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Plasticity of cell identity and its role in cancer progression

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DECLARATION

I hereby declare that I worked on this thesis independently using the sources listed in the bibliography.

Brno, 2024

Mgr. Karel Souček, Ph.D.

> “Not everything that can be counted counts, and not everything that counts can be counted.”

William Bruce Cameron

To A2I

ABSTRACT

Cancer remains a formidable global health challenge, contributing significantly to morbidity and mortality worldwide. Despite substantial progress in cancer research and treatment, the intricate nature of this disease presents significant obstacles in developing effective therapies. In cancer biology, the concepts of cancer cell plasticity and tumor heterogeneity have garnered considerable attention. This attention is driven by recognizing their crucial roles in cancer progression, resistance to treatment, metastasis, and the recurrence of the disease. This thesis briefly summarizes our current understanding of cell plasticity and tumor heterogeneity and specifies the author's contribution to this topic.

ABSTRAKT

Nádorová onemocnění zůstávají obrovským celosvětovým zdravotním problémem, který významně přispívá k nemocnosti a úmrtnosti na celém světě. Navzdory značnému pokroku ve výzkumu a léčbě nádorů představuje složitá povaha tohoto onemocnění významné překážky při vývoji účinných léčebných postupů. Mezi novými koncepty v biologii nádorů zaujala značnou pozornost plasticita nádorových buněk a heterogenita nádorů, a to vzhledem k její zásadní roli při progresi, rezistenci na léčbu, metastazování a recidivě onemocnění. Tato práce stručně shrnuje naše současné poznatky o plasticitě buněk a nádorové heterogenitě a specifikuje přínos autora k tomuto tématu.

PODĚKOVÁNÍ

Měl jsem a mám štěstí a privilegium, že se v mém životě objevily osobnosti, bez kterých by mé individuální úsilí bylo marné. Za to jim náleží velké poděkování a zasloužený kredit. Jejich seznam je dlouhý a omlouvám se těm, které jsem níže nevyjmenoval. I vám děkuji.

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CONTENTS

ABBREVIATIONS

ACCA	Automatic Cell Clonning Assay
ADT	Androgen Deprivation Therapy
AML	Acute Myeloid Leukemia
AR	Androgen Receptor
CSC	Cancer Stem Cell
DDR	DNA Damage Response
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMP	Epithelial-Mesenchymal Plasticity
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
EnR	Endoplasmatic Reticulum
FACS	Fluorescence-Activated Cell Sorting
FC	Flow Cytometry
FCB	Fluorescent Cell Barcoding
FGF	Fibroblast Growth Factor
GDF-15	Growth/Differention Factor - 15
HGF	Hepatocyte Growth Factor
HR	Homologous Recombination
HTCP	High-Throughput Cytometry-Based Profiling
IκB	IκB kinase
IL-6	Interleukin - 6
JAK	Janus Kinase
MAPK	Mitogen-Activated Protein Kinase
MC	Mass Cytometry
MCSF	Macrophage Colony-Stimulating Factor
MDM2	Mouse Double Minute 2
MDMX	Mouse Double Minute X
MET	Mesenchymal-Epithelial Transition
MYB	MYB (myeloblastosis) family of transcription factors
NE	Neuroendocrine
NED	Neuroendocrine Differentiation

NEP	Neuroendocrine Phenotype
NEPC	Neuroendocrine Prostate Cancer
NF-κB	Nuclear Factor κ-B
NOTCH	Notch signaling pathway
p38	p38 mitogen-activated protein kinase
PCa	Prostate Cancer
Ras	Ras (Rat Sarcoma) GTPase
RANKL	Receptor Activator of Nuclear factor κ-B Ligand
Rb	Retinoblastoma
SASP	Senescence-Associated Secretory Phenotype
SMAD	homologs to the Caenorhabditis elegans SMA ("small" worm phenotype) and MAD family ("Mothers Against Decapentaplegic") of genes in Drosophila
Slug	Zinc finger protein SNAI2
Snail	Zinc finger protein SNAI1
Sox	SRY-related HMG-box genes
p53	Tumor Protein 53
TGF-β	Transforming Growth Factor β
TME	Tissue Microenvironment
TNBC	Triple-Negative Breast Cancer
TNM	Tumor, Node, Metastasis (Staging System)
Trop2	Tumor-Associated Calcium Signal Transducer 2
t-SNE	t-distributed Stochastic Neighbor Embedding
TUSC3	Tumor Suppressor Candidate 3
TWIST	Twist-related protein
UPR	Unfolded Protein Response
ZEB1	Zinc finger E-box-binding homeobox 1
Wnt	Wnt signalling pathway

INTRODUCTION

Cancer is the leading cause of morbidity and mortality worldwide. In 2020, there were approximately 18 million new cases and 10 million deaths from cancer. Moreover, by 2040, it is projected to rise further to 28 million new cancer cases per year. (Cancer_Research_UK, 2023). The leading cause of death in solid cancers relates to metastasis, a complex process involving multiple steps. This process consists of the escape of cancer cells from the primary site, their entry into the bloodstream, extravasation, and subsequent colony formation in distant organs. Clonal selection and the high phenotypic plasticity of tumor cells play a crucial role in the colonization process, allowing for reversible switching between cellular states (Massague and Obenauf, 2016). This plasticity of cancer cell identity contributes to diverse cell populations in the tumor, leading to intratumoral heterogeneity and increased cancer fitness. Although traditionally associated with epithelial-mesenchymal transition (EMT), recent research has expanded the definition of tumor cell plasticity to include a variety of other biological processes (Jehanno et al., 2022).

The heterogeneity of tumor populations is further associated with cellular differentiation. In the cancer stem cell (CSC) model, tumors exhibit a hierarchical organization characterized by cells with stem cell-like properties. The CSC population maintains tumor growth through processes of self-renewal and differentiation (Shibue and Weinberg, 2017). In addition, the tumor microenvironment, comprised of a non-malignant stroma that includes fibroblasts, endothelial cells, immune cells, and extracellular matrix (ECM), introduces additional intratumoral heterogeneity to the tumor. This heterogeneity is induced by varying selection pressures exerted in different tumor regions. These patterns are not mutually exclusive; collectively, they constitute a complex system with numerous layers of heterogeneity stemming from diverse genetic, epigenetic, transcriptomic, proteomic, and functional properties exhibited by distinct cell types. Plasticity, a significant impediment to effective anticancer therapy, emerges from intrinsic and extrinsic cellular factors (Lawson et al., 2018). Single-cell technologies have contributed to unraveling tumor heterogeneity and cell plasticity (Marusyk et al., 2012; Hinohara and Polyak, 2019). However, only new approaches to drug development, using knowledge of the complexity of the tumor ecosystem combined with advances in clinical trial design and precision medicine, will improve patient outcomes in the future (McGranahan and Swanton, 2017).

In organogenesis, cells develop, determine, and organize into tissues, culminating in terminal differentiation, where progenitor cells cease growth. This anti-proliferative outcome poses a barrier to neoplasia. Evidence suggests that unlocking restricted phenotypic plasticity is crucial in cancer pathogenesis, allowing cells to evade terminal differentiation (Yuan et al., 2019). Cancer cells may dedifferentiate from a mature state to a progenitor-like state or maintain a partially differentiated state, deviating from the end-stage pathway. Additionally, transdifferentiation may occur, leading cells down a different developmental program (Figure 1). The various manifestations of cellular plasticity collectively represent a distinct hallmark capability in cancer (Hanahan, 2022).

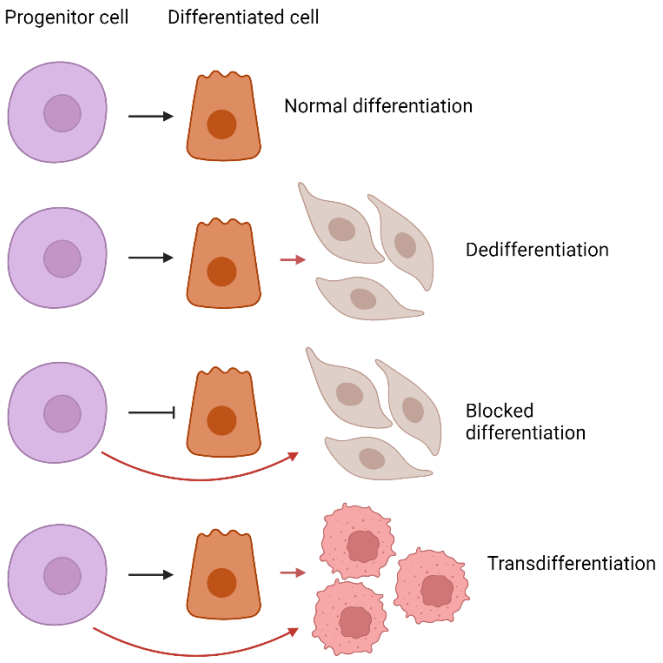

Figure 1. Unlocking phenotypic plasticity. (i) The process of dedifferentiation, involving the transition from mature states to progenitor states, (ii) the block of terminal differentiation in progenitor cell states, and (iii) the phenomenon of transdifferentiation into diverse cell lineages are recognized as three notable modes of perturbed differentiation intrinsic to the pathogenesis of cancer. Adapted from (Hanahan, 2022). Created with BioRender.com

CHAPTER 1 REGULATION OF CELL SIGNALING AND TUMOR SUPPRESSION IN THE CONTEXT OF EPITHELIAL-MESENCHYMAL PLASTICITY

Epithelial-mesenchymal plasticity (EMP) is a process through which epithelial cells can change their phenotype and acquire mesenchymal characteristics. Various signaling pathways (Figure 2 (Haerinck et al., 2023; Perez-Gonzalez et al., 2023)), including the TGF-β family of cytokines, regulate this process (Katsuno and Derynck, 2021). TGF-β signaling is pivotal in inducing epithelial cell plasticity, encompassing EMT, a critical process in fibrosis and tumor progression (Fabregat and Caballero-Díaz, 2018). Chronic long-term inflammation is also implicated in fibrosis and cancer, with TGF-β as a primary mediator in these processes (Katsuno and Derynck, 2021). Furthermore, TGF-β1 exhibits potent immunosuppressive and anti-inflammatory effects (Nixon et al., 2022). Interestingly, it plays a dual role in the process of carcinogenesis. TGF-β1, as a tumor suppressor, can inhibit the proliferation of cancer cells (Massague, 2008); on the other hand, it can support cancer progression by stimulating angiogenesis or through induction of EMT (Massague, 2008). It has been demonstrated that there is a communication between TGF-β1 and IL-6 pathways, which are both involved in the development of prostate cancer and inflammation (Culig and Puhr, 2018). This communication affects the expression of miR34a (as summarized in Appendix 12, (Slabakova et al., 2017)). Additionally, TGF-β1 can decrease the signaling of IL-6, as seen in intestinal epithelial cells or AML blast cells. In the prostate epithelial cells, where particularly TGF-β and IL-6 signaling pathways are crucial for tissue homeostasis, we demonstrated that TGF-β1 inhibits IL-6-induced STAT3 activation and expression of the cancer-associated gene MUC1 through down-regulation of Jak2 expression (Appendix 2, (Starsíchová et al., 2010)). These findings uncovered a new interaction between TGF-β1 and IL-6 signaling, suggesting an additional way defects in TGF-β signaling, commonly linked to prostate issues, may disrupt tissue homeostasis. Growth/differentiation factor-15 (GDF-15), a unique member of the TGF-β family, is a stress-induced cytokine with suggested immunomodulatory roles (Soucek et al., 2010), often highly expressed in cancer progression, including PCa (Savita Wakchoure et al., 2009). However, there is still a lack of studies clearly illustrating the mechanisms for signal transduction and functions related to cell interaction, cancer progression, and therapy (summarized in review Appendix 6, (Vanhara et al., 2012)). Interestingly, GDF-15 has been recognized for its role in influencing the development of osteoclasts and its potential use in treating bone

metastases (Savita Wakchoure et al., 2009). Our study demonstrates that both pure GDF-15 and the growth medium containing GDF-15 from prostate adenocarcinoma LNCaP cells, treated with 1,25(OH)(2)-vitamin D(3), hinder the formation of mature osteoclasts derived from RAW264.7 macrophages and bone-marrow precursors through M-CSF/RANKL in a dose-dependent manner.

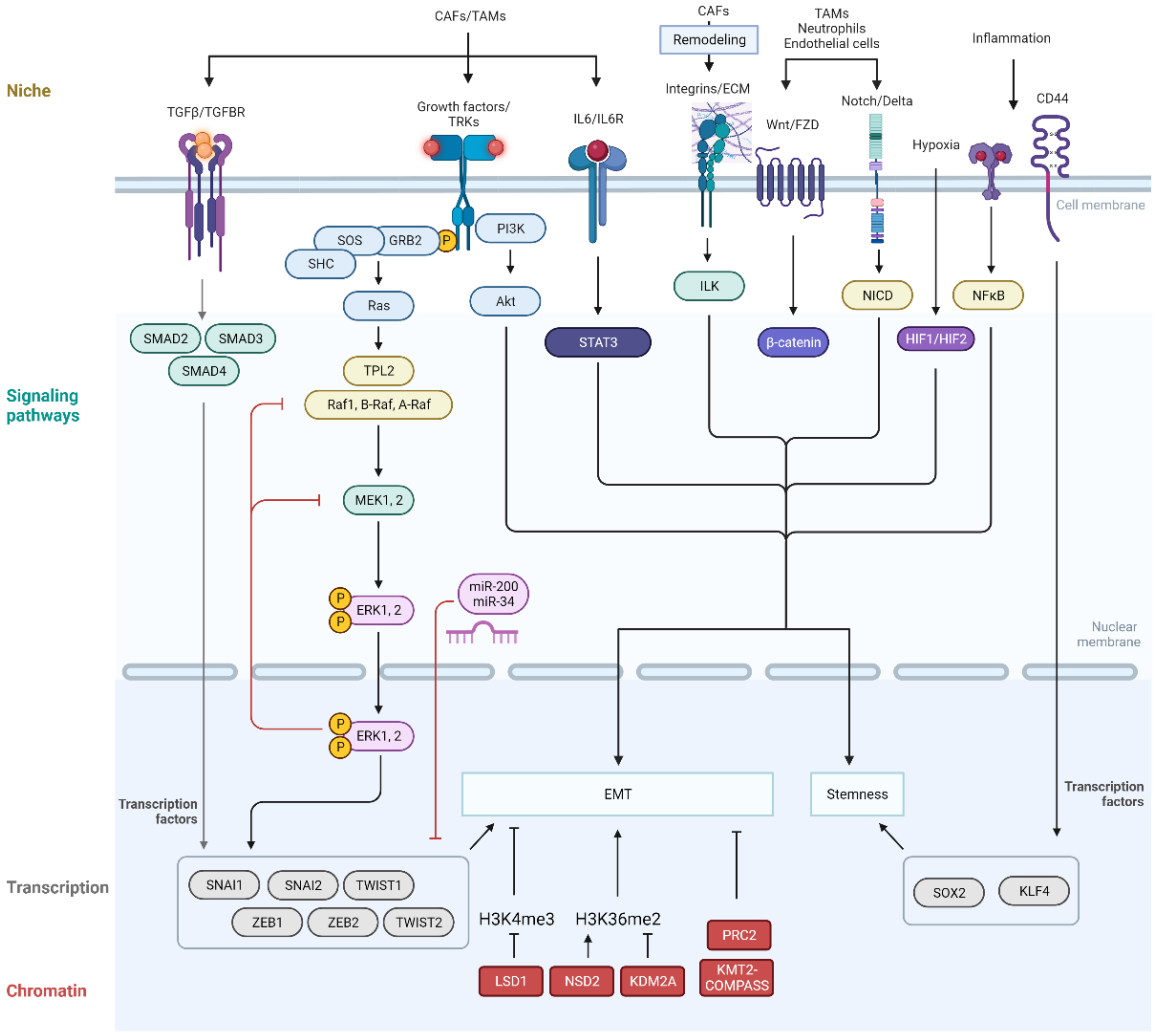

Figure 2. Molecular mechanisms regulating plasticity. Cancer cell plasticity is influenced extrinsically, through signals from the microenvironment, and intrinsically, via various signaling pathways, transcriptional programs, and chromatin remodeling. Key pathways, such as TGFβ, RAS–MAPK, CD44, Wnt, Notch, JAK–STAT, and integrins, regulate EMT and context-dependent stemness. Factors like hypoxia and NF-κB further contribute to plasticity and inflammation. Transcriptional programs involve crucial factors like SNAI1, SNAI2, ZEB1, ZEB2, Twist1, Twist2, SOX2, and KLF4, and are modulated by microRNAs and chromatin landscape. Enzymes like LSD1, NSD2, and KDM2A play roles in histone modifications, influencing EMT. Polycomb repressive complex 2 (PRC2) and type 2 lysine methyltransferase (KMT2) – the complex of proteins associated with Set1 (COMPASS) are

vital for regulating the epithelial state. Adapted from (Perez-Gonzalez et al., 2023). Created with BioRender.com

GDF-15 suppresses the expression of c-Fos and the activity of NFκB by delaying the degradation of IκB. Additionally, GDF-15 inhibits the expression of crucial osteoclast enzymes, carbonic anhydrase II, and cathepsin K while causing changes in SMAD and p38 signaling. The absence of functional osteoclasts may contribute to the accumulation of bone matrix by reducing bone resorption. These findings reveal a new role for GDF-15 in influencing the differentiation of osteoclasts and potentially in the treatment of bone metastases (See Appendix 1, (Vanhara et al., 2009)).

c-Myb is a transcription factor that plays a role in cell plasticity and adaptation during the formation of bone metastasis (Fang and Kang, 2022), primarily through its essential role in controlling the balance between cell proliferation, differentiation, and survival (Ramsay and Gonda, 2008). Recent investigations have uncovered the presence of c-Myb expression in osteosarcoma cell lines, suggesting its functional significance in bone development (Guo et al., 2016). A retrospective clinical study found high c-Myb levels correlated with poor overall survival and metastatic progression in young osteosarcoma patients. Depleting MYB in metastatic cell lines reduced growth, colony-forming capacity, migration, and increased chemosensitivity. MYB knock-out diminished metastatic activity *in vivo*. Transcriptomic analysis has unveiled that c-Myb significantly impacts cell growth, stress response, adhesion, and cell differentiation, with the Wnt signaling pathway being recognized as a target.

