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Autoreferát disertační práce


# Molekulární mechanismy nádorové patogeneze signální cesty Hedgehog u vybraných nádorových typů

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**ABSTRAKT**

Předkládaná dizertační práce se zaměřuje na roli signální cesty Hedgehog (HH) v nádorové patogenezi. Signální dráha HH je evolučně velmi konzervovaná signální dráha, která hraje zásadní roli v embryonálním vývoji. V dospělosti je její aktivita silně omezená, aktivovaná je především v kmenových a progenitorových buňkách například mozku, plic, kůže nebo prostaty. Důležitou roli hraje v udržování homeostázy tkání a v jejich regeneraci. Aberantně aktivovaná dráha HH je klíčová v progresi nádorů.

Cílem předkládané práce bylo objasnit nové detaily týkající se signalizace HH dráhy. Podařilo se nám identifikovat nový cílový gen HH dráhy – anti-apoptotický protein survivin, jehož exprese je považována za významný nádorový marker spojovaný se špatnou prognózou pacientů. Prokázali jsme, že inhibitor GANT61, který blokuje koncové proteiny HH dráhy GLI1 a GLI2, snižuje hladinu survivinu v nádorových buňkách. Následně jsme GANT61 spolu s inhibitorem BCL2 proteinové rodiny obatoclaxem použili k inhibici růstu melanomových buněk. Tato kombinace se ukázala velmi efektivní v eradikaci melanomových buněk. Prokázali jsme také, že GANT61 spouští v melanomových buňkách proces apoptózy.

Zjistili jsme také, že signální dráha Hedgehog je aktivovaná u velkého množství buněčných kultur odvozených od různých typů nádorů.

Dále jsme testovali takzvaný reostatový model transkripčního faktoru MITF u melanomu, podle kterého jsou vysoké hladiny MITF spjaté s vysokou diferenciací a malou invazivitou melanomových buněk a nízké hladiny MITF jsou spojené s malou mírou diferenciace, proliferace a vysokou mírou invazivity. Vytvořili jsme buněčný model s inducibilně regulovatelnou hladinou MITF. Pozorovali jsme, že snížení hladiny MITF se neodrazilo na vlastnostech buněk – nesnížila se míra proliferace, ani se nezvýšila invazivita, ale snížila se exprese diferenciačních markerů. To naznačuje, že role transkripčního faktoru MITF musí být dále zkoumána a lépe definována.

Předložené výsledky ukazují na důležitost dráhy HH v nádorové progresi a ukazují na důležitost kombinované cílené terapie.

**ABSTRACT**

The presented doctoral thesis is focused on the role of the Hedgehog (HH) signaling pathway in cancer pathogenesis. HH signaling pathway is an evolutionarily conserved signaling pathway that plays an essential role in embryonic development. Its activity is strictly limited to stem and progenitor cells for example in brain, lung, skin or prostate. HH pathway also plays a key role in tissue homeostasis and regeneration. Aberrantly activated HH pathway is essential in cancer progression.

The aim of the presented thesis was to elucidate new details about the HH signaling pathway. We identified a new target gene of the HH pathway – the anti-apoptotic protein survivin. Survivin is considered to be an important tumor marker associated with a poor prognosis of patients. We showed that the inhibitor of HH pathway effectors GLI1 and GLI2 GANT61 reduced the survivin level in cancer cells. Subsequently, we used GANT61 and the inhibitor of the anti-apoptotic BCL2 protein family obatoclax to inhibit melanoma cells growth. We showed that the combination of these inhibitors was very effective in the eradication of melanoma cells in vitro. We also proved that GANT61 triggers the process of apoptosis in melanoma cells.

