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The Role of Sonic Hedgehog Pathway in Neuro- and Tumorigenesis

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Declaration
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

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CONTENTS

INTRODUCTION

The Hedgehog (Hh) signaling pathway is essential for the regulation of vital vertebrate embryonic processes and for the organogenesis (Ingham and McMahon, 2001). Hh is a secreted as diffusible protein and is a critical signaling molecule for the pattern formation of the anterior-posterior axis. In vertebrates, Hh protein family consists of three proteins: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh). In rodents, targeted disruption of Shh leads to multiple defects in embryonic tissues, including notochord, floor plate and limb structures (Chiang et al., 1996). The role of Hh during development is not limited to patterning. Hh regulates the proliferation of neuronal precursors, epidermal stem cells and somatic stem cells

Hh signaling remains important in the adulthood. Shh has been shown to promote the proliferation of adult stem cells from various tissues, including primitive haemathopoetic cells, mammary, retina and neural stem cells (Ahn and Joyner, 2005; Bhardwaj et al., 2001; Jian et al., 2009; Liu et al., 2006). Recent studies have demonstrated that Shh regulates adult neural progenitor proliferation in hippocampus (Lai et al., 2003).

Alterations in Hh signaling are implicated in many types of malignancies. Aberrant activation of the Hh pathway in cancerogenesis is caused by ligand-independent mutations in the pathway or through ligand-dependent Hh overexpression (for reviews see (Chari and McDonnell, 2007; Evangelista et al., 2006). Overexpression of Hh has been shown in basal cell carcinoma (BCC), medulloblastoma (MB), pancreatic cancer, small cell lung cancer (SCLC), breast cancer, prostate cancer and digestive tract tumors (for reviews see (Chari and McDonnell, 2007; Evangelista et al., 2006; Lauth et al., 2007; Rubin and de Sauvage, 2006). The growth of some tumors can be effectively suppressed by various pathway inhibitors, such as Hh-neutralizing antibodies or Smoothened (Smo) antagonists.

Accumulated data suggest that alteration of Hh signaling pathway may be used as a unique mechanism-based therapy (1) to block tumor growth or stimulate its regression and (2) to stimulate the adult neurogenesis. For these reasons, Hh pathway remains the target of continuous investigation and became the theme of this thesis.

ORIGINAL PUBLICATIONS

1. Speek M., **Njunkova O.**, Pata I., Valdre E., Kogerman P. (2006). A potential role of alternative splicing in the regulation of the transcriptional activity of human GLI2 in gonadal tissues. *BMC Molecular Biology*, 7, 1 - 13.

2. Hunt R., **Bragina O.**, Drews M., Kasak L., Timmusk S., Valkna A., Kogerman P., Järvekülg L. (2007). Generation and Characterization of mouse monoclonal antibody 5E1 against human transcription factor Gli3. *Hybridoma*, 26, 131 - 138.

3. **Bragina O.**, Njunkova N., Sergejeva S., Järvekülg L., Kogerman P. (2010). Sonic hedgehog pathway activity in prostate cancer. *Oncology letters*, 1(2), 319 - 327.

4. **Bragina O.**, Sergejeva S., Serg M., Žarkovsky T., Maloverjan A., Kogerman P., Žarkovsky A. (2010). Smoothened agonist augments proliferation and survival of neural cells. Accepted for publication in *Neuroscience letters*.

ABBREVIATIONS

Genes are indicated with *italics* (e.g. *Ptch1*), human proteins with capital letters (e.g. PTCH1), mouse proteins with an initial capital letter (e.g. Ptch1) and Drosophila proteins with small letters (e.g.ptc)

ALK5	Activin receptor-like kinase 5
BCC	Basal Cell Carcinoma
BrdU	5-bromo-2'-deoxyuridine
cos2	Costal2
ci	Cubitus interruptus
DNA	Deoxyribonucleic acid
Dhh	Desert Hedgehog
DG	Dentate Gyrus
Disp	Dispatched
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ECS	Electroconvulsive Seizure
Fu	Fused
GCPS	Greig's cephalopolysyndactyly
GFAP	Glial fibrillary acidic protein
GLIA	GLI protein activator form
GLIFL	GLI protein full lenght
GLIR	GLI protein repressor form
GS	Gorlin's syndrome
Hh	Hedgehog
Hh-Ag	Hedgehog agonist
HPE	Holoprosencephaly
Ihh	Indian Hedgehog
KAAD-cyclopamine	3-keto-N-(aminoethyl-aminocaproyl-dihydrocinnamoyl)cyclopamine
MB	Medulloblastoma
mRNA	Messenger RNA
MS	Multiple Sclerosis
NBCCS	Nevoid Basal Cell Carcinoma Syndrome
PD	Parkinson's disease
PHS	Pallister-Hall syndrome
Ptch	Patched
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
RNA	Ribonucleic acid
qRT-PCR	Quantitative reverse transcriptase PCR