In summary, c-Myb acts as an adverse prognostic factor in osteosarcoma, influencing cell behavior and the occurrence of metastasis (Appendix 25, (Rihova et al., 2022)). Furthermore, we showed that c-Myb controls the invasive tendencies of breast cancer cells, relying on the type of matrix involved, potentially through a new signaling pathway that disrupts the expression of MMP1, MMP9, and cathepsin D (Appendix 5, (Knopfova et al., 2012)). In this context, c-Myb controls the inflammatory response and determines the metastatic capability of breast cancer cells. The recognized inflammatory pattern enhances the ability to identify high-risk patients for lung relapse in most analyzed datasets compared to just assessing MYB alone. Significantly, these markers predict lung relapses in breast cancer patients, regardless of estrogen receptor expression. Blocking the c-Myb-driven paracrine inflammatory pathway could enhance the targeting of tumor

cells that are not easily reached by standard anti-proliferative therapy, potentially reducing the occurrence of metastasis (Appendix 14, (Knopfova et al., 2018)).

The tumor suppressor candidate 3 (TUSC3 or N33) gene is in the chromosomal region 8p22, commonly lost in various epithelial cancers like breast, prostate, oral squamous, and ovarian cancer (Yu et al., 2017). Additionally, TUSC3 has been identified as a promoter of cancer growth and inducer of EMT in non-small-cell lung cancer cells (Feng et al., 2018). Loss of TUSC3 has been linked to modified endoplasmic reticulum (EnR) stress response and increased growth of prostate cancer *in vivo* (Horak et al., 2014). We establish TUSC3 as a newly identified EnR-associated tumor suppressor involved in the pathobiology of ovarian cancer. Both at the morphological and molecular levels, our findings indicate that TUSC3 plays a role in maintaining rough EnR balance, and its absence triggers markers of EMT and boosts tumor growth *in vivo* (Appendix 10, (Kratochvilova et al., 2015)).

In conclusion, epithelial plasticity, driven by processes such as EMT, is a dynamic phenomenon regulated by signaling pathways, notably the TGF-β family. The dual role of TGF-β in suppressing and promoting cancer highlights its complexity. The interaction between TGF-β1 and IL-6 adds a layer of complexity to prostate epithelial cells, impacting tissue homeostasis. GDF-15, a member of the TGF-β family, demonstrates potential in modulating osteoclast differentiation and presents a novel avenue for therapy in bone metastases. The transcription factor c-Myb emerges as a critical player in cellular plasticity, influencing metastasis in osteosarcoma and breast cancer, with potential implications for predictive assessments. TUSC3 is a significant EnR-associated tumor suppressor implicated in cancer growth and EMT, particularly in ovarian cancer. These findings contribute valuable insights into the intricate molecular mechanisms underlying cancer progression and show possible avenues for targeted therapeutic interventions.

CHAPTER 2 NEUROENDOCRINE DIFFERENTIATION AND CELLULAR SENESCENCE IN THE CONTEXT OF ANDROGEN DEPLETION IN PROSTATE CANCER

Pathologists have long acknowledged the concept of transdifferentiation through tissue metaplasia, where cells with a specific differentiated phenotype undergo marked morphological changes, exemplified by Barrett's esophagus. In this condition, chronic inflammation induces transdifferentiation of the stratified squamous epithelium into a simple columnar epithelium resembling the intestine (Yuan et al., 2019; Hanahan, 2022). This transformation facilitates the development of adenocarcinomas rather than the expected squamous cell carcinomas. Molecular determinants are now uncovering transdifferentiation mechanisms in various cancers, whether characterized by evident tissue metaplasia or more subtle transformations, as illustrated in the example of neuroendocrine (trans)differentiation (NED) (Hanahan, 2022).

NED refers to the process by which cancer cells acquire characteristics of neuroendocrine cells, which are specialized cells that release hormones and neurotransmitters. This phenomenon has been documented in diverse cancer types, such as prostate cancer, breast cancer, and colorectal cancer (Yuan et al., 2007a; Kleist and Poetsch, 2015; Tsang and Tse, 2021). Neuroendocrine differentiation is associated with increased tumor aggressiveness, resistance to therapy, and poor prognosis (Parimi et al., 2014). The presence of neurosecretory granules in neoplastic cells is a hallmark of neuroendocrine differentiation, and these cells express neuroendocrine markers and exhibit architectural patterns and cytologic features like nonneoplastic neuroendocrine cells (Tsang and Tse, 2021). Mechanistically, NED is associated with altered expression and activity of several transcription factors, including, for example, those of the SOX family. They are broadly implicated in cell fate specification and lineage switching during development (Parimi et al., 2014) while also exhibiting connections to various tumor-associated phenotypes (Grimm et al., 2020).

In neuroendocrine tumors, the loss of RB and p53 tumor suppressors represents a necessary yet insufficient condition for transforming well-differentiated prostate cancer cells into carcinoma cells with molecular and histologic characteristics resembling neuroendocrine cells. Notably, these transformed cells exhibit a notable absence of androgen receptor expression. Beyond the loss of RB and p53, resistance to antiandrogen therapy involves the heightened expression of the developmental

regulatory gene SOX2. SOX2 plays a pivotal role in driving the transdifferentiation of adenocarcinoma cells responsive to therapy into derivatives adopting a neuroendocrine cell state unresponsive to the treatment (Mu et al., 2017; Hanahan, 2022). In this context, there is a notable dearth of experimentally relevant models to comprehensively elucidate the mechanisms underlying aggressive prostate carcinoma with a neuroendocrine phenotype (Faugeroux et al., 2020).

Androgen deprivation therapy (ADT) is a crucial treatment for advanced-stage prostate cancer, accomplished by blocking the androgen receptor (AR) or performing medical or surgical castration (Harris et al., 2009; Sharifi et al., 2010). While ADT is initially highly effective, tumors under treatment eventually advance to castrate-resistant prostate cancer (CRPC), which is presently incurable and fatal. Therefore, understanding the mechanism by which ADT leads to androgen independence is of significant clinical importance (Pernicova et al., 2011). The plasticity of prostate cancer cells in the context of ADT is a well-documented phenomenon. The plasticity of prostate cancer cells results in resistance to ADT, during which cells can transdifferentiate into neuroendocrine prostate cancer (NEPC) (Tiwari et al., 2020). This reprogramming, involving phenotype switching, is part of the cellular plasticity that enables prostate cancer cells to adapt to the absence of androgens and develop resistance to ADT (De Velasco et al., 2014). The tumor microenvironment (TME) plays a critical role in regulating and inducing NEPC formation during androgen deprivation therapy, further highlighting the impact of the TME on prostate cancer cell plasticity (Zhou et al., 2022). NEPC is an example of the diverse levels of tumor cell heterogeneity and plasticity, posing a significant challenge for effective clinical diagnosis and therapy (Pernicova et al., 2011). Since normal prostate neuroendocrine (NE) cells are postmitotic and do not divide (Abrahamsson, 1999), new cells with NE-like properties are assumed to emerge through a process known as neuroendocrine differentiation (NED) from existing epithelial tumor cells (Yuan et al., 2007b). These tumor cells acquire an NE-like phenotype, meaning they can release different neuropeptides and are independent of androgens. Research has suggested that NED can be induced *in vitro* by various triggering factors such as androgen depletion (Ismail et al., 2002; Yuan et al., 2006), increased IL-6 levels (Deeble et al., 2001), Wnt activation (Yang et al., 2005) and EGF (Cortes et al., 2012), activation of the cyclic adenosine 3', 5'-monophosphate (cAMP) signaling pathway (Cox et al., 2000; Zelivianski et al., 2001; Cantile et al., 2005), or exposure to ionizing radiation (Deng et al., 2008; Deng et al., 2011). Additionally, various genes and transcription factors, like protocadherin-PC and the transcription factors Foxa2 and Neuro D1, as well as miRNAs,

were found to be involved in NED (Summarized in (Cindolo et al., 2007; Slabakova et al., 2021)). Androgen depletion, known to induce NED, is linked to a halt in the cell cycle at the G1 phase (Knudsen et al., 1998; Balk and Knudsen, 2008). This cell cycle arrest is associated with adjustments in well-known regulators of cell cycle progression from the G1 to S phase (Knudsen et al., 1998; Fribourg et al., 2000). Another contributing factor to cell cycle arrest is contact inhibition. Cells in high-density arrest in the G1 phase, accompanied by decreased Cdk2 and Cdk4 activity, even in cancer cells that typically do not follow the normal contact inhibition seen in normal cells (Pernicova et al., 2014). Furthermore, cell density can also affect intracellular signaling, as demonstrated by density-dependent changes in the distribution of cAMP inside and outside the cells (Orbo et al., 1994). Therefore, we focused on understanding how cell cycle adjustments regulate NED in prostate cancer cells. Our study showed that both androgen depletion and cell cycle adjustments due to high cell density encourage NED. It was evidenced by increased expression of characteristic markers in both AR-positive and AR-negative prostate epithelial cell lines of different origins. We identified a crucial role of Cdk1 and Cdk2 activity in promoting NED through cell cycle slowing and suggested that the activation of cAMP signaling plays a role in promoting NED in AR-positive prostate cancer cell (Appendix 9, (Pernicova et al., 2014)). The activation of senescence in cancer cells is thought to be a powerful way to suppress tumors (Schmitt et al., 2022). However, senescent cells, while inactive in terms of cell division, stay metabolically active. They release a variety of substances, influencing the tissue surroundings, and potentially encouraging the development of tumors in nearby malignant cells. It is crucial to note that some growth factors and cytokines produced by senescent cells might contribute to the development of CRPC and the induction of NED (Figure 3, for review see Appendix 8, (Pernicova et al., 2013)).

The induction of the senescence-associated secretory phenotype (SASP) due to androgen depletion is closely linked to the control of the cell cycle machinery by reducing the levels of S-phase kinase-associated protein 2 (refer to Appendix 3 (Pernicova et al., 2011)). Skp2 is a crucial component of the SCF^{Skp2} E3 ubiquitin ligase and is often excessively expressed in various cancer types, including PCa (Wang et al., 2012). Our research discovered that the nuclear presence of Skp2 was higher in PCa patients than those with benign hyperplasia and was associated with a higher Gleason score in PCa patients. Elevated Skp2 expression was also noted in PCa cell lines with a mesenchymal and cancer stem cell (CSC) - like phenotype compared to their epithelial counterparts. Conversely, when SKP2 expression was suppressed, the CSC-like phenotype diminished. Additionally,

we observed that lowering Skp2 levels decreased the subpopulation of $CD44^{+}CD24^{-}$ cancer stem-like cells (see Appendix 18, (Šimečková et al., 2019)).

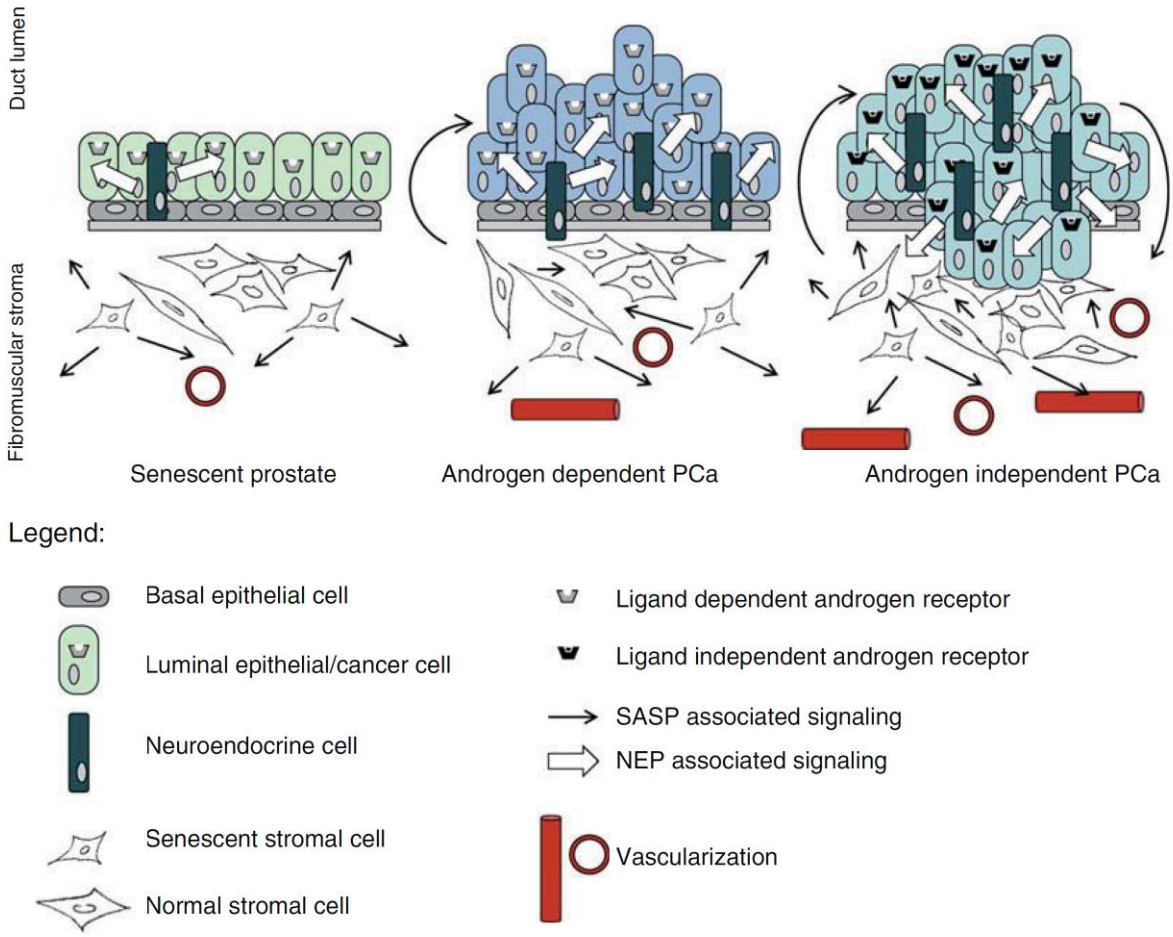

Figure 3. Senescent stromal and neuroendocrine cells modify the prostate microenvironment with autocrine and paracrine soluble molecules. In PCa, cancer cells acquire both SASP and neuroendocrine phenotype (NEP) that promote the formation of epithelial neoplasia and the development of the androgen-independent phenotype. Reproduced from (Pernicova et al., 2013).

Apart from employing ADT, treatment approaches encompass chemotherapeutic agents like docetaxel, exhibiting restricted efficacy, which could eventually develop resistance and result in a metastatic state (Marin-Aguilera et al., 2012). Recently, increased focus has shifted towards using poly (ADP-ribose) polymerase inhibitors and immunotherapy (Slovin, 2023), aiming to suppress tumor growth and enhance clinical outcomes. Toll-like receptors (TLRs) play a crucial role in the immune response. Over the past decade, more studies have focused on the potential involvement of TLRs in either promoting or inhibiting cancer progression (Muresan et al., 2020). Specifically, TLR3, expressed in

various cancer types, has been linked to patient context-dependent prognoses. Recent investigations have revealed its expression in PCa (summarized in (Muresan et al., 2020)).

Conversely, emerging evidence suggests that TLR3 stimulation might impact the metabolism of PCa cells and sustain tumor progression (Magnifico et al., 2019). These findings imply that TLR3 could be significant in both the progression and regression of PCa, although its specific role in each pathway remains to be defined. Our study proved that the exogenous activation of TLR3 in PCa cell lines induces a significant increase in the secretion of cytokines such as IL-6, IL-8, and interferon-β. The specific cytokine response depended on the model and the chemoresistance status. Transcriptomic analysis of cells overexpressing TLR3 unveiled a functional program enriched with genes regulating cell motility, migration, and tumor invasiveness. Augmented motility, migration, and invasion in the TLR3-overexpressing cell line were confirmed through various *in vitro* assays and validated using an orthotopic prostate xenograft model *in vivo*. These findings suggest that TLR3 may play a role in the progression and metastasis of prostate cancer. However, it also raises the possibility that TLR3 could be a vulnerability in PCa, presenting an opportunity for targeted therapeutic interventions (refer to Appendix 24, (Muresan et al., 2022)).

In summary, ADT is a crucial treatment for advanced prostate cancer, achieved through androgen receptor blockade or castration. However, the inevitable progression to CRPC poses a significant clinical challenge. Prostate cancer cells exhibit plasticity during ADT, often transdifferentiating into NEPC, contributing to treatment resistance. The TME plays a pivotal role in inducing NEPC after ADT, highlighting the impact of TME on cancer cell plasticity. NED in prostate cancer cells can be caused by various stimuli, including androgen depletion, IL-6, Wnt, EGF signaling pathways, cAMP signaling, and ionizing radiation.

Additionally, cell cycle modulation, senescence, and the SASP are implicated in promoting NEPC, further complicating therapeutic strategies. The roles of Skp2 and TLR3 overexpressed in PCa add other layers to the intricate mechanisms involved in prostate cancer progression. These findings underscore the complexity of prostate cancer plasticity and emphasize the need for comprehensive approaches to diagnosis and therapy.

CHAPTER 3 EPITHELIAL-MESENCHYMAL PLASTICITY AND METASTASIS

EMT is a cellular process where epithelial cells take on mesenchymal characteristics and behavior by reducing their typical features (Yang et al., 2020). This dynamic process is crucial for normal embryonic development and maintenance of tissue homeostasis via tissue regeneration and wound healing (Katsuno and Derynck, 2021).

In neoplasias, EMT imparts increased tumor-initiating and metastatic potential to cancer cells, rendering them more resistant to various therapeutic regimens (see Figure 4) (Chaffer et al., 2016). EMT also plays a pivotal role in the progression of tumors, facilitating metastatic spread and leading to the development of tumor cells with stem cell characteristics. These cells contribute significantly to resistance against cancer treatment (Shibue and Weinberg, 2017). The emerging mesenchymal-like cells can undergo a reverse change to an epithelial state, called mesenchymal-epithelial transition (MET) (Katsuno and Derynck, 2021). During EMT, cells lose their adhesion and polarity, becoming more mobile and invasive (Chaffer et al., 2016).

