prostate cancer and therefore should opt for diagnostic procedure from the low risk individuals?

Today, we are working on a new area of "liquid biopsy" to help clinicians decide if patients are likely to harbor high grade prostate cancer that require intervention and those that do not. The test examines a class of particles called extracellular vesicles (EVs) that contains proteins and genomic information within and they can be found in all biological fluids which renders them highly accessible for collection. EVs are about one thousand times smaller than the width of human hair. In that size range, many naturally occurring biological molecules can masquerade as EVs. The challenge for researchers is to isolate and purity these particles in an efficient and repeatable manner.

In collaboration with faulty of biomedical engineering, we have applied a microfluidic technology termed "acoustic trapping" that facilitated the process of isolating EVs from plasma and urine samples. Acoustic trapping is based on particle manipulation by ultrasound embedded into a microfluidic device. Using this technology, we have successfully enriched EVs from human plasma and urine samples. Furthermore, we have optimized a method in order to study a type of regulatory genomic information called microRNA (miRNA) that have important function in human. miRNA is consistently deregulated in many types of cancer including prostate cancer and importantly they can be found in EVs.

At the current version, the system can be fully automated and do not require significant operator input which makes it an ideal instrument for setting that requires high throughput sample processing such as clinics.

With the developed technology, we began exploring the question of whether miRNA found in urinary EVs can be used to predict high grade prostate cancer. We screened 200 urine samples from patients with or without detectable prostate cancer and found many candidates that could discriminate between patients harboring high grade and low grade prostate cancer. Though the finding is at an early stage, we hope to validate and translate the finding into routine clinical management of prostate cancer.

As part of the effort to understand the progression of prostate cancer, we studied the response of prostate cancer cells under reduced oxygen level also know as hypoxia. Hypoxia occurs in many different tumor types. In our study, we investigated the change in miRNA in EVs and cells as a result of hypoxic exposure. Our finding suggest that prostate cancers generally do not respond to prolong hypoxic insult while others that lacks a critical receptor known as androgen receptor do. These responder cells could arise from different cell population in the prostate or from the prostate cancer treatment. The hypoxia responsive cells tend to secrete more EVs and their miRNA abundance is altered. This translate to possible more aggressive tumors therefore, clinical detection hypoxia could help stratify high risk patients. Our work had shown that thought hypoxia do exist in prostate cancer, it may not be easily detectable. An alternative strategy of screening for miR-210 could be more sensitive.

Acknowledgements

If there is ever a time to acknowledge the improbable existence of this thesis, it would be now. There are many individuals that made these pages a reality. Countless men and women whom in their own way have deeply shaped my experience here in Sweden. That said, I will start with the most important people:

Yvonne, the past five years *possibly* could not have happened if you had not taken me into your lab (see what I did there?). I appreciate you picking me up from the train station on the first day I arrived in January and all of the things, both in science and in life you taught me since. I am eternally grateful!

Thomas, it was truly an honor to have you as my co-supervisor. You have not only taught me how to think about problems but more importantly, your positivity gave me lots of hope in times of sinking despair. Thank you for your openness and ready to engage in discussions!

Hans, this opportunity is not possible with your support for these past five years. More than that, your scientific insights and your drive for perfection in our work is truly inspiring. I usually walk away from our meetings feeling like I gained IQ points. I will bring your standard of science with me from here on. I am and will always be grateful for the past five years.

Stephan, thank you for all the insightful discussions we had through these years. Your input had definite shaped the way I think about our work.

To the beloved current lab members, **Margareta**, what can I say, without you I would have never found my head in the lab. Thank you for all the positive energy, fun conversation and always willing to help! **Gjendine**, your wits, positivity and enthusiasm are infectious. I have definitely learned a lot from you and feel blessed that we had shared an office together for the past years. Will miss the banter and your baking ☺. **James**, thank you for being an exceptionally kind soul, you have infused the lab with energy, laughter and delicious smelling food. Will miss that tons. **Roger**, a part of Ceders lab by proximity. Thank you for your welcoming attitude and help with all the HIFs stuff. The tea you bought is fantastic!

Past members of Ceder's lab. **Tina**, you have such wisdom at such young age. Thank you for dispensing pointers about life and work, learned a great deal!

David U, you are one of my first mentor in research that I had the pleasure of working with. You have made this all possible and words cannot describe my gratitude. P.S, I still prefer StarBucks coffee.

Rene, tack för lär mig Svenska och Nederländsk. You are such a treat to have in the lab and always fun to talk to. Your determination and hard work are really inspiring. Cooking Thai is still on my agenda for you and Peter, let this me a record. Ska sagna din smaklig kakor!

Camilla, Elinn and **Vasiliki**, thank you being so welcoming and for all guidance in a field that I knew absolutely nothing about. I was a premature-hypoxia-field infant and you raised me to the point of a talking (babbling?) toddler, ya'll did the best you can!

TCR, you have shown me what a well-oiled, efficient and cohesive department could accomplish. Thank you to the silent heroes of TCR, **Elisabth** and **Kristin** whom made much of the work we do possible.

Mikael, if I can split this degree with ya'll I would, you deserve as much of it as I do, the paramedics of the Acoutrap! You have such high dimensionality to your character that makes it really engaging to be around. Thank you for not giving up my attempts at the sour dough starter. I did kill the last one, maybe this might be a good time to ask for the third.

Jessica, thank you for being so supportive with the instrumentation. Your ease and steadiness were soothing during stressful times. P.S, keep in mind, I still have your ab roller record to beat.

Olof, you are like a Chinese brother to me. Your depth of knowledge, intellect and inquisitive nature made for some really fun and interesting conversations. Thank you for all the dinner, philosophy nights and camping trips. Definitely missed knowing whether something is edible or not in the woods. Importantly, thank you for introducing me to the next dude.

Vignesh, it is funny that you think of me as an Indian because I see you as an American with your effortless cool. I admire your intellect, determination, moral compass and kindness towards everyone. Thank you for the advice and encouragement, this couldn't happen with it. Missed all the outdoor trips into the woods, fishing, dinner parties and more importantly our conversations. Also, super cool that we can share twins stories !!!

Emila, you are one of the most generous people I know in Sweden. Your *fun*tastic atitude have been a delight. Thank you for all the fun trips to the summer house and random banter.

Shamik, I am sure you are plugged into the encyclopedia at all time. Your breath of knowledge and intellect is unsurpassed. Thank you for all the amazing

conversations and 24/7 bioinformatic support but more importantly, all the great dinner conversations.

Minerva, **Rolf**, **Jeanette** where do I begin? Thank you for being so welcoming especially with the help on sequencing. A big part of this is possible because of your guidance. I will miss our lunch discussions about everything under the sun.

Franzie, **Eva**, **Cecillia**, **Andrea**, **Anke,** BMC was super fun with you guys around. I love our banter around the FACS and NanoSight. Thank you for all of the encouragements throughout the years!

Hooi Ching, thank you for your guidance and advice during these past years. You taught me a lot about publishing and science in general.

Becca, you have been one of the first people I had the pleasure of meeting and became a lynchpin during my residence here. I love your sense of adventure will miss our forage for Vietnamese food. Definitely the best person to get into trouble with!

Andreea, you always manage to bring peace in my soul with your calm presence. Sweden without your scones will not be the same. I look forward more of it and boardgames with you and hopefully Oscar!

Andy, my brother from a different mother. Your goofiness and chillax attitude

Marina, Markus, **Phuc**, **Thomas T**, **Itzia**, **Saul** and **Natalia**, you guys have made KC and climbing in general so fun. I will miss our banter in between crushing problems. Thank you for being an inspiration and the good vibes.

Hugo, **Astrid**, **Axel**, **Adam,** you guys have been such a cornerstone during my time here. Thank you for the endless laughter, climbing inspiration, dinners and game nights.

Nils, a Swede by birth and name but an American by palate. Thank you for the chill and welcoming attitude! Needless to say, fun climbing trips both local and abroad. Look forward to you coming to the States.

Robert (**Bobby**), you have truly made Sweden a fun and exciting place. Thank you for introducing me to the full body exercise of off-widthing. I thought I knew fear but with you, things can always go to the next level (please delete that picture permanently). **Maria**, you are such a delight, I love your chill and easy laughter!

My family, this is dedicated to you whom with your sacrifice have made this possible. **Sharon** and **David**, I am so blessed to have you guys in my life. **Thomas**, there are thoughts that words can't convey, this is one, so I won't.

Your name will be the last word of this thesis (not including the reference which no one reads) **Adam**.

References

1. Prins GS, Putz O. Molecular signaling pathways that regulate prostate gland development. Differentiation; research in biological diversity. 2008;76(6):641-59.
2. Cunha GR, Chung LW. Stromal-epithelial interactions--I. Induction of prostatic phenotype in urothelium of testicular feminized (Tfm/y) mice. J Steroid Biochem. 1981;14(12):1317-24.
3. Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins SJ, et al. The endocrinology and developmental biology of the prostate. Endocr Rev. 1987;8(3):338-62.
4. Marker PC, Donjacour AA, Dahiya R, Cunha GR. Hormonal, cellular, and molecular control of prostatic development. Dev Biol. 2003;253(2):165-74.
5. Szczyrba J, Niesen A, Wagner M, Wandernoth PM, Aumuller G, Wennemuth G. Neuroendocrine Cells of the Prostate Derive from the Neural Crest. J Biol Chem. 2017;292(5):2021-31.
6. Cunha GR, Vezina CM, Isaacson D, Ricke WA, Timms BG, Cao M, et al. Development of the human prostate. Differentiation; research in biological diversity. 2018;103:24-45.
7. McNeal JE. The zonal anatomy of the prostate. The Prostate. 1981;2(1):35-49.
8. Lee CH, Akin-Olugbade O, Kirschenbaum A. Overview of prostate anatomy, histology, and pathology. Endocrinology and metabolism clinics of North America. 2011;40(3):565-75, viii-ix.
9. Moad M, Hannezo E, Buczacki SJ, Wilson L, El-Sherif A, Sims D, et al. Multipotent Basal Stem Cells, Maintained in Localized Proximal Niches, Support Directed Long-Ranging Epithelial Flows in Human Prostates. Cell Rep. 2017;20(7):1609-22.
10. Zhang D, Zhao S, Li X, Kirk JS, Tang DG. Prostate Luminal Progenitor Cells in Development and Cancer. Trends in cancer. 2018;4(11):769-83.
11. Henry GH, Malewska A, Joseph DB, Malladi VS, Lee J, Torrealba J, et al. A Cellular Anatomy of the Normal Adult Human Prostate and Prostatic Urethra. Cell Rep. 2018;25(12):3530-42 e5.