We found out that the HH signaling pathway is canonically activated in many cell lines of various tumor origins. Next, we tested the so-called "rheostat model" of MITF transcription factor in melanoma. According to the model, a high-MITF level is associated with high differentiation and low invasion and a low-MITF level is connected with a low differentiation and proliferation rate and high invasion. We established cell lines with inducibly regulated MITF levels. We observed that cell characteristics did not reflect the reduction of MITF level – neither proliferation rate nor invasion decreased. But the expression of differentiation markers decreased. It implies that the role of MITF needs to be more researched and defined better.

The presented results highlight the role of HH signaling in tumor progression and point out the importance of combined therapy.

**CONTENT**

# 1 INTRODUCTION

## 1.1HEDGEHOG SIGNALING PATHWAY

Hedgehog (HH) pathway is an evolutionarily conserved signaling cascade that plays a crucial role in the development of all vertebrates.

Human HH signaling pathway begins in "HH ligand producing cells" secreting one variant of HH ligand - sonic hedgehog (SHH), Indian Hedgehog (IHH) or desert hedgehog (DHH). The expression of HH ligand is tissue-specific. Secreted HH ligand binds to the Patched1 or Patched2 receptor (PTCH1, PTCH2) on the "receptor cell" membrane. In the absence of HH ligand, PTCH1 is localized to the base of primary cilia (PC), special non-motile cilia on the surface of epithelial cells. PTCH1 represses and excludes receptor Smoothened (SMO) from the PC. SMO is a 7-pass transmembrane receptor, a member of G protein-coupled receptor superfamily. Protein Suppressor of Fused (SUFU) blocks effectors of HH pathway - Glioma-associated oncogene (GLI) transcription factors. The SUFU/GLI complex is localized in the tip of PC. Protein kinase A(PKA) and glycogen synthase kinase 3β (GSK3β) promote the formation of repressor forms of GLI factors that are subsequently translocated to the nucleus, where they bind to the HH target gene promoters and block their transcription. When the HH ligand binds to PTCH1, the repression of SMO is relieved. SMO enters into the primary cilia and represses the activity of SUFU. It results in SUFU/GLI complex dissociation. GLI factors are post-translationally modified and form activator forms that are translocated to the nucleus, where they activate the expression of target genes (reviewed in Briscoe and Thérond, 2013; Cochrane et al., 2015).

HH signaling pathway has three effectors GLI1, GLI2 and GLI3. They are zinc-finger transcription factors. GLI1 is considered an activator of the pathway. GLI2 is considered an activator of the pathway and GLI3 is mainly thought to be a repressor (Skoda et al., 2018).

HH pathway regulates the transcription of many target genes involved in many cellular processes such as cell cycle (e.g. *CCND1, CCND2, FOXM1*), regulation of apoptosis (*BCL2, CFLAR*), epithelial-to-mesenchymal transition (*FOXC2, SNAI1, TWIST2, ZEB1, ZEB2*), stem-cells signaling (e.g. *WNT2B, WNT5A, JAG2*). HH pathway also regulates the expression of stem cell markers (*CD44, CD133, LGR5*) (Katoh and Katoh, 2009).

HH signaling pathway can also be activated non-canonically, which means that HH signaling differs from the standard HH-PTCH-SMO-GLI axis. The first type of non-canonical signaling is often called signaling crosstalk. It means that GLI factors are activated by proteins from other signaling pathways and the HH pathway upstream of GLI factors is excluded from signalization. The second type of non-canonical signaling occurs in a GLI-

independent manner. SMO does not activate GLI factors. Instead, SMO coupled with G-protein of the Gαi family modulates $Ca^{2+}$ flux, activation of the small GTPases RHOA and RAC1 and Warburg-like metabolism (Arensdorf et al., 2016).

## 1.2 HEDGEHOG SIGNALING IN CANCER

HH signaling is essential in embryonic development. In an adult organism, the HH pathway is activated in tissue-specific manner. HH expression is localized to the stem and progenitor cells for example in brain, skin or prostate. HH signaling is important for tissue homeostasis and regeneration (Petrova and Joyner, 2014). Increased expression of SHH and activated HH signaling pathway play an important role in cancer progression. In tumors, HH signaling deregulates many cellular and tissue processes, such as proliferation, escape from apoptosis, reactivation of telomerase activity, angiogenesis, deregulation of energetic metabolism, epithelial-to-mesenchymal transition, escaping the immune system, activation of invasion and metastasis or genomic instability (Hanna and Shevde, 2016).