The processes of EMT and MET, which were initially thought to be binary processes involving a transition between epithelial and mesenchymal phenotypes, have undergone a paradigm shift (Haerinck et al., 2023). Recent profiling at the single-cell level using both RNA and protein analyses (Pastushenko et al., 2018) revealed that cells can exist in transitional states along the epithelial-mesenchymal axis (Pastushenko and Blanpain, 2019). These partial EMT states simultaneously exhibit features of both epithelial and mesenchymal (E/M) phenotypes. Depending on the specific context, cells with hybrid E/M phenotypes can either be fixed (Kroger et al., 2019; Pastushenko et al., 2021) or flexibly undergo a diverse range of alternative states, a phenomenon referred to as epithelial-mesenchymal plasticity (EMP) (Yang et al., 2020).

EMT is not a uniform phenomenon but manifests as a localized event in each context (Nieto et al., 2016). Each cellular entity exists in and is shaped by a microenvironment of neighboring cells, the extracellular matrix, and cytokine gradients. Epithelial cells can initiate EMT only by recognizing changes in this microenvironment. Ligands such as TGFβ (Slabakova et al., 2011), IL-6/STAT (Xie et al., 2012), HGF (Farrell et al., 2014), FGF (Sun and Stathopoulos, 2018), EGF, Wnt/β-catenin and Notch (Sun and Stathopoulos, 2018) have demonstrated the ability to induce or enhance EMT (Figure 3, (Haerinck et al., 2023; Perez-Gonzalez et al., 2023)).

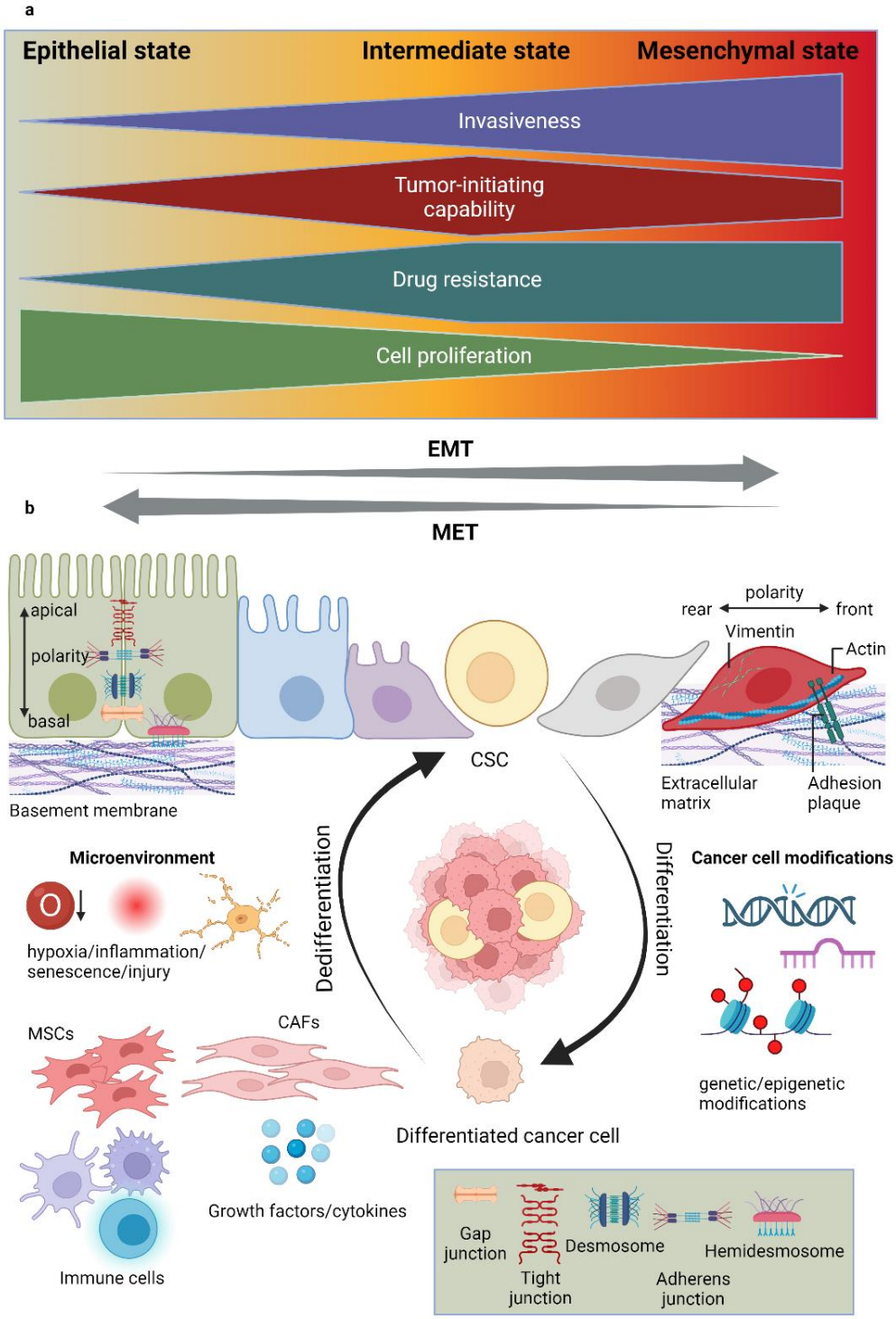

Figure 4. (Patho)physiological and morphological changes in EMT/MET. a - summary of pathophysiological outcomes of EMT/EMT in cancer. b – An overview of EMT/EMT-associated changes and cellular level. Adapted from (Shibue and Weinberg, 2017; Fanelli et al., 2020). Created with BioRender.com

Additional microenvironmental factors, including hypoxia (Jiang et al., 2007), nutrient availability (Recouvreux et al., 2020), shear forces (Heise et al., 2011), and matrix rigidity (Deng et al., 2021), also contribute to EMT. It is crucial to note that investigations into these parameters have predominantly occurred under diverse artificial *in vitro* conditions employing fully transformed and genetically unstable cancer cell lines. The use of these models has been one reason why EMT has long been seen as controversial and questioned (Ledford, 2011). The controversy primarily arose from the skepticism voiced by pathologists, as they had not encountered compelling evidence of EMT in human carcinoma samples with sufficient frequency to confidently affirm the widespread implication of the EMT program in the pathogenesis of numerous cancer types (Tarin, 2005; Shibue and Weinberg, 2017). Nonetheless, these studies underscore the profound impact of the microenvironment in dictating a cell's susceptibility to EMT or even driving EMT itself. The microenvironmental influences may act synergistically or antagonistically; however, the intricate nuances of these interactions are yet to be fully elucidated (Haerinck et al., 2023). An asymmetry arises when comparing the interactions of epithelial cells with mesenchymal cells in the microenvironment. Epithelial cells, anchored in place by their strong adhesive properties, are "overcome" by their microenvironment. In contrast, migrating mesenchymal cells, guided by chemotactic signals, actively seek out specific environments. These chemotactic mechanisms play a crucial role during development and contribute to organotropism in cancer cell metastasis (Gao et al., 2019). Cells in a state of partial EMT may associate motility with some environmental stability by migrating in clusters. In such cases, the migratory unit involves a "traveling microenvironment". In addition to cancer cells, these clusters may include platelets, immune cells, cancer-associated fibroblasts, and extracellular matrix (Kan et al., 2020). These clusters enable partial EMT state maintenance through persistent TGFβ signaling (Kan et al., 2020). In addition, clustering prevents anoikis (a specific mechanism of programmed cell death observed in anchorage-dependent cells when they detach from the ECM). It protects tumor cells from immune system attacks, shear forces, and other external stressors (Tiwari et al., 2012). In metastasis, clusters are very effective in colonizing secondary tumors (Aceto et al., 2014; Haerinck et al., 2023).

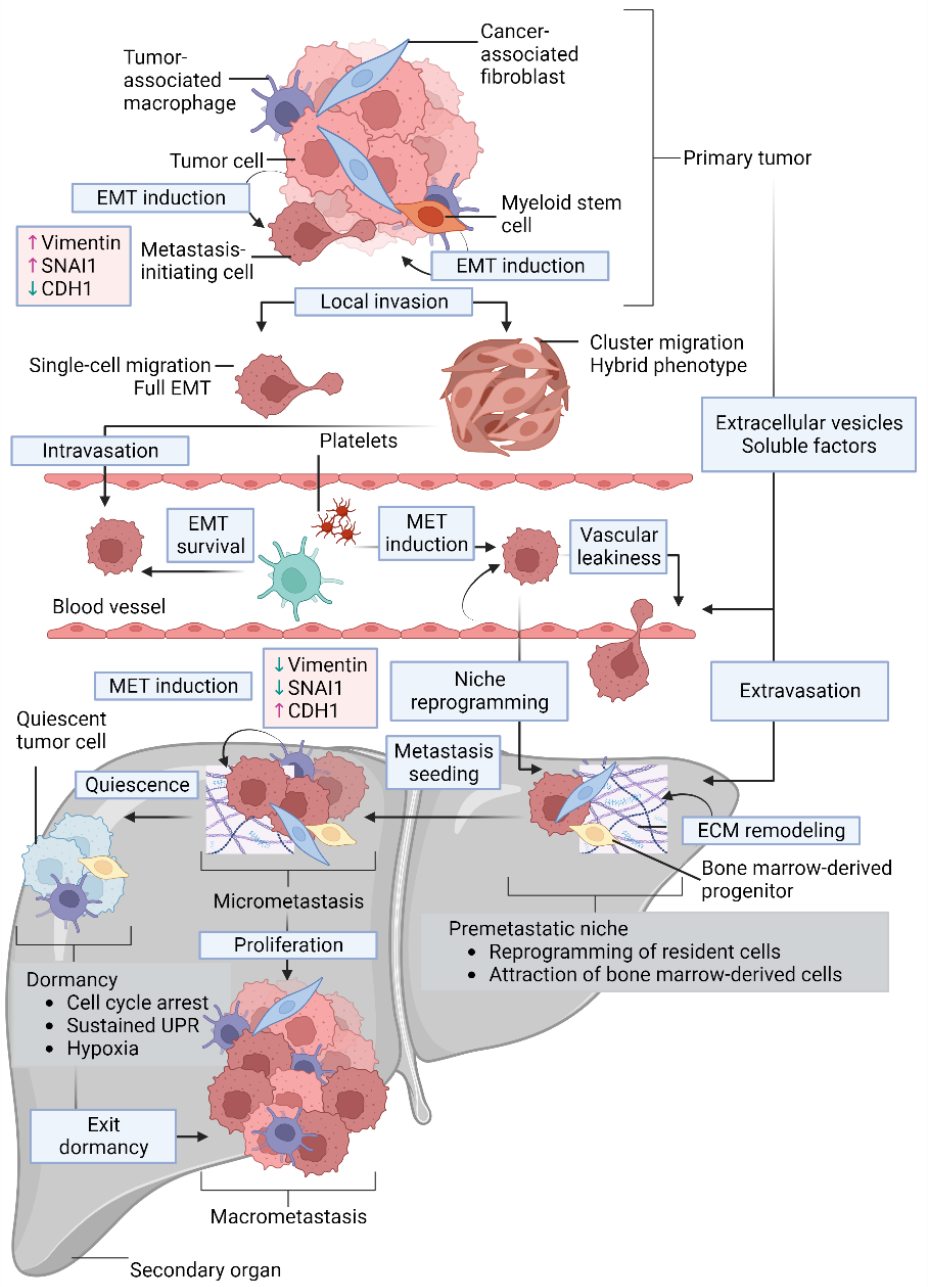

Figure 5. Cellular plasticity and metastasis cascade. Tumor cells acquire metastasis-initiating properties through internal and external stimuli that induce EMT. EMT enables metastasis-initiating cells to detach from the primary tumor and intravasate into the bloodstream. Circulating tumor cells, either single or clustered, display high plasticity and hybrid EMT. Interactions with platelets and macrophages enhance plasticity, and platelet coating protects CTCs. The primary tumor primes the secondary organ by secreting extracellular vesicles and soluble factors, establishing a permissive microenvironment. Colonization of the metastatic site involves the reversion of tumor cells to the epithelial state, influenced by signals from the metastatic niche. Post-seeding, tumor cells may enter dormancy, gain immune evasion traits and therapy resistance, or proliferate to form macroscopic metastases. CDH1 - Cadherin 1), SNAI1 – SNAIL, UPR - unfolded protein response. Adapted from (Perez-Gonzalez et al., 2023). Created with BioRender.com

As mentioned above, cellular plasticity and tumor heterogeneity are some of the significant obstacles to the successful treatment of advanced and disseminated tumors (Figure 5). Its contribution to the capacity of cancer cells to adapt to changing conditions, evade therapy, and promote metastasis is leading to poor patient outcomes (Fanelli et al., 2020; Lüönd et al., 2021). Therefore, developing treatment strategies to target cancer cell plasticity directly is reasonable and promising (Shibue and Weinberg, 2017). Residual carcinoma cells that survive diverse therapies, including chemotherapy, molecularly targeted therapy, and immunotherapy, often exhibit signs of EMT activation. Targeting cancer cells with activated EMT components may enhance these therapies' efficacy for durable clinical responses. However, the mechanisms responsible for maintaining or inducing the EMT program in these residual cells are poorly understood.

The complexity and redundancy of the mechanisms that drive EMT in cancer cells pose a significant challenge to this program's selective and efficient targeting. Overcoming these challenges is essential for developing treatment strategies with minimal risk of resistance, ultimately leading to curative cancer treatment. Many biological properties of cancer cells are influenced by non-genetic mechanisms, highlighting the limitations of information derived from cancer genome sequencing and the need for integrated approaches such as proteomics, epigenomics, and transcriptomics to gain a comprehensive view of cancer cell biology (Shibue and Weinberg, 2017; Jehanno et al., 2022; Perez-Gonzalez et al., 2023).

EMT is a crucial mechanism in embryonic development and maintenance of tissue homeostasis. However, in carcinoma cells, this process gives rise to harmful traits, such as invasive behavior, cancer stem cell activity, and increased resistance to chemotherapy (Lu and Kang, 2019). EMP plasticity refers to the ability of epithelial cells to undergo a transition to a mesenchymal state, which is characterized by increased motility and invasiveness, and vice versa (Summarized in Appendix 22, (Kvokackova et al., 2021)). Cancer cells in transitional states between epithelial and mesenchymal (known as partial EMT) exhibit high adaptability throughout cancer progression, making them critical contributors to tumor development (Kvokackova et al., 2021). Cells displaying hybrid epithelial and mesenchymal characteristics are more effective at entering the bloodstream and establishing metastases (Lu and Kang, 2019; Kvokackova et al., 2021). Understanding the underlying molecular and cellular mechanisms of EMP offers essential insights into the origins of cancer and can potentially guide the development of new therapeutic approaches (Lu and Kang, 2019; Kvokackova et al., 2021).

As mentioned above, the TGF-β family is one of the essential inducers of EMT (Lee and Massagué, 2022). The spectrum of mechanisms in this context is broad and includes canonical (SMAD-dependent) and non-canonical signaling (Katsuno and Derynck, 2021; Lee and Massagué, 2022). We previously described that benign prostate hyperplasia cells undergo EMT upon TGF-β1 treatment (Appendix 2, (Starsíchová et al., 2010)). Next, we focused on the kinetics of expression of EMT markers, transcription factors, and miRNAs regulating the EMT process in the benign and derived tumorigenic clones. In these cells, the transition to EMT was marked by a swift increase in SNAI2/Slug and ZEB1 transcription factors, with alterations in the expression of ZEB2 and miR-200 family members observed over more extended periods. Our findings suggest that SNAI2/Slug is essential for the TGF-β1-induced increase in vimentin and migration in benign prostate epithelial cells. Additionally, the ZEB family of transcription factors, in collaboration with the miR-200 family, may play a role in suppressing the TGF-β1-induced EMT phenotype (See Appendix 4, (Slabakova et al., 2011)). Next, we explored how the TGF-β family regulates Sca-1, a widely used marker for adult stem cells (Upadhyay et al., 2011). Our evidence suggests that TGF-β disrupts lineage commitment and encourages the accumulation of tumor-initiating cells in pre-neoplastic cells. Brief exposure of these cells to TGF-β resulted in the loss of Sca-1 and the selection or enrichment of cells with enhanced tumorigenic potential. While endogenous TGF-β signaling suppressed Sca-1 through Smad2/3/4, the inhibition of Sca-1 expression due to external TGF-β stimuli was Smad2/3-independent. Our study demonstrates that TGF-β signaling regulates Sca-1 expression, tumorigenicity, and plasticity in mammary epithelial and cancer stem cells (Refer to Appendix 21 (Remsik et al., 2020)).

Stimuli inducing the EMT process include cytokines, oncogenes, and TME-associated molecules (Lamouille et al., 2014). EMT can stem from defects in the tumor suppressor p53, with gain-of-function mutations and loss associated with EMT (Kogan-Sakin et al.; Lu and Kang, 2019).