12. Singh M, Jha R, Melamed J, Shapiro E, Hayward SW, Lee P. Stromal androgen receptor in prostate development and cancer. Am J Pathol. 2014;184(10):2598-607.
13. Cunha GR, Hayward SW, Wang YZ. Role of stroma in carcinogenesis of the prostate. Differentiation; research in biological diversity. 2002;70(9-10):473-85.
14. Olapade-Alaopa EO, MacKay EH, Taub NA, Sandhu DPS, Terry TR, Habib FK. Malignant transformation of human prostatic epithelium is associated with the loss of androgen receptor immunoreactivity in the surrounding stromal. Clinical Cancer Research. 1999;5(3):569-76.
15. Tan MH, Li J, Xu HE, Melcher K, Yong EL. Androgen receptor: structure, role in prostate cancer and drug discovery. Acta Pharmacol Sin. 2015;36(1):3-23.
16. Schaufele F, Carbonell X, Guerbadot M, Borngraeber S, Chapman MS, Ma AA, et al. The structural basis of androgen receptor activation: intramolecular and intermolecular amino-carboxy interactions. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(28):9802-7.
17. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature. 1991;352(6330):77-9.
18. Giovannucci E, Stampfer MJ, Krithivas K, Brown M, Dahl D, Brufsky A, et al. The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(7):3320-3.
19. Callewaert L, Christiaens V, Haelens A, Verrijdt G, Verhoeven G, Claessens F. Implications of a polyglutamine tract in the function of the human androgen receptor. Biochem Biophys Res Commun. 2003;306(1):46-52.
20. Irvine RA, Ma H, Yu MC, Ross RK, Stallcup MR, Coetzee GA. Inhibition of p160-mediated coactivation with increasing androgen receptor polyglutamine length. Hum Mol Genet. 2000;9(2):267-74.
21. Callewaert L, Van Tilborgh N, Claessens F. Interplay between two hormone-independent activation domains in the androgen receptor. Cancer research. 2006;66(1):543-53.
22. Nadal M, Prekovic S, Gallastegui N, Helsen C, Abella M, Zielinska K, et al. Structure of the homodimeric androgen receptor ligand-binding domain. Nat Commun. 2017;8:14388.
23. Yeap BB, Alfonso H, Chubb SA, Handelsman DJ, Hankey GJ, Norman PE, et al. Reference ranges and determinants of testosterone, dihydrotestosterone, and estradiol levels measured using liquid chromatography-tandem mass spectrometry in a population-based cohort of older men. J Clin Endocrinol Metab. 2012;97(11):4030-9.
24. Wilson EM, French FS. Binding properties of androgen receptors. Evidence for identical receptors in rat testis, epididymis, and prostate. J Biol Chem. 1976;251(18):5620-9.

25. Sartorius G, Spasevska S, Idan A, Turner L, Forbes E, Zamojska A, et al. Serum testosterone, dihydrotestosterone and estradiol concentrations in older men self-reporting very good health: the healthy man study. Clin Endocrinol (Oxf). 2012;77(5):755-63.
26. Khan FU, Ihsan AU, Khan HU, Jana R, Wazir J, Khongorzul P, et al. Comprehensive overview of prostatitis. Biomed Pharmacother. 2017;94:1064-76.
27. Parsons JK. Benign Prostatic Hyperplasia and Male Lower Urinary Tract Symptoms: Epidemiology and Risk Factors. Curr Bladder Dysfunct Rep. 2010;5(4):212-8.
28. Mochtar CA, Kiemeney LALM, van Riemsdijk MM, Barnett GS, Laguna MP, Debruyne FMJ, et al. Prostate-specific antigen as an estimator of prostate volume in the management of patients with symptomatic benign prostatic hyperplasia. European urology. 2003;44(6):695-700.
29. Platz EA, Joshu CE, Mondul AM, Peskoe SB, Willett WC, Giovannucci E. Incidence and progression of lower urinary tract symptoms in a large prospective cohort of United States men. J Urol. 2012;188(2):496-501.
30. Chughtai B, Forde JC, Thomas DD, Laor L, Hossack T, Woo HH, et al. Benign prostatic hyperplasia. Nat Rev Dis Primers. 2016;2:16031.
31. American Cancer Society. Cancer Facts & Figures 2019. Atlanta: American Cancer Society; 2019. [
32. SEER Research Data 1975-2016 -- ASCII Text Data:

Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) Research Data (1975-2016), National Cancer Institute, DCCPS, Surveillance Research Program, released April 2019, based on the November 2018 submission. [
33. Taitt HE. Global Trends and Prostate Cancer: A Review of Incidence, Detection, and Mortality as Influenced by Race, Ethnicity, and Geographic Location. Am J Mens Health. 2018;12(6):1807-23.
34. Madersbacher S, Alcaraz A, Emberton M, Hammerer P, Ponholzer A, Schroder FH, et al. The influence of family history on prostate cancer risk: implications for clinical management. BJU Int. 2011;107(5):716-21.
35. Chen YC, Page JH, Chen R, Giovannucci E. Family history of prostate and breast cancer and the risk of prostate cancer in the PSA era. The Prostate. 2008;68(14):1582-91.
36. Munoz M, Pong-Wong R, Canela-Xandri O, Rawlik K, Haley CS, Tenesa A. Evaluating the contribution of genetics and familial shared environment to common disease using the UK Biobank. Nat Genet. 2016;48(9):980-+.
37. Mucci LA, Hjelmborg JB, Harris JR. Familial Risk and Heritability of Cancer Among Twins in Nordic Countries (vol 315, pg 68, 2016). Jama-J Am Med Assoc. 2016;315(8):822-.
38. Al Olama AA, Kote-Jarai Z, Berndt SI, Conti DV, Schumacher F, Han Y, et al. A meta-analysis of 87,040 individuals identifies 23 new susceptibility loci for prostate cancer. Nat Genet. 2014;46(10):1103-9.

39. Taylor RA, Fraser M, Rebello RJ, Boutros PC, Murphy DG, Bristow RG, et al. The influence of BRCA2 mutation on localized prostate cancer. Nat Rev Urol. 2019;16(5):281-90.
40. Castro E, Goh C, Leongamornlert D, Saunders E, Tymrakiewicz M, Dadaev T, et al. Effect of BRCA Mutations on Metastatic Relapse and Cause-specific Survival After Radical Treatment for Localised Prostate Cancer. European urology. 2015;68(2):186-93.
41. Mateo J, Boysen G, Barbieri CE, Bryant HE, Castro E, Nelson PS, et al. DNA Repair in Prostate Cancer: Biology and Clinical Implications. European urology. 2017;71(3):417-25.
42. Ben-Shlomo Y, Evans S, Ibrahim F, Patel B, Anson K, Chinegwundoh F, et al. The risk of prostate cancer amongst black men in the United Kingdom: the PROCESS cohort study. European urology. 2008;53(1):99-105.
43. Lin PH, Aronson W, Freedland SJ. Nutrition, dietary interventions and prostate cancer: the latest evidence. BMC Med. 2015;13:3.
44. Chen J, Song Y, Zhang L. Lycopene/tomato consumption and the risk of prostate cancer: a systematic review and meta-analysis of prospective studies. J Nutr Sci Vitaminol (Tokyo). 2013;59(3):213-23.
45. De Nunzio C, Andriole GL, Thompson IM, Jr., Freedland SJ. Smoking and Prostate Cancer: A Systematic Review. Eur Urol Focus. 2015;1(1):28-38.
46. Zhao J, Stockwell T, Roemer A, Chikritzhs T. Is alcohol consumption a risk factor for prostate cancer? A systematic review and meta-analysis. Bmc Cancer. 2016;16(1):845.
47. Li J, Wang Z. The pathology of unusual subtypes of prostate cancer. Chin J Cancer Res. 2016;28(1):130-43.
48. Inamura K. Prostatic cancers: understanding their molecular pathology and the 2016 WHO classification. Oncotarget. 2018;9(18):14723-37.
49. McNeal JE, Redwine EA, Freiha FS, Stamey TA. Zonal distribution of prostatic adenocarcinoma. Correlation with histologic pattern and direction of spread. The American journal of surgical pathology. 1988;12(12):897-906.
50. Andreoiu M, Cheng L. Multifocal prostate cancer: biologic, prognostic, and therapeutic implications. Hum Pathol. 2010;41(6):781-93.
51. Bostwick DG, Shan A, Qian J, Darson M, Maihle NJ, Jenkins RB, et al. Independent origin of multiple foci of prostatic intraepithelial neoplasia: comparison with matched foci of prostate carcinoma. Cancer. 1998;83(9):1995-2002.
52. Wei L, Wang J, Lampert E, Schlanger S, DePriest AD, Hu Q, et al. Intratumoral and Intertumoral Genomic Heterogeneity of Multifocal Localized Prostate Cancer Impacts Molecular Classifications and Genomic Prognosticators. European urology. 2017;71(2):183-92.
53. Berger MF, Lawrence MS, Demichelis F, Drier Y, Cibulskis K, Sivachenko AY, et al. The genomic complexity of primary human prostate cancer. Nature. 2011;470(7333):214-20.