### 1.2.1 HEDGEHOG SIGNALING IN APOPTOSIS

Apoptosis (programmed cell death) is often pathologically blocked in cancers. The connection between the HH pathway and apoptosis in development and cancer has been shown in various studies. HH pathway affects the expression of proteins playing a role in apoptosis. The expression of pro-apoptotic protein Noxa is downregulated by GLI1 (Meister et al., 2018), the expression of anti-apoptotic proteins BCL2 (B-cell lymphoma 2) and XIAP (X-linked inhibitor of apoptosis protein) is activated by HH signaling pathway (Regl et al., 2004; Bigelow et al., 2004, Kurita et al., 2011).

Moreover, the HH pathway can modulate the switch between intrinsic and extrinsic apoptotic pathways. XIAP protein (transcriptional target of GLI2) represses the extrinsic apoptotic pathways, but blocking HH signaling (and XIAP) by anticancerous drug leads to activation of extrinsic mitochondria- independent pathway (Kurita et al., 2011).

It seems that the activated HH pathway acts as a suppressor of apoptosis in cancer cells, but more research is needed.

## 1.3 MALIGNANT MELANOMA

Malignant melanoma is the most aggressive type of skin cancer. Melanoma originates from the special skin cells, melanocytes, that in response to DNA damage in skin

keratinocytes produce melanin protecting the skin from the UV-light. (Lin and Fisher, 2007). Melanocytes can escape from the control of keratinocytes, proliferate and spread and form a naevus or common mole. Melanoma derives from preexisting nevi in 25 % and transformed melanocytes in 75 % cases (Berlotto, 2013).

MITF (microphthalmia-associated transcription factor) is essential in melanocyte development and is also a key factor in melanoma development. Therefore, MITF can be termed as lineage survival oncogene (Garraway et al., 2005). MITF regulates the expression of many target genes that play role in melanocytes differentiation and melanin synthesis, cell cycle and proliferation, cell survival and apoptosis or epithelial-to mesenchymal transition (Cheli et al., 2010).

MITF expression levels were linked with tumor characteristics, such as tumor growth, survival and proliferation of tumor cells, invasion and metastasis. Therefore, the so-called "MITF rheostat model" was created (Carreira et al., 2006; Hoek and Goding, 2010). According to the model, there are subpopulations of tumor cells with distinct MITF levels and with distinct characteristics in the tumor. Tumor cells with high expression of MITF are associated with differentiation and increased proliferation but low invasion. Oppositely, tumor cells expressing low levels of MITF are more invasive and their proliferation is decreased. Cells with depleted MITF (for example by RNAi) are associated with cell senescence and increased apoptosis.

### 1.3.1 HEDGEHOG SIGNALING IN MELANOMA

Even though HH signaling is not in the focus of melanoma research, it was shown that HH signaling affects processes linked to invasion, proliferation and tumorigenesis of melanoma cells.

The RAS-MEK/AKT signaling pathway regulates GLI1 activity in melanoma, highlighting the role of HH-GLI1 signaling in RAS-induced melanomas (Stecca et al., 2007). It was also proved that HH signaling modulates the expression of E2F1 (E2F transcription factor 1) protein that is crucial in cell cycle progression, DNA damage response and apoptosis and is aberrantly activated in various cancers (Pandolfi et al., 2015). Interestingly, there is a connection between TGF-β (transforming growth factor β) and GLI2 in melanoma. GLI2 is a direct target of TGF-β (Dennler et al., 2007 and 2009) and TGF-β plays an essential role in melanoma tumorigenicity and invasion (Javelaud et al., 2007; 2005). Alexaki et al. (2010) showed that GLI2 has an important role in invasion and metastasis in melanoma.