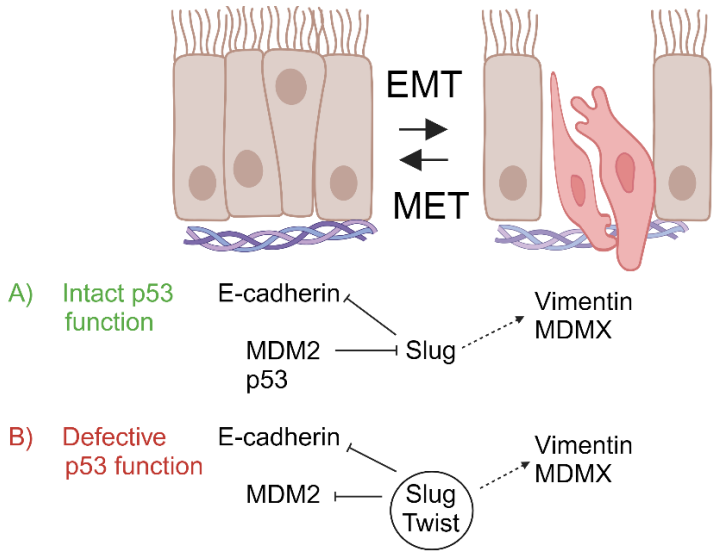

Figure 6. Crosstalk between MDM2, Slug, and Twist in EMT. A. In the case of intact p53 function, MDM2 cooperates with p53 in the degradation of Slug, thereby preserving the epithelial phenotype. B. When p53 function is impaired, elevated expression of MDM2 correlates with the epithelial phenotype. Slug and Twist inhibit the expression of MDM2 and promote EMT, which is associated with upregulated expression of MDMX. Solid lines delineate direct effects; dotted lines represent indirect mechanisms. Adapted from (Slabakova et al., 2015). Created with BioRender.com

Proper p53 function is vital for maintaining the epithelial phenotype, regulated tightly by MDM2 and MDMX. MDM2, through various mechanisms, controls p53 functions, while MDMX lacks E3 ubiquitin ligase activity but regulates p53 and MDM2 (Wade et al., 2010). Inhibiting MDM2 sensitizes cancer cell lines to chemotherapy, and clinical trials explore p53 reactivation via MDM2 inhibitors (Wade and Wahl, 2009). We demonstrated that the EMT phenotype is linked to a reduction in MDM2 and an increase in MDMX expression in various cellular models and clinical prostate and breast cancer samples. Modulating EMT-associated alterations in MDM2 expression in benign and transformed prostate epithelial cells impacts their migration capacity and responsiveness to docetaxel. Examination of potential mechanisms controlling MDM2 expression reveals that, in the presence of impaired p53 function, EMT-inducing transcription factors Slug and Twist regulate MDM2 expression. These findings unveil an alternative, context-specific role of MDM2 in EMT, cell migration, metastasis, and resistance to therapy (Figure 6, Appendix 11, (Slabakova et al., 2015)).

A growing body of research indicates that EMT is a pivotal process influencing the responsiveness of cancer cells to chemotherapy. Zinc finger E-box binding homeobox 1 (ZEB1) plays a central role within a network of transcription factors governing EMT.

Additionally, it has been recognized as a significant molecule in regulating DNA damage, cancer cell differentiation, and metastasis (Summarized in Figure 7, Appendix 19, (Drapela et al., 2020)).

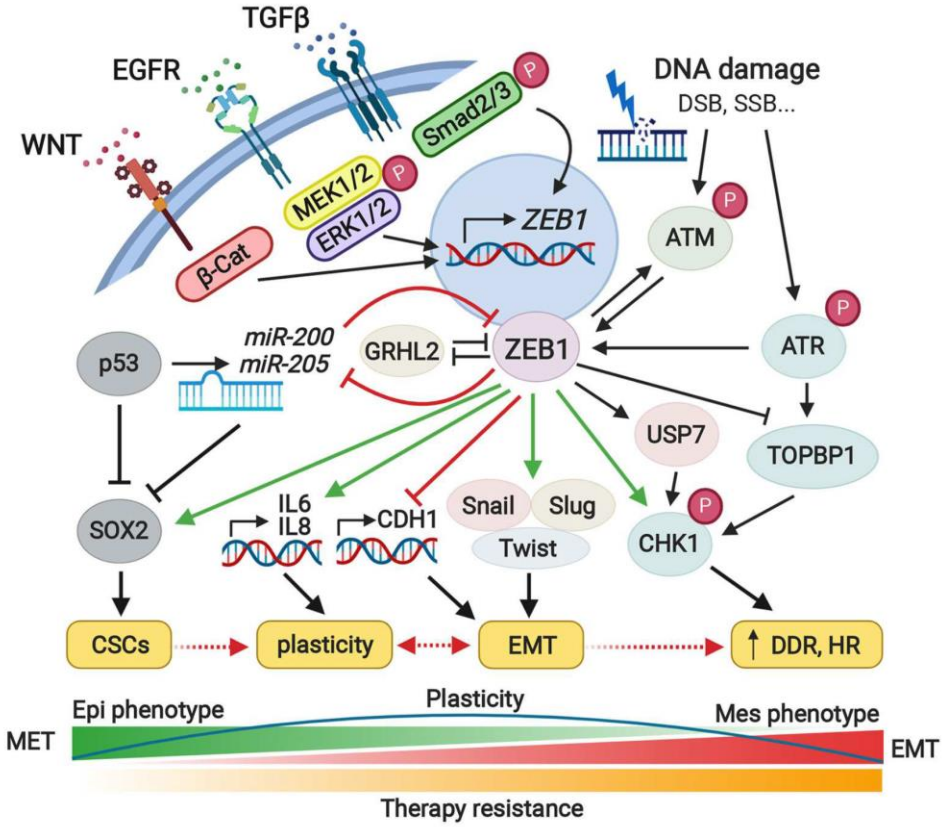

Figure 7. Pleiotropic roles of ZEB1 in the cell plasticity, EMT, and therapy resistance. The ZEB1 represents a core transcriptional factor and central determinant of cell fate, which controls fundamental intracellular processes, including cell plasticity, EMT, or therapy resistance. Downstream signaling pathways triggered by ZEB1 regulate the activity of the proteins and miRNAs involved in cell differentiation, proliferation, or motility. ZEB1 overexpression is accompanied by an overall changeover of the cell phenotype, higher tumorigenic potential, and increased migratory character. ZEB1 also promotes immune escape as well as contributes to the formation of a pre-metastatic niche. Given the tumor heterogeneity, ZEB1 plays an essential role in the stemness of cancer cells and increased radio- and chemoresistance. The green and red arrows illustrate the significant activating or inhibitory effects of ZEB1, respectively. CSCs, cancer stem cells; EMT, epithelial-to-mesenchymal transition; MET, mesenchymal-to-epithelial transition; DDR, DNA damage response; HR, homologous recombination. Created with Biorender.com. Reproduced from (Drapela et al., 2020).

Trop2, also known as trophoblast cell-surface antigen 2, is a cell surface glycoprotein that has been found to play a role in the promotion of EMT in various types of cancer (Summarized in Appendix 20 (Lenart et al., 2020)). Most studies characterize Trop2 as an

oncogene and a mediator of metastasis in prostate cancer, attributing its impact to regulating focal adhesions and integrins (Trerotola et al., 2015). Conversely, a separate study demonstrated that the loss of Trop2 in mice lacking Arf results in the development of squamous cell carcinoma with features of EMT (Wang et al., 2011). Despite the approval of therapies targeting Trop2-expressing cells, the functional role of Trop2 within the tumor mass remains unknown or subject to controversy (Lenart et al., 2020; Yao et al., 2023). Our research indicates that the membrane expression of Trop2 is positively correlated with E-cadherin expression and negatively correlated with the mesenchymal gene signature in a broad range of human and murine breast and prostate cancer cell lines, as well as human tumors (See Appendix 15, (Remšík et al., 2018)). Our findings suggest that in breast and prostate cancers, the surface expression of Trop-2 is associated with the epithelial phenotype. Additionally, we demonstrated that Trop-2 expression is either epigenetically suppressed through DNA hypermethylation or EMT transcription factors, such as ZEB1 during EMT. Furthermore, our data suggest that intratumoral heterogeneity in Trop-2 expression, driven by cancer plasticity, may significantly contribute to both the response and resistance to therapies targeting Trop2-expressing cells (Appendix 15 (Remšík et al., 2018)).

To understand EMP-driven intratumoral heterogeneity, we implemented a high-throughput screening platform to identify surface antigens that are linked to EMP in distinct pairs of epithelial cell lines and their mesenchymal counterparts (see Chapter below and Appendix 16 & Appendix 21 (Remsik et al., 2018; Remsik et al., 2020; Drápela et al., 2021)). We identified ten surface antigens that reflect EMP *in vitro* using antibody-based high-throughput profiling and verified their expression in dissociated breast cancer patient samples. The depicted antigens showed heterogeneous expression in clinical specimens. CD9, CD29, CD49c, and integrin β5 (ITGB5) exhibited significant downregulation in a subset of breast cancer cells undergoing EMT *in vivo*. CD9, a tetraspanin with various functions in development and disease, consistently experienced reduced levels both *in vitro* and *in vivo* despite being commonly overexpressed in breast cancer (Ondrussek et al., 2023). We correlated the expression of CD9 with the epithelial phenotype, EMP, and a favorable prognosis in extensive cohorts of breast cancer patients. The identification of molecules associated with cancer plasticity and the comprehension of mechanisms involved in transcriptional reprogramming may uncover potential biomarkers and therapies targeting tumor-initiating cells, thereby preventing cancer relapse and the growth of macrometastases in the future (Refer to Appendix 16, (Remsik et al., 2018)).

As a continuation of this study, and considering the acknowledged inaccuracies in identifying cell subtypes, such as fibroblasts using single markers (refer to Appendix 13 (Kahounova et al., 2018)), we have delineated creating a comprehensive mass cytometry panel. This panel is specifically designed for the multiparametric identification of 23 phenotypic markers and 13 signaling molecules. Employing this single-cell proteomic approach, we investigated the landscape of heterogeneity in triple-negative breast cancer (TNBC), focusing on the tumor microenvironment (Figure 11). Our prospective profiling involved the examination of freshly resected tumors from 26 TNBC patients, unveiling unique subsets of cancer and stromal cells with connections to the patient's clinical condition at the time of primary tumor resection. We also examined the EMP of tumor cells and molecularly delineated various phenotypes within the tumor-associated stroma. Furthermore, in a retrospective tissue-microarray cohort of TNBC, we revealed the prognostic capability of CD97 levels (Refer to Appendix 26, (Kvokackova et al., 2023)).

Understanding EMP in cancer reveals a complex regulatory network involving factors like TGF-β, p53, MDM2, and Trop2. These findings provide better insight into cancer development and suggest a pathway for new therapeutic strategies targeting tumor-initiating cells. Developing a comprehensive mass cytometry panel is a significant technological opportunity to accurately identify cell subtypes and phenotypic markers and improve our understanding of tumor heterogeneity and microenvironment (see next chapter).

CHAPTER 4 CYTOMETRIC ANALYSIS OF HETEROGENEITY AND PLASTICITY OF CELL IDENTITY

The complexity of cancer is underscored by the intricate interplay of diverse molecular mechanisms and the remarkable tumor heterogeneity exhibited within and among cancer types, challenging conventional therapeutic approaches and necessitating a comprehensive understanding of the complex biological landscapes underlying this formidable disease. This heterogeneity can manifest as variations in morphology, gene expression, metabolism, and metastatic potential (Jehanno et al., 2022). It occurs at both inter-tumor and intra-tumor levels, posing significant challenges to effective treatment and contributing to tumor resistance and aggressive metastasizing (Bhatia et al., 2012; Boyd et al., 2012).

The TME plays a vital role in the complexity of cancer. This complex ecosystem surrounding the tumor includes various non-tumor components, including endothelial and immune cells, fibroblasts, soluble factors, extracellular vesicles, and ECM (Witz, 2008; Monteran et al., 2023). Dynamic interactions between tumor cells and their microenvironment are essential for inducing tumor cell heterogeneity, clonal evolution and promoting multidrug resistance, ultimately resulting in tumor cell progression and metastasis (Baghban et al., 2020). Much of our current knowledge of the TME is based on studies conducted in primary tumors. Nevertheless, when tumor cells disseminate to distant organs, they must adapt to distinct organ-specific microenvironments characterized by variations in stromal cell composition and ECM compared to the primary site. It necessitates the utilization of unique molecular and cellular interactions to either support or counteract the growth of disseminated cancer cells (Massague and Obenauf, 2016; Monteran et al., 2023). Notably, the reprogramming and activating of stromal cells in metastatic organs precede the actual formation of metastases (Hu et al., 2023; Monteran et al., 2023). Stromal cells receive multiple activating signals throughout the metastatic cascade, leading to functional impairment. Instead of resolving the tissue damage caused by disseminated cancer cells, these stromal cells transform into facilitators of metastatic growth (Lavie et al., 2022; Monteran et al., 2023).

Additionally, activated stromal cells are pivotal in attracting tumor cells, fostering cancer cell dormancy and awakening, and promoting tumor cell invasion, survival, and proliferation. Ultimately, these stromal cells support metastatic outgrowth (Lawson et al., 2015; Monteran et al., 2023). Considering these observations, targeting the TME emerges as an appealing and innovative strategy to indirectly disrupt the intricate interplay among cancer cells, potentially contributing to developing effective cancer therapies (Anderson and Simon, 2020). Most tumors originate from a single mutated cell and accrue additional mutations during their progression to advanced disease through Darwinian evolution (Marusyk et al., 2012; Hinohara and Polyak, 2019). Mutations facilitating tumor progression and conferring therapeutic resistance may be present in early preinvasive or premalignant lesions (Spira et al., 2017). Topographic single-cell sequencing applied to both in situ and invasive regions within the same breast tumor tissue sections unveiled the evolution of multiple mutated clones within ducts before the invasion. Specific subclones were more prevalent in the invasive areas, indicating multiclonal invasion (Casasent et al., 2018).

Microenvironmental factors, such as hypoxia, tissue stiffness, and chronic inflammation, exert both direct and indirect influences on the epigenetic and phenotypic heterogeneity of cancer cells (Lawson et al., 2018). Another source contributing to intratumoral heterogeneity is the epigenetic variation associated with the inherent differentiation hierarchy in normal tissues. The differentiation state of the transformed cell and its normal cell-of-origin collectively determine the molecular subtypes of the tumor. In breast cancer, for instance, luminal A, luminal B, basal-like, and $HER2^{+}$ subtypes are believed to originate from distinct luminal and basal progenitors (Perou et al., 2000; Hinohara and Polyak, 2019).

EMT plays an essential role in regulating cellular heterogeneity, as cells undergoing EMT acquire stem cell-like features (refer to the subsequent chapter, (Mani et al., 2008). While many studies on intratumoral heterogeneity primarily focus on genetic changes, treatment resistance often stems from a malfunction of pathways targeted by the therapy (McGranahan and Swanton, 2017). Nevertheless, non-genetic variability, such as epigenetic heterogeneity, impacts therapeutic resistance through diverse mechanisms (Brock et al., 2009; Saxena et al., 2022).

The master regulator of EMT, ZEB1, induces the expression of histone methyltransferase, establishing a positive feedback loop that sustains ZEB1 expression and maintains the invasive EMT regulatory state (Lindner et al., 2020). A previous study similarly

demonstrated that the induction of EMT by upregulated expression of the related transcription factor SNAIL1 led to significant alterations in the chromatin landscape. This induction activated several chromatin modifiers, and their activity was indispensably necessary for maintaining the phenotypic state (Javaid et al., 2013; Hanahan, 2022).

These findings underscore the complexity and interconnectedness of individual processes and modes of regulation that give rise to dynamic changes and the demonstration of different tumor phenotypes. Consequently, the combined manipulation of the microenvironment, genomic, and epigenetic landscape in cancer cells could be a potent therapeutic tool, enhancing the efficacy of targeted therapies or chemotherapies (Hinohara and Polyak, 2019).

Cytometry methods, particularly single-cell analysis, have played a significant role in understanding cancer heterogeneity and cell plasticity. These methods have enabled researchers to isolate and characterize different tumor cell populations, assess tumor cell heterogeneity, and investigate the dynamics of immune cells and tumor-associated fibroblasts in the TME (Luo et al., 2022; Zhao and Rosen, 2022). Single-cell techniques such as RNA sequencing and single-cell proteomics have yielded valuable insights into the complexity of cancer cell heterogeneity and plasticity, empowering researchers to devise more effective strategies for cancer diagnosis, treatment, and personalized medicine.

The proven technology applied in this field is undoubtedly flow cytometry. It is a powerful technology that enables rapid multiparametric analysis of single cells in suspension (McKinnon, 2018). Flow cytometry proves valuable in various fields, including immunology, molecular biology, and cancer biology (McKinnon, 2018). Essential for quantifying the expression of cell surface and intracellular molecules, assessing the purity of isolated cell subpopulations, and sorting different cell populations through fluorescence-activated cell sorting (FACS), flow cytometry has no full-fledged alternative to date. Its capacity for multiparametric analysis, high speed, and the ability to separate complex, defined populations or single cells remains unmatched.

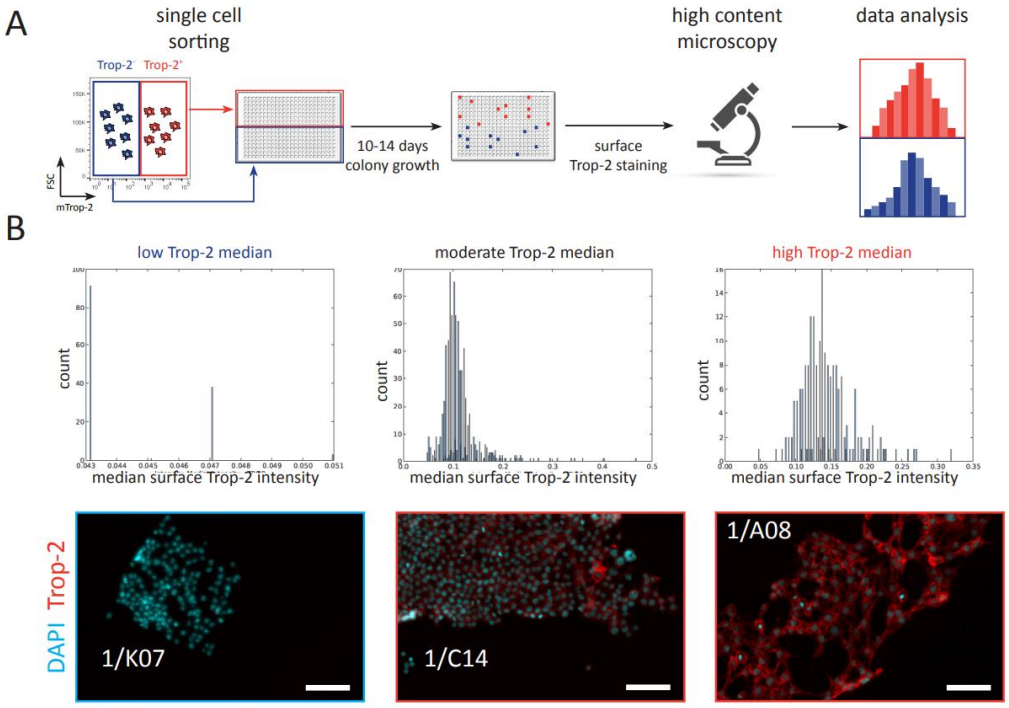

Figure 8. (A) The illustration shows the experimental workflow used to determine the *in vitro* plasticity of parental 4T1 cells with high-content microscopy. (B) Histograms show the single-cell Trop2 surface intensity of colonies derived from sorted Trop2$^-$ and Trop2$^+$ cells. Representative images show Trop2 (red) and DAPI (blue) staining of the colonies (scale = 100 µm). Reproduced with modification from (Remšík et al., 2018).