SAG	Smoothened Agonist
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Smo	Smoothened
Shh	Sonic Hedgehog
SGZ	Subgranular Zone
SVZ	Subventricular Zone
Su(fu)	Suppressor of fused
TGF-β	Transforming growth factor beta
TRAMP	TRansgenic Adnocarcinoma Mouse Prostate model
TUJ1	Neuron-specific class III beta-tubulin
UTR	Untranslated Region
WT	Wild type

1. REVIEW OF THE LITERATURE

1.1 The canonical Hedgehog signaling pathway

The Hedgehog (Hh) signaling pathway has conserved role in the embryonic development of species ranging from *Drosophila* to human. Although the Hh signaling pathway is well conserved through evolution (Burglin, 2008), a fraction of the pathway components underwent duplications and divergence of the Hh signaling mechanisms have been reported between flies and mammals (Huangfu and Anderson, 2006) (Table 1). For example, divergence of Smoothened structure, the role of Fused and Suppressor of Fused has been reported (Burglin, 2008).

Drosophila has a single hh ligand, but in mammals there are three Hh ligands with different patterns of expression: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh) and at least two Hh receptor, *Patched,* genes: *Ptch1* and *Ptch2*. The glioma-associated Gli family of zinc finger transcription factors, including Gli1, Gli2 and Gli3, are responsible for the activation or repression of Hedgehog target genes in vertebrates (Lee et al., 1997; Ruiz i Altaba, 1998). In *Drosophila* there is only one homolog of Gli proteins – cubitus interruptus (ci).

Table 1. Divergence of Hh signaling pathway components in *Drosophila melanogaster* and vertebrates.

Drosophila melanogaster	Vertebrates
hedgehog (hh)	*Sonic hedgehog (Shh), Indian hedgehog (Ihh), Desert hedgehog (Dhh)*
disp (dispatched)	*Disp1*
patched (ptc)	*Ptch1, Ptch2*
smoothened (smo)	*Smo*
fused (fu)	*Fu*
suppressor of fused (Su(fu))	*Su(Fu)*
costal 2 (cos)	*KIF27, KIF7*
cubitus interruptus (ci)	*Gli1, Gli2, Gli3*

Hh signaling is initiated by binding of the secreted cholesterol- and palmitoyl-modified Hh peptide to the 12-span transmembrane protein Patched (Ptch), resulting in loss of Ptch activity and consequent phosphorylation and posttranscriptional stabilization of 7-span transmembrane protein Smoothened (Smo) (Fig. 1) (Osterlund and Kogerman, 2006). In both, *Drosophila* and

mammals, release of Hh from producing cells requires the transmembrane protein Dispatched (Disp) (Burke e t a l., 1 999; C aspary e t a l., 2 002; Kawakami e t a l., 2002).