Moreover, GLI2 blocks the expression of melanoma transcription factor MITF. It was suggested that GLI2 and MITF are inversely correlated (Javelaud et al., 2011).

## 1.4 TARGETING THE HEDGEHOG PATHWAY

Targeted therapy is a type of drug treatment that specifically blocks target genes involved in cancer cells' growth and survival. Specific inhibitors can block the HH pathway at different levels depending on the protein of HH pathway that is inhibited. Most of the inhibitors target the SMO receptor. The first promising agent inhibiting SMO was cyclopamine, a steroidal alkaloid isolated from the plant *Veratrum californicum* (Chen et al., 2002). The use of cyclopamine in clinical practice has been limited due to low water solubility and low stability in acids and not optional pharmacokinetics. These problems led to the synthesis of more stable derivates of cyclopamine, such as vismodegib (GDC-0449), sonidegib (erismodegib, LDE-225) or saridegib (patidegib, IPI-926). Three SMO inhibitors were approved by the United States Food and Drug Administration (FDA) - vismodegib (treatment of recurrent, locally advanced or metastatic basal cell carcinoma (BCC)), sonidegib (treatment of locally advanced BCC not suitable for surgery or radiotherapy) and glasdegib (to be used with low doses of cytarabine for the treatment of acute myeloid leukemia in patients older than 75 years or with comorbidities not enabling intensive induction chemotherapy) (Axelson et al., 2013; Casey et al., 2017; Norsworthy et al., 2019). SMO inhibitors sonidegib, glasdegib, patidegib and itraconazole are in phase III of clinical trials (www.clinicaltrials.gov).

The problem of SMO inhibitors is acquired resistance. SMO is often mutated in tumors or develops adaptive mutations after the treatment with SMO inhibitors (Pricl et al., 2015). Therefore, inhibitors targeting downstream of SMO, especially drugs blocking GLI factors, are in the focus of the research. GANT58 and GANT61 are direct inhibitors of GLI1 and GLI2 transcription factors. GANT61 is intensively investigated in preclinical studies of various cancer types as rhabdomyosarcoma, neuroblastoma, colon, pancreas, leukemia, prostate or melanoma (Gonnissen et al., 2015).

SMO-acquired resistance is also possible to solve by combination therapy using HH pathway inhibitors together with inhibitors of other signaling pathways, ionizing radiation or chemotherapy. Combination treatment strategies, including HH pathway inhibition combined with standard cancer treatment, are in the focus of clinical trials (Xie et al., 2019; www.clinicaltrials.gov).

## 2 HYPOTHESIS AND AIMS

This study is focused on the role of the Hedgehog signaling pathway in cancers. The study aimed at clarifying new aspects of HH signaling in cancer

**Major aims:**

**1/** HH pathway is aberrantly activated in many types of cancer. The effectors of the HH pathway, GLI transcription factors, activate a wide range of target genes in tumors. Anti-apoptotic proteins BCL2 and XIAP are direct targets of the HH pathway. Thus, it seems HH signaling has a certain role in disbalancing pro-apoptotic and anti-apoptotic signals in tumor cells**. Our aim was to identify new pro-/anti-apoptotic target gene(s) of the HH pathway and shed more light on the role of HH signaling in apoptosis in cancer cells.**

**2/** In recent decades, there are efforts to treat patients suffering from various cancers with a therapy specifically targeted to the tumor. The FDA approved three SMO inhibitors to be used in patients suffering from basal cell carcinoma and acute myeloid leukemia. However, there is a problem with SMO acquired resistance. Therefore, inhibitors of other components of the HH pathway are intensively studied. The research is also focused on therapy combining two or more inhibitors that target different signaling pathways. **We aimed to examine the HH signaling inhibition by the inhibitor of GLI transcription factors GANT61.**

**3/ Our next aim was to find an appropriate combination of inhibitor GANT61 and inhibitor of another signaling pathway that will eliminate cancer cells more effectively than the monotherapy of each agent.**

**4/ We were also interested in the effect of GANT61 on cancer cells. We aimed to study which tumor characteristics and cellular processes are altered by GANT61 treatment**.