54. Mosquera JM, Mehra R, Regan MM, Perner S, Genega EM, Bueti G, et al. Prevalence of TMPRSS2-ERG fusion prostate cancer among men undergoing prostate biopsy in the United States. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15(14):4706-11.
55. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, et al. Integrative genomic profiling of human prostate cancer. Cancer Cell. 2010;18(1):11-22.
56. Barbieri CE, Bangma CH, Bjartell A, Catto JW, Culig Z, Gronberg H, et al. The mutational landscape of prostate cancer. European urology. 2013;64(4):567-76.
57. Robinson D, Van Allen EM, Wu YM, Schultz N, Lonigro RJ, Mosquera JM, et al. Integrative Clinical Genomics of Advanced Prostate Cancer. Cell. 2015;161(5):1215-28.
58. Cancer Genome Atlas Research N. The Molecular Taxonomy of Primary Prostate Cancer. Cell. 2015;163(4):1011-25.
59. Grasso CS, Wu YM, Robinson DR, Cao X, Dhanasekaran SM, Khan AP, et al. The mutational landscape of lethal castration-resistant prostate cancer. Nature. 2012;487(7406):239-43.
60. Cancer Genome Atlas Research Network. Electronic address scmo, Cancer Genome Atlas Research N. The Molecular Taxonomy of Primary Prostate Cancer. Cell. 2015;163(4):1011-25.
61. Hayes JH, Barry MJ. Screening for prostate cancer with the prostate-specific antigen test: a review of current evidence. JAMA. 2014;311(11):1143-9.
62. Martin RM, Donovan JL, Turner EL, Metcalfe C, Young GJ, Walsh EI, et al. Effect of a Low-Intensity PSA-Based Screening Intervention on Prostate Cancer Mortality: The CAP Randomized Clinical Trial. JAMA. 2018;319(9):883-95.
63. Schroder FH, Hugosson J, Roobol MJ, Tammela TL, Zappa M, Nelen V, et al. Screening and prostate cancer mortality: results of the European Randomised Study of Screening for Prostate Cancer (ERSPC) at 13 years of follow-up. Lancet. 2014;384(9959):2027-35.
64. Godtman RA, Holmberg E, Lilja H, Stranne J, Hugosson J. Opportunistic Testing Versus Organized Prostate-specific Antigen Screening: Outcome After 18 Years in the Goteborg Randomized Population-based Prostate Cancer Screening Trial. European urology. 2015;68(3):354-60.
65. Loeb S, Vonesh EF, Metter EJ, Carter HB, Gann PH, Catalona WJ. What Is the True Number Needed to Screen and Treat to Save a Life With Prostate-Specific Antigen Testing? Journal of Clinical Oncology. 2011;29(4):464-7.
66. Cohn JA, Wang CE, Lakeman JC, Silverstein JC, Brendler CB, Novakovic KR, et al. Primary care physician PSA screening practices before and after the final U.S. Preventive Services Task Force recommendation. Urol Oncol. 2014;32(1):41.e23-30.
67. Bhindi B, Mamdani M, Kulkarni GS, Finelli A, Hamilton RJ, Trachtenberg J, et al. Impact of the U.S. Preventive Services Task Force

recommendations against prostate specific antigen screening on prostate biopsy and cancer detection rates. J Urol. 2015;193(5):1519-24.
68. Houston KA, King J, Li J, Jemal A. Trends in Prostate Cancer Incidence Rates and Prevalence of Prostate Specific Antigen Screening by Socioeconomic Status and Regions in the United States, 2004 to 2013. J Urol. 2018;199(3):676-82.
69. Banerji JS, Wolff EM, Massman JD, 3rd, Odem-Davis K, Porter CR, Corman JM. Prostate Needle Biopsy Outcomes in the Era of the U.S. Preventive Services Task Force Recommendation against Prostate Specific Antigen Based Screening. J Urol. 2016;195(1):66-73.
70. Hamdy FC, Donovan JL, Lane JA, Mason M, Metcalfe C, Holding P, et al. 10-Year Outcomes after Monitoring, Surgery, or Radiotherapy for Localized Prostate Cancer. N Engl J Med. 2016;375(15):1415-24.
71. Fenton JJ, Weyrich MS, Durbin S, Liu Y, Bang H, Melnikow J. Prostate-Specific Antigen-Based Screening for Prostate Cancer: Evidence Report and Systematic Review for the US Preventive Services Task Force. JAMA. 2018;319(18):1914-31.
72. Lilja H, Christensson A, Dahlen U, Matikainen MT, Nilsson O, Pettersson K, et al. Prostate-specific antigen in serum occurs predominantly in complex with alpha 1-antichymotrypsin. Clin Chem. 1991;37(9):1618-25.
73. Lilja H. Significance of different molecular forms of serum PSA. The free, noncomplexed form of PSA versus that complexed to alpha 1-antichymotrypsin. Urol Clin North Am. 1993;20(4):681-6.
74. Lilja H, Ulmert D, Vickers AJ. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. Nature reviews Cancer. 2008;8(4):268-78.
75. Harvey P, Basuita A, Endersby D, Curtis B, Iacovidou A, Walker M. A systematic review of the diagnostic accuracy of prostate specific antigen. Bmc Urol. 2009;9:14.
76. Thompson IM, Pauler DK, Goodman PJ, Tangen CM, Lucia MS, Parnes HL, et al. Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. N Engl J Med. 2004;350(22):2239-46.
77. Franlund M, Arnsrud Godtman R, Carlsson SV, Lilja H, Mansson M, Stranne J, et al. Prostate cancer risk assessment in men with an initial P.S.A. below 3 ng/mL: results from the Goteborg randomized population-based prostate cancer screening trial. Scand J Urol. 2018;52(4):256-62.
78. Stattin P, Vickers AJ, Sjoberg DD, Johansson R, Granfors T, Johansson M, et al. Improving the Specificity of Screening for Lethal Prostate Cancer Using Prostate-specific Antigen and a Panel of Kallikrein Markers: A Nested Case-Control Study. European urology. 2015;68(2):207-13.
79. Ahmed HU, El-Shater Bosaily A, Brown LC, Gabe R, Kaplan R, Parmar MK, et al. Diagnostic accuracy of multi-parametric MRI and TRUS biopsy in prostate cancer (PROMIS): a paired validating confirmatory study. Lancet. 2017;389(10071):815-22.

80. Serefoglu EC, Altinova S, Ugras NS, Akincioglu E, Asil E, Balbay MD. How reliable is 12-core prostate biopsy procedure in the detection of prostate cancer? Can Urol Assoc J. 2013;7(5-6):E293-8.
81. van der Leest M, Cornel E, Israel B, Hendriks R, Padhani AR, Hoogenboom M, et al. Head-to-head Comparison of Transrectal Ultrasound-guided Prostate Biopsy Versus Multiparametric Prostate Resonance Imaging with Subsequent Magnetic Resonance-guided Biopsy in Biopsy-naive Men with Elevated Prostate-specific Antigen: A Large Prospective Multicenter Clinical Study. European urology. 2019;75(4):570-8.
82. Loeb S, Vellekoop A, Ahmed HU, Catto J, Emberton M, Nam R, et al. Systematic review of complications of prostate biopsy. European urology. 2013;64(6):876-92.
83. Roth JA, Gulati R, Gore JL, Cooperberg MR, Etzioni R. Economic Analysis of Prostate-Specific Antigen Screening and Selective Treatment Strategies. JAMA Oncol. 2016;2(7):890-8.
84. Gleason DF, Mellinger GT. Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. J Urol. 1974;111(1):58-64.
85. Shariat SF, Kattan MW, Vickers AJ, Karakiewicz PI, Scardino PT. Critical review of prostate cancer predictive tools. Future Oncol. 2009;5(10):1555-84.
86. Sanda MG, Cadeddu JA, Kirkby E, Chen RC, Crispino T, Fontanarosa J, et al. Clinically Localized Prostate Cancer: AUA/ASTRO/SUO Guideline. Part I: Risk Stratification, Shared Decision Making, and Care Options. J Urol. 2018;199(3):683-90.
87. Mottet N, Bellmunt J, Bolla M, Briers E, Cumberbatch MG, De Santis M, et al. EAU-ESTRO-SIOG Guidelines on Prostate Cancer. Part 1: Screening, Diagnosis, and Local Treatment with Curative Intent. European urology. 2017;71(4):618-29.
88. Nawaz M, Camussi G, Valadi H, Nazarenko I, Ekstrom K, Wang XQ, et al. The emerging role of extracellular vesicles as biomarkers for urogenital cancers. Nature Reviews Urology. 2014;11(12):688-701.
89. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75(5):843-54.
90. Ludwig N, Leidinger P, Becker K, Backes C, Fehlmann T, Pallasch C, et al. Distribution of miRNA expression across human tissues. Nucleic Acids Res. 2016;44(8):3865-77.
91. Liang Y, Ridzon D, Wong L, Chen C. Characterization of microRNA expression profiles in normal human tissues. Bmc Genomics. 2007;8:166.
92. Bartel DP. Metazoan MicroRNAs. Cell. 2018;173(1):20-51.
93. Roden C, Gaillard J, Kanoria S, Rennie W, Barish S, Cheng J, et al. Novel determinants of mammalian primary microRNA processing revealed by systematic evaluation of hairpin-containing transcripts and human genetic variation. Genome Res. 2017;27(3):374-84.

94. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, et al. Argonaute2 is the catalytic engine of mammalian RNAi. Science. 2004;305(5689):1437-41.
95. Broughton JP, Lovci MT, Huang JL, Yeo GW, Pasquinelli AE. Pairing beyond the Seed Supports MicroRNA Targeting Specificity. Mol Cell. 2016;64(2):320-33.
96. Gebert LFR, MacRae IJ. Regulation of microRNA function in animals. Nat Rev Mol Cell Bio. 2019;20(1):21-37.
97. Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. Science. 2004;304(5670):594-6.
98. Meijer HA, Kong YW, Lu WT, Wilczynska A, Spriggs RV, Robinson SW, et al. Translational repression and eIF4A2 activity are critical for microRNA-mediated gene regulation. Science. 2013;340(6128):82-5.
99. Fukaya T, Iwakawa HO, Tomari Y. MicroRNAs block assembly of eIF4F translation initiation complex in Drosophila. Mol Cell. 2014;56(1):67-78.
100. Eichhorn SW, Guo H, McGeary SE, Rodriguez-Mias RA, Shin C, Baek D, et al. mRNA destabilization is the dominant effect of mammalian microRNAs by the time substantial repression ensues. Mol Cell. 2014;56(1):104-15.
101. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature. 2010;466(7308):835-40.
102. Jakymiw A, Lian S, Eystathioy T, Li S, Satoh M, Hamel JC, et al. Disruption of GW bodies impairs mammalian RNA interference. Nat Cell Biol. 2005;7(12):1267-74.
103. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19(1):92-105.
104. Moore MJ, Scheel TKH, Luna JM, Park CY, Fak JJ, Nishiuchi E, et al. miRNA-target chimeras reveal miRNA 3 '-end pairing as a major determinant of Argonaute target specificity. Nature Communications. 2015;6.
105. Hafner M, Landthaler M, Burger L, Khorshid M, Hausser J, Berninger P, et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. Cell. 2010;141(1):129-41.
106. Neilsen CT, Goodall GJ, Bracken CP. IsomiRs--the overlooked repertoire in the dynamic microRNAome. Trends Genet. 2012;28(11):544-9.
107. Yang WD, Chendrimada TP, Wang QD, Higuchi M, Seeburg PH, Shiekhattar R, et al. Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nat Struct Mol Biol. 2006;13(1):13-21.
108. Kawahara Y, Zinshteyn B, Sethupathy P, Iizasa H, Hatzigeorgiou AG, Nishikura K. Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. Science. 2007;315(5815):1137-40.
109. Yang A, Bofill-De Ros X, Shao TJ, Jiang M, Li K, Villanueva P, et al. 3' Uridylation Confers miRNAs with Non-canonical Target Repertoires. Mol Cell. 2019;75(3):511-22.e4.

110. Kawahara Y, Zinshteyn B, Chendrimada TP, Shiekhattar R, Nishikura K. RNA editing of the microRNA-151 precursor blocks cleavage by the Dicer-TRBP complex. EMBO Rep. 2007;8(8):763-9.
111. Wang YM, Liang H. When MicroRNAs Meet RNA Editing in Cancer: A Nucleotide Change Can Make a Difference. Bioessays. 2018;40(2).
112. Ruegger S, Grosshans H. MicroRNA turnover: when, how, and why. Trends in Biochemical Sciences. 2012;37(10):436-46.
113. Reichholf B, Herzog VA, Fasching N, Manzenreither RA, Sowemimo I, Ameres SL. Time-Resolved Small RNA Sequencing Unravels the Molecular Principles of MicroRNA Homeostasis. Mol Cell. 2019;75(4):756-68 e7.
114. La Rocca G, Olejniczak SH, Gonzalez AJ, Briskin D, Vidigal JA, Spraggon L, et al. In vivo, Argonaute-bound microRNAs exist predominantly in a reservoir of low molecular weight complexes not associated with mRNA. Proceedings of the National Academy of Sciences of the United States of America. 2015;112(3):767-72.
115. Pitchiaya S, Heinicke LA, Park JI, Cameron EL, Walter NG. Resolving Subcellular miRNA Trafficking and Turnover at Single-Molecule Resolution. Cell Rep. 2017;19(3):630-42.
116. Smibert P, Yang JS, Azzam G, Liu JL, Lai EC. Homeostatic control of Argonaute stability by microRNA availability. Nat Struct Mol Biol. 2013;20(7):789-95.
117. Yang M, Haase AD, Huang FK, Coulis G, Rivera KD, Dickinson BC, et al. Dephosphorylation of tyrosine 393 in argonaute 2 by protein tyrosine phosphatase 1B regulates gene silencing in oncogenic RAS-induced senescence. Mol Cell. 2014;55(5):782-90.
118. Rudel S, Wang Y, Lenobel R, Korner R, Hsiao HH, Urlaub H, et al. Phosphorylation of human Argonaute proteins affects small RNA binding. Nucleic Acids Res. 2011;39(6):2330-43.
119. McKenzie AJ, Hoshino D, Hong NH, Cha DJ, Franklin JL, Coffey RJ, et al. KRAS-MEK Signaling Controls Ago2 Sorting into Exosomes. Cell Rep. 2016;15(5):978-87.
120. Pritchard CC, Cheng HH, Tewari M. MicroRNA profiling: approaches and considerations. Nat Rev Genet. 2012;13(5):358-69.
121. Metpally RP, Nasser S, Malenica I, Courtright A, Carlson E, Ghaffari L, et al. Comparison of Analysis Tools for miRNA High Throughput Sequencing Using Nerve Crush as a Model. Front Genet. 2013;4:20.
122. Yehudai-Resheff S, Schuster G. Characterization of the E.coli poly(A) polymerase: nucleotide specificity, RNA-binding affinities and RNA structure dependence. Nucleic Acids Res. 2000;28(5):1139-44.
123. Zhuang F, Fuchs RT, Sun Z, Zheng Y, Robb GB. Structural bias in T4 RNA ligase-mediated 3'-adapter ligation. Nucleic Acids Res. 2012;40(7):e54.
124. Fuchs RT, Sun Z, Zhuang F, Robb GB. Bias in ligation-based small RNA sequencing library construction is determined by adaptor and RNA structure. PLoS One. 2015;10(5):e0126049.

125. Dabney J, Meyer M. Length and GC-biases during sequencing library amplification: a comparison of various polymerase-buffer systems with ancient and modern DNA sequencing libraries. Biotechniques. 2012;52(2):87-94.
126. Williamson V, Kim A, Xie B, McMichael GO, Gao Y, Vladimirov V. Detecting miRNAs in deep-sequencing data: a software performance comparison and evaluation. Brief Bioinform. 2013;14(1):36-45.
127. Campbell JD, Liu G, Luo L, Xiao J, Gerrein J, Juan-Guardela B, et al. Assessment of microRNA differential expression and detection in multiplexed small RNA sequencing data. RNA. 2015;21(2):164-71.
128. Costa-Silva J, Domingues D, Lopes FM. RNA-Seq differential expression analysis: An extended review and a software tool. PLoS One. 2017;12(12):e0190152.
129. Rosenfeld N, Aharonov R, Meiri E, Rosenwald S, Spector Y, Zepeniuk M, et al. MicroRNAs accurately identify cancer tissue origin. Nat Biotechnol. 2008;26(4):462-9.
130. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(7):2257-61.
131. Yang CH, Yue J, Fan M, Pfeffer LM. IFN induces miR-21 through a signal transducer and activator of transcription 3-dependent pathway as a suppressive negative feedback on IFN-induced apoptosis. Cancer research. 2010;70(20):8108-16.
132. Coppola V, Musumeci M, Patrizii M, Cannistraci A, Addario A, Maugeri-Sacca M, et al. BTG2 loss and miR-21 upregulation contribute to prostate cell transformation by inducing luminal markers expression and epithelial-mesenchymal transition. Oncogene. 2013;32(14):1843-53.
133. Tian L, Fang YX, Xue JL, Chen JZ. Four microRNAs promote prostate cell proliferation with regulation of PTEN and its downstream signals in vitro. PLoS One. 2013;8(9):e75885.
134. Ribas J, Ni X, Haffner M, Wentzel EA, Salmasi AH, Chowdhury WH, et al. miR-21: an androgen receptor-regulated microRNA that promotes hormone-dependent and hormone-independent prostate cancer growth. Cancer research. 2009;69(18):7165-9.
135. Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, Ciafre SA, et al. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. J Biol Chem. 2007;282(32):23716-24.
136. Hsu TI, Hsu CH, Lee KH, Lin JT, Chen CS, Chang KC, et al. MicroRNA-18a is elevated in prostate cancer and promotes tumorigenesis through suppressing STK4 in vitro and in vivo. Oncogenesis. 2014;3:e99.
137. Dong Q, Meng P, Wang T, Qin W, Qin W, Wang F, et al. MicroRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2. PLoS One. 2010;5(4):e10147.