These features find simple applications in an automated cell clonogenic assay (ACCA). We employed ACCA to evaluate the cloning ability of different prostate and colon cancer cell subsets, considering the expression of specific markers associated with stem cells (CD44 and CD133) and cancer stem cells (Trop2, CD49f, and CD44). Our findings demonstrated that the newly introduced ACCA is an effective method for measuring the clonogenic capacity of cancer stem cells, as identified in both cell lines and patient samples (See Appendix 7, (Fedr et al., 2013). This methodology, in conjunction with high-content microscopy and image analysis, was applied to assess the variability of Trop2 expression within a diverse *in vitro* 4T1 cell model containing both Trop2$^-$ and Trop2$^+$ cells (Appendix 15 (Remšík et al., 2018)). Initially, we sorted both cellular factions as single cells based on their surface Trop2 levels and allowed them to develop colonies. Subsequently, we assessed the quality of the colonies and the surface Trop2 expression intensity at a single-cell level through high-content microscopy (see Figure 8). Intriguingly, both cell types generated colonies with low and moderate surface Trop2 intensity, but only Trop2$^+$ cells proliferated into colonies exhibiting high Trop2 staining. It confirms, among other

things, that this experimental approach is suitable for uncovering the link between cell identity and function (See Appendix 15, (Remšík et al., 2018)).

Complex analysis of the cellular response and cell fate (cytokinetics) following experimental treatment is essential for screening and understanding treatment (drug) effects and identifying sensitive or resistant cell types with a characteristic phenotype. For such analyses, a variety of methodological approaches might be used. Typically, these can involve adapting several parallel methods, which may have different readouts. Most frequently used single readout methods (e.g., western blot or qPCR) cannot detect cell-to-cell heterogeneity in response to the tested compound unless performed at the single-cell level (Heath et al., 2016). A modern cell analysis method should take full advantage of flow cytometry's multicolor character to simultaneously measure several types of cell responses and analyze the treated cells' phenotype and functional characteristics. In this perspective, we have established a multiparametric flow cytometric assay for the complex analysis of cellular phenotypes in response to experimental therapy (Appendix 17 (Simeckova et al., 2017)). Using this assay, we can simultaneously detect changes in viability, cell cycle profile, DNA synthesis and damage, and apoptosis in cells characterized by the expression of two surface markers. The main benefit of this approach is that various biological responses to a specific treatment can be evaluated in a single assay on a single-cell level.

Furthermore, we demonstrate the possible application of this established procedure to analyze heterogeneous samples and to compare the effects of drugs on different subsets of cell populations (Figure 9). Moreover, the protocol can be applied to both human and mouse and adherent and non-adherent cells. It creates an opportunity to analyze mixed cell populations efficiently, including infiltrating host cells in xenografts.

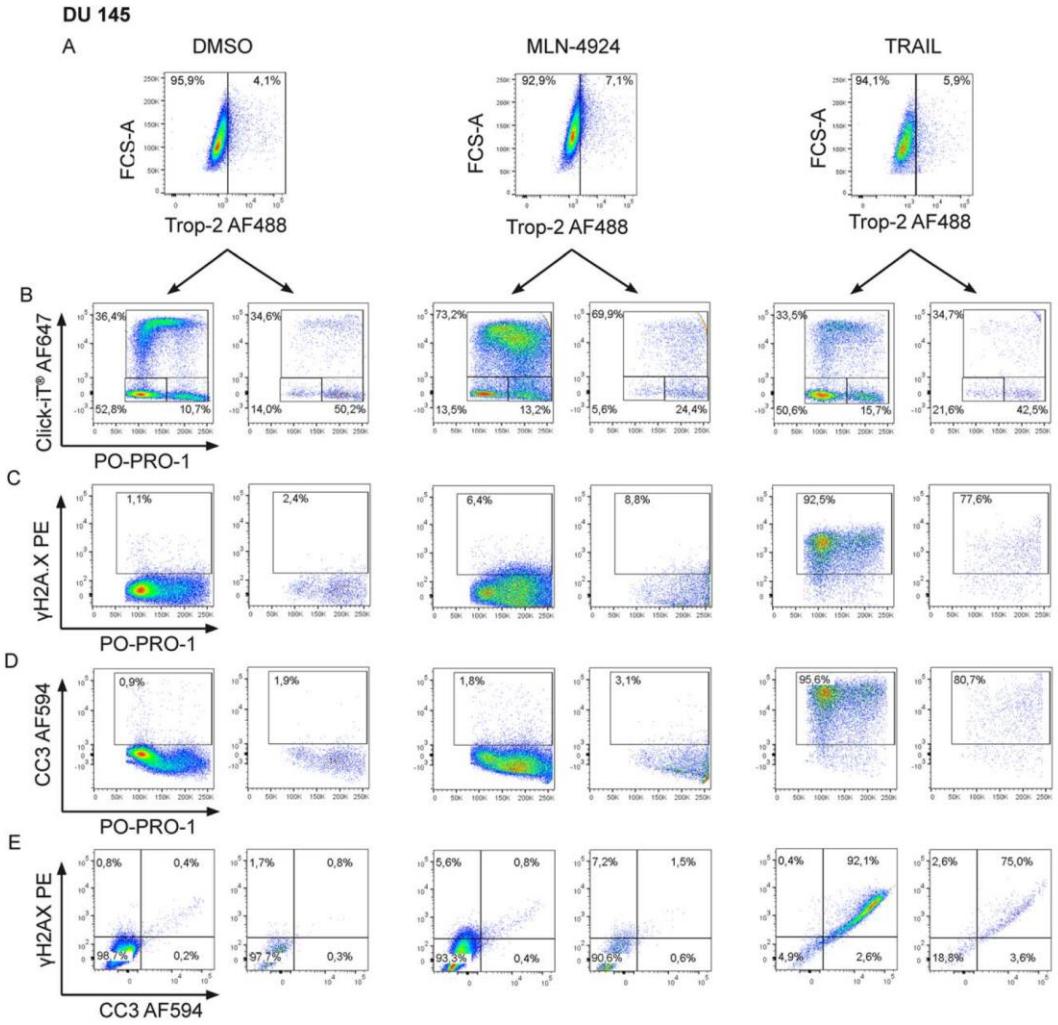

Figure 9. Comparison of complex responses to treatment among Trop2^{-} and Trop2^{+} subpopulations. Response to treatment was analyzed separately in the Trop2^{-} and Trop2^{+} subpopulations in the DU 145 cell line (A). Differences in proliferation (B), DNA damage (C, E), and induction of apoptosis (D, E) were examined. Reproduced from (Simeckova et al., 2017).

Fluorescent cell barcoding (FCB) is a method that facilitates high-throughput and high-content flow cytometry through the multiplexing of samples before specific staining and acquisition (Krutzik and Nolan, 2006; Giudice et al., 2017). The general concept behind the FCB utilizes one, two, or more fluorescent dyes at different concentrations - "barcodes"—allowing for subsequent discrimination of pooled cell samples using flow cytometry based on their distinct fluorescence emission wavelengths and corresponding intensities (Krutzik and Nolan, 2006). This method reduces reagent usage, shortens the time per sample (including staining, handling, and acquisition), and eliminates technical variability related to differences in staining volume and antibody concentration (batch

effect). It maximizes data robustness and assay efficiency (Krutzik and Nolan, 2006; Giudice et al., 2017). Applications that already demonstrated the effectivity of FCB methods include the analysis of intracellular phosphoproteome (Manohar et al., 2019), large-scale drug screenings (Krutzik and Nolan, 2006), or investigation of intratumoral heterogeneity within various cell samples (Appendix 16 & Appendix 21, (Remsik et al., 2018; Remsik et al., 2020; Drápela et al., 2021)). The absence of technologies enabling cost-effective profiling of a substantial number of cells using an antibody-based approach led us to create a high-throughput cytometry-based platform for surface profiling (see Figure 10). We combined fluorescent cell barcoding with existing commercially available screening tools to analyze cell surface fingerprints on a large scale (Refer to Appendix 23, (Drapela et al., 2022)). This robust approach allowed characterizing cell surface fingerprints linked to epithelial plasticity (See Appendix 16 & Appendix 21, (Remsik et al., 2018; Remsik et al., 2020; Drápela et al., 2021) and has the potential to identify novel biomarkers and druggable targets.

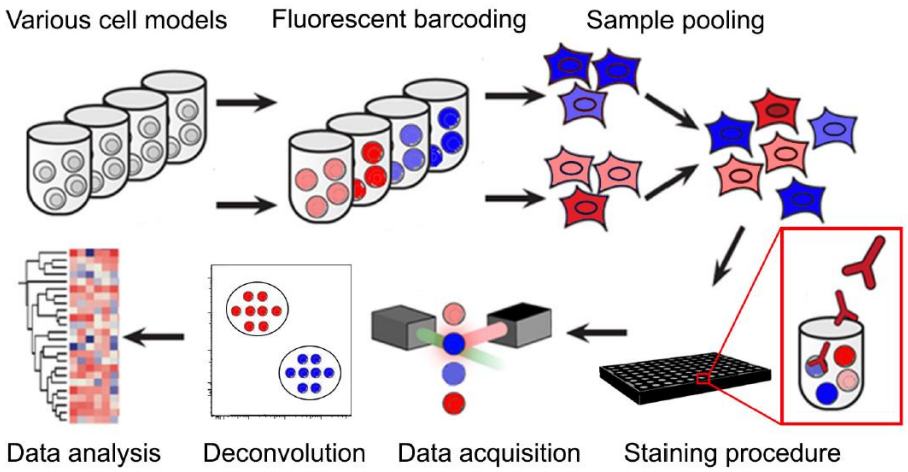

Figure 10. The experimental workflow of fluorescent barcoding and high-throughput flow cytometry screening of surface markers includes data acquisition, deconvolution of cell lines based on the fluorescent barcode, and data analysis. Reproduced from (Drápela et al., 2021).

Mass cytometry is a flow cytometry-based technique (CyTOF) that combines flow cytometry principles with elemental mass spectrometry to detect and quantify multiple markers on single cells (Iyer et al., 2022). In the context of flow cytometry, mass cytometry offers several advantages, including higher parameter detection. Mass cytometry allows for the simultaneous detection of up to 50 markers, compared to the

limitations of traditional flow cytometry, which are typically restricted to the detection of around a dozen parameters due to spectral overlap between fluorochromes. Several studies utilized single-cell mass cytometry to reveal the inter and intra-tumor heterogeneity. For example, Petrilli *et al.* demonstrated the significant phenotypic heterogeneity of pediatric-type diffuse high-grade gliomas among the analyzed tumor cell lines. They highlighted the degree of plasticity within the tumors (Petrilli et al., 2022).

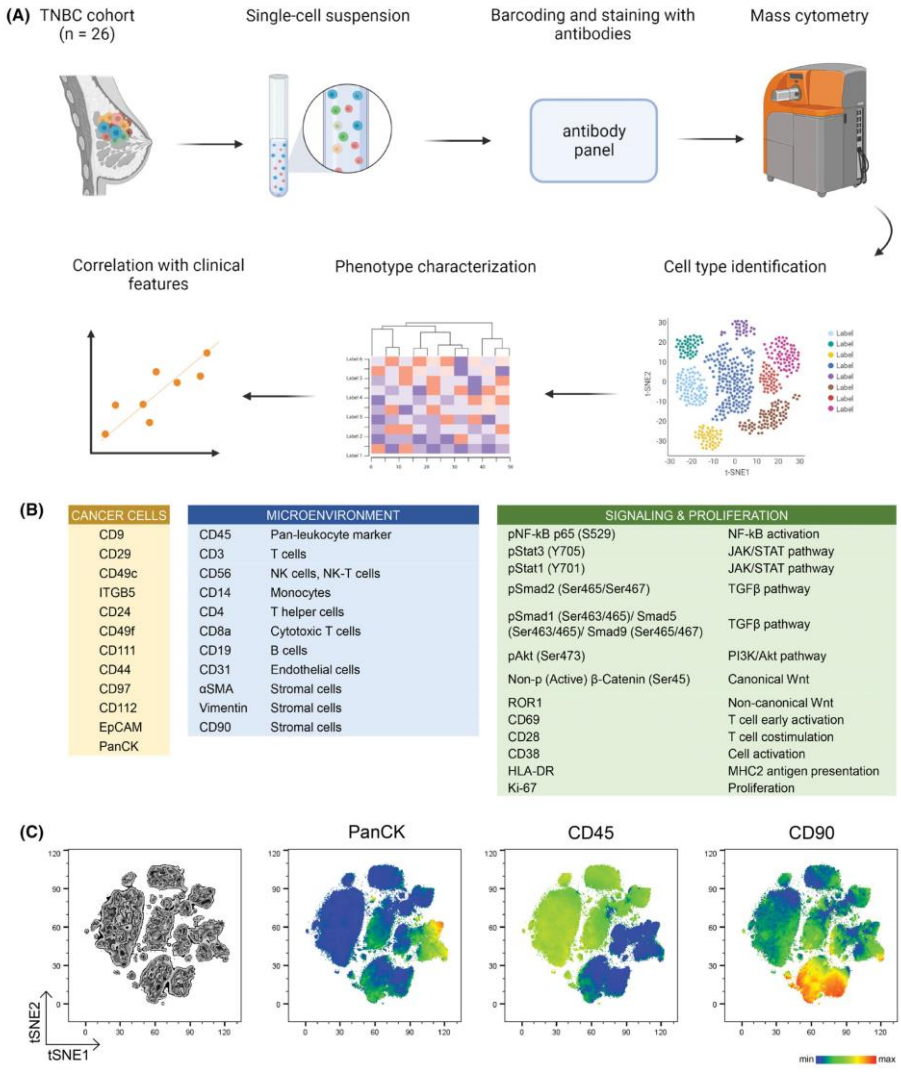

Figure 11. Characterization of tumor heterogeneity in TNBC samples by mass cytometry. (A) The scheme depicting the experimental approach and analytical workflow for primary TNBC patient samples was used in this study. (B) List of cell surface and signaling molecules selected to characterize tumor and microenvironmental compartments. (C) Two-dimensional t-SNE visualization of PanCK, CD45, and CD90 expression in all cells and all samples (n = 26). The combination of these three markers was used for the identification of cancer ($PanCK^+$), immune ($CD45^+$), and stromal ($PanCK^-CD90^+$) cells, respectively. Reproduced from (Kvokackova et al., 2023).

Moreover, a study published in Nature Cancer in 2021 introduced the development of three-dimensional imaging mass cytometry for multiplexed 3D tissue analysis at single-cell resolution (Kuett et al., 2022). Mass cytometry is an excellent tool for comprehensively understanding cancer heterogeneity and plasticity, particularly in the tumor microenvironment and the spatial organization of cells within tissues. Recently, we employed this technology and described the generation of a comprehensive mass cytometry (CyTOF) panel for multiparametric detection of 23 phenotypic markers and 13 signaling molecules. This proteomic approach at the single-cell level allowed us to investigate TNBC heterogeneity with a specific focus on the tumor microenvironment (Figure 11, Appendix 26, (Kvokackova et al., 2023)).

In summary, cytometric methods, encompassing single-cell analysis, flow cytometry, FCB, and mass cytometry, play a crucial role in revealing the heterogeneity and plasticity of cancer cells. These techniques provide valuable insights into tumor dynamics, facilitating the development of more effective cancer diagnosis and treatment strategies. With its multiparametric analysis, flow cytometry remains indispensable for studying cell surface markers, intracellular molecules, and clonogenic capacity. FCB streamlines high-throughput analyses, while mass cytometry offers a powerful tool for comprehensively investigating the proteomics of single cells. These advanced cytometric methods significantly contribute to our understanding of cancer biology and hold promise for biomarker discovery and targeted therapeutic approaches.

FUTURE PERSPECTIVES

Future perspectives in the context of cellular plasticity and tumor heterogeneity include understanding the complex interactions between different cell types and the tumor microenvironment and developing personalized treatment strategies targeting tumor cell plasticity. Advances in this research area are closely tied to progress in analytical cytometry and data analysis methods. Flow cytometry, single-cell RNA sequencing, mass cytometry, and spatial cytometry provide relevant answers to novel biological and clinical questions.

The presented research opens avenues for future investigation in several areas:

Precision targeting: Identified signaling pathways and molecules, such as TGF-β, c-Myb, and Trop2, offer potential targets for precision therapy in the context of cellular plasticity and cancer heterogeneity. A more precise understanding of the biology associated with these molecules is needed for further research to develop targeted interventions exploiting their dual roles in tumor suppression and progression. Given the immunosuppressive effects of TGF-β1 and its interactions with inflammatory pathways, exploring immunomodulatory strategies could reveal novel approaches to manipulate the tumor microenvironment and enhance the anti-tumor immune response. It is essential to elucidate the functional roles of Trop2 and its interactions in various aspects of tumor biology, including proliferation, invasion, and metastasis. Understanding these roles could reveal new tumor vulnerabilities and therapeutic opportunities.

Clinical biomarkers: Surface molecules associated with epithelial-mesenchymal plasticity, such as CD9, CD29, CD49c, and CD97, are promising clinical biomarkers. Validating their significance in large patient cohorts may lead to developing prognostic tools to predict cancer progression and response to treatment.

Cell plasticity, cancer heterogeneity, and resistance: Exploring the role of cellular heterogeneity, particularly concerning surface antigen expression, provides insight into resistance mechanisms and may guide the development of combination therapies targeting different tumor subpopulations. Elucidating the molecular mechanisms underlying treatment resistance and cellular plasticity is essential for developing strategies to overcome resistance and prevent cancer recurrence. Understanding these processes can increase treatment efficacy for resistant tumors and address the challenges of heterogeneity and plasticity in cancer therapy.

Spatial analysis: Advances in mass cytometry, genomic techniques, and three-dimensional imaging techniques allow spatial analysis of the tumor microenvironment. Understanding the spatial organization of different cell types within tumors can unravel critical interactions influencing cancer progression. Together with the massive advances in spectral cytometry and the opening of new possibilities in multi-parameter flow cytometry with image analysis and intracellular signal localization, it brings entirely new tools for revealing and understanding cellular phenotypes and their roles in cancer development.