**5/** There are several different mechanisms of activation of HH signaling pathway, for example HH ligand can be aberrantly overproduced, gain-of-function or loss-of-function mutations of HH pathway components are found. GLI factors can be activated non-canonically. Thus, the expression pattern of the HH pathway components could indicates whether the HH pathway is active (and how is activated) in cancer cells. **Our aim was to examine the completeness of expression of HH pathway components in the cell lines of various tumor origins**.

**Minor aims:**

5/ MITF transcription factor is crucial for the survival of melanocytes as well as melanoma cancer cells. In melanoma, the so-called "rheostat model" of MITF expression was established. According to the model, within tumors, there are subpopulations of melanoma cells with distinct MITF levels and cellular characteristics. Tumor cells expressing high levels of MITF are associated with differentiation and increased proliferation and low invasiveness, melanoma cells with low levels of MITF are more invasive and their proliferation is decreased. **Our aim was to regulate MITF expression in melanoma cells and monitor changes in proliferation, differentiation, invasiveness and expression of MITF target genes.**

6/ GLI2 expression was described to be inversely correlated with the expression of MITF in melanomas. **Therefore, the results obtained in the "rheostat study" would be discussed from the point of view that GLI2 negatively correlates with MITF expression level.**

## 3 METHODS

I personally worked with these methods:

Cell culturing, Cloning (plasmids, promoter-reporter constructs, expression constructs, site-directed mutagenesis), Colony outgrowth assay, Chromatin immunoprecipitation, Detection of apoptosis (detection of apoptotic nuclei, TUNEL assay), Flow cytometry, Immunofluorescence microscopy, Invasivity assay, promoter-reporter assays, shRNA knockdown, Statistical analysis, Transfections, Viability assay, Western blotting, Working with lentiviruses - inducible (production of lentivirus, lentiviral infection), Wound healing assay

## 4 RESULTS AND DISCUSSION

In the presented doctoral thesis, I focus on the role of HH signaling in tumorigenesis. I present new findings of HH target genes, the activity of HH pathway in tumor cells, targeting the HH pathway and discuss the role of HH pathway in melanoma cell lines. Since our research group is focused on melanoma research, the main experiments were performed on melanoma cell lines and supplemented by cell lines of various tumor origins.

In the first paper **"Survivin, a novel target of the Hedgehog/GLI signaling pathway in human tumor cells."** we newly demonstrated that the anti-apoptotic protein survivin (also called BIRC5) is a direct target of the HH signaling pathway. We found 11 potential GLI binding sites in *survivin* promoter. However, none of them had the consensus sequence GACCACCCA defined by Kinzler and Vogelstein (1990). However, GLI binding sites with two or more substitutions were previously found in promoters of many known GLI target genes (Winklmayr et al., 2010). We determined that no single binding site exhibits the capability to activate the *survivin* promoter. However, GLI binding site occupying +2 to +10 area of the promoter (relative to the start of translation) seems to have an inhibitory character in reporter assays. We did not find the specific combination of GLI binding sites crucial for survivin expression.

The activity of the survivin promoter decreased after cyclopamine (inhibitor of SMO) or GANT61 (inhibitor of GLI1 and GLI2) treatment and GLI2 increased the activity of survivin promoter in promoter-reporter assays. Moreover, endogenous survivin mRNA and protein levels decreased after GANT61 treatment in a majority of cell lines from a large panel of 40 cancer cell lines of various origins. It was proved by chromatin immunoprecipitation that ΔNGLI2 (lacks the N-terminal suppressor domain) bound to the endogenous survivin promoter. Ectopic expression of ΔNGLI2 increased the expression of endogenous survivin in human fibroblast IMR-90. The overlap of GLI2 and survivin expression was observed in immunohistochemically stained tumor sections. Similarly, the co-localization of GLI2 and survivin protein was detected in tumor sections under immunofluorescence microscopy.