138. Karatas OF, Guzel E, Suer I, Ekici ID, Caskurlu T, Creighton CJ, et al. miR-1 and miR-133b are differentially expressed in patients with recurrent prostate cancer. PLoS One. 2014;9(6):e98675.
139. Liu YN, Yin JJ, Abou-Kheir W, Hynes PG, Casey OM, Fang L, et al. MiR-1 and miR-200 inhibit EMT via Slug-dependent and tumorigenesis via Slug-independent mechanisms. Oncogene. 2013;32(3):296-306.
140. Qin W, Shi Y, Zhao B, Yao C, Jin L, Ma J, et al. miR-24 regulates apoptosis by targeting the open reading frame (ORF) region of FAF1 in cancer cells. PLoS One. 2010;5(2):e9429.
141. Ambs S, Prueitt RL, Yi M, Hudson RS, Howe TM, Petrocca F, et al. Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. Cancer research. 2008;68(15):6162-70.
142. Haflidadottir BS, Larne O, Martin M, Persson M, Edsjo A, Bjartell A, et al. Upregulation of miR-96 enhances cellular proliferation of prostate cancer cells through FOXO1. PLoS One. 2013;8(8):e72400.
143. Yao J, Xu C, Fang Z, Li Y, Liu H, Wang Y, et al. Androgen receptor regulated microRNA miR-182-5p promotes prostate cancer progression by targeting the ARRDC3/ITGB4 pathway. Biochem Biophys Res Commun. 2016;474(1):213-9.
144. Majid S, Dar AA, Saini S, Yamamura S, Hirata H, Tanaka Y, et al. MicroRNA-205-directed transcriptional activation of tumor suppressor genes in prostate cancer. Cancer. 2010;116(24):5637-49.
145. Reis ST, Pontes-Junior J, Antunes AA, Dall'Oglio MF, Dip N, Passerotti CC, et al. miR-21 may acts as an oncomir by targeting RECK, a matrix metalloproteinase regulator, in prostate cancer. Bmc Urol. 2012;12:14.
146. Voss G, Haflidadottir BS, Jaremo H, Persson M, Catela Ivkovic T, Wikstrom P, et al. Regulation of Cell-Cell Adhesion in Prostate Cancer Cells by microRNA-96 through Upregulation of E-Cadherin and EpCAM. Carcinogenesis. 2019.
147. Clape C, Fritz V, Henriquet C, Apparailly F, Fernandez PL, Iborra F, et al. miR-143 interferes with ERK5 signaling, and abrogates prostate cancer progression in mice. PLoS One. 2009;4(10):e7542.
148. Andersen S, Richardsen E, Moi L, Donnem T, Nordby Y, Ness N, et al. Fibroblast miR-210 overexpression is independently associated with clinical failure in Prostate Cancer - a multicenter (in situ hybridization) study. Scientific reports. 2016;6:36573.
149. Taddei ML, Cavallini L, Comito G, Giannoni E, Folini M, Marini A, et al. Senescent stroma promotes prostate cancer progression: the role of miR-210. Mol Oncol. 2014;8(8):1729-46.
150. Ostling P, Leivonen SK, Aakula A, Kohonen P, Makela R, Hagman Z, et al. Systematic analysis of microRNAs targeting the androgen receptor in prostate cancer cells. Cancer research. 2011;71(5):1956-67.
151. Amir S, Ma AH, Shi XB, Xue L, Kung HJ, Devere White RW. Oncomir miR-125b suppresses p14(ARF) to modulate p53-dependent and p53-independent apoptosis in prostate cancer. PLoS One. 2013;8(4):e61064.

152. Aakula A, Leivonen SK, Hintsanen P, Aittokallio T, Ceder Y, Borresen-Dale AL, et al. MicroRNA-135b regulates ERalpha, AR and HIF1AN and affects breast and prostate cancer cell growth. Mol Oncol. 2015;9(7):1287-300.
153. Larne O, Hagman Z, Lilja H, Bjartell A, Edsjo A, Ceder Y. miR-145 suppress the androgen receptor in prostate cancer cells and correlates to prostate cancer prognosis. Carcinogenesis. 2015;36(8):858-66.
154. Larne O, Ostling P, Haflidadottir BS, Hagman Z, Aakula A, Kohonen P, et al. miR-183 in prostate cancer cells positively regulates synthesis and serum levels of prostate-specific antigen. European urology. 2015;68(4):581-8.
155. Deatherage BL, Cookson BT. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. Infect Immun. 2012;80(6):1948-57.
156. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. The Journal of cell biology. 2013;200(4):373-83.
157. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol. 2014;30:255-89.
158. Baietti MF, Zhang Z, Mortier E, Melchior A, Degeest G, Geeraerts A, et al. Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nat Cell Biol. 2012;14(7):677-85.
159. Colombo M, Moita C, van Niel G, Kowal J, Vigneron J, Benaroch P, et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. J Cell Sci. 2013;126(Pt 24):5553-65.
160. Baietti MF, Zhang Z, Mortier E, Melchior A, Degeest G, Geeraerts A, et al. Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nat Cell Biol. 2012;14(7):677-85.
161. Abrami L, Brandi L, Moayeri M, Brown MJ, Krantz BA, Leppla SH, et al. Hijacking multivesicular bodies enables long-term and exosome-mediated long-distance action of anthrax toxin. Cell Rep. 2013;5(4):986-96.
162. Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. J Biol Chem. 1998;273(32):20121-7.
163. Nazarenko I, Rana S, Baumann A, McAlear J, Hellwig A, Trendelenburg M, et al. Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation. Cancer research. 2010;70(4):1668-78.
164. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, et al. Ceramide triggers budding of exosome vesicles into multivesicular Endosomes. Science. 2008;319(5867):1244-7.
165. Laulagnier K, Grand D, Dujardin A, Hamdi S, Vincent-Schneider H, Lankar D, et al. PLD2 is enriched on exosomes and its activity is correlated to the release of exosomes. Febs Lett. 2004;572(1-3):11-4.

166. Villarroya-Beltri C, Gutierrez-Vazquez C, Sanchez-Cabo F, Perez-Hernandez D, Vazquez J, Martin-Cofreces N, et al. Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. Nat Commun. 2013;4:2980.
167. Temoche-Diaz MM, Shurtleff MJ, Nottingham RM, Yao J, Fadadu RP, Lambowitz AM, et al. Distinct mechanisms of microRNA sorting into cancer cell-derived extracellular vesicle subtypes. Elife. 2019;8.
168. Koppers-Lalic D, Hackenberg M, Bijnsdorp IV, van Eijndhoven MAJ, Sadek P, Sie D, et al. Nontemplated nucleotide additions distinguish the small RNA composition in cells from exosomes. Cell Rep. 2014;8(6):1649-58.
169. Shurtleff MJ, Temoche-Diaz MM, Karfilis KV, Ri S, Schekman R. Y-box protein 1 is required to sort microRNAs into exosomes in cells and in a cell-free reaction. Elife. 2016;5.
170. Teng Y, Ren Y, Hu X, Mu J, Samykutty A, Zhuang X, et al. MVP-mediated exosomal sorting of miR-193a promotes colon cancer progression. Nat Commun. 2017;8:14448.
171. Kowal J, Tkach M, Thery C. Biogenesis and secretion of exosomes. Curr Opin Cell Biol. 2014;29:116-25.
172. Martens S, McMahon HT. Mechanisms of membrane fusion: disparate players and common principles. Nat Rev Mol Cell Biol. 2008;9(7):543-56.
173. Andreu Z, Yanez-Mo M. Tetraspanins in extracellular vesicle formation and function. Front Immunol. 2014;5:442.
174. Stenmark H. Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol. 2009;10(8):513-25.
175. Nabhan JF, Hu R, Oh RS, Cohen SN, Lu Q. Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(11):4146-51.
176. Muralidharan-Chari V, Clancy J, Plou C, Romao M, Chavrier P, Raposo G, et al. ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. Curr Biol. 2009;19(22):1875-85.
177. Bolukbasi MF, Mizrak A, Ozdener GB, Madlener S, Strobel T, Erkan EP, et al. miR-1289 and "Zipcode"-like Sequence Enrich mRNAs in Microvesicles. Mol Ther Nucleic Acids. 2012;1:e10.
178. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. Nature reviews Immunology. 2014;14(3):195-208.
179. Stenqvist AC, Nagaeva O, Baranov V, Mincheva-Nilsson L. Exosomes secreted by human placenta carry functional Fas ligand and TRAIL molecules and convey apoptosis in activated immune cells, suggesting exosome-mediated immune privilege of the fetus. J Immunol. 2013;191(11):5515-23.
180. Feng Q, Zhang C, Lum D, Druso JE, Blank B, Wilson KF, et al. A class of extracellular vesicles from breast cancer cells activates VEGF receptors and tumour angiogenesis. Nat Commun. 2017;8:14450.

181. Webber J, Steadman R, Mason MD, Tabi Z, Clayton A. Cancer exosomes trigger fibroblast to myofibroblast differentiation. Cancer research. 2010;70(23):9621-30.
182. Kalluri R. The biology and function of fibroblasts in cancer. Nature reviews Cancer. 2016;16(9):582-98.
183. Vu LT, Peng B, Zhang DX, Ma V, Mathey-Andrews CA, Lam CK, et al. Tumor-secreted extracellular vesicles promote the activation of cancer-associated fibroblasts via the transfer of microRNA-125b. J Extracell Vesicles. 2019;8(1):1599680.
184. Dvorak HF. Tumors: wounds that do not heal-redux. Cancer Immunol Res. 2015;3(1):1-11.
185. Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR. Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. Clinical cancer research : an official journal of the American Association for Cancer Research. 2002;8(9):2912-23.
186. Wendler F, Favicchio R, Simon T, Alifrangis C, Stebbing J, Giamas G. Extracellular vesicles swarm the cancer microenvironment: from tumor-stroma communication to drug intervention. Oncogene. 2017;36(7):877-84.
187. Nabet BY, Qiu Y, Shabason JE, Wu TJ, Yoon T, Kim BC, et al. Exosome RNA Unshielding Couples Stromal Activation to Pattern Recognition Receptor Signaling in Cancer. Cell. 2017;170(2):352-66 e13.
188. Admyre C, Bohle B, Johansson SM, Focke-Tejkl M, Valenta R, Scheynius A, et al. B cell-derived exosomes can present allergen peptides and activate allergen-specific T cells to proliferate and produce TH2-like cytokines. J Allergy Clin Immunol. 2007;120(6):1418-24.
189. Qazi KR, Gehrmann U, Domange Jordo E, Karlsson MC, Gabrielsson S. Antigen-loaded exosomes alone induce Th1-type memory through a B-cell-dependent mechanism. Blood. 2009;113(12):2673-83.
190. Thery C, Duban L, Segura E, Veron P, Lantz O, Amigorena S. Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. Nat Immunol. 2002;3(12):1156-62.
191. Huber V, Fais S, Iero M, Lugini L, Canese P, Squarcina P, et al. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. Gastroenterology. 2005;128(7):1796-804.
192. Andreola G, Rivoltini L, Castelli C, Huber V, Perego P, Deho P, et al. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. J Exp Med. 2002;195(10):1303-16.
193. Chen G, Huang AC, Zhang W, Zhang G, Wu M, Xu W, et al. Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. Nature. 2018;560(7718):382-6.
194. Ricklefs FL, Alayo Q, Krenzlin H, Mahmoud AB, Speranza MC, Nakashima H, et al. Immune evasion mediated by PD-L1 on glioblastoma-derived extracellular vesicles. Sci Adv. 2018;4(3):eaar2766.
195. Szajnik M, Czystowska M, Szczepanski MJ, Mandapathil M, Whiteside TL. Tumor-derived microvesicles induce, expand and up-regulate

