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Autoreferát disertační práce

UNIVERZITA KARLOVA
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Regulace exprese markerů nádorových kmenových buněk (CSC) včetně epigenetických mechanismů, exprese/aktivita transkripčních faktorů GLI a cílený experimentální zásah proti CSC subpopulaci nádoru jako účinná protinádorová terapie u vybraných nádorových typů.

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Abstrakt

Hedgehog signální dráha (HH) je evolučně konzervovaná signální dráha, nezbytná nejen pro správný vývoj mozku, plic, kůže a prostaty, ale take pro udržení homeostázy ve tkáních a jejich regeneraci. HH je také zapojena do vývoje a progrese melanomu, což je agresivní a smrtelná forma kožního karcinomu, která obsahuje subpopulaci buněk známou jako rakovinné kmenové buňky (CSCs). Dle dostupných dat o CSCs se předpokládá, že jsou zodpovědné za iniciaci, progresi a odolnost nádoru vůči terapii. V této práci se zaměřujeme na roli CSCs v progresi nádoru a na to, jak HH přispívá k udržení fenotypu kmenových buněk. Budou diskutovány současné analytické a terapeutické strategie zaměřené na CSCs a HH. Naše zjištění naznačují, že zaměření se na CSCs a HH může přinést naději pro vývoj účinných terapií pro léčbu melanomu.

Cílem práce bylo poskytnout nové poznatky o HH a jejích interakcích uvnitř buňky. Podařilo se nám identifikovat zcela nový transkripční cíl této dráhy - transkripční faktor Slug. Protein Slug je zapojen do vývoje neurální lišty a udržování kmenového fenotypu rakovinných buněk. Zjistili jsme, že inhibice efektorových proteinů HH - transkripčních faktorů GLI - pomocí

GANT61 vede k významné redukci koncentrace Slugu uvnitř buňky. Dále ukazujeme, že prvky signální dráhy HH jsou přítomny ve více než 50 liniích nádorových buněk, což naznačuje jejich aktivování HH klasickou cestou.

Dále jsme analyzovali buněčné linie s regulovatelnými hladinami Microphthalmia-associated transkriúčního faktoru (MITF), abychom zjistili, jak jeho měnící se koncentrace ovlivňuje rakovinný fenotyp. Tato závislost byla již dříve popsána a označena jako "reostatový model", který tvrdí, že vysoká koncentrace MITF se projevuje zrychlenou diferenciací a nízkou invazivitou. Naopak nízké hladiny MITF jsou provázeny pomalejší diferenciací, rychlým růstem a vysokou invazivitou. Naše data však toto nepotvrzují, protože jsme zjistili, že s klesajícími hladinami MITF se snižuje exprese markerů diferenciace, zatímco rychlost proliferace a invazivita se nesnižují. "Reostatový model" byl již od doby své formulace zpochybňován a naše data naznačují, že je třeba další výzkum tohoto transkripčního faktoru, aby jeho funkce byly přesněji popsány a platnost modelu mohla být správně posouzena. Také bylo ukázáno, že MITF je jedním z klíčových regulátorů ubikvitinace v melanomech.

Abstract

Hedgehog signaling pathway (HH) is evolutionarily conserved signaling pathway, essential for proper brain, lung, skin and prostate development, tissue homeostasis and regeneration. HH has also been implicated in the development and progression of melanoma, which is a highly aggressive and deadly form of skin cancer that has been shown to contain a subpopulation of cells known as cancer stem cells (CSCs). CSCs are thought to be responsible for tumor initiation, progression, and resistance to therapy. In this review, we discuss the role of CSCs in tumor progression and how the hedgehog signaling pathway contributes to their maintenance and survival. We also explore current therapeutic strategies targeting CSCs and the hedgehog signaling pathway in melanoma treatment. Our findings suggest that targeting CSCs and the hedgehog signaling pathway may hold promise for the development of effective therapies for the treatment of melanoma.

We aim to provide novel insights to HH signaling pathway and its interactions within the cell. We have succeeded to identify a brand-new transcriptional target of this pathway – Slug transcription factor. Slug protein involved in development of the

neural crest and maintenance of the Cancer Stem Cell (CSC) phenotype. We found out that inhibition of HH effector proteins – the GLI transcription factors – by GANT61 leads to significant reduction of Slug protein levels. Further, we show that elements of HH signaling pathway are present in more than 50 cancer cell lines implying its canonical activation.

We also analyzed cell lines with inducibly regulated Microphthalmia-associated transcription factor (MITF) levels to find out how its varying levels will affect the cancerous phenotype. This dependency has been described in the past and nicknamed the "rheostat model", which states that high-MITF levels are manifested high differentiation rate and low invasiveness and the low-MITF level is associated with low differentiation and proliferation rates combined with high invasiveness. Our data disprove of this postulate as we report that with decreasing MITF differentiation markers decreased while proliferation rate and invasiveness did not decrease. The "rheostat model" has been questioned ever since its definition and our data suggest that more extensive research is needed to properly assess its validity. Lastly, it was shown, that MITF is a key regulator of ubiquitination in melanoma cells.