Integration of omics data: Integrating data from diverse "omics" approaches, including genomics, transcriptomics, proteomics, and cytometry, will provide a holistic view of cancer biology. This integration can uncover novel therapeutic targets and enhance our understanding of the molecular networks driving cancer progression.

In conclusion, the future of cancer research lies in unraveling the complexities of tumor heterogeneity and plasticity, translating these findings into precision therapies, and exploring innovative technologies that allow a deeper understanding of cancer biology in both experimental and clinical settings.

LIST OF SELECTED PUBLICATIONS

List of articles representing the author´s contribution to understanding cell plasticity and cancer heterogeneity. Author's articles in the Appendix below represents a selection of 19 primary research articles, two book chapters, and five reviews (out of 140 author's publication records available on WOS on January 31st, 2024), which are related to the topic of the habilitation.

1. Vanhara P, Lincova E, Kozubik A, Jurdic P, **Soucek K***, Smarda J. Growth/differentiation factor-15 inhibits differentiation into osteoclasts-A novel factor involved in control of osteoclast differentiation. ***Differentiation*** 2009;**78**: 213-22. ** shared corresponding author*
2. Starsichova A, Lincova E, Pernicova Z, Kozubik A, **Soucek K**. TGF-β 1 suppresses IL-6-induced STAT3 activation through regulation of Jak2 expression in prostate epithelial cells. ***Cellular Signalling*** 2010;**22**: 1734-44.
3. Pernicova Z, Slabakova E, Kharaishvili G, Bouchal J, Kral M, Kunicka Z, Machala M, Kozubik A, **Soucek K**. Androgen Depletion Induces Senescence in Prostate Cancer Cells through Down-regulation of Skp2. ***Neoplasia*** 2011;**13**: 526-36.
4. Slabakova E, Pernicova Z, Slavickova E, Starsichova A, Kozubik A, **Soucek K**. TGF-β1-Induced EMT of Non-Transformed Prostate Hyperplasia Cells Is Characterized by Early Induction of SNAI2/Slug. ***Prostate*** 2011;**71**: 1332-43.
5. Knopfova L, Benes P, Pekarcikova L, Hermanova M, Masarik M, Pernicova Z, **Soucek K**, Smarda J. c-Myb regulates matrix metalloproteinases 1/9, and cathepsin D: implications for matrix-dependent breast cancer cell invasion and metastasis. ***Molecular Cancer*** 2012;**11**.
6. Vanhara P, Hampl A, Kozubik A, **Soucek K.** Growth/differentiation factor-15: prostate cancer suppressor or promoter? ***Prostate Cancer and Prostatic Diseases*** 2012;**15**: 320-8.
7. Fedr R, Pernicova Z, Slabakova E, Strakova N, Bouchal J, Grepl M, Kozubik A, Soucek K. Automatic cell cloning assay for determining the clonogenic capacity of cancer and cancer stem-like cells. ***Cytometry A*** 2013;**83A**: 472-82.
8. Pernicova Z, Vanhara P, **Soucek K**. Formation of secretory senescent cells in prostate tumors: the role of androgen receptor activity and cell cycle regulation. In: *Tumor Dormancy, Quiescence, and Senescence, Volume 1:* ***Aging, Cancer, and Noncancer Pathologies,*** Springer Netherlands, 2013: 303-16.
9. Pernicova Z, Slabakova E, Fedr R, Simeckova S, Jaros J, Suchankova T, Bouchal J, Kharaishvili G, Kral M, Kozubik A, **Soucek K.** The role of high cell density in the

promotion of neuroendocrine transdifferentiation of prostate cancer cells. ***Molecular Cancer*** 2014;**13**: 113.

10. Kratochvilova K, Horak P, Esner M, **Soucek K**, Pils D, Anees M, Tomasich E, Drafi F, Jurtikova V, Hampl A, Krainer M, Vanhara P. Tumor suppressor candidate 3 (TUSC3) prevents the epithelial-to-mesenchymal transition and inhibits tumor growth by modulating the endoplasmic reticulum stress response in ovarian cancer cells. ***Int J Cancer*** 2015;137: 1330-1340.
11. Slabakova E, Kharaishvili G, Smejova M, Pernicova Z, Suchankova T, Remsik J, Lerch S, Strakova N, Bouchal J, Kral M, Culig Z, Kozubik A, **Soucek K**. Opposite regulation of MDM2 and MDMX expression in acquisition of mesenchymal phenotype in benign and cancer cells. ***Oncotarget*** 2015;**6**: 36156-71.
12. Slabakova E, Culig Z, Remsik J, **Soucek K.** Alternative mechanisms of miR-34a regulation in cancer. ***Cell Death & Disease*** 2017;**8**.
13. Kahounova Z, Kurfurstova D, Bouchal J, Kharaishvili G, Navratil J, Remsik J, Simeckova S, Student V, Kozubik A, **Soucek K.** The fibroblast surface markers FAP, anti-fibroblast, and FSP are expressed by cells of epithelial origin and may be altered during epithelial-to-mesenchymal transition. ***Cytometry A*** 2018;**93**: 941-51.
14. Knopfova L, Biglieri E, Volodko N, Masarik M, Hermanova M, Glaus Garzon JF, Ducka M, Kucirkova T, **Soucek K**, Smarda J, Benes P, Borsig L. Transcription factor c-Myb inhibits breast cancer lung metastasis by suppression of tumor cell seeding. ***Oncogene*** 2018;**37**: 1020-30.
15. Remsik J, Bino L, Kahounova Z, Kharaishvili G, Simeckova S, Fedr R, Kucirkova T, Lenart S, Muresan XM, Slabakova E, Knopfova L, Bouchal J, Kral M, Benes P, **Soucek K.** Trop-2 plasticity is controlled by epithelial-to-mesenchymal transition. ***Carcinogenesis*** 2018;39: 1411-1418.
16. Remsik J, Fedr R, Navratil J, Bino L, Slabakova E, Fabian P, Svoboda M, **Soucek K.** Plasticity and intratumoural heterogeneity of cell surface antigen expression in breast cancer. ***British Journal of Cancer*** 2018;**118**: 813-819.
17. Simeckova S, Fedr R, Remsik J, Kahounova Z, Slabakova E, **Soucek K.** Multiparameter cytometric analysis of complex cellular response. ***Cytometry A*** 2018;**93**: 239-248.
18. Simeckova S, Kahounova Z, Fedr R, Remsik J, Slabakova E, Suchankova T, Prochazkova J, Bouchal J, Kharaishvili G, Kral M, Benes P, **Soucek K.** High Skp2 expression is associated with a mesenchymal phenotype and increased tumorigenic potential of prostate cancer cells. ***Sci Rep*** 2019;**9**: 5695.
19. Drapela S, Bouchal J, Jolly MK, Culig Z, **Soucek K.** ZEB1: A Critical Regulator of Cell Plasticity, DNA Damage Response, and Therapy Resistance. ***Front Mol Biosci*** 2020;**7**: 36.
20. Lenart S, Lenart P, Smarda J, Remsik J, **Soucek K**, Benes P. Trop2: Jack of All Trades, Master of None. ***Cancers (Basel)*** 2020;**12:** 3328.

21. Remsik J, Pickova M, Vacek O, Fedr R, Bino L, Hampl A, **Soucek K.** TGF-β regulates Sca-1 expression and plasticity of pre-neoplastic mammary epithelial stem cells. ***Sci Rep*** 2020;**10**: 11396.

22. Kvokackova B, Remsik J, Jolly MK, **Soucek K.** Phenotypic Heterogeneity of Triple-Negative Breast Cancer Mediated by Epithelial-Mesenchymal Plasticity. ***Cancers (Basel)*** 2021;**13**: 2188.

23. Drapela S, Fedr R, Vacek O, Remsik J, **Soucek K.** High-Throughput, Parallel Flow Cytometry Screening of Hundreds of Cell Surface Antigens Using Fluorescent Barcoding. ***Methods Mol Biol*** 2022;**2543**: 99-111.

24. Muresan XM, Slabakova E, Prochazkova J, Drapela S, Fedr R, Pickova M, Vacek O, Vichova R, Suchankova T, Bouchal J, Kurfurstova D, Kral M, Hulinova T, Sykora RP, Student V, Hejret V, van Weerden WM, Puhr M, Pustka V, Potesil D, Zdrahal Z, Culig Z, **Soucek K.** Toll-Like Receptor 3 Overexpression Induces Invasion of Prostate Cancer Cells, whereas Its Activation Triggers Apoptosis. ***Am J Pathol*** 2022;**192**: 1321-35.

25. Rihova K, Ducka M, Zambo IS, Vymetalova L, Sramek M, Trcka F, Verner J, Drapela S, Fedr R, Suchankova T, Pavlatovska B, Ondrouskova E, Kubelkova I, Zapletalova D, Tucek S, Mudry P, Krakorova DA, Knopfova L, Smarda J, **Soucek K**, Borsig L, Benes P. Transcription factor c-Myb: novel prognostic factor in osteosarcoma. ***Clin Exp Metastasis*** 2022;**39**: 375-90.

26. Kvokackova B, Fedr R, Kuzilkova D, Stuchly J, Vavrova A, Navratil J, Fabian P, Ondrussek R, Ovesna P, Remsik J, Bouchal J, Kalina T, **Soucek K.** Single-cell protein profiling defines cell populations associated with triple-negative breast cancer aggressiveness. ***Molecular Oncology*** 2023;**17**: 1024-40.

APPENDIX: COMMENTED OVERVIEW OF SELECTED PUBLICATIONS

Commented list of articles representing the author´s contribution to understanding cell plasticity and cancer heterogeneity. Articles below represents a selection of 19 primary research articles, two book chapters, and five reviews (out of 140 author's publication records available on WOS on January 31st, 2024), which are related to the topic of the habilitation.

Appendix 1 *Vanhara P, Lincova E, Kozubik A, Jurdic P, **Soucek K***, Smarda J: **Growth/differentiation factor-15 inhibits differentiation into osteoclasts-a novel factor involved in control of osteoclast differentiation**. Differentiation 2009, 78:213-22.*

Significance: Survival and capability of cancer cells to form metastases fundamentally depend on interactions with their microenvironment. Secondary tumors originating from prostate carcinomas affect the remodeling of bone tissue and can induce both osteolytic and osteocondensing lesions. However, molecular mechanisms responsible for selective homing and activity of cancer cells in bone microenvironment have not been clarified yet. Growth/differentiation factor-15 (GDF-15), a distant member of the TGF-β protein family, has recently been associated with many human cancers, including prostate. In this work, we unveiled the new role of GDF-15 in the modulation of osteoclast differentiation and possibly in the therapy of bone metastases.

DOI: 10.1016/j.diff.2009.07.008

Journal: Differentiation

Impact factor (WOS, 2008): 3,180, Q2

Number of citations (WOS, 2023): 36

Most important citation:

1. Amin A, Karpowicz PA, Carey TE, Arbiser J, Nahta R, Chen ZG, et al: **Evasion of anti-growth signaling: A key step in tumorigenesis and potential target for treatment and prophylaxis by natural compounds**. *Seminars in Cancer Biology* 2015, **35**:S55-S77. **IF(2014): 9,330**

2. Siddiqui JA, et al: **GDF15 promotes prostate cancer bone metastasis and colonization through osteoblastic CCL2 and RANKL activation**. *Bone Research* 2022; 10(1). **IF(2021): 13,362**

3. Silva-Bermudez LS, et al. **Titanium Nanoparticles Enhance Production and Suppress Stabilin-1-Mediated Clearance of GDF-15 in Human Primary Macrophages**. *Frontiers in Immunology* 2021; 12. **IF(2020): 7,561**

Contribution of the author/author's team: *Shared corresponding author. Conceptualization and data interpretation, manuscript revision.

Appendix 2 *Starsichova A, Lincova E, Pernicova Z, Kozubik A, Soucek K: TGF-β1 suppresses IL-6-induced STAT3 activation through regulation of Jak2 expression in prostate epithelial cells.* *Cell Signal 2010, 22:1734-44.*

Significance: Chronic inflammation plays an essential role in the initiation and progression of various human diseases, including benign prostatic hyperplasia or prostate cancer. Here, we show the interaction between TGF-β1 and IL-6 signaling and suggest another mechanism of how defects in TGF-β signaling, frequently associated with prostate pathologies, can contribute to the disruption of tissue homeostasis.

DOI: 10.1016/j.cellsig.2010.06.014

Journal: CELLULAR SIGNALLING

Impact factor (WOS, 2009): 4,094, Q2

Number of citations (WOS, 2023): 25

Most important citation:

1. de Boer HR, Pool M, Joosten E, Everts M, Samplonius DF, Helfrich W, et al. **Quantitative proteomics analysis identifies MUC1 as an effect sensor of EGFR inhibition**. *Oncogene* 2019, **38**(9):1477-1488. **IF(2018): 6,634**

2. De Nunzio C, Kramer G, Marberger M, Montironi R, Nelson W, Schröder F, et al. **The Controversial Relationship Between Benign Prostatic Hyperplasia and Prostate Cancer: The Role of Inflammation**. *European Urology* 2011, **60**(1):106-117. **IF(2010): 8,843**

3. Menon DR, Wels C, Rad EB, Joshi S, Knausz H, Lade-Keller J, et al. **TGF-β1 and TNF-α differentially regulate Twist1 mediated resistance towards BRAF/MEK inhibition in melanoma**. *Pigment Cell & Melanoma Research* 2013, **26**(6). **IF(2012): 5,839**

Contribution of the author/author's team: Corresponding author. Conceptualization and data interpretation, manuscript revision.

Appendix 3 *Pernicova Z, Slabakova E, Kharaishvili G, Bouchal J, Kral M, Kunicka Z, Machala M, Kozubik A,* ***Soucek K****:* ***Androgen depletion induces senescence in prostate cancer cells through down-regulation of Skp2****. Neoplasia 2011, 13:526-36.*

Significance: Although the induction of senescence in cancer cells is a potent mechanism of tumor suppression, senescent cells remain metabolically active and may secrete a broad spectrum of factors that promote tumorigenicity in neighboring malignant cells. Here, we showed that androgen deprivation therapy (ADT), a widely used treatment for advanced prostate cancer, induces a senescence-associated secretory phenotype in prostate cancer epithelial cells. We revealed a previously unrecognized link between inhibition of androgen receptor signaling, down-regulation of S-phase kinase-associated protein 2, and the appearance of secretory, tumor-promoting senescent cells in prostate tumors.

DOI: 10.1593/neo.11182

Journal: NEOPLASIA

Impact factor (WOS, 2010): 5,476, Q1

Number of citations (WOS, 2023): 59

Most important citation:

1. de Bono JS, Guo C, Gurel B, De Marzo AM, Sfanos KS, Mani RS, et al: **Prostate carcinogenesis: inflammatory storms**. *Nature Reviews Cancer* 2020, **20**(8):455-469. **IF(2019): 53,030**

2. Fiard G, Stavrinides V, Chambers ES, Heavey S, Freeman A, Ball R, et al. **Cellular senescence as a possible link between prostate diseases of the ageing male**. *Nature Reviews Urology* 2021, **18**(10):597-610. **IF(2020): 14,432**

3. Galsky MD. **Resistance to prostate-cancer treatment is driven by immune cells**. *Nature* 2018, **559**(7714):338-339. **IF(2017): 41,577**

Contribution of the author/author's team: Corresponding author. Conceptualization and data interpretation, manuscript revision.

Appendix 4 *Slabakova E, Pernicova Z, Slavickova E, Starsichova A, Kozubik A, Soucek K: TGF-β1-induced EMT of non-transformed prostate hyperplasia cells is characterized by early induction of SNAI2/Slug. Prostate 2011, 71:1332-43.*

Significance: Epithelial-mesenchymal transition (EMT) underlying cancer cell invasion and metastasis has been thoroughly studied in prostate cancer. Although EMT markers have been clinically observed in benign prostate hyperplasia, molecular events underlying the onset and progression of EMT in benign prostate cells have not been described. In this study, we suggest that in benign prostate hyperplasia cells, the transcription factor SNAI2/Slug is essential for EMT initiation. In contrast, in cooperation with the miR-200 family, the ZEB family of transcription factors may oppose the EMT phenotype's reversal.

DOI: 10.1002/pros.21350

Journal: PROSTATE

Impact factor (WOS, 2010): 3,377, Q1

Number of citations (WOS, 2023): 83

Most important citation:

1. Fang YX, Gao WQ. **Roles of microRNAs during prostatic tumorigenesis and tumor progression**. *Oncogene* 2014, **33**(2):135-147. **IF(2013): 8,559**

2. Jung HY, Fattet L, Yang J. **Molecular Pathways: Linking Tumor Microenvironment to Epithelial-Mesenchymal Transition in Metastasis**. *Clinical Cancer Research* 2015, **21**(5):962-968. **IF(2014): 8,722**

3. Jurj A, Ionescu C, Berindan-Neagoe I, Braicu C. **The extracellular matrix alteration, implication in modulation of drug resistance mechanism: friends or foes?** *Journal of Experimental & Clinical Cancer Research* 2022, **41**(1). **IF(2022): 12,658**

Contribution of the author/author's team: Corresponding author. Conceptualization and data interpretation, manuscript revision.

Appendix 5 *Knopfova L, Benes P, Pekarcikova L, Hermanova M, Masarik M, Pernicova Z, Soucek K, Smarda J:* ***c-Myb regulates matrix metalloproteinases 1/9, and cathepsin D: implications for matrix-dependent breast cancer cell invasion and metastasis.*** *Molecular Cancer 2012; 11:15.*

Significance: The significance of this study lies in its contribution to the understanding of the mechanisms behind tumor development and metastasis, as well as the potential implications for future cancer treatment strategies. The research highlights the importance of c-Myb in regulating tumor growth and metastasis, specifically in the context of MMP-19. This knowledge can contribute to developing more effective cancer treatments that target the c-Myb pathway and inhibit tumor growth and metastasis.