biological activities of human regulatory T cells (Treg). PLoS One. 2010;5(7):e11469.
196. Valenti R, Huber V, Filipazzi P, Pilla L, Sovena G, Villa A, et al. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. Cancer research. 2006;66(18):9290-8.
197. Hakulinen J, Sankkila L, Sugiyama N, Lehti K, Keski-Oja J. Secretion of active membrane type 1 matrix metalloproteinase (MMP-14) into extracellular space in microvesicular exosomes. J Cell Biochem. 2008;105(5):1211-8.
198. Bobrie A, Krumeich S, Reyal F, Recchi C, Moita LF, Seabra MC, et al. Rab27a Supports Exosome-Dependent and -Independent Mechanisms That Modify the Tumor Microenvironment and Can Promote Tumor Progression. Cancer research. 2012;72(19):4920-30.
199. Di Vizio D, Morello M, Dudley AC, Schow PW, Adam RM, Morley S, et al. Large oncosomes in human prostate cancer tissues and in the circulation of mice with metastatic disease. Am J Pathol. 2012;181(5):1573-84.
200. Fan D, Wang Y, Qi P, Chen Y, Xu P, Yang X, et al. MicroRNA-183 functions as the tumor suppressor via inhibiting cellular invasion and metastasis by targeting MMP-9 in cervical cancer. Gynecol Oncol. 2016;141(1):166-74.
201. Zhang L, Dong Y, Zhu N, Tsoi H, Zhao Z, Wu CW, et al. microRNA-139-5p exerts tumor suppressor function by targeting NOTCH1 in colorectal cancer. Mol Cancer. 2014;13:124.
202. Liu G, Jiang C, Li D, Wang R, Wang W. MiRNA-34a inhibits EGFR-signaling-dependent MMP7 activation in gastric cancer. Tumour Biol. 2014;35(10):9801-6.
203. Sung BH, Ketova T, Hoshino D, Zijlstra A, Weaver AM. Directional cell movement through tissues is controlled by exosome secretion. Nat Commun. 2015;6:7164.
204. Eddy RJ, Weidmann MD, Sharma VP, Condeelis JS. Tumor Cell Invadopodia: Invasive Protrusions that Orchestrate Metastasis. Trends Cell Biol. 2017;27(8):595-607.
205. Steffen A, Le Dez G, Poincloux R, Recchi C, Nassoy P, Rottner K, et al. MT1-MMP-dependent invasion is regulated by TI-VAMP/VAMP7. Curr Biol. 2008;18(12):926-31.
206. Patsialou A, Wang Y, Lin J, Whitney K, Goswami S, Kenny PA, et al. Selective gene-expression profiling of migratory tumor cells in vivo predicts clinical outcome in breast cancer patients. Breast cancer research : BCR. 2012;14(5):R139.
207. Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nature Medicine. 2012;18(6):883-+.

208. Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nat Cell Biol. 2015;17(6):816-26.
209. Zhang L, Zhang S, Yao J, Lowery FJ, Zhang Q, Huang WC, et al. Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. Nature. 2015;527(7576):100-4.
210. Godoy PM, Bhakta NR, Barczak AJ, Cakmak H, Fisher S, MacKenzie TC, et al. Large Differences in Small RNA Composition Between Human Biofluids. Cell Reports. 2018;25(5):1346-58.
211. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. Clin Chem. 2010;56(11):1733-41.
212. Musante L, Tataruch DE, Holthofer H. Use and isolation of urinary exosomes as biomarkers for diabetic nephropathy. Front Endocrinol (Lausanne). 2014;5:149.
213. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(36):13368-73.
214. Gonzales PA, Pisitkun T, Hoffert JD, Tchapyjnikov D, Star RA, Kleta R, et al. Large-scale proteomics and phosphoproteomics of urinary exosomes. J Am Soc Nephrol. 2009;20(2):363-79.
215. Wang Y-h, Zhang Y-g. Kidney and innate immunity. Immunology Letters. 2017;183:73-8.
216. Micanovic R, LaFavers K, Garimella PS, Wu XR, El-Achkar TM. Uromodulin (Tamm-Horsfall protein): guardian of urinary and systemic homeostasis. Nephrol Dial Transplant. 2020;35(1):33-43.
217. Weichhart T, Zlabinger GJ, Saemann MD. The multiple functions of Tamm-Horsfall protein in human health and disease: a mystery clears up. Wien Klin Wochenschr. 2005;117(9-10):316-22.
218. Fernandez-Llama P, Khositseth S, Gonzales PA, Star RA, Pisitkun T, Knepper MA. Tamm-Horsfall protein and urinary exosome isolation. Kidney Int. 2010;77(8):736-42.
219. Franzen CA, Blackwell RH, Foreman KE, Kuo PC, Flanigan RC, Gupta GN. Urinary Exosomes: The Potential for Biomarker Utility, Intercellular Signaling and Therapeutics in Urological Malignancy. J Urology. 2016;195(5):1331-9.
220. Tomlins SA, Aubin SM, Siddiqui J, Lonigro RJ, Sefton-Miller L, Miick S, et al. Urine TMPRSS2:ERG fusion transcript stratifies prostate cancer risk in men with elevated serum PSA. Science translational medicine. 2011;3(94):94ra72.
221. Overbye A, Skotland T, Koehler CJ, Thiede B, Seierstad T, Berge V, et al. Identification of prostate cancer biomarkers in urinary exosomes. Oncotarget. 2015;6(30):30357-76.
222. Rodriguez M, Bajo-Santos C, Hessvik NP, Lorenz S, Fromm B, Berge V, et al. Identification of non-invasive miRNAs biomarkers for prostate

cancer by deep sequencing analysis of urinary exosomes. Mol Cancer. 2017;16(1):156.
223. Foj L, Ferrer F, Serra M, Arevalo A, Gavagnach M, Gimenez N, et al. Exosomal and Non-Exosomal Urinary miRNAs in Prostate Cancer Detection and Prognosis. The Prostate. 2017;77(6):573-83.
224. Fredsoe J, Rasmussen AKI, Thomsen AR, Mouritzen P, Hoyer S, Borre M, et al. Diagnostic and Prognostic MicroRNA Biomarkers for Prostate Cancer in Cell-free Urine. Eur Urol Focus. 2018;4(6):825-33.
225. Huang XY, Yuan TZ, Liang MH, Du MJ, Xia S, Dittmar R, et al. Exosomal miR-1290 and miR-375 as Prognostic Markers in Castration-resistant Prostate Cancer. European urology. 2015;67(1):33-41.
226. Bryant RJ, Pawlowski T, Catto JW, Marsden G, Vessella RL, Rhees B, et al. Changes in circulating microRNA levels associated with prostate cancer. British journal of cancer. 2012;106(4):768-74.
227. Srivastava A, Goldberger H, Dimtchev A, Ramalinga M, Chijioke J, Marian C, et al. MicroRNA Profiling in Prostate Cancer - The Diagnostic Potential of Urinary miR-205 and miR-214. PLOS ONE. 2013;8(10):e76994.
228. Fredsoe J, Rasmussen AKI, Mouritzen P, Borre M, Orntoft T, Sorensen KD. A five-microRNA model (pCaP) for predicting prostate cancer aggressiveness using cell-free urine. Int J Cancer. 2019;145(9):2558-67.
229. Yanez-Mo M, Siljander PRM, Andreu Z, Zavec AB, Borras FE, Buzas EI, et al. Biological properties of extracellular vesicles and their physiological functions. Journal of Extracellular Vesicles. 2015;4.
230. Liao Z, Jaular LM, Soueidi E, Jouve M, Muth DC, Schoyen TH, et al. Acetylcholinesterase is not a generic marker of extracellular vesicles. J Extracell Vesicles. 2019;8(1):1628592.
231. Witwer KW, Thery C. Extracellular vesicles or exosomes? On primacy, precision, and popularity influencing a choice of nomenclature. J Extracell Vesicles. 2019;8(1):1648167.
232. Thery C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles. 2018;7(1):1535750.
233. Simonsen JB. What Are We Looking At? Extracellular Vesicles, Lipoproteins, or Both? Circ Res. 2017;121(8):920-2.
234. Royo F, Zuniga-Garcia P, Sanchez-Mosquera P, Egia A, Perez A, Loizaga A, et al. Different EV enrichment methods suitable for clinical settings yield different subpopulations of urinary extracellular vesicles from human samples. J Extracell Vesicles. 2016;5:29497.
235. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol. 2006;Chapter 3:Unit 3 22.