CONTENTS

I INTRODUCTION

A CANCER STEM CELLS (CSCS)

Initially discovered by John Dick in the 1990s in leukemia, CSCs have been extensively studied for their unique characteristics (Bonnet, 1997). They were described as possessing a unique surface marker profile (CD34+/CD38-) and were thought to be able to initiate tumor growth when transplanted into compatible hosts. CSCs are responsible for tumor heterogeneity. They possess a longer cell cycle compared to other tumor cells, contributing to the rapid growth and complexity of tumors. CSCs are resistant to standard therapies that target rapidly dividing cells, leading to potential tumor recurrence (Baker et al., 2009).

The stochastic model proposes that all tumor cells have the potential to contribute to tumor growth. Every cell within the tumor has the potential to contribute to tumor growth. In contrast, the CSC model suggests that only a small subset of cells, the CSCs, are crucial for tumor maintenance and growth. These CSCs lack self-renewal capacity but form the bulk of the tumor (Metz et al., 1995).

Other theory postulates that CSCs originate from normal stem cells that undergo mutations or from differentiated cells that acquire stem cell-like properties. This transition is influenced by genetic and epigenetic changes, as well as by the tumor microenvironment (Shackleton et al., 2009).

The tumor microenvironment (TME), comprising various cell types and signaling molecules, is crucial for CSC maintenance and tumor progression (Oskarsson et al., 2014). It influences the behavior of CSCs and contributes to the dynamic equilibrium between different types of tumor cells. The interaction between CSCs and the TME is bidirectional. CSCs can shape the TME, while the TME influences CSC properties. This dynamic plays a key role in tumor growth, progression, and resistance to treatment (Cabarcas et al., 2011).

CSCs express specific markers that vary across cancer types. These markers include cell surface proteins (like CD44, CD133) and enzymes (like ALDHs). They are instrumental in identifying and isolating CSCs (Walcher et al, 2020; Zhao et al., 2017).

Transcription factors like SOX2 (Al-Mamun et al., 2018), OCT4 (Zhang et al., 2020), and NANOG (Gong et al., 2015) are central to CSC biology. They regulate genes associated with

stemness and tumorigenicity, influencing CSC behavior and characteristics.

Epigenetic changes, such as DNA methylation and histone modifications, play a significant role in controlling gene expression related to CSC properties. These modifications can either activate oncogenic pathways or suppress tumor-inhibiting genes (French and Pauklin., 2020).

Post-Transcriptional Regulation involves the regulation of CSC marker expression through mechanisms such as miRNAs, lncRNAs, and RNA-binding proteins. These elements affect the stability and translation efficiency of mRNAs encoding CSC markers (Bryl et al., 2022).

Post-Translational Regulation comprises of modifications like phosphorylation, ubiquitination, and glycosylation of CSC marker proteins. These modifications can alter the stability, location, and function of CSC markers, thereby influencing CSC behavior and characteristics (Wang et al., 2015).

This detailed examination underscores the complexity of CSCs in cancer biology and highlights the potential of targeting CSCs for more effective cancer therapies.

B HEDGEHOG SIGNALING PATHWAY

The Hedgehog signaling pathway (HH) under physiological circumstances regulates the morphogenesis of various organs during embryogenesis (Ingham and McMahon, 2001). This pathway is conserved in vertebrates and highly active in mammals during prenatal development, especially in the formation of the neural tube, lungs, and skeleton. Subsequently, this pathway becomes inactive in most adult tissues. However, even postnatally, some organs (central nervous system and lungs) and their proper functioning rely on the continued signaling of the Hedgehog pathway, which mediates cellular renewal and organ homeostasis (Beachy et al., 2004; Merchant and Matsui, 2010). The molecular mechanisms of the Hedgehog signaling pathway are complex and intricate. In the case of canonical activation (starting with ligand binding to the receptor), three types of ligands activating this pathway were identified (Merchant and Matsui, 2010) - Sonic Hedgehog, Indian Hedgehog and Desert Hedgehog. These ligands can bind to a membrane receptor called Patched (PTCH1). This is a G-protein coupled transmembrane receptor, whose polypeptide chain passes through the lipid bilayer of the cell membrane 12 times. In the absence of a bound ligand, the Patched receptor constitutively suppresses the activity of

another transmembrane protein (also from the family of G-protein-coupled receptors) called Smoothened (SMO). It should be noted that, in addition to the binding site on the Patched receptor, ligands also bind to co-receptors on the cell membrane surface (CDO, BOC, and GAS1), thereby facilitating the pathway's receptivity to activation. Upon binding of one of the three ligands to the Patched receptor (and co-receptors), a change in its conformation occurs, thereby stopping the suppression of the transmembrane protein Smoothened. This allows its accumulation in the primary cilium and phosphorylation at the cytoplasmic end. The activity of Smoothened then leads to inhibitory influence on the protein Suppressor of Fused (SUFU). Under normal circumstances (inactivated Hedgehog pathway, without bound ligand), it acts as a repressor of GLI transcription factors. Thanks to the activity of Smoothened, the GLI transcription factors are released from the repressive influence of SUFU, phosphorylated, and move into the cell nucleus, where they influence the transcription of many genes including some components of the Hedgehog pathway itself (such as Patched or GLI1). SUFU is subsequently proteolytically degraded (Abe and Tanaka, 2016).