We conclude that survivin is a direct target gene of the HH signaling pathway. It is consistent with a study performed by Brun et al. (2015), who showed that survivin is overexpressed in HH-driven medulloblastoma. Another study demonstrated a positive correlation between survivin and GLI2 in ovarian carcinoma samples (Ozretić et al., 2017). Our results highlight the role of the HH signaling pathway in the regulation of apoptosis.

Anti-apoptotic protein XIAP was previously described as a direct target of the HH pathway (Kurita et al., 2011). It was also described that survivin together with XIAP inhibits caspase-9 (Garg et al., 2016). It implies that this XIAP/survivin anti-apoptotic pathway could be under Thus, it seems that the HH signaling pathway has a crucial role in anti-apoptotic signaling.

Moreover, our findings can be important for the following research, for example in the targeted therapy.

We aimed to find a potent combination of GANT61 and an inhibitor of another important signaling pathway in our following study **"GLI inhibitor GANT61 kills melanoma cells and acts in synergy with obatoclax".**

The treatment of melanoma with MEK (Mitogen-activated *protein* kinase kinase) kinase inhibitors or kinase inhibitors directed against mutated BRAF(V6000E) seemed to be very promising but acquired resistance invariably appeared after a few months of treatment (Davies and Kopetz, 2013; Kozar et al., 2019). In the recent decade, the role of HH signaling in melanoma has been elucidated. It was shown that GLI2 is inversely correlated with MITF, a crucial factor in melanoma transcription circuitry (Javelaud et al., 2011). Thus, we examined the effect of inhibitor of GLI factors GANT61 alone or in combination with obatoclax (inhibitor of BCL-2 protein family) on melanoma cells.

We showed that MITF and GLI2 are inversely correlated in most of tested cell lines as described by Javelaud et al. (2011). As it was previously described that GLI2 represses transcription of MITF (Pierrat et al., 2012), we expected an increase of MITF after GANT61 treatment. However, no increase of MITF expression was observed in any of the four tested melanoma cell lines. These results were quite surprising because they do not reflect the consensual GLI2/MITF inversion model proposed by Javelaud et al. (2011) and confirmed by Faião-Flores et al. (2017). Thus we can only discuss why MITF does not respond to GANT61 treatment in our experiment. We propose that GLI2 may not be the repressor of MITF transcription in every cell context. We also suggest that GLI2 can possibly regulate the MITF level indirectly.

We demonstrated that GANT61 strongly decreased the viability of melanoma cells. GANT61 also partially blocked the colony formation in BEU and SK-MEL-3 cell lines. The combination of GANT61 and obatoclax inhibited colony formation completely. Moreover, we proved that GANT61 causes apoptosis in melanoma cell lines. At last, we were looking for an efficient combination of drugs decreasing the survival of melanoma cells. We used 20 μM GANT61 alone or with 100 nm obatoclax. Obatoclax alone had no effect on melanoma cells

but enhanced the effect of GANT61 treatment in 6 tested melanoma cell lines. Calculation of the combination index revealed the strong synergistic effect of obatoclax and GANT61.

Our finding of a novel potent combination of anticancerous drugs that dramatically decreases proliferation of melanoma cells is important from the point of view that monotherapies are often followed by acquired resistance. The benefit of using inhibitor obatoclax was showen by Haq et al. (2013)., who determined that deregulated expression of proteins from the anti-apoptotic BCL2 family plays a crucial role in melanoma resistance to apoptosis and observed that combination treatment with obatoclax and BRAF inhibitors overcomes the resistance to BRAF inhibitors. Generally, the treatment with combined therapy is believed to be more efficient and to have a better long-term effect than monotherapies.