236. Linares R, Tan S, Gounou C, Arraud N, Brisson AR. High-speed centrifugation induces aggregation of extracellular vesicles. J Extracell Vesicles. 2015;4:29509.
237. Momen-Heravi F, Balaj L, Alian S, Trachtenberg AJ, Hochberg FH, Skog J, et al. Impact of biofluid viscosity on size and sedimentation efficiency of the isolated microvesicles. Front Physiol. 2012;3:162.
238. Konoshenko MY, Lekchnov EA, Vlassov AV, Laktionov PP. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. Biomed Res Int. 2018;2018:8545347.
239. Cvjetkovic A, Lotvall J, Lasser C. The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles. J Extracell Vesicles. 2014;3.
240. Livshts MA, Khomyakova E, Evtushenko EG, Lazarev VN, Kulemin NA, Semina SE, et al. Isolation of exosomes by differential centrifugation: Theoretical analysis of a commonly used protocol. Scientific reports. 2015;5.
241. Taylor DD, Shah S. Methods of isolating extracellular vesicles impact down-stream analyses of their cargoes. Methods. 2015;87:3-10.
242. Boing AN, van der Pol E, Grootemaat AE, Coumans FA, Sturk A, Nieuwland R. Single-step isolation of extracellular vesicles by size-exclusion chromatography. J Extracell Vesicles. 2014;3.
243. Alvarez ML, Khosroheidari M, Ravi RK, DiStefano JK. Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. Kidney Int. 2012;82(9):1024-32.
244. Lobb RJ, Becker M, Wen SW, Wong CS, Wiegmans AP, Leimgruber A, et al. Optimized exosome isolation protocol for cell culture supernatant and human plasma. J Extracell Vesicles. 2015;4:27031.
245. He M, Crow J, Roth M, Zeng Y, Godwin AK. Integrated immunoisolation and protein analysis of circulating exosomes using microfluidic technology. Lab on a chip. 2014;14(19):3773-80.
246. Cheruvanky A, Zhou H, Pisitkun T, Kopp JB, Knepper MA, Yuen PS, et al. Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. Am J Physiol Renal Physiol. 2007;292(5):F1657-61.
247. Kornilov R, Puhka M, Mannerstrom B, Hiidenmaa H, Peltoniemi H, Siljander P, et al. Efficient ultrafiltration-based protocol to deplete extracellular vesicles from fetal bovine serum. J Extracell Vesicles. 2018;7(1):1422674.
248. Gyuris A, Navarrete-Perea J, Jo A, Cristea S, Zhou S, Fraser K, et al. Physical and Molecular Landscapes of Mouse Glioma Extracellular Vesicles Define Heterogeneity. Cell Rep. 2019;27(13):3972-87 e6.
249. Crescitelli R, Lasser C, Szabo TG, Kittel A, Eldh M, Dianzani I, et al. Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. J Extracell Vesicles. 2013;2.
250. Yang JS, Lee JC, Byeon SK, Rha KH, Moon MH. Size Dependent Lipidomic Analysis of Urinary Exosomes from Patients with Prostate Cancer by

Flow Field-Flow Fractionation and Nanoflow Liquid Chromatography-Tandem Mass Spectrometry. Anal Chem. 2017;89(4):2488-96.
251. Flack K, Jimenez LA, Zhong W. Analysis of the Distribution Profiles of Circulating MicroRNAs by Asymmetrical Flow Field Flow Fractionation. Methods Mol Biol. 2017;1509:161-8.
252. Zhang H, Freitas D, Kim HS, Fabijanic K, Li Z, Chen H, et al. Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. Nat Cell Biol. 2018;20(3):332-43.
253. Willms E, Cabanas C, Mager I, Wood MJA, Vader P. Extracellular Vesicle Heterogeneity: Subpopulations, Isolation Techniques, and Diverse Functions in Cancer Progression. Front Immunol. 2018;9:738.
254. Chen C, Skog J, Hsu CH, Lessard RT, Balaj L, Wurdinger T, et al. Microfluidic isolation and transcriptome analysis of serum microvesicles. Lab on a chip. 2010;10(4):505-11.
255. Davies RT, Kim J, Jang SC, Choi EJ, Gho YS, Park J. Microfluidic filtration system to isolate extracellular vesicles from blood. Lab on a chip. 2012;12(24):5202-10.
256. Yasui T, Yanagida T, Ito S, Konakade Y, Takeshita D, Naganawa T, et al. Unveiling massive numbers of cancer-related urinary-microRNA candidates via nanowires. Sci Adv. 2017;3(12):e1701133.
257. Wunsch BH, Smith JT, Gifford SM, Wang C, Brink M, Bruce RL, et al. Nanoscale lateral displacement arrays for the separation of exosomes and colloids down to 20 nm. Nat Nanotechnol. 2016;11(11):936-40.
258. Contreras-Naranjo JC, Wu HJ, Ugaz VM. Microfluidics for exosome isolation and analysis: enabling liquid biopsy for personalized medicine. Lab on a chip. 2017;17(21):3558-77.
259. Iliescu FS, Vrtacnik D, Neuzil P, Iliescu C. Microfluidic Technology for Clinical Applications of Exosomes. Micromachines (Basel). 2019;10(6).
260. Dyson M, Woodward B, Pond JB. Flow of red blood cells stopped by ultrasound. Nature. 1971;232(5312):572-3.
261. Hammarstrom B, Laurell T, Nilsson J. Seed particle-enabled acoustic trapping of bacteria and nanoparticles in continuous flow systems. Lab on a chip. 2012;12(21):4296-304.
262. Evander M, Nilsson J. Acoustofluidics 20: applications in acoustic trapping. Lab on a chip. 2012;12(22):4667-76.
263. Wu M, Chen C, Wang Z, Bachman H, Ouyang Y, Huang PH, et al. Separating extracellular vesicles and lipoproteins via acoustofluidics. Lab on a chip. 2019;19(7):1174-82.
264. Dragovic RA, Gardiner C, Brooks AS, Tannetta DS, Ferguson DJ, Hole P, et al. Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. Nanomedicine. 2011;7(6):780-8.
265. Gruia F, Parupudi A, Polozova A. Practical Considerations for Detection and Characterization of Sub-Micron Particles in Protein Solutions by Nanoparticle Tracking Analysis. PDA J Pharm Sci Technol. 2015;69(3):427-39.

266. Filipe V, Hawe A, Jiskoot W. Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. Pharm Res. 2010;27(5):796-810.
267. Cizmar P, Yuana Y. Detection and Characterization of Extracellular Vesicles by Transmission and Cryo-Transmission Electron Microscopy. In: Kuo WP, Jia S, editors. Extracellular Vesicles: Methods and Protocols. New York, NY: Springer New York; 2017. p. 221-32.
268. McKeown SR. Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. Br J Radiol. 2014;87(1035):20130676.
269. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000;407(6801):249-57.
270. Baeriswyl V, Christofori G. The angiogenic switch in carcinogenesis. Seminars in Cancer Biology. 2009;19(5):329-37.
271. Stapor P, Wang X, Goveia J, Moens S, Carmeliet P. Angiogenesis revisited - role and therapeutic potential of targeting endothelial metabolism. J Cell Sci. 2014;127(Pt 20):4331-41.
272. Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, et al. Normalization of the vasculature for treatment of cancer and other diseases. Physiol Rev. 2011;91(3):1071-121.
273. Jain RK. Normalization of Tumor Vasculature: An Emerging Concept in Antiangiogenic Therapy. Science. 2005;307(5706):58.
274. Muz B, de la Puente P, Azab F, Azab AK. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia. 2015;3:83-92.
275. Parker C, Milosevic M, Toi A, Sweet J, Panzarella T, Bristow R, et al. Polarographic electrode study of tumor oxygenation in clinically localized prostate cancer. Int J Radiat Oncol Biol Phys. 2004;58(3):750-7.
276. Movsas B, Chapman JD, Horwitz EM, Pinover WH, Greenberg RE, Hanlon AL, et al. Hypoxic regions exist in human prostate carcinoma. Urology. 1999;53(1):11-8.
277. Zhong H, Semenza GL, Simons JW, De Marzo AM. Up-regulation of hypoxia-inducible factor 1alpha is an early event in prostate carcinogenesis. Cancer Detect Prev. 2004;28(2):88-93.
278. Rupp NJ, Schuffler PJ, Zhong Q, Falkner F, Rechsteiner M, Ruschoff JH, et al. Oxygen supply maps for hypoxic microenvironment visualization in prostate cancer. J Pathol Inform. 2016;7:3.
279. Hompland T, Hole KH, Ragnum HB, Aarnes EK, Vlatkovic L, Lie AK, et al. Combined MR Imaging of Oxygen Consumption and Supply Reveals Tumor Hypoxia and Aggressiveness in Prostate Cancer Patients. Cancer research. 2018;78(16):4774-85.
280. Supiot S, Rousseau C, Dore M, Cheze-Le-Rest C, Kandel-Aznar C, Potiron V, et al. Evaluation of tumor hypoxia prior to radiotherapy in intermediate-risk prostate cancer using (18)F-fluoromisonidazole PET/CT: a pilot study. Oncotarget. 2018;9(11):10005-15.