The GLI family of Hedgehog pathway effector transcription factors in vertebrates consists of three proteins: GLI1, GLI2, and GLI3. All GLI proteins contain an activation domain (GLI-A), GLI2 and GLI3 also contain a repressor domain (GLI-R). GLI2 is probably the main activator of the Hedgehog signaling pathway, while GLI3 is the main repressor. GLI1 then serves more as an amplifier of the signal for GLI2 (Abe and Tanaka, 2016). The current ratio between the activation and repressive forms of the GLI family proteins leads to the expression of respective target genes. These genes include those coding for anti-apoptotic proteins (e.g., BCL-2), proteins accelerating the cell cycle, or for example SOX2, which is a marker of pluripotent stem cells.

The Hedgehog signaling pathway plays a crucial role during both embryonic and postnatal lung development (Kugler et al., 2015). During embryonic development, signaling molecules of the Hedgehog pathway significantly alter patterns and rates of gene expression. The pattern of gene expression activated by the Sonic Hedgehog ligand between the 10th and 17th day of embryonic development is important for all branching and growth of bronchi, then it is limited only to a subset of epithelial cells (Miller et al., 2001). Although the rate of

expression of Sonic Hedgehog and Patched proteins is lower around birth, it persists in the epithelial cells of the lungs (Bellusci et al., 1997). Experimental suppression of the Hedgehog signaling pathway in postnatal lungs even leads to abnormal lung maturation. Thus, the Hedgehog signaling pathway is also involved in postnatal maturation of the lungs (Liu et al., 2013; Hyman et al., 2009).

C ABERRANT HEDGEHOG ACTIVATION IN CSC SUBPOPULATION AND IN MALIGNANT MELANOMA

HH activates survival and anti-apoptotic pathways in CSCs, enhancing their viability and resistance to conventional therapies (Cochrane et al., 2015; Lu et al., 2021). It upregulates anti-apoptotic genes like BCL-2 (Cochrane et al., 2015) and MCL-1 (Barakat et al., 2010), aiding CSC survival in adverse conditions and facilitating tumor progression. These proteins prevent apoptosis, contributing to the challenges of cancer treatment. The HH pathway also influences the TME, promoting CSC maintenance and activity, and it is implicated in the epithelial-mesenchymal transition (EMT), a process linked to

increased cancer cell stemness, migration, and invasion (Takabatake et al., 2019).

In melanoma, over 40% of cell lines show higher levels of HH mediators compared to normal melanocytes. Inhibiting the SMO component of the HH pathway suppresses melanoma growth and induces apoptosis (O´Reilly et al., 2013; Shamsoon et al., 2023). However, this leads to compensatory activation of other pathways like Notch and Wnt. Increased SMO expression and decreased GLI3 expression correlate with shorter survival in metastatic melanoma patients, suggesting potential targeted therapy benefits (Jalili et al., 2013).

Overall, the HH pathway's role in CSC maintenance, resistance to therapy, and tumor progression makes it a promising target for cancer therapy. However, challenges remain in modulating this pathway effectively and safely in clinical settings. Further research is needed to understand this pathway's complexities in CSCs and develop novel strategies to overcome associated therapeutic challenges.

Early clinical trials with HH pathway antagonists have shown the potential of targeting this pathway as an effective anticancer strategy across various human tumors (Dusek and

Hadden., 2020). However, challenges remain in understanding the pathway's basic biology in human cancers, such as how specific oncogenic events affect HH signal transduction and the most effective methods for inhibiting aberrant pathway activity in clinical settings. Due to the pathway's diverse roles in different types of cancer, disease-specific considerations are crucial for optimizing the use of novel HH pathway inhibitors (Didiášová et al., 2018).

Numerous agents targeting the HH pathway, along with other critical pathways like Wnt and Notch signaling, have been developed for cancer treatment (Kumar et al., 2021). These treatments aim to inhibit the pathway's influence on CSCs, with the goal of reducing tumor growth and spread and overcoming drug resistance.

Overall, the ongoing research into the HH signaling pathway underscores its importance in cancer biology, particularly concerning CSCs. This research highlights the potential for developing targeted therapies that could significantly impact cancer treatment strategies.

II AIMS

This doctoral thesis is focused on the role of GLI transcription factors in cancer and is aimed to unveil their relation to other genes known for their role in tumor upkeep and prosperity.