DOI: 10.1186/1476-4598-11-15

Journal: MOLECULAR CANCER

Impact factor (WOS, 2011): 3,993, Q1

Number of citations (WOS, 2023): 53

Most important citation:

1. Abhange K, Makler A, Wen Y, Ramnauth N, Mao WJ, Asghar W, et al. **Small extracellular vesicles in cancer**. *Bioactive Materials* 2021, **6**(11):3705-3743. **IF(2020): 14,593**

2. Anand S, Vikramdeo KS, Sudan SK, Sharma A, Acharya S, Khan MA, et al. **From modulation of cellular plasticity to potentiation of therapeutic resistance: new and emerging roles of MYB transcription factors in human malignancies**. *Cancer and Metastasis Reviews* 2023. **IF(2022): 9,2**

3. van Gogh M, Garzon JFG, Sahin D, Knopfova L, Benes P, Boyman O, et al. **Tumor Cell-Intrinsic c-Myb Upregulation Stimulates Antitumor Immunity in a Murine Colorectal Cancer Model**. *Cancer Immunology Research* 2023, **11**(10):1432-1444. **IF(2022): 10,1**

Contribution of the author/author's team: Co-author. Methodology and manuscript revision.

Appendix 6 *Vanhara P, Hampl A, Kozubik A, Soucek K: Growth/differentiation factor-15: prostate cancer suppressor or promoter? Prostate Cancer Prostatic Dis 2012, 15:320-8.*

Significance: In this review, we focused on growth/differentiation factor-15 (GDF-15), a divergent member of the TGF-β family. This stress-induced cytokine has been proposed to possess immunomodulatory functions, and its high expression is often associated with cancer progression, including prostate cancer (PCa). We discussed studies that focus on regulating GDF-15 expression and its role in tissue homeostasis, repair, and the immune response, emphasizing the role in PCa development.

DOI: 10.1038/pcan.2012.6

Journal: PROSTATE CANCER AND PROSTATIC DISEASES

Impact factor (WOS, 2011): 2,421, Q2

Number of citations (WOS, 2023): 50

Most important citation:

1. Ge RB, Wang ZW, Cheng L. **Tumor microenvironment heterogeneity an important mediator of prostate cancer progression and therapeutic resistance**. *Npj Precision Oncology* 2022, **6**(1). **IF(2021): 10,123**

2. Mullican SE, Rangwala SM. **Uniting GDF15 and GFRAL: Therapeutic Opportunities in Obesity and Beyond**. *Trends in Endocrinology and Metabolism* 2018, **29**(8):560-570. **IF(2027): 10,769**

3. Ratnam NM, Peterson JM, Talbert EE, Ladner KJ, Rajasekera PV, Schmidt CR, et al. **NF-κB regulates GDF-15 to suppress macrophage surveillance during early tumor development**. *Journal of Clinical Investigation* 2017, **127**(10):3796-3809. **IF(2016): 12,784**

Contribution of the author/author's team: Corresponding author. Conceptualization and manuscript revision.

Appendix 7 *Fedr R, Pernicova Z, Slabakova E, Strakova N, Bouchal J, Grepl M, Kozubik A, Soucek K: Automatic cell cloning assay for determining the clonogenic capacity of cancer and cancer stem-like cells. Cytometry A 2013, 83:472-82.*

Significance: The clonogenic assay is a well-established *in vitro* method for testing cells' survival and proliferative capability. It can be used to determine the cytotoxic effects of various treatments, including chemotherapeutics and ionizing radiation. However, this approach can also characterize cells with different phenotypes and biological properties, such as stem cells or cancer stem cells. Here, we implemented a faster and more precise method for assessing the cloning efficiency of cancer stem-like cells characterized and separated using a high-speed cell sorter. We demonstrated a straightforward approach for determining the clonogenic capacity of cancer stem-like cells identified in both cell lines and patient samples.

DOI: 10.1002/cyto.a.22273

Journal: CYTOMETRY PART A

Impact factor (WOS, 2012): 3,711, Q2

Number of citations (WOS, 2023): 26

Most important citation:

1. Dayal JHS, Mason SM, Salas-Alanis JC, McGrath JA, Taylor RG, Mellerio JE, et al. **Heterogeneous addiction to transforming growth factor-beta signalling in recessive dystrophic epidermolysis bullosa-associated cutaneous squamous cell carcinoma**. *British Journal of Dermatology* 2021, **184**(4):697-708. **IF(2020): 9,302**

2. Liu NQ, Maresca M, van den Brand T, Braccioli L, Schijns M, Teunissen H, et al. **WAPL maintains a cohesin loading cycle to preserve cell-type-specific distal gene regulation**. *Nature Genetics* 2021, **53**(1). **IF(2020): 38,330**

3. ter Huurne M, Chappell J, Dalton S, Stunnenberg HG. **Distinct Cell-Cycle Control in Two Different States of Mouse Pluripotency**. *Cell Stem Cell* 2017, **21**(4):449. **IF(2016): 23,394**

Contribution of the author/author's team: Corresponding author. Conceptualization, data interpretation and manuscript revision.

Appendix 8 *Pernicova Z, Vanhara P, Soucek K: Formation of secretory senescent cells in prostate tumors: the role of androgen receptor activity and cell cycle regulation. Tumor Dormancy, Quiescence, and Senescence, Volume 1: Aging, Cancer, and Noncancer Pathologies. Edited by MA H. Springer Netherlands, 2013. pp. 303-16.*

Significance: The induction of senescence in cancer cells is believed to be a potent mechanism of tumor suppression; however, senescent cells remain metabolically active and secrete a broad spectrum of factors, modulate the tissue microenvironment, and potentially promote tumorigenicity in neighboring malignant cells. In this chapter, we summarized possible mechanisms and consequences of the formation of the secretory phenotypes in prostate tumors and the role of the androgen receptor and cell cycle regulation in these processes.

DOI: 10.1007/978-94-007-5958-9_26

Book: Tumor Dormancy, Quiescence, and Senescence, Volume 1: Aging, Cancer, and Noncancer Pathologies.

Impact factor (WOS): n-a

Number of citations (WOS): n-a

Most important citation: n-a

Contribution of the author/author's team: Corresponding author. Conceptualization and manuscript revision.

Appendix 9 *Pernicova Z, Slabakova E, Fedr R, Simeckova S, Jaros J, Suchankova T, Bouchal J, Kharaishvili G, Kral M, Kozubik A, Soucek K: The role of high cell density in the promotion of neuroendocrine transdifferentiation of prostate cancer cells. Mol Cancer 2014, 13:113.*

Significance: Neuroendocrine transdifferentiation (NED) and the emergence of neuroendocrine-like cancer cells in prostate tumors frequently arise from androgen-depleted prostate adenocarcinoma and are associated with the development of castration-resistant prostate cancer and poor prognosis. In this study, we showed a new relationship between cell cycle attenuation and the promotion of NED. We suggested high cell density as a trigger for cAMP signaling that can mediate reversible NED in prostate cancer cells.

DOI: 10.1186/1476-4598-13-113

Journal: MOLECULAR CANCER

Impact factor (WOS, 2013): 5,397, Q1

Number of citations (WOS, 2023): 25

Most important citation:

1. Friedrich M, Wiedemann K, Reiche K, Puppel SH, Pfeifer G, Zipfel I, et al. **The Role of lncRNAs TAPIR-1 and-2 as Diagnostic Markers and Potential Therapeutic Targets in Prostate Cancer**. *Cancers* 2020, **12**(5). **IF(2019): 6,126**

2. Jorda R, Hendrychová D, Voller J, Reznícková E, Gucky T, Krystof V. **How Selective Are Pharmacological Inhibitors of Cell-Cycle-Regulating Cyclin-Dependent Kinases?** *Journal of Medicinal Chemistry* 2018, **61**(20):9105-9120. **IF(2017): 6,253**

3. Santer FR, Erb HHH, McNeill RV. **Therapy escape mechanisms in the malignant prostate**. *Seminars in Cancer Biology* 2015, **35**:133-144. **IF(2014): 9,330**

4. Santer, F. R., Erb, H. H. H. & Mcneill, R. V. **Therapy escape mechanisms in the malignant prostate**. *Seminars in Cancer Biology*, 35, 133-144, (2015). Doi: 10.1016/j.semcancer.2015.08.005. **IF (2014): 9,330**

Contribution of the author/author's team: Corresponding author. Conceptualization, data interpretation and manuscript revision.

Appendix 10 *Kratochvilova K, Horak P, Esner M, Soucek K, Pils D, Anees M, Tomasich E, Drafi F, Jurtikova V, Hampl A, Krainer M, Vanhara P:* ***Tumor suppressor candidate 3 (TUSC3) prevents the epithelial-to-mesenchymal transition and inhibits tumor growth by modulating the endoplasmic reticulum stress response in ovarian cancer cells****. Int J Cancer 2015, 137:1330-40.*

Significance: Tumor suppressor candidate 3 (TUSC3) is a putative tumor suppressor gene located at chromosomal region 8p22, which is often lost in epithelial cancers. Epigenetic silencing of TUSC3 has been associated with poor prognosis, and hypermethylation of its promoter provides an independent biomarker of overall and disease-free survival in ovarian cancer patients. In this study, we establish TUSC3 as a novel ovarian cancer tumor suppressor using a xenograft mouse model and demonstrate that loss of TUSC3 alters the molecular response to endoplasmic reticulum stress and induces hallmarks of the epithelial-to-mesenchymal transition in ovarian cancer cells.

DOI: 10.1002/ijc.29502

Journal: INTERNATIONAL JOURNAL OF CANCER

Impact factor (WOS, 2014): 5,085, Q1

Number of citations (WOS, 2023): 37

Most important citation:

1. Deng RX, et al. **Downregulation of TUSC3 promotes EMT and hepatocellular carcinoma progression through LIPC/AKT axis**. *Journal of Translational Medicine* 2022, **20**(1). **IF(2021): 8,448**

2. Jeon YJ, et al. **miRNA-mediated TUSC3 deficiency enhances UPR and ERAD to promote metastatic potential of NSCLC**. *Nature Communications* 2018, **9**. **IF(2017): 12,353**

3. Zhang Y, et al. **Scavenger Receptor A1 Prevents Metastasis of Non-Small Cell Lung Cancer via Suppression of Macrophage Serum Amyloid A1**. *Cancer Research* 2017, **77**(7):1586-1598. **IF(2016): 9,122**

Contribution of the author/author's team: EMT data interpretation and manuscript revision.

Appendix 11 *Slabakova E, Kharaishvili G, Smejova M, Pernicova Z, Suchankova T, Remsik J, Lerch S, Strakova N, Bouchal J, Kral M, Culig Z, Kozubik A, Soucek K: Opposite regulation of MDM2 and MDMX expression in acquisition of mesenchymal phenotype in benign and cancer cells. Oncotarget 2015, 6:36156-71.*

Significance: The plasticity of cancer cells, manifested by transitions between epithelial and mesenchymal phenotypes, represents a challenging issue in treating neoplasias. Both epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) are implicated in metastasis formation and acquiring stem cell-like properties. Mouse double minute (MDM) 2 and MDMX are essential players in cancer progression, as they act as regulators of p53, but their function in EMT and metastasis may be contradictory. Here, we showed that the EMT phenotype in multiple cellular models and clinical prostate and breast cancer samples is associated with a decrease in MDM2 and an increase in MDMX expression and provided an alternative context-specific role of MDM2 in EMT, cell migration, metastasis, and therapy resistance.

DOI: 10.18632/oncotarget.5392

Journal: ONCOTARGET

Impact factor (WOS, 2014): 6,359, Q1

Number of citations (WOS, 2023): 16

Most important citation:

1. Gao C, Xiao G, Piersigilli A, Gou JT, Ogunwobi O, Bargonetti J. **Context-dependent roles of MDMX (MDM4) and MDM2 in breast cancer proliferation and circulating tumor cells**. *Breast Cancer Research* 2019, **21**. **IF(2018): 5,676**

2. Giridhar PV, Williams K, VonHandorf AP, Deford PL, Kasper S. **Constant Degradation of the Androgen Receptor by MDM2 Conserves Prostate Cancer Stem Cell Integrity**. *Cancer Research* 2019, **79**(6):1124-1137. **IF(2018): 8,378**

Contribution of the author/author's team: Corresponding author. Conceptualization, data interpretation, and manuscript revision.

Appendix 12 *Slabakova E, Culig Z, Remsik J, Soucek K: Alternative mechanisms of miR-34a regulation in cancer. Cell Death Dis 2017, 8:e3100.*

Significance: MicroRNA miR-34a is recognized as a master regulator of tumor suppression. In this work, we reviewed p53-independent mechanisms regulating the expression of miR-34a, including multiple mechanisms that operate in the context of cancer-associated phenomena, such as aberrant oncogene signaling, EMT, or inflammation.

DOI: 10.1038/cddis.2017.495

Journal: CELL DEATH & DISEASE

Impact factor (WOS, 2016): 5,965, Q1

Number of citations (WOS, 2023): 183

Most important citation:

1. Chen S, et al. **Non-coding RNAs, guardians of the p53 galaxy**. *Seminars in Cancer Biology* 2021**, 75**:72-83. **IF(2020): 15,707**

2. Joo JI, et al. **Realizing Cancer Precision Medicine by Integrating Systems Biology and Nanomaterial Engineering**. *Advanced Materials* 2020, **32**(35). **IF(2019): 27,398**

3. Pathak GA, et al. **Two-stage Bayesian GWAS of 9576 individuals identifies SNP regions that are targeted by miRNAs inversely expressed in Alzheimer's and cancer**. *Alzheimers & Dementia* 2020, **16**(1):162-177. **IF(2019): 17,127**

4. Proença C, et al. **The role of flavonoids in the regulation of epithelial-mesenchymal transition in cancer: A review on targeting signaling pathways and metastasis**. *Medicinal Research Reviews* 2023, **43**(6):1878-1945. **IF(2022): 13,3**

5. Sehgal K, Barbie DA. **Targeting the mutant p53 secretome**. *Journal of Clinical Investigation* 2021, **131**(1). **IF(2020): 14,808**

Contribution of the author/author's team: Corresponding author. Conceptualization, and manuscript revision.

Appendix 13 *Kahounova Z, Kurfurstova D, Bouchal J, Kharaishvili G, Navratil J, Remsik J, Simeckova S, Student V, Kozubik A, Soucek K: The fibroblast surface markers FAP, anti-fibroblast, and FSP are expressed by cells of epithelial origin and may be altered during epithelial-to-mesenchymal transition. Cytometry A 2018, 93:941-51.*

Significance: This study supports the findings that the widely used fibroblast markers are not fibroblast specific and may also be expressed by cells of epithelial origin (e.g., cells undergoing EMT). Therefore, the expression of these markers should be interpreted with caution, and the combination of several epitopes for both positive (anti-fibroblast or fibroblast activation protein alpha) and negative (EpCAM) identification of fibroblasts from breast and prostate tumor tissues is advised.

DOI: 10.1002/cyto.a.23101

Journal: CYTOMETRY PART A

Impact factor (WOS, 2017): 3,260, Q2

Number of citations (WOS, 2023): 45

Most important citation:

1. Hettiarachchi SU, Li YH, Roy J, Zhang FH, Puchulu-Campanella E, Lindeman SD, et al. **Targeted inhibition of PI3 kinase/mTOR specifically in fibrotic lung fibroblasts suppresses pulmonary fibrosis in experimental models**. *Science Translational Medicine* 2020, **12**(567). **IF(2019): 16,304**

2. Wan ZP, Zhang S, Zhong AX, Shelton SE, Campisi M, Sundararaman SK, et al. **A robust vasculogenic microfluidic model using human immortalized endothelial cells and Thy1 positive fibroblasts**. *Biomaterials* 2021, **276**. **IF(2020): 12,479**

3. Yang DK, Liu J, Qian H, Zhuang Q. **Cancer-associated fibroblasts: from basic science to anticancer therapy**. *Experimental and Molecular Medicine* 2023, **55**(7):1322-1332. **IF(2022): 12,8**

4. Pure, E. & Blomberg, R. **Pro-tumorigenic roles of fibroblast activation protein in cancer: back to the basics.** *Oncogene* 2018, 37(32), 4343-4357. **IF(2018): 6,854**

Contribution of the author/author's team: Corresponding author. Conceptualization, data interpretation, and manuscript revision.

Appendix 14 *Knopfova L, Biglieri E, Volodko N, Masarik M, Hermanova M, Glaus Garzon JF, Ducka M, Kucirkova T, Soucek K, Smarda J, Benes P, Borsig L:* ***Transcription factor c-Myb inhibits breast cancer lung metastasis by suppression of tumor cell seeding****. Oncogene 2018;37: 1020-30.*

Significance: Here, we investigate the role of the transcription factor c-Myb in breast cancer (BC) lung metastasis and its potential as a prognostic marker and therapeutic target. We showed increased c-Myb expression in breast cancer (BC) cells inhibits spontaneous lung metastasis through impaired tumor cell extravasation. BC cells with increased lung metastatic capacity exhibit low c-Myb levels. These findings demonstrate the potential role of c-Myb as a prognostic marker for lung metastasis in BC patients and suggest that it could serve as a therapeutic target for improving patient outcomes.

DOI: 10.1038/onc.2017.392

Journal: ONCOGENE

Impact factor (WOS, 2017): 6,854, Q1

Number of citations (WOS, 2023): 15

Most important citation:

1. Bayley R, Ward C, Garcia P. **MYBL2 amplification in breast cancer: Molecular mechanisms and therapeutic potential**. *Biochimica Et Biophysica Acta-Reviews on Cancer* 2020, **1874**(2). **IF(2019): 7,365**

2. Hashemi M, et al. **Biological functions and molecular interactions of Wnt/13-catenin in breast cancer: Revisiting signaling networks**. *International Journal of Biological Macromolecules* 2023, **232**. **IF(2022): 8,2**

3. van Gogh M, et al. **Tumor Cell-Intrinsic c-Myb Upregulation Stimulates Antitumor Immunity in a Murine Colorectal Cancer Model**. *Cancer Immunology Research* 2023, **11**(10):1432-1444. **IF(2022): 10,1**

Contribution of the author/author's team: Co-author. Methodology and manuscript revision.