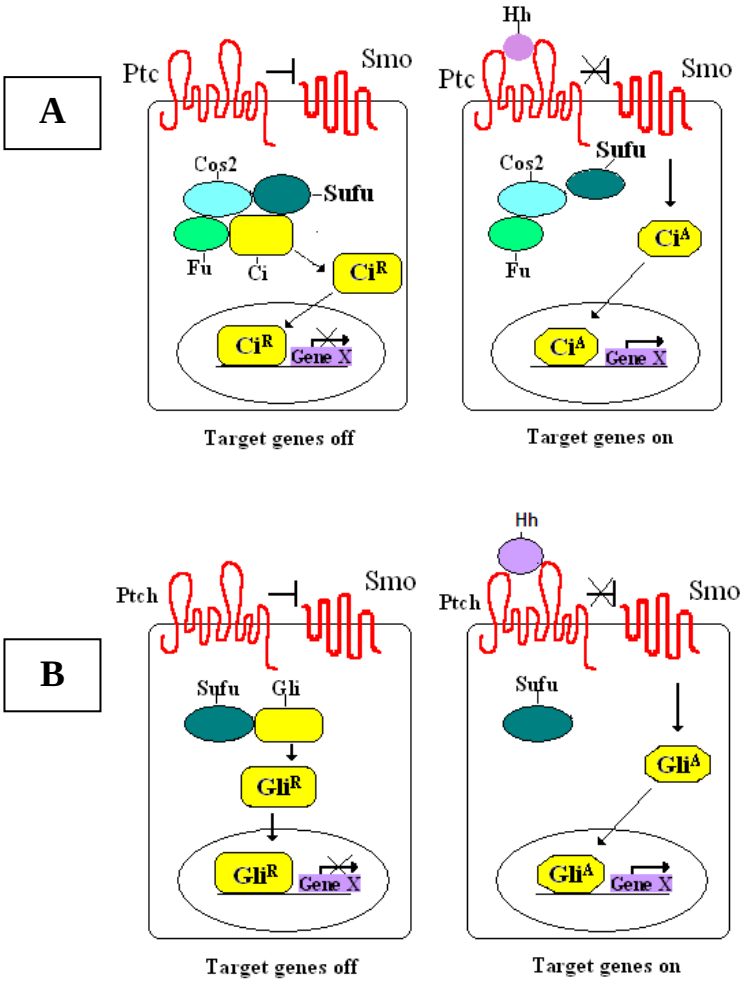

Figure 1. Schematic r epresentation of t he H edgehog s ignaling i n *Drosophila melanogaster* (A) and ma mmals (B). In t he a bsence of H h, Ptch i nhibits t he activity of Smo. In *Drosophila*, Ci is processed into a repressor Ci^R, which inhibits the transcription of the target genes. In the presence of Hh, full-length Ci induces transcription of target genes.
(B) In mammals, Gli is processed to Gli^R form, which inhibits transcription of the target genes. In the presence of Hh, the inhibition of Smo is relieved and Gli^A is produced, leading to the activation of target gene transcription.

Smo transduces the Hh signal across the plasma membrane. In *Drosophila*, Hh induces multiple phosphorylation in the Smo C-terminal cytoplasmic tail, leading to its cell surface accumulation and activation (Denef et al., 2000; Jia et al., 2004; Zhang et al., 2004). Smo transmits the signal to a cytoplasmic complex composed of the kinase Fu, the kinesin/like protein Cos2, the protein Sufu and the transcription factor Ci (Monnier et al., 1998; Robbins et al., 1997; Ruel et al., 2003; Stegman et al., 2000). This complex controls the processing, activity and subcellular distribution of the Ci transcription factor responsible for Hh target gene activation.

Drosophila and mammalian Hh signaling have diverged between Smo and Ci/Gli. Based on functional analysis and sequence conservation of putative Cos2 orthologues, Sufu, Smo and Ci/Gli it was found that major Cos2-like activities are absent in mammalian cells and that the inhibition of the Hh pathway in the absence of ligand depends on Sufu (Varjosalo et al., 2006).

The function of Fu is also different. In *Drosophila*, Hh-induced Smo accumulation is inhibited in *fu* mutants (Ascano and Robbins, 2004). Mice deficient in Fu do not exhibit phenotypes indicative of defective Hh signaling during embryonic development (Chen et al., 2005).

Sufu, like Ptch, is a negative regulator of Hh signaling pathway (Kogerman et al., 1999; Methot and Basler, 2000). *Drosophila* Sufu appears to inhibit Ci by blocking nuclear accumulation of full-length Ci (Methot and Basler, 2000; Wang et al., 2000b). In humans, *Sufu* is a tumor suppressor gene (Taylor et al., 2002). Sufu binds directly to the Gli proteins (Dunaeva et al., 2003; Stone et al., 1999). In the absence of signaling, Sufu retain Gli3 in the cytoplasm and promote its processing into a repressor form (Humke et al.) Initiation of Hh signaling allows dissociation of Gli proteins and Sufu, and the full-length Gli2/Gli3 proteins enter to the nucleus and work like transcriptional activators (Humke et al.).

In *Drosophila*, in response to Hh pathway activation, the zinc finger transcription factor Ci activate or repress the Hh target genes (Von Ohlen et al., 1997). The Ci activity control occur mainly at the post-transcriptional level (Aza-Blanc et al., 1997). In the absence of Hh ligand, full-length Ci is found in the cytoplasmic complex with Cos2, Fu and Sufu and it is a target for processing (Chen et al., 1999; Wang et al., 1999). Truncated Ci (CiR) translocates to the nucleus and repress expression of target genes (Wang et al., 1999). In the presence of Hh, processing of Ci is inhibited and the cytoplasmic complex dissociate. Ci translocates to the nucleus and activates expression of target genes.

In mammals, in the absence of Hh, the full-length Gli (Gli2/3) zinc finger transcriptional factors are proteolytically processed by the proteosome to generate C-terminally truncated GliR that actively represses a subset of Hh target genes (Pan et al., 2006; Pan et al., 2009; Rohatgi et al., 2007). The activation of Hh signaling suppresses Gli cleavage and allows the activator forms of Gli2A/Gli3A and Gli1 activate transcription of target genes such as *Ptch1, Gli1, CyclinD*. Expression of Ptch1 starts a negative feedback loop that shuts down Hh signaling.