1) The GLI transcription factors, the effectors of the Hedgehog signaling pathway, are known to target multiple genes associated with tumor development, progression, and metastasis. Our group already expanded the list of GLI targets while identifying Survivin as a GLI-regulated gene. Here we present data linking GLIs to Slug transcription factor, which is involved in embryogenesis and tumor cell invasion. **Our aim is to define the relation between HH pathway and Slug, a known asset in EMT and anti-apoptotic activity.**

2) Recent years brough novel therapies based on SMO inhibition. This treatment was developed to help patients suffering from acute myeloid leukemia and basal cell carcinoma. Unfortunately, this therapy often leads to SMO acquired resistance. We understand that the signaling crosstalk between

HH pathway and others is robust and can bridge over a singular interference, but **we aim to investigate the regulatory effect of GLI inhibition by GANT61 with outlook to clinical applications.**

3) Lastly, although the MITF gene was isolated almost some 30 years ago and has been studied extensively for most of this period, we are far from describing its functions in its entirety. Its involvement in melanoma invasiveness has been reported, so **we aim to analyze how varying levels of MITF influence other key features of melanomas, such as, proliferation, differentiation, and ubiquitination.**

III METHODS

In this part I disclose the list of methods I adopted during my research and lab work. These are further elaborated in published papers.

Adopted techniques include:

Cell cultivating, Cell migration assay, Cell proliferation assay, Chromatin Immunoprecipitation, Colony outgrowth assay, growth curves, Detection of apoptosis – detection of apoptotic nuclei, TUNEL assay, Flow cytometry, Gene expression profiling, Immunofluorescence microscopy, Impassivity assay, mRNA preparation and real-time quantitative PCR, Plasmid engineering, expression vectors creation, promoter-reporter constructs transfection, Statistical analysis, Transient transfection of siRNA, Viability assay, Western blotting,

Techniques employed by other lab team members include:

Animal experimentation, Immunohistochemical analysis, Proteomics analysis and nano-HPLC-MALDI-TOF/TOF analysis, shRNA knock-down, production of lentivirus, lentiviral infection, Wound healing assay

IV RESULTS AND DISCUSSION

In the first paper "**The Hedgehog/GLI signaling pathway activates transcription of Slug (Snail2) in melanoma cells**" we demonstrate that the Hedgehog signaling pathway directly targets the transcription factor Slug (Snail2) in melanoma cells. Slug, a C2H2-type zinc-finger transcription factor, is involved in various cellular processes, such as osteoblast maturation, neural crest cell migration, and transcriptional repression of certain proteins (Ganesan et al., 2015). Although the Slug promoter lacks the full GLI consensus sequence, over 80 potential GLI binding sites were identified, indicating HH-mediated transcriptional regulation.

The study analyzed the response of the Slug promoter to different levels of GLI transcription factors. Key findings include the discovery that the middle part of the promoter is the most active, and its removal significantly decreases luciferase expression. Additionally, the study found that inhibition of the HH pathway with cyclopamine and GANT61 reduced the activity of the Slug promoter in various melanoma cell lines.

Chromatin immunoprecipitation experiments revealed GLI factor binding to the Slug promoter, suggesting a complex regulatory mechanism. Moreover, western blot assays confirmed that inhibition of the HH pathway decreases Slug protein levels and affects other proteins involved in EMT and cancer stem cell markers (Naber et al., 2013).

Furthermore, the study investigated the relationship between MITF and Slug expression. While wild-type MITF did not affect Slug promoter activity, a hyperactive form of MITF increased it. However, Slug expression appeared independent of MITF in melanoma cells, as shown by experiments with anti-MITF shRNA. Immunohistochemical analysis of skin, nevus, and melanoma metastasis samples indicated a correlation between GLI2 and Slug expression but not with MITF, supporting the findings of Slug regulation by GLI2 rather than MITF in melanoma cells.

In the paper titled "**Widespread Expression of Hedgehog Pathway Components in a Large Panel of Human Tumor Cells and Inhibition of Tumor Growth by GANT61: Implications for Cancer Therapy**", a comprehensive study was conducted on 56 cell lines (53 cancerous and 3 non-cancerous) to

assess the presence of key components of the HH pathway. The study revealed a universal expression of primary HH pathway components across all cancer cell lines, suggesting the pathway's pervasive role in these cells. The presence of GLI effector transcription factors was particularly notable, indicating potential non-canonical HH activation and signaling crosstalk (Pietrobono et al., 2019).

The study also explored the effects of GANT61, an HH pathway inhibitor, on cell proliferation. It found that certain cell lines, like SK-MEL-3 and U-2 OS, were highly sensitive to GANT61, while others showed partial or no sensitivity. Surprisingly, pancreatic cancer cell lines, previously reported as sensitive to HH signaling blockage, showed resistance to GANT61. However, a combination of GANT61 with rapamycin affected the PANC-1 cell line, indicating potential for combined therapy. Melanomas were generally sensitive to GANT61, especially when combined with obatoclax, a BCL-2 family inhibitor.

Further investigations using the TUNEL apoptotic assay confirmed that GANT61 induces apoptosis in melanoma cells. This aligns with previous findings on the anti-apoptotic role of HH signaling in melanoma. The study also observed a link

between MITF regulation and anti-apoptotic proteins like BCL2 and BIRC7 (McGill et al., 2002).