In publication **"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."** we examined the expression of individual components of the HH signaling pathway in 56 cell lines of various origin (53 cancer cell lines, 3 control non-cancerous cell lines) by Western blot analysis. We examined the expression of proteins SHH, PTCH1, SMO, SUFU, GLI1, GLI2 and GLI3. In general, we determined that all mentioned proteins are expressed in the vast majority of cell lines. The effectors of the HH pathway and SHH were expressed in all cell lines. It implicated that the HH signaling pathway was canonically activated by SHH in all cell lines in an autocrine or paracrine manner.

Next, we studied how treatment with GANT61 affects various cell lines. In proliferation assay, GANT61 treatment completely eradicated cells of melanoma, osteosarcoma, neuroblastoma and small cell lung cancer cell lines. Oppositely, non-small cell lung cancer cell line A549 and pancreatic cancer cell lines were GANT61 resistant.

We proved that GANT61 caused apoptosis in melanoma cells by TUNEL assay, we also detected apoptotic nuclei in GANT61 treated cells. Similarly, Faião-Flores et al. (2017) has recently shown that GANT61 induced apoptosis in melanoma cells (but they used different cell lines). These observations are in accordance with the role of HH pathway in anti-apoptotic signaling in cancer cells. However, it is believed that in melanoma the anti-apoptotic characteristics are primarily maintained by MITF, a key regulator of many transcripts in melanoma. MITF positively regulates the expression of anti-apoptotic protein BCL2 and it seems that it also regulates the expression of anti-apoptotic protein BIRC7 (Goding and Arnheiter, 2019).

Our following study was focused on melanoma. It was described that the different levels of MITF expression are linked to melanoma cell growth, proliferation, survival, differentiation, invasion and metastasis. It was described as MITF "rheostat model" or "phenotype switching" (Carreira et al., 2006; Hoek and Goding, 2010). According to the model, phenotypically distinct populations of cells with different MITF levels are found in melanoma: high-MITF cells are associated with differentiation and increased proliferation but low invasion, low-MITF cells are more invasive and their proliferation is decreased. In our study **"Inducibly decreased MITF levels do not affect proliferation and phenotype switching but reduce differentiation of melanoma cells."** we established cell lines with inducibly regulated MITF levels. We aimed to characterize better the role of MITF in phenotype switching. The inducible regulation of MITF was achieved by using doxycycline (DOX)-based inducible lentiviral system (Tet-on system) in six melanoma cell lines with a high or average basal level of MITF.

DOX in doses 0-1 μg/ml decreased the protein expression of MITF, albeit sometimes not completely. Surprisingly, DOX-decreased expression of MITF did not cause any (or a very slight) effect on cell proliferation, migration or invasion. These results differ from the rheostat model, in which high-MITF level was connected to high rate of proliferation, low invasion and the low-MITF level was linked to decreased proliferation and higher invasion. (Carreira et al, 2006; Hoek and Goding, 2010; Goding and Arnheiter, 2019). Moreover, the proliferation rate did not correlate with MITF levels in six native melanoma cell lines, which implies that no specific basal level of MITF is connected to high or low proliferation rate.

The mRNA level of differentiation markers melastatin and tyrosinase (direct targets of MITF) lowered in response to decreasing level of MITF in accordance with the rheostat model. Western blot analysis revealed that the expression of selected MITF target genes (livin, p27, AXL) changed as could be expected in MITF decreasing manner. The expression of BCL2 did not react on decreasing level of MITF (discussed below). Only minimal changes in protein levels of EMT markers were detected in MITF decreasing manner. Similarly, we analyzed the expression of stemness markers and showed that the level of SOX2 protein increases with decreasing MITF. SOX2 is essential for the cell-renewal and tumorigenicity of melanoma-initiating cells. Interestingly, the HH signaling pathway plays an important role in the regulation of *SOX2* gene (Santini et al., 2014).