1.2 Hedgehog signaling and Gli transcription factors

In vertebrates, the Hh signal transduction occurs via activation of a set of transcription factors: Gli1, Gli2 and Gli3. The *GLI1* gene was first isolated as an amplified gene of human in glioblastoma (Kinzler et al., 1987). By virtue of sequence similarity, two *GLI*-related genes, *GLI2* and *GLI3*, were subsequently identified (Ruppert et al., 1988). All Gli proteins bind to DNA through five zinc-finger domains that recognize the consensus Gli-selective sequence 5'-TGGGTGGTC-3' (Kinzler and Vogelstein, 1990).

Gli1 does not contain a repressor domain and is not processed (Dai et al., 1999; Kaesler et al., 2000; Sasaki et al., 1999), whereas Gli2 and Gli3 processing is phosphorylation- and proteasome-dependent. In the absence of Hh ligand, Gli3 is processed to the Gli3 transcriptional repressor, Gli3R (Wang et al., 2000a). In the presence of a Hh signal, Gli3 processing is inhibited and the full-length protein is activated (Huangfu and Anderson, 2005; Litingtung et al., 2002; Wang et al., 2000a; Wen et al.).

In contrast to Gli3, Gli2 is generally thought to act as a transcriptional activator. Expression of Gli1 in place of *Gli2* locus can rescue *Gli2* mutant phenotypes (Bai and Joyner, 2001). In the absence of Hh signaling, full-length Gli2 is processed via the ubiquitin-proteasome pathway to generate Gli2 repressor, Gli2R (Pan and Wang, 2007). Hh stimulation represses this processing and is thought to result in a predominance of full-length (presumably activator) forms of Gli2A.

GLI2 is reported to be the primary activator of Hh signaling and Gli1 is a secondary target, downstream of Gli2, which also acts as a transcriptional activator (Dai et al., 1999). Because Gli1 itself is a transcriptional target of the Hh pathway, Gli1 mRNA expression serves as a reliable indicator of activated Hh signaling, and elevated Gli1 expression was linked with cancer development and progression (Karhadkar et al., 2004; Sanchez et al., 2004).

1.3 Cilia in mammalian Hedgehog signaling

Genetic studies in mice revealed that a number of components of the intraflagellar transport (IFT) machinery are required for mammalian Hh signaling (Huangfu and Anderson, 2005; Huangfu et al., 2003). IFT proteins are essential for assembly and maintenance of cilia and flagella (Rosenbaum and Witman, 2002) and Hh signaling pathway components including Ptch1, Smo, Sufu and all Gli proteins have been found to localize to cilia (Corbit et al., 2005; Haycraft et al., 2005). In the abcense of Hh signaling, Smo is not released from Ptch1 and GLI3 is constantly proteolytically cleaved into the repressor GLI3R. In response to Hh ligand, Smo moves into cilia, where it suppresses Gli3 processing and so activates downstream signaling events (Corbit et al., 2005) (Fig. 2). In IFT mutants the localization of Smo to cilia is disrupted (May et al., 2005). The Gli transcription

factors and the negative regulator Suppressor of Fused (Sufu) are localized to cilia tips both in the presence and absence of ligand (Haycraft et al., 2005). GLI2 and full-length (the activator form) GLI3 functions are disrupted in the *IFT* mutants, but that GLI1 and GLI3R (the repressor form) can induce or repress the Hh pathway, respectively, regardless of IFT function (Haycraft et al., 2005). .

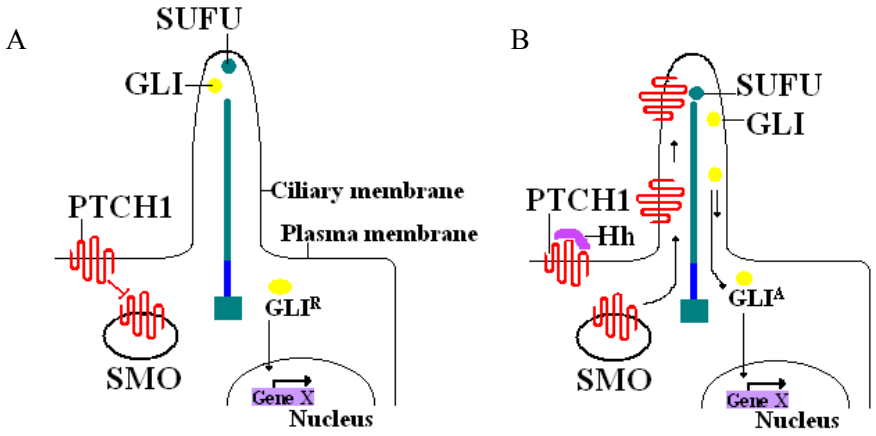

Figure 2. Hh signaling pathway in mammals cilia.
(A) No signaling. GLI is processed to create a transcriptional repressor, which is transported back to the cell body. (B) Signaling. Binding of Hh turns off Gli-repressive (GLI^R) processing and the active form of Gli (GLI^A) activates transcription of target genes. Adapted from (Fliegauf et al., 2007).