A 12xGLI-luciferase reporter assay was used to measure GLI-responsive promoter activity in various cell lines. The assay showed a correlation between HH pathway inhibition by GANT61 or cyclopamine and reduced cell proliferation, suggesting that HH signaling plays a significant role in preventing apoptosis in many tumor cell lines.

In summary, this study highlights the critical role of HH signaling in cancer biology and therapy, demonstrating that HH components are universally expressed in cancer cell lines and that inhibition of this pathway by GANT61 can lead to apoptosis in certain cancer types, especially melanoma. This underscores the potential of targeting the HH pathway in cancer treatment.

The study titled "**Inducibly decreased MITF levels do not affect proliferation and phenotype switching but reduce differentiation of melanoma cells**" examines the role of MITF in melanoma. MITF, a key regulator in melanocytes and melanomas, influences various cellular functions. The study used a doxycycline (DOX)-based inducible system to control MITF

expression in six melanoma cell lines and explored its impact on melanoma phenotype switching.

Contrary to expectations, lowering MITF expression did not significantly impact cell proliferation. This observation challenges the rheostat model, which suggests a direct correlation between MITF levels and proliferation rates. For instance, some cell lines continued proliferating normally even with decreased MITF, revealing heterogeneity at a single-cell level (Carreira et al., 2006, Hoek and Goding, 2010).

The study found no direct correlation between MITF levels and cell invasiveness or migration, contradicting the rheostat model's assertion that low MITF levels enhance invasiveness and EMT capability.

Reduced MITF levels led to a decrease in melastatin and tyrosinase mRNA levels, indicating a reduction in differentiation. Variations were observed in the levels of proteins associated with EMT and stem cell markers, such as an increase in SOX2 in some cell lines, suggesting that MITF influences these factors.

The findings question the rheostat model’s validity, as the expected correlations between MITF levels and various cellular behaviors (proliferation, invasion, migration, EMT) were not observed. This suggests that MITF's effect on cellular processes

might be more complex and context-dependent than previously thought.

The study's results were reviewed and approved by Professor Colin Goding, a prominent researcher whose work contributed to the development of the rheostat model. This acknowledgment indicates a growing recognition of the complexities in MITF regulation in melanoma.

In summary, the study reveals that while decreased MITF levels reduce differentiation in melanoma cells, they do not significantly affect proliferation, invasion, migration, or EMT, challenging the traditional understanding of MITF's role in melanoma biology and indicating a need for further research in this area.

The study "**FBXO32 links ubiquitination to epigenetic reprogramming in melanoma cells**" investigates the role of FBXO32, a component of the SCF E3 ubiquitin ligase complex, in melanoma cells, particularly in relation to its regulation by MITF and its impact on gene expression and melanoma progression.

MITF directly regulates the levels of FBXO32. Silencing of MITF resulted in a reduction of the overall ubiquitination rate

in melanoma cell lines and a decrease in FBXO32 expression. This regulation was consistent in several cell lines, except in a BRAF inhibitor-resistant culture, where MITF silencing did not affect FBXO32 expression.

Altering FBXO32 levels influenced melanoma cell behavior. Silencing FBXO32 reduced migration in high-baseline expression cells and inhibited proliferation, while forced expression of FBXO32 in cells with low baseline levels enhanced migration, colony formation, and proliferation. *In vivo* experiments using xenografts showed that manipulating FBXO32 expression affected tumor growth.

Transcriptomic analysis following FBXO32 knockdown revealed over 300 affected genes, with the most upregulated ones associated with inhibition of cell proliferation and migration. The analysis suggested involvement of epigenetic regulation, indicated by the activation of microRNAs and alterations in the expression of histone-modifying enzymes like KDM5B and HDAC3.

Tandem affinity purification and mass spectrometry identified proteins that bind to FBXO32, linking it to chromatin remodeling, chromosome organization, and transcription machinery. Notably, proteins like SMARCA4 were identified,

suggesting a connection between FBXO32 and transcriptional regulation.

Immunofluorescence assays and ChIP-qPCR confirmed the interaction between FBXO32 and SMARCA4, with SMARCA4 binding to promoters of genes like CDK6 and HDAC3, which are involved in cell proliferation and epigenetic regulation.

The study concludes that FBXO32 plays a significant role in melanoma progression by influencing cell migration, proliferation, and potentially through epigenetic reprogramming. The findings highlight the complex interplay between ubiquitination, transcriptional regulation, and epigenetic mechanisms in melanoma cells and underscore the potential of targeting these pathways for melanoma therapy.

V CONCLUSIONS

This thesis provides a significant advancement in understanding the role of the HH in the maintenance of CSC subpopulations. CSCs, known for their resistance to conventional therapies and elusive nature, pose a considerable challenge in cancer treatment. The parallels between CSCs and induced pluripotent stem cells (iPSCs), particularly regarding their pluripotency and self-renewal capabilities, add a layer of complexity to this research, enhancing the understanding of stem cell biology and aiding in the identification and targeting of CSCs.

The research underscores the critical role of the HH pathway in targeting CSCs, clarifying its mechanisms in CSC regulation, and highlighting its potential as a therapeutic target. These findings have profound implications for developing more effective, targeted cancer therapies.