Appendix 15 *Remsik J, Bino L, Kahounova Z, Kharaishvili G, Simeckova S, Fedr R, Kucirkova T, Lenart S, Muresan XM, Slabakova E, Knopfova L, Bouchal J, Kral M, Benes P,* ***Soucek K: Trop2 plasticity is controlled by epithelial-to-mesenchymal transition****. Carcinogenesis 2018, 39:1411-8.*

Significance: The cell surface glycoprotein Trop2 is commonly overexpressed in carcinomas and represents an exceptional antigen for targeted therapy. Here, we provide evidence that surface Trop2 expression is functionally connected with an epithelial phenotype in breast and prostate cell lines and patient tumor samples. Moreover, our data suggest that the cancer plasticity-driven intratumoral heterogeneity in Trop2 expression may significantly contribute to response and resistance to therapies targeting Trop2-expressing cells.

DOI: 10.1093/carcin/bgy095

Journal: CARCINOGENESIS

Impact factor (WOS, 2017): 5,072, Q1

Number of citations (WOS, 2023): 18

Most important citation:

1. Bardia A, Tolaney SM, Punie K, Loirat D, Oliveira M, Kalinsky K, et al. **Biomarker analyses in the phase III ASCENT study of sacituzumab govitecan versus chemotherapy in patients with metastatic triple-negative breast cancer**. *Annals of Oncology* 2021, **32**(9):1148-1156. **IF(2020): 32,976**

2. Liu HC, Bai LL, Huang L, Ning N, Li L, Li YJ, et al. **Bispecific antibody targeting TROP2xCD3 suppresses tumor growth of triple negative breast cancer**. *Journal for Immunotherapy of Cancer* 2021, **9**(10). **IF(2020): 13,751**

3. Liu XL, Deng JW, Yuan Y, Chen WJ, Sun WS, Wang YH, et al. **Advances in Trop2-targeted therapy: Novel agents and opportunities beyond breast cancer**. *Pharmacology & Therapeutics* 2022, **239**. **IF(2022): 13,5**

Contribution of the author/author's team: Corresponding author. Conceptualization, data interpretation, and manuscript revision.

Appendix 16 *Remsik J, Fedr R, Navratil J, Bino L, Slabakova E, Fabian P, Svoboda M, Soucek K: **Plasticity and intratumoural heterogeneity of cell surface antigen expression in breast cancer**. Br J Cancer 2018, 118:813-9.*

Significance: The intratumoral heterogeneity, often driven by epithelial-to-mesenchymal transition (EMT), significantly contributes to chemoresistance and disease progression in adenocarcinomas. Here, we introduced a high-throughput screening platform to identify surface antigens associated with epithelial-mesenchymal plasticity in well-defined pairs of epithelial cell lines and their mesenchymal counterparts. We found that surface CD9, CD29, CD49c, and integrin β5 are lost in breast cancer cells undergoing EMT in vivo. We propose that the overall landscape of the 10-molecule surface signature expression reflects the epithelial-mesenchymal plasticity in breast cancer.

DOI: 10.1038/bjc.2017.497

Journal: BRITISH JOURNAL OF CANCER

Impact factor (WOS, 2017): 5,922, Q1

Number of citations (WOS, 2023): 17

Most important citation:

1. Abou-Fadel J, et al. **CCM signaling complex (CSC) couples both classic and non-classic Progesterone receptor signaling**. *Cell Communication and Signaling* 2022, **20**(1). **IF(2021): 7,54**

2. Kopylov AT, et al. **Convolutional neural network in proteomics and metabolomics for determination of comorbidity between cancer and schizophrenia**. *Journal of Biomedical Informatics* 2021, **122**. **IF(2020): 6,317**

3. Morales E, Olson M, Iglesias F, Dahiya S, Luetkens T, Atanackovic D. **Role of immunotherapy in Ewing sarcoma**. *Journal for Immunotherapy of Cancer* 2020, **8**(2). **IF(2019): 10,252**

Contribution of the author/author's team: Corresponding author. Conceptualization, data interpretation and manuscript revision.

Appendix 17 *Simeckova S, Fedr R, Remsik J, Kahounova Z, Slabakova E,* ***Soucek K:*** ***Multiparameter cytometric analysis of complex cellular response****. Cytometry A 2018;93: 239-48.*

Significance: The study focuses on using flow cytometry to analyze the effects of various treatments on cellular responses by introducing a multiparametric protocol for complex analysis of cytokinetics by simultaneously detecting seven fluorescence parameters. We demonstrated that this protocol has the potential to provide complex and simultaneous analysis of cytokinetics and analyze the heterogeneity of the response at the single-cell level.

DOI: 10.1002/cyto.a.23295

Journal: CYTOMETRY PART A

Impact factor (WOS, 2018): 3,260

Number of citations (Google Scholar, 2023): 2

Contribution of the author/author's team: Corresponding author. Conceptualization, data interpretation and manuscript revision.

Appendix 18 *Simeckova S, Kahounova Z, Fedr R, Remsik J, Slabakova E, Suchankova T, Prochazkova J, Bouchal J, Kharaishvili G, Kral M, Benes P,* ***Soucek K: High Skp2 expression is associated with a mesenchymal phenotype and increased tumorigenic potential of prostate cancer cells.*** *Sci Rep 2019, 9:5695.*

Significance: Skp2 is a crucial component of SCF^{Skp2} E3 ubiquitin ligase and is often overexpressed in various types of cancer, including prostate cancer (PCa). This study uncovered the Skp2-mediated CSC-like phenotype with oncogenic functions in PCa.

DOI: 10.1038/s41598-019-42131-y

Journal: SCIENTIFIC REPORTS

Impact factor (WOS, 2018): 4,011, Q1

Number of citations (WOS, 2023): 20

Most important citation:

1. Asmamaw MD, Liu Y, Zheng YC, Shi XJ, Liu HM. **Skp2 in the ubiquitin-proteasome system: A comprehensive review**. *Medicinal Research Reviews* 2020, **40**(5):1920-1949. **IF(2019): 9,3**

2. Celada SI, Li GL, Celada LJ, Lu WF, Kanagasabai T, Feng WR, et al. **Lysosome-dependent FOXA1 ubiquitination contributes to luminal lineage of advanced prostate cancer**. *Molecular Oncology* 2023. **IF(2022): 6,6**

3. Khan AQ, Al-Tamimi M, Uddin S, Steinhoff M. **F-box proteins in cancer stemness: An emerging prognostic and therapeutic target**. *Drug Discovery Today* 2021, **26**(12):2905-2914. **IF(2020): 7,851**

Contribution of the author/author's team: Corresponding author. Conceptualization, data interpretation and manuscript revision.

Appendix 19 Drapela S, Bouchal J, Jolly MK, Culig Z, Soucek K: ZEB1: A Critical Regulator of Cell Plasticity, DNA Damage Response, and Therapy Resistance. Front Mol Biosci 2020, 7:36.

Significance: Zinc finger E-box binding homeobox 1 (ZEB1) is a prime element of a network of transcription factors controlling EMT and has been identified as an essential molecule in regulating DNA damage, cancer cell differentiation, and metastasis. In this review, we focused on the role of ZEB1 in regulating DDR and described the mechanisms of ZEB1-dependent chemoresistance.

DOI: 10.3389/fmolb.2020.00036

Journal: FRONT MOL BIOSCI

Impact factor (WOS, 2019): 4,188, Q2

Number of citations (WOS, 2023): 90

Most important citation:

1. Ervin EH, et al. **Inside the stemness engine: Mechanistic links between deregulated transcription factors and stemness in cancer**. *Seminars in Cancer Biology* 2022, **87**:48-83. **IF(2021): 17,012**

2. Pommier RM, et al. **Comprehensive characterization of claudin-low breast tumors reflects the impact of the cell-of-origin on cancer evolution**. *Nature Communications* 2020, **11**(1). **IF(2019): 12,121**

3. Proença C, et al. **The role of flavonoids in the regulation of epithelial-mesenchymal transition in cancer: A review on targeting signaling pathways and metastasis**. *Medicinal Research Reviews* 2023, **43**(6):1878-1945. **IF(2022): 13,3**

4. Zhang N, et al. **Novel therapeutic strategies: targeting epithelial-mesenchymal transition in colorectal cancer**. *Lancet Oncology* 2021, **22**(8):E358-E368. **IF(2020): 41,316**

5. Zhang QM, et al. **Adipocyte-Derived Exosomal MTTP Suppresses Ferroptosis and Promotes Chemoresistance in Colorectal Cancer**. *Advanced Science* 2022, **9**(28). **IF(2021): 17,521**

Contribution of the author/author's team: Corresponding author. Conceptualization and manuscript revision.

Appendix 20 *Lenart S, Lenart P, Smarda J, Remsik J, Soucek K, Benes P: **Trop2: Jack of All Trades, Master of None**. Cancers (Basel) 2020, 12.*

Significance: Trophoblast cell surface antigen 2 (Trop2) is a widely expressed glycoprotein and an epithelial cell adhesion molecule (EpCAM) family member. In this article, we review the current knowledge about the yet controversial function of Trop2 in homeostasis and pathology.

DOI: 10.3390/cancers12113328

Journal: CANCERS

Impact factor (WOS, 2020): 6,126, Q1

Number of citations (WOS, 2023): 46

Most important citation:

1. Lei ZN, Teng QX, Tian Q, Chen W, Xie YH, Wu KM, et al. **Signaling pathways and therapeutic interventions in gastric cancer**. *Signal Transduction and Targeted Therapy* 2022, **7**(1). **IF(2021): 38,120**

2. Li CC, Liu J, Yang X, Yang Q, Huang WP, Zhang MY, et al. **Theranostic application of ^{64}Cu/^{177}Lu-labeled anti-Trop2 monoclonal antibody in pancreatic cancer tumor models**. *European Journal of Nuclear Medicine and Molecular Imaging* 2022, **50**(1):168-183. **IF(2021): 10,057**

3. Micalizzi DS, Che D, Nicholson BT, Edd JF, Desai N, Lang ER, et al. **Targeting breast and pancreatic cancer metastasis using a dual-cadherin antibody**. *Proceedings of the National Academy of Sciences of the United States of America* 2022, **119**(43). **IF(2021): 12,779**

Contribution of the author/author's team: Manuscript revision.

Appendix 21 *Remsik J, Pickova M, Vacek O, Fedr R, Bino L, Hampl A, Soucek K: TGF-β regulates Sca-1 expression and plasticity of pre-neoplastic mammary epithelial stem cells. Sci Rep 2020, 10:11396.*

Significance: The epithelial-mesenchymal plasticity, in tight association with stemness, contributes to the mammary gland homeostasis, evolution of early neoplastic lesions, and cancer dissemination. In this study, we mechanistically dissected the TGF-β family-driven regulation of Sca-1, one of the most commonly used adult stem cell markers. We further proved that TGF-β disrupts the lineage commitment and promotes the accumulation of tumor-initiating cells in pre-neoplastic cells.

DOI: 10.1038/s41598-020-67827-4

Journal: SCI REP

Impact factor (WOS, 2020): 3,998, Q1

Number of citations (WOS, 2023): 4

Most important citation:

1. Chhetri D, Vengadassalapathy S, Venkadassalapathy S, Balachandran V, Umapathy VR, Veeraraghavan VP, et al. **Pleiotropic effects of DCLK1 in cancer and cancer stem cells**. *Frontiers in Molecular Biosciences* 2022, **9**. **IF(2021): 6,113**

Contribution of the author/author's team: Corresponding author. Conceptualization of data interpretation and manuscript revision.

Appendix 22 *Kvokackova B, Remsik J, Jolly MK, Soucek K: Phenotypic Heterogeneity of Triple-Negative Breast Cancer Mediated by Epithelial-Mesenchymal Plasticity. Cancers (Basel) 2021, 13.*

Significance: Triple-negative breast cancer (TNBC) is a subtype of breast carcinoma known for its unusually aggressive behavior and poor clinical outcome. Besides the lack of molecular targets for therapy and profound intratumoral heterogeneity, the quick overt metastatic spread remains a significant obstacle to effective clinical management. In this review, we focus on cellular drivers underlying EMT/MET phenotypic plasticity and its detrimental consequences in the context of TNBC cancer.

DOI: 10.3390/cancers13092188

Journal: CANCERS

Impact factor (WOS, 2020): 6,639, Q1

Number of citations (WOS, 2023): 31

Most important citation:

1. Chen CR, Shen M, Wan XF, Sheng LL, He Y, Xu ML, et al. **Activated T cell-derived exosomes for targeted delivery of AXL-siRNA loaded paclitaxel-poly-L-lysine prodrug to overcome drug resistance in triple-negative breast cancer**. *Chemical Engineering Journal* 2023, **468**. **IF(2022): 15,1**

2. Li F, Sun HZ, Yu YH, Che N, Han JY, Cheng RF, et al. **RIPK1-dependent necroptosis promotes vasculogenic mimicry formation via eIF4E in triple-negative breast cancer**. *Cell Death & Disease* 2023, **14**(5). **IF(2022): 9,0**

3. Queen A, Bhutto HN, Yousuf M, Syed MA, Hassan MI. **Carbonic anhydrase IX: A tumor acidification switch in heterogeneity and chemokine regulation**. *Seminars in Cancer Biology* 2022, **86**:899-913. **IF(2021): 17,012**

Contribution of the author/author's team: Corresponding author. Conceptualization and manuscript revision.

Appendix 23 *Drapela S, Fedr R, Vacek O, Remsik J,* ***Soucek K****:* ***High-Throughput, Parallel Flow Cytometry Screening of Hundreds of Cell Surface Antigens Using Fluorescent Barcoding.*** *Apoptosis and Cancer: Methods in Molecular Biology. Edited by Barcenilla H, Diaz D. New York, USA: Springer Nature, 2022.*

Significance: Multicolor flow cytometry allows the analysis of tens of cellular parameters in millions of cells at a single-cell resolution within minutes. Here, we developed a high-throughput cytometry-based platform for profiling the expression of surface molecules using immunofluorescence. This powerful approach will help to identify novel biomarkers and druggable targets and facilitate the discovery of new concepts in immunology, oncology, and developmental biology.

DOI: 10.1007/978-1-0716-2553-8_9

Book: Apoptosis and Cancer: Methods and Protocol.

Impact factor (WOS, 2022): n-a

Number of citations (WOS, 2023): n-a

Most important citation: n-a

Contribution of the author/author's team: Corresponding author. Conceptualization and manuscript revision.

*Appendix 24 Muresan XM, Slabakova E, Prochazkova J, Drapela S, Fedr R, Pickova M, Vacek O, Vichova R, Suchankova T, Bouchal J, Kurfurstova D, Kral M, Hulinova T, Sykora RP, Student V, Hejret V, van Weerden WM, Puhr M, Pustka V, Potesil D, Zdrahal Z, Culig Z, Soucek K: **Toll-like receptor 3 (TLR3) overexpression induces invasion of prostate cancer cells, whereas its activation triggers apoptosis.** Am J Pathol 2022.*

Significance: Toll-like receptor 3 (TLR3) is an endosomal receptor expressed in several immune and epithelial cells. Recent studies have highlighted its expression also in solid tumors, including prostate cancer (PCa), and described its role mainly in the pro-inflammatory response and induction of apoptosis. It has been found to be upregulated in some castration-resistant prostate cancers. However, the role of TLR3 in prostate cancer progression remains largely unknown. In this study, we demonstrated that TLR3 may be involved in prostate cancer progression and metastasis. However, it might also represent an Achilles' heel of PCa, which can be exploited for targeted therapy.

DOI: 10.1016/j.ajpath.2022.05.009

Journal: AM J PATHOL

Impact factor (WOS, 2022): 5,770, Q1

Number of citations (WOS, 2023): 2

Most important citation:

1. Zapletal E, Vasiljevic T, Busson P, Glavan TM. **Dialog beyond the Grave: Necrosis in the Tumor Microenvironment and Its Contribution to Tumor Growth**. *International Journal of Molecular Sciences* 2023, **24**(6). **IF(2022): 5,6**

Contribution of the author/author's team: Corresponding author. Conceptualization, data interpretation and manuscript revision.

Appendix 25 *Rihova K, Ducka M, Zambo IS, Vymetalova L, Sramek M, Trcka F, Verner J, Drapela S, Fedr R, Suchankova T, Pavlatovska B, Ondrouskova E, Kubelkova I, Zapletalova D, Tucek S, Mudry P, Krakorova DA, Knopfova L, Smarda J, Soucek K, Borsig L, Benes P:* ***Transcription factor c-Myb: novel prognostic factor in osteosarcoma.*** *Clin Exp Metastasis 2022;39: 375-90.*

Significance: We have shown that c-Myb plays a role in cell cycle progression and differentiation in various types of cancer, including osteosarcoma. It has also been associated with stemness-related genes and the WNT/β-catenin signaling pathway in cancer cells. Additionally, c-Myb has been linked to calcium storage and the regulation of differentiation in both normal and tumoral-developing nervous systems. These findings suggest that c-Myb may affect the prognosis and therapeutic opportunities in osteosarcoma and other cancers.

DOI: 10.1007/s10585-021-10145-4

Impact factor (WOS, 2021): 4,510, Q2

Number of citations (WOS, 2023): 3

Most important citation:

1. Li YW, Azmi AS, Mohammad RM. **Deregulated transcription factors and poor clinical outcomes in cancer patients**. *Seminars in Cancer Biology* 2022, **86**:122-134. **IF(2021): 17,012**

2. van Gogh M, Garzon JFG, Sahin D, Knopfova L, Benes P, Boyman O, et al. **Tumor Cell-Intrinsic c-Myb Upregulation Stimulates Antitumor Immunity in a Murine Colorectal Cancer Model**. *Cancer Immunology Research* 2023, **11**(10):1432-1444. **IF(2022): 10,1**

Contribution of the author/author's team: Co-author. Methodology and manuscript revision.

Appendix 26 *Kvokackova B, Fedr R, Kuzilkova D, Stuchly J, Vavrova A, Navratil J, Fabian P, Ondrussek R, Ovesna P, Remsik J, Bouchal J, Kalina T, Soucek K: Single-cell protein profiling defines cell populations associated with triple-negative breast cancer aggressiveness. Molecular Oncology, 2022;17(6):1024-1040.*

Significance: The generation of a comprehensive mass cytometry panel for multiparametric detection of 23 phenotypic markers and 13 signaling molecules is described, and it is shown that the level of CD97 at the time of surgery has predictive potential.

DOI: 10.1002/1878-0261.13365

Impact factor (WOS, 2021): 6,6, Q1

Number of citations (WOS, 2023): 1

Contribution of the author/author's team: Corresponding author. Conceptualization and manuscript revision.

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