1.4 Hedgehog signaling in embryonic development

The importance of Hedgehog signaling as well as the identity and function of key Hedgehog signal transduction mediators was first established by work in *Drosophila*, where it contributes to the segmentation of embryos and the patterning of imaginal-disk outgrowth (Nusslein-Volhard and Wieschaus, 1980). Hedgehog signaling has since been proved to be essential for the regulation of vital vertebrate embryonic processes as well as for the development of many organ systems. These include differentiation of visceral endoderm, the establishment of left - right asymmetry (Levin et al., 1995), somite patterning (Johnson et al., 1994) and differentiation (Fan et al., 1995; Tellet et al., 1998), central nervous system patterning and differentiation (Echelard et al., 1993; Roelink et al., 1994), spermatogenesis (Bitgood et al., 1996) , specification of haemathopoietic and endothelial cells (Dyer et al., 2001), lung (Bellusci et al., 1997; Motoyama et al.,

1998), pancreatic (Hebrok et al., 2000) and intestinal development (Motoyama et al., 1998), hair cycle regulation (St-Jacques et al., 1998), tooth development (Hardcastle et al., 1998), limb patterning and outgrowth (Riddle et al., 1993), regulation of chondrocyte (St-Jacques et al., 1999; Vortkamp et al., 1996) and osteoblast differentiation (Chung et al., 2001; St-Jacques et al., 1999) and brain development (Hynes et al., 1997).

The Hh ligand function as morphogen is well-known in neural tube (Dessaud et al., 2008) and in the limbs (Butterfield et al., 2009). At the cellular level, the effect of Hh ranges from growth and self-renewal to cell survival, differentiation and/or migration (Jacob and Lum, 2007; Jiang and Hui, 2008). Thus, Hh signaling drives the proliferation of precursor cells in organs such as the skin (Ambler and Maatta, 2009) and cerebellum (Wechsler-Reya, 2003), and mediates interactions between epithelial and mesenchymal compartments that sculpt organs such as lung (Kimura and Deutsch, 2007).

1.5 Hedgehog signaling in adulthood

Hh signaling is also involved in adult tissue homeostasis. Thus, Hh plays a central role in the control of proliferation and differentiation of both embryonic stem cells and adult stem cells and stem-like progenitors. Studies in mice central nervous system have shown that Shh is required not only for patterning, but also for the proliferation of neuronal precursors (Rowitch et al., 1999). Inhibition of Shh signaling decreases proliferation of stem cells in the subventricular zone of the brain, while addition of Shh increases proliferation of neurospheres derived from subventricular zone cultures (Palma et al., 2005). Hh also regulates the proliferation of other stem cells, like human bone marrow-derived mesenchymal stem cells (Warzecha et al., 2006), epidermal stem cells (Adolphe et al., 2004) and somatic stem cells of the gonad (Zhang and Kalderon, 2001). Two papers established a strong role for Hh signaling in adult cardiovascular pathophysiology (Pola et al., 2003; Pola et al., 2001). More recent studies have demonstrated that Shh is required for cell proliferation in the subventricular zone, tuberculum olfactorium, and dentate gyrus (DG) of the hippocampal formation in adult animals (Blaess et al., 2006; Palma et al., 2005).

1.6 Hedgehog signaling in neurogenesis

Neurogenic stem cells are restricted to two specific brain regions in adult central nervous system (CNS): the subventricular zone (SVZ) and the hippocampal subgranular zone (SGZ) (Gage, 2000). Intensity of hippocampal neurogenesis is associated with learning abilities, memory strength and regulation of emotions and mood (Gould et al., 1999; Shors et al., 2001). Neurogenesis in the DG dramatically

decreases with age, and may contribute to age-related memory deficits (Drapeau et al., 2003; Kuhn et al., 1996).