While elucidating CSC maintenance complexities via the HH pathway, the research opens new avenues for exploration in overcoming resistance to current treatments and understanding the interplay between CSCs and their microenvironments. This

foundational work paves the way for nuanced and effective approaches to cancer treatment.

The study enhances the understanding of CSC maintenance and suggests potential strategies for their detection and eradication, with substantial implications for developing more effective cancer therapies. It emphasizes the need for continued research into the HH pathway's role in overcoming the challenges posed by CSCs, setting a promising direction for future oncological breakthroughs.

- Our data imply that an important regulator of EMT and embryonic neural development, Slug transcription factor, which is associated with tumors´ propensity to metastasis and poor prognosis for cancer patients is directly regulated by HH signaling pathway. It has been clearly proven that Slug is a transcriptional target of GLI transcription factors and notably GLI2.

- We confirmed that all the main constituents of the HH signaling pathway are being produced in virtually all of cancer cell lines that were analyzed (56 cell lines in total)., leading us to a conclusion that HH signaling pathway is aberrantly

activated in cancers regardless of its origin and as such contributes to the CSC phenomenon.

- We found that GANT61, a known inhibitor of GLI1 and GLI2 transcription factors, reduces intracellular levels of Slug protein in melanoma significantly.

- We also aimed to elucidate how varying levels of MITF influence actual phenotype of melanoma cells. Conclusions drawn from our data, put us in opposition to the "rheostat" model, a theory which states that proliferation rate, migration and invasiveness are all regulated by presence, or absence of MITF. What we observed was that these parameters remained invariant to decreasing MITF levels. On the other hand, we gathered data that establish a link between MITF and seemingly unrelated, yet essential process – protein ubiquitination in melanoma.

In summary, this thesis significantly contributes to oncology by exploring novel approaches in cancer therapy, particularly in strategies aimed at CSC identification and eradication. It opens promising avenues for future exploration in

the intersecting realms of stem cell and cancer research, proposing new directions for overcoming the challenges posed by CSCs in cancer treatment.

VI REFERRENCES

Abe, Y., & Tanaka, N. (2016). The Hedgehog Signaling Networks in Lung Cancer: The Mechanisms and Roles in Tumor Progression and Implications for Cancer Therapy. *BioMed Research International*, *2016*, 1–11. https://doi.org/10.1155/2016/7969286

Al-Mamun, M., Kaiissar Mannoor, Cao, J., Qadri, F., & Song, X. (2018). SOX2 in cancer stemness: tumor malignancy and therapeutic potentials. *Journal of Molecular Cell Biology*, *12*(2), 85–98. https://doi.org/10.1093/jmcb/mjy080

Baker, M. (2009). Cancer stem cells resemble healthy stem cells, resist chemotherapy. *Nature Reports*. https://doi.org/10.1038/stemcells.2009.110

Barakat, M. T., Humke, E. W., & Scott, M. P. (2010). Learning from Jekyll to control Hyde: Hedgehog signaling in development and cancer. *Trends in Molecular Medicine*, *16*(8), 337–348. https://doi.org/10.1016/j.molmed.2010.05.003

Beachy, P. A., Karhadkar, S. S., & Berman, D. M. (2004). Tissue repair and stem cell renewal in carcinogenesis. *Nature*, *432*(7015), 324–331. https://doi.org/10.1038/nature03100

Bellusci, S., Furuta, Y., Rush, M., Henderson, R., Winnier, G. E., & Brigid L.M. Hogan. (1997). Involvement of Sonic hedgehog (*Shh*) in mouse embryonic lung growth and morphogenesis. *Development*, *124*(1), 53–63. https://doi.org/10.1242/dev.124.1.53

Berabez, N., Durand, S., & Mathieu Gabut. (2019). Post-transcriptional regulations of cancer stem cell homeostasis. *Current Opinion in Oncology*, *31*(2), 100–107. https://doi.org/10.1097/cco.0000000000000503

Bonnet, D., & Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine*, *3*(7), 730–737. https://doi.org/10.1038/nm0797-730

Bryl, R., Oliwia Piwocka, Kawka, E., Mozdziak, P., Kempisty, B., & Agnieszka Knopik-Skrocka. (2022). Cancer Stem Cells—The Insight into Non-Coding RNAs. *Cells*, *11*(22), 3699–3699. https://doi.org/10.3390/cells11223699

Cabarcas, S. M., Mathews, L., & Farrar, W. L. (2011). The cancer stem cell niche-there goes the neighborhood? *International Journal of Cancer*, *129*(10), 2315–2327. https://doi.org/10.1002/ijc.26312

Carreira, S., Goodall, J., Denat, L., Rodríguez, M., Paolo Nuciforo, Hoek, K. S., Alessandro Testori, Larue, L., & Goding, C. R. (2006). Mitf regulation of Dia1 controls melanoma proliferation and invasiveness. *Genes & Development*, *20*(24), 3426–3439. https://doi.org/10.1101/gad.406406