281. Lalonde E, Ishkanian AS, Sykes J, Fraser M, Ross-Adams H, Erho N, et al. Tumour genomic and microenvironmental heterogeneity for integrated prediction of 5-year biochemical recurrence of prostate cancer: a retrospective cohort study. Lancet Oncol. 2014;15(13):1521-32.
282. Vergis R, Corbishley CM, Norman AR, Bartlett J, Jhavar S, Borre M, et al. Intrinsic markers of tumour hypoxia and angiogenesis in localised prostate cancer and outcome of radical treatment: a retrospective analysis of two randomised radiotherapy trials and one surgical cohort study. Lancet Oncol. 2008;9(4):342-51.
283. Movsas B, Chapman JD, Hanlon AL, Horwitz EM, Greenberg RE, Stobbe C, et al. Hypoxic prostate/muscle pO2 ratio predicts for biochemical failure in patients with prostate cancer: preliminary findings. Urology. 2002;60(4):634-9.
284. Yang SL, Wu C, Xiong ZF, Fang X. Progress on hypoxia-inducible factor-3: Its structure, gene regulation and biological function (Review). Mol Med Rep. 2015;12(2):2411-6.
285. Graham AM, Presnell JS. Hypoxia Inducible Factor (HIF) transcription factor family expansion, diversification, divergence and selection in eukaryotes. PloS one. 2017;12(6):e0179545-e.
286. Illingworth CJ, Loenarz C, Schofield CJ, Domene C. Chemical basis for the selectivity of the von Hippel Lindau tumor suppressor pVHL for prolyl-hydroxylated HIF-1alpha. Biochemistry-Us. 2010;49(32):6936-44.
287. Bhandari V, Hoey C, Liu LY, Lalonde E, Ray J, Livingstone J, et al. Molecular landmarks of tumor hypoxia across cancer types. Nat Genet. 2019;51(2):308-18.
288. Kulshreshtha R, Ferracin M, Wojcik SE, Garzon R, Alder H, Agosto-Perez FJ, et al. A microRNA signature of hypoxia. Mol Cell Biol. 2007;27(5):1859-67.
289. Choudhry H, Harris AL, McIntyre A. The tumour hypoxia induced non-coding transcriptome. Molecular Aspects of Medicine. 2016;47-48:35-53.
290. Camps C, Saini HK, Mole DR, Choudhry H, Reczko M, Guerra-Assunção JA, et al. Integrated analysis of microRNA and mRNA expression and association with HIF binding reveals the complexity of microRNA expression regulation under hypoxia. Mol Cancer. 2014;13(1):28.
291. Huang X, Ding L, Bennewith KL, Tong RT, Welford SM, Ang KK, et al. Hypoxia-Inducible mir-210 Regulates Normoxic Gene Expression Involved in Tumor Initiation. Mol Cell. 2009;35(6):856-67.
292. Yang Y, Zhang J, Xia T, Li G, Tian T, Wang M, et al. MicroRNA-210 promotes cancer angiogenesis by targeting fibroblast growth factor receptor-like 1 in hepatocellular carcinoma. Oncol Rep. 2016;36(5):2553-62.
293. He J, Wu J, Xu N, Xie W, Li M, Li J, et al. MiR-210 disturbs mitotic progression through regulating a group of mitosis-related genes. Nucleic Acids Res. 2013;41(1):498-508.
294. Crosby ME, Kulshreshtha R, Ivan M, Glazer PM. MicroRNA regulation of DNA repair gene expression in hypoxic stress. Cancer research. 2009;69(3):1221-9.

295. Noman MZ, Janji B, Berchem G, Chouaib S. miR-210 and hypoxic microvesicles: Two critical components of hypoxia involved in the regulation of killer cells function. Cancer Lett. 2016;380(1):257-62.
296. Kosaka N, Iguchi H, Hagiwara K, Yoshioka Y, Takeshita F, Ochiya T. Neutral sphingomyelinase 2 (nSMase2)-dependent exosomal transfer of angiogenic microRNAs regulate cancer cell metastasis. J Biol Chem. 2013;288(15):10849-59.
297. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol. 2018;19(4):213-28.
298. Mateescu B, Kowal EJK, van Balkom BWM, Bartel S, Bhattacharyya SN, Buzás EI, et al. Obstacles and opportunities in the functional analysis of extracellular vesicle RNA – an ISEV position paper. Journal of Extracellular Vesicles. 2017;6(1):1286095.
299. Retraction notice to: Cell adhesion-dependent control of microRNA decay. Molecular Cell 43, 1005-1014; September 16, 2011. Mol Cell. 2012;46(6):896.
300. van Dijk EL, Jaszczyszyn Y, Thermes C. Library preparation methods for next-generation sequencing: tone down the bias. Exp Cell Res. 2014;322(1):12-20.
301. Hafner M, Renwick N, Brown M, Mihailovic A, Holoch D, Lin C, et al. RNA-ligase-dependent biases in miRNA representation in deep-sequenced small RNA cDNA libraries. RNA. 2011;17(9):1697-712.
302. Giraldez MD, Spengler RM, Etheridge A, Godoy PM, Barczak AJ, Srinivasan S, et al. Comprehensive multi-center assessment of small RNA-seq methods for quantitative miRNA profiling. Nat Biotechnol. 2018;36(8):746-57.
303. Vlachos IS, Zagganas K, Paraskevopoulou MD, Georgakilas G, Karagkouni D, Vergoulis T, et al. DIANA-miRPath v3.0: deciphering microRNA function with experimental support. Nucleic Acids Res. 2015;43(W1):W460-6.
304. Hugosson J, Godtman RA, Carlsson SV, Aus G, Grenabo Bergdahl A, Lodding P, et al. Eighteen-year follow-up of the Goteborg Randomized Population-based Prostate Cancer Screening Trial: effect of sociodemographic variables on participation, prostate cancer incidence and mortality. Scand J Urol. 2018;52(1):27-37.
305. Dhondt B, Van Deun J, Vermaerke S, de Marco A, Lumen N, De Wever O, et al. Urinary extracellular vesicle biomarkers in urological cancers: From discovery towards clinical implementation. Int J Biochem Cell Biol. 2018;99:236-56.
306. Lin HM, Mahon KL, Spielman C, Gurney H, Mallesara G, Stockler MR, et al. Phase 2 study of circulating microRNA biomarkers in castration-resistant prostate cancer. British journal of cancer. 2017;116(8):1002-11.
307. McDonald AC, Vira M, Walter V, Shen J, Raman JD, Sanda MG, et al. Circulating microRNAs in plasma among men with low-grade and high-grade prostate cancer at prostate biopsy. The Prostate. 2019;79(9):961-8.
308. Ghorbanmehr N, Gharbi S, Korsching E, Tavallaei M, Einollahi B, Mowla SJ. miR-21-5p, miR-141-3p, and miR-205-5p levels in urine-promising

biomarkers for the identification of prostate and bladder cancer. The Prostate. 2019;79(1):88-95.
309. Cheng L, Sun X, Scicluna BJ, Coleman BM, Hill AF. Characterization and deep sequencing analysis of exosomal and non-exosomal miRNA in human urine. Kidney Int. 2014;86(2):433-44.
310. Ku A, Ravi N, Yang M, Evander M, Laurell T, Lilja H, et al. A urinary extracellular vesicle microRNA biomarker discovery pipeline; from automated extracellular vesicle enrichment by acoustic trapping to microRNA sequencing. PLoS One. 2019;14(5):e0217507.
311. Pasqualini L, Bu H, Puhr M, Narisu N, Rainer J, Schlick B, et al. miR-22 and miR-29a Are Members of the Androgen Receptor Cistrome Modulating LAMC1 and Mcl-1 in Prostate Cancer. Mol Endocrinol. 2015;29(7):1037-54.
312. Li J, Wan X, Qiang W, Li T, Huang W, Huang S, et al.

MiR-29a suppresses prostate cell proliferation and induces apoptosis via KDM5B protein regulation. Int J Clin Exp Med. 2015;8(4):5329-39.
313. Shang A, Yang M, Shen F, Wang J, Wei J, Wang W, et al. MiR-1-3p Suppresses the Proliferation, Invasion and Migration of Bladder Cancer Cells by Up-Regulating SFRP1 Expression. Cell Physiol Biochem. 2017;41(3):1179-88.
314. Geng H, Xue CH, Mendonca J, Sun XX, Liu Q, Reardon PN, et al. Interplay between hypoxia and androgen controls a metabolic switch conferring resistance to androgen/AR-targeted therapy. Nature Communications. 2018;9.
315. Peyromaure M, Camparo P, Badoual C, Descazeaud A, Dinh-Xuan AT. The expression of vascular endothelial growth factor is associated with the risk of cancer progression after radical prostatectomy. BJU Int. 2007;99(5):1150-3.
316. Panigrahi GK, Praharaj PP, Peak TC, Long J, Singh R, Rhim JS, et al. Hypoxia-induced exosome secretion promotes survival of African-American and Caucasian prostate cancer cells. Scientific reports. 2018;8(1):3853.
317. Ramteke A, Ting H, Agarwal C, Mateen S, Somasagara R, Hussain A, et al. Exosomes secreted under hypoxia enhance invasiveness and stemness of prostate cancer cells by targeting adherens junction molecules. Mol Carcinog. 2015;54(7):554-65.
318. Geng H, Xue CH, Mendonca J, Sun XX, Liu Q, Reardon PN, et al. Interplay between hypoxia and androgen controls a metabolic switch conferring resistance to androgen/AR-targeted therapy (vol 9, 4972, 2018). Nature Communications. 2019;10.
319. Forristal CE, Wright KL, Hanley NA, Oreffo RO, Houghton FD. Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions. Reproduction. 2010;139(1):85-97.
320. Covello KL, Kehler J, Yu H, Gordan JD, Arsham AM, Hu CJ, et al. HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. Genes Dev. 2006;20(5):557-70.
321. Mitchell PJ, Welton J, Staffurth J, Court J, Mason MD, Tabi Z, et al. Can urinary exosomes act as treatment response markers in prostate cancer? Journal of Translational Medicine. 2009;7(1):4.

322. Santangelo L, Giurato G, Cicchini C, Montaldo C, Mancone C, Tarallo R, et al. The RNA-Binding Protein SYNCRIP Is a Component of the Hepatocyte Exosomal Machinery Controlling MicroRNA Sorting. Cell Rep. 2016;17(3):799-808.

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