It has been previously shown that Shh is a powerful regulator of adult hippocampal neurogenesis and is essential for the maintenance of the adult stem cell niches (Lai et al., 2003; Machold et al., 2003; Palma et al., 2005). In adult brain, Shh mRNA is expressed in the Purkinje cells of the cerebellum, SVZ, in motor neurons and in the forebrain structures, where it is thought to be anterogradely transported to the hippocampus (Traiffort et al., 1999). Within the hippocampus, expression of the Shh receptor Patched is seen within the hilar region, the pyramidal cell and the neurogenic niche of SGZ (Lai et al., 2003; Traiffort et al., 1999). Smo mRNA is found in the granule cells of the DG (Traiffort et al., 1998). Interestingly, nonpeptidyl Smo antagonists have been shown to inhibit the growth of medulloblastoma, whereas Smo agonists have been proved to be a potential therapeutic approach for Parkinson's disease and peripheral nerve damage (Borzillo and Lippa, 2005). Recently it has been shown that increase in adult hippocampal neurogenesis can be induced by electroconvulsive seizure (ECS) (Scott et al., 2000). The ECS-induced increase in proliferation of adult hippocampal progenitors was completely blocked in rats treated with cyclopamine, a pharmacological inhibitor of Shh signaling (Banerjee et al., 2005).

1.7 Hedgehog signaling pathway in genetic diseases

As it was previously discussed, Hh signaling pathway is important during embryogenesis. Mutations in Hh pathway components have been associated with genetic disorders such as holoprosencephaly (HPE), Greig's cephalopolysyndactyly (GCPS), Pallister–Hall syndrome (PHS) and Gorlin's syndrome (GS) (nevoid basal cell carcinoma syndrome) (Table 2). Patients carrying heterozygous mutations in *SHH* results in HPE, which affects the forebrain and face to various degrees, from the most extreme, lethal alobar type, to milder microforms that include small midline facial defects (Muenke and Beachy, 2000). 30–40% of GS have familial loss-of-function mutations in the *PTCH1* gene. Clinically, GS patients present congenital abnormalities with variable penetrance that include skeletal defects (e.g. general overgrowth, polydactyly, fused or bifid ribs), early onset of multiple BCCs and a higher-than-normal rate of other tumors, including medulloblastomas of the cerebellum (reviewed in (Goodrich and Scott, 1998; Ruiz i Altaba et al., 2002)). PHS has been shown to be caused by mutations in the middle third of the GLI3 gene, which have been predicted to result in a truncated GLI3 protein (Johnston et al., 2005; Kang et al., 1997; Wild et al., 1997). PHS is a pleiotropic disorder of human development that comprises a multitude of symptoms ranging from skeletal dysplasia to life-threatening malformations of the inner organs. Moreover, translocations as well as point mutations affecting one allele of the zinc finger gene *GLI3* has been demonstrated to be associated with GCPS, characterized by craniofacial and limb anomalies (Johnston et al., 2005).

Table 2. Genetic disorders associated with germline and/or somatic mutations in the Shh pathway components.

Genetic disorders	Mutation in	Reference
Holoprosencephaly	*SHH, PTCH1, GLI2*	(Ming et al., 2002; Odent et al., 1999)
Greig's cephalopolysyndactyly	*GLI3*	(Wild et al., 1997; Vortkamp et al., 1991)
Pallister–Hall syndrome	*GLI3*	(Kang et al., 1997)
Gorlin's syndrome (basal cell nevoid)	*SUFU PTCH1*	(Goodrich and Scott, 1998)

1.8 Hedgehog signaling pathway in cancer

Mutations in Hh pathway components are implicated in the develoment of variety of cancers. Hyperactivation of Hh signaling pathway, caused by mutation in *PTCH1*, leads to the development of the BCCs (Hahn et al., 1996). Mutations in *SMO* have also been associated with sporadic BCCs and primitive neuroectodermal tumors (Ruiz i Altaba et al., 2007). Overexpression of Shh in mouse skin produces basal cell carcinoma-like tumors (Oro et al., 1997). *SU(FU)* is located in a chromosomal region linked to several types of tumors, including glioblastoma multiforme, prostate cancer, malignant melanoma and endometrial cancer (Stone et al., 1999). Correlation between mutations in *SU(FU)* and predisposition to desmoplastic medulloblastoma has been established in children (Taylor et al., 2002). The involvement of GLI1 in brain tumors has been described (Kinzler et al., 1987). Also, increased expression of Gli1 was found in colon and lung cancer (Varnat et al., 2009; Watkins et al., 2003).

Overall, Hh pathway activation has been described in tumors of the brain/ cerebellum (glioma and medulloblastoma), the prostate, the oral cavity (oral squamous cell carcinoma), the muscle (rhabdomyosarcoma) and in cell lines derived from lung, digestive tract and pancreatic tumors and melanomas (Karhadkar et al., 2004; Kinzler et al., 1987; Sheng et al., 2004; Stecca et al., 2007; Thayer et al., 2003; Thompson et al., 2006; Watkins et al., 2003) (Fig. 3). The mechanisms of pathway activation include loss of SU(FU) function, missense mutations in *SMO*, overexpression of GLI1/GLI2, *GLI1* chromosomal translocation or GLI2 protein stabilization.