Cochrane, C. R., Szczepny, A., Watkins, N., & Cain, J. E. (2015). Hedgehog Signaling in the Maintenance of Cancer Stem Cells. *Cancers*, *7*(3), 1554–1585. https://doi.org/10.3390/cancers7030851

Didiášová, M., Schaefer, L., & Małgorzata Wygrecka. (2018). Targeting GLI Transcription Factors in Cancer. *Molecules*, *23*(5), 1003–1003. https://doi.org/10.3390/molecules23051003

Dusek, C. O., & M. Kyle Hadden. (2020). Targeting the GLI family of transcription factors for the development of anti-cancer drugs. *Expert Opinion on Drug Discovery*, *16*(3), 289–302. https://doi.org/10.1080/17460441.2021.1832078

French, R., & Siim Pauklin. (2020). Epigenetic regulation of cancer stem cell formation and maintenance. *International Journal of Cancer*, *148*(12), 2884–2897. https://doi.org/10.1002/ijc.33398

Ganesan, R., Mallets, E. R., & Julián Gómez-Cambronero. (2015). The transcription factors Slug (SNAI2) and Snail (SNAI1) regulate phospholipase D (PLD) promoter in opposite ways towards cancer cell invasion. *Molecular Oncology*, *10*(5), 663–676. https://doi.org/10.1016/j.molonc.2015.12.006

Gong, S., Li, Q., Jeter, C., Fan, Q., Tang, D. G., & Liu, B. (2015). Regulation of NANOG in cancer cells. *Molecular Carcinogenesis*, *54*(9), 679–687. https://doi.org/10.1002/mc.22340

Hoek, K. S., & Goding, C. R. (2010). Cancer stem cells versus phenotype-switching in melanoma. *Pigment Cell & Melanoma Research*, *23*(6), 746–759. https://doi.org/10.1111/j.1755-148x.2010.00757.x

Hyman, J. M., Firestone, A. J., Heine, V. M., Zhao, Y., Ocasio, C. A., Han, K., Sun, M., Rack, P. D., Sinha, S., Wu, J., Solow-Cordero, D. E., Jen, J., Rowitch, D. H., & Chen, J. (2009). Small-molecule inhibitors reveal multiple strategies for Hedgehog pathway blockade. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(33), 14132–14137. https://doi.org/10.1073/pnas.0907134106

Ingham, P. W., & McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes & Development*, *15*(23), 3059–3087. https://doi.org/10.1101/gad.938601

Jalili, A., Mertz, K. D., Romanov, J., Wagner, C., Kalthoff, F., Stuetz, A., Gaurav Pathria, Gschaider, M., Stingl, G., & Wagner, S. N. (2013). NVP-LDE225, a Potent and Selective SMOOTHENED Antagonist Reduces Melanoma Growth In Vitro and In Vivo. *PLOS ONE*, *8*(7), e69064–e69064. https://doi.org/10.1371/journal.pone.0069064

Kugler, M. C., Joyner, A. L., Loomis, C. A., & Munger, J. S. (2015). Sonic Hedgehog Signaling in the Lung. From

Development to Disease. *American Journal of Respiratory Cell and Molecular Biology*, *52*(1), 1–13. https://doi.org/10.1165/rcmb.2014-0132tr

Kumar, V., Mohit Vashishta, Kong, L., Wu, X., Lu, J. J., Guha, C., & Dwarakanath, B. S. (2021). The Role of Notch, Hedgehog, and Wnt Signaling Pathways in the Resistance of Tumors to Anticancer Therapies. *Frontiers in Cell and Developmental Biology*, *9*. https://doi.org/10.3389/fcell.2021.650772

Liu, L., Kugler, M. C., Loomis, C. A., Rashmi Samdani, Zhao, Z., Chen, G. J., Brandt, J. P., Brownell, I., Joyner, A. L., Rom, W. N., & Munger, J. S. (2013). Hedgehog Signaling in Neonatal and Adult Lung. *American Journal of Respiratory Cell and Molecular Biology*, *48*(6), 703–710. https://doi.org/10.1165/rcmb.2012-0347oc

Lu, Y., Zhu, Y., Deng, S., Chen, Y., Li, W., Sun, J., & Xu, X. (2021). Targeting the Sonic Hedgehog Pathway to Suppress the Expression of the Cancer Stem Cell (CSC)—Related Transcription Factors and CSC-Driven Thyroid Tumor Growth. *Cancers*, *13*(3), 418–418. https://doi.org/10.3390/cancers13030418

McGill, G., Horstmann, M., Widlund, H. R., Du, J., Motyckova, G., Nishimura, E. K., Lin, Y.-L., Ramaswamy, S., Avery, W., Ding, H., Jordan, S. A., Jackson, I. J., Korsmeyer, S. J., Golub, T. R., & Fisher, D. E. (2002). Bcl2 Regulation by the Melanocyte Master Regulator Mitf Modulates Lineage Survival and Melanoma Cell Viability. *Cell*, *109*(6), 707–718. https://doi.org/10.1016/s0092-8674(02)00762-6