We conclude that our results are not entirely in accordance with the rheostat model. In this respect, we somewhat revised the rheostat model. We showed that decreasing levels of MITF protein do not correlate with changes in proliferation rate, invasion, migration or EMT.

The pitfalls of MITF rheostat model were discussed from many points of view (Seberg et al., 2017; Vachtenheim and Ondrušová, 2015) . Wellbrock and Arozarena (2015) conclude that the expression level of MITF is not the only "characteristic" that defines the activity of MITF, although it is well measurable and changeable. They point out that MITF often acts in an opposite manner at similar expression levels but in a different context. Thus, it is clear that more research is needed to define the role of MITF in "the rheostat model" more precisely. Our results were discussed by Goding and Arnheiter (2019) in an extensive MITF review.

The results from the presented rheostat model study can also be discussed from the point of view of HH signaling pathway context. It was shown that GLI2 is inversely correlated with MITF (though in different cell lines than we used), a crucial factor in melanoma transcription circuitry (Javelaud et al., 2011; Faião-Flores et al., 2017). Thus, we can discuss the obtained result from the point of view that decreased expression of MITF is linked to increased expression of GLI2. For example, BCL2 (direct target of MITF) protein expression did not decrease in response to lowering MITF expression. However, BCL2 was also described to be a target of GLI2 (Regl et al., 2004). Thus we speculate that both transcription factors MITF and GLI2 affect the expression of BCL2 in melanoma cells. Since GLI2 and MITF are negatively correlated, GLI2 could compensate the downregulated expression of MITF that could result in the continual expression of BCL2 irrespective of MITF expression level.

The expression of CSC marker SOX2 increased in response to lowering MITF expression. The expression of SOX2 is regulated by GLI1 and GLI2 (Santini et al., 2014). Therefore, it seems that increasing expression of GLI2 could be the mechanism that could explain the higher expression of SOX2 in low-MITF cells.

Our hypothesis about the role of GLI2 should be investigated in the following research. Other similar studies of MITF should be performed in the GLI2 context.

## 5 CONCLUSION

Hedgehog signaling pathway plays a crucial role in embryonic development and when aberrantly activated in cancer initiation and progression. The results presented in this doctoral thesis bring new findings about the role of HH signaling and HH targeted therapy in cancer, especially in melanoma.

- We found that the anti-apoptotic protein survivin, associated with a poor prognosis of cancer patients, is regulated by the HH signaling pathway. We proved that the survivin gene is a direct target of the GLI2 transcription factor.

- We showed that the inhibitor of GLI1 and GLI2 transcription factors GANT61 can inhibit survivin in cancer cells.

- We found a new combination of anticancerous drugs that eliminate melanoma cells in vitro. We found that obatoclax, the inhibitor of the BCL2 protein family, has no effect if used alone. We found that a combination of obatoclax and GANT61 eradicates melanoma cells in few days in vitro.

- We conclude that all main components of the HH pathway are expressed in the most of cancer cell lines that we tested (56 cell lines). It implies that the canonical Hh pathway is active in cancer cell lines of various origins

- We show that GLI1 and GLI2 inhibitor GANT61 eradicates melanoma cells in vitro by the process of apoptosis.

- We established cell lines with inducibly regulated MITF level by shRNA and aimed to characterize better the role of MITF in phenotype switching. We show that proliferation, migration and invasion do not change with decreasing MITF expression level. Our results differ from the “rheostat model” proposed for MITF transcription factor in melanoma cells. We suggest that only changes in MITF expression level do not explain the changes of cellular characteristics such as proliferation or invasion.

- We observed that the expression of SOX2 increases with decreasing levels of MITF. We propose that the increase is caused by the high expression of GLI2, the effector of HH signaling pathway.

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## 7 LIST OF PUBLICATIONS

**Publications related to the thesis with impact factor:**

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