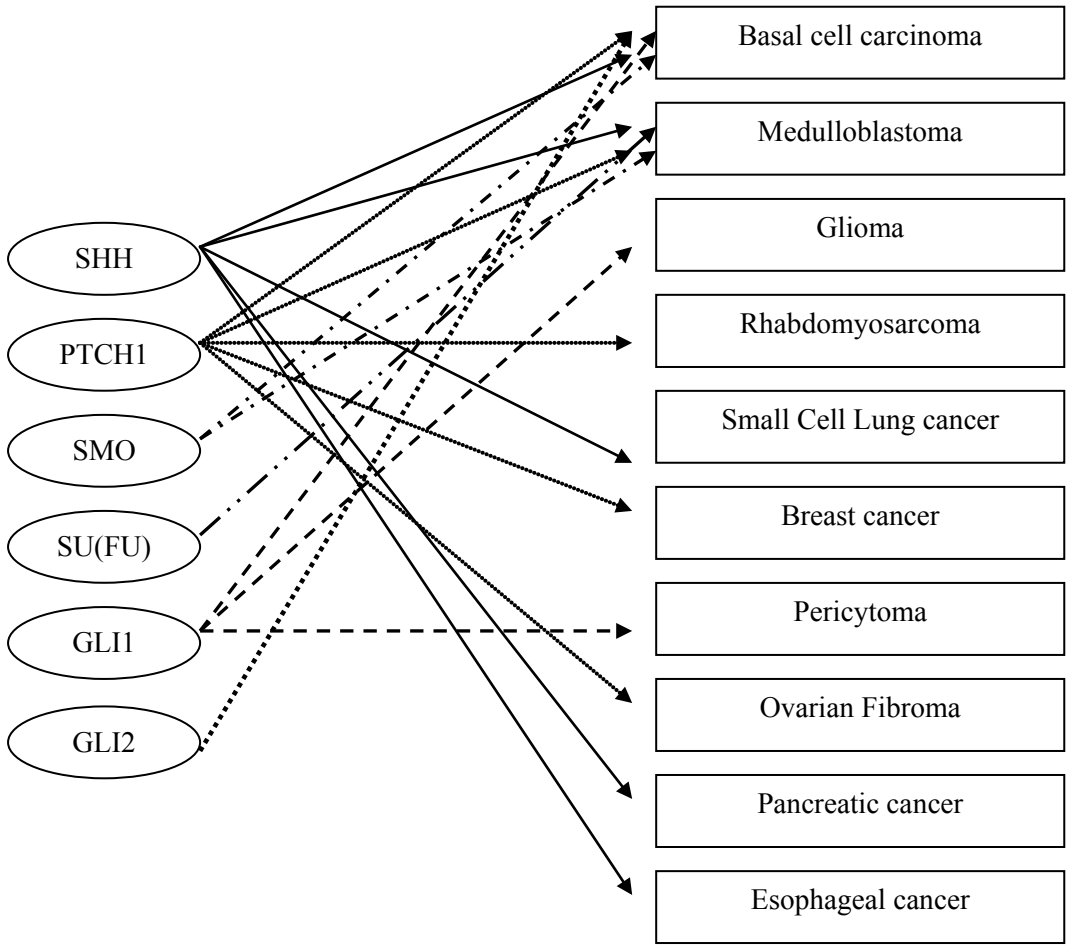

Figure 3. Cancers, associated with mutations in the Shh pathway components.

1.8.1 Hedgehog signaling pathway and prostate cancer

In the male reproductive tract, Hh signaling is necessary for the formation of the external genitalia and for the development of the prostate (Podlasek et al., 1999). SHH is expressed in the developing prostatic epithelium and inhibition of Hh signaling causes defects of ductal patterning and in the reduction of epithelial cell proliferation (Berman et al., 2004; Freestone et al., 2003).

In adults, there is compelling evidence on the involvement of Hh signaling in prostate tumorigenesis. A series of articles defined the role of Hh signaling in

the growth and metastasis of advanced prostate cancer (Fan et al., 2004; Karhadkar et al., 2004; Sanchez et al., 2004; Sheng et al., 2004). Expression degree of Hh pathway components and targets has been reported to be elevated in high-grade or metastatic prostate cancers (Karhadkar et al., 2004; Shaw et al., 2008; Sheng et al., 2004).

Bioinformatic analysis of data from genetic studies of familial prostate cancer showed mutations in genes, coding components of Hh signaling pathway, including *GLI1*, *GLI3*, *SMOH* and *SU(FU)* (Easton et al., 2003; Sanchez et al., 2004; Xu et al., 2003) (Fig. 4).