Merchant, A., & Matsui, W. (2010). Targeting Hedgehog — a Cancer Stem Cell Pathway. *Clinical Cancer Research*, *16*(12), 3130–3140. https://doi.org/10.1158/1078-0432.ccr-09-2846

Metz, J., Geritz, S., Meszéna, G., Jacobs, F., & Van Heerwaarden, J. (1995). *Adaptive Dynamics: A*

Geometrical Study of the Consequences of Nearly Faithful Reproduction. https://warwick.ac.uk/fac/sci/sbdtc/msc/ch927/notes/metz_et_al_adaptive_dynamics.pdf

Miller, L.-A. D., Wert, S. E., & Whitsett, J. A. (2001). Immunolocalization of Sonic Hedgehog (Shh) in Developing Mouse Lung. *Journal of Histochemistry and Cytochemistry*, *49*(12), 1593–1603. https://doi.org/10.1177/002215540104901213

Naber, H., Drabsch, Y., B. Ewa Snaar-Jagalska, Peter ten Dijke, & Theo van Laar. (2013). Snail and Slug, key regulators of TGF-β-induced EMT, are sufficient for the induction of single-cell invasion. *Biochemical and Biophysical Research Communications*, *435*(1), 58–63. https://doi.org/10.1016/j.bbrc.2013.04.037

O'Reilly, K. E., Eleazar, Segura, M. F., Friedman, E. B., Poliseno, L., Sung Won Han, Zhong, J., Zavadil, J., Pavlick, A. C., Hernando, E., & Osman, I. (2013). Hedgehog Pathway Blockade Inhibits Melanoma Cell Growth in Vitro and in Vivo. *Pharmaceuticals*, *6*(11), 1429–1450. https://doi.org/10.3390/ph6111429

Oskarsson, T., Batlle, E., & Massagué, J. (2014). Metastatic Stem Cells: Sources, Niches, and Vital Pathways. *Cell Stem Cell*, *14*(3), 306–321. https://doi.org/10.1016/j.stem.2014.02.002

Pietrobono, S., Gagliardi, S., & Stecca, B. (2019). Non-canonical Hedgehog Signaling Pathway in Cancer: Activation of GLI Transcription Factors Beyond Smoothened. *Frontiers in Genetics*, *10*. https://doi.org/10.3389/fgene.2019.00556

Shackleton, M., Quintana, E., Fearon, E. R., & Morrison, S. J. (2009). *Heterogeneity in Cancer: Cancer Stem Cells versus Clonal Evolution*. *138*(5), 822–829. https://doi.org/10.1016/j.cell.2009.08.017

Shamsoon, K., Hiraki, D., Yoshida, K., Kiyofumi Takabatake, Hiroaki Takebe, Kenji Yokozeki, Horie, N., Fujita, N., Nisrina Ekayani Nasrun, Tatsuo Okui, Hitoshi Nagatsuka, Yoshihiro Abiko, Akihiro Hosoya, Saito, T., & Shimo, T. (2023). The Role of Hedgehog Signaling in the Melanoma Tumor Bone Microenvironment. *International Journal of Molecular Sciences*, *24*(10), 8862–8862. https://doi.org/10.3390/ijms24108862

Takabatake, K., Shimo, T., Murakami, J., Anqi, C., Kawai, H., Yoshida, S., May Wathone Oo, Omori, H., Shintaro Sukegawa, Hidetsugu Tsujigiwa, Nakano, K., & Hitoshi Nagatsuka. (2019). The Role of Sonic Hedgehog Signaling in the Tumor Microenvironment of Oral Squamous Cell Carcinoma. *International Journal of Molecular Sciences*, *20*(22), 5779–5779. https://doi.org/10.3390/ijms20225779

Walcher, L., Kistenmacher, A.-K., Suo, H., Reni Kitte, Dluczek, S., Strauß, A., André-René Blaudszun, Tetyana Yevsa, Stephan, F., & Uta Kossatz-Boehlert. (2020). Cancer Stem Cells—Origins and Biomarkers: Perspectives for Targeted Personalized Therapies. *Frontiers in Immunology*, *11*. https://doi.org/10.3389/fimmu.2020.01280

Wang, Z., Fei, X., Dai, X., Chen, H., Tian, H., Wang, A., Dong, J., & Huang, Q. (2015). Differentiation of Glioma Stem Cells and Progenitor Cells into Local Host Cell-Like Cells: A Study Based on Choroidcarcinoma Differentiation of Choroid Plexus of GFP Transgenic Nude Mouse. *Cancer Biotherapy and Radiopharmaceuticals*, *30*(5), 225–232. https://doi.org/10.1089/cbr.2014.1722

Zhang, Q., Han, Z., Zhu, Y., Chen, J., & Li, W. (2020). The Role and Specific Mechanism of OCT4 in Cancer Stem Cells: A Review. *International Journal of Stem Cells*, *13*(3), 312–325. https://doi.org/10.15283/ijsc20097

Zhao, W., Li, Y., & Zhang, X. (2017). Stemness-related markers in cancer. *Cancer Translational Medicine*, *3*(3), 87–87. https://doi.org/10.4103/ctm.ctm_69_16