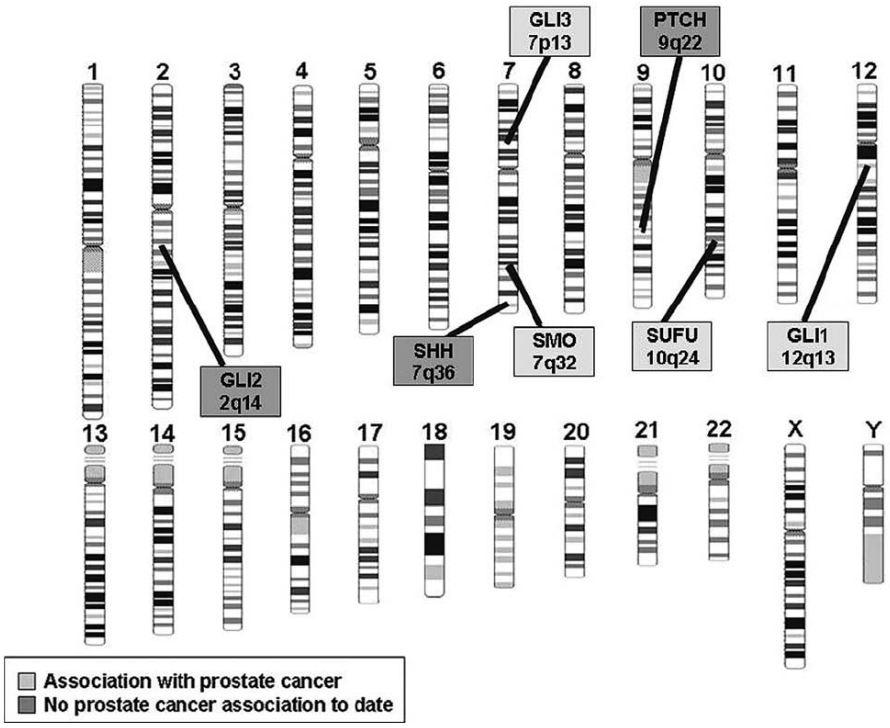

Figure 4. Prostate cancer genetic associations and the Hh pathway (Datta and Datta, 2006).

Knockdown of transcription factor *GLI2* by GLI2-specific small hairpin RNA in the prostate cancer cells resulted in significant down-regulation of the Hh signaling pathway, followed by inhibition of colony formation and growth of prostate cancer cell-line xenografts *in vivo* (Thiyagarajan et al., 2007). Ectopic expression of Gli2 in normal prostate epithelial cells resulted in accelerated cell cycle progression, especially transition through G_2-M phase and increased cell proliferation (Thiyagarajan et al., 2007).

Growth of the prostate cancer can be inhibited using specific Hh antagonists that block the pathway at three different levels: ligand, receptor and transcription factors. Thus, antibody against Hh was proven to inhibit proliferation

of primary prostate tumors and cell lines (Karhadkar et al., 2004; Sanchez et al., 2004). Cyclopamine treatment via blocking SMO activity have been reported to decrease viability and proliferation of prostate cancer *in vitro* (Karhadkar et al., 2004; Sanchez et al., 2004; Sheng et al., 2004) and in a xenograft mouse model (Karhadkar et al., 2004). Specific small-interfering RNAs against *GLI1* coding sequence was found to inhibit the growth of metastatic prostate tumor cell lines (Sanchez et al., 2004).

On the contrary, some studies have demonstrated that for instance prostate cancer cell line, PC-3, is not susceptible for activation or repression of Hh signaling pathway (McCarthy and Brown, 2008; Zhang et al., 2007). Also, recent *in vivo* data from LADY prostate cancer mouse model have shown that the expression level of Shh and other components of Hh signaling pathway (Ptc1, Gli1) are not altered during prostate tumor development (Gipp et al., 2007).

1.9 Hedgehog signaling pathway in neurological diseases

Hh signal transduction determines success in embryonic organogenesis and postnatal tissue repair throughout adulthood. However, little is known about the molecular mechanisms by which alterations in the cell signal transduction cause age-related pathologies.

Several studies suggested that endogenous Shh signaling is diminished by aging. It has been shown that angiogenesis is dependent on Shh activity in an age-specific manner (Riobo et al., 2006). Thus, injecting Shh into ischemic mice hind limbs, or Shh DNA into myocardial ischemia models, resulted in enhanced revascularization and organ salvage (Muller et al., 2000). In diabetic rats suffering from diabetic neuropathy, Shh treatment induced arteriogenesis and restored nerve function (Kusano et al., 2004). Moreover, Hh signaling was found to play a regulatory role in atherosclerosis development and progression, and its inhibition reduced plasma cholesterol levels (Beckers et al., 2007).

At the level of coordination between nervous and immune systems, age-specific changes in Hh signaling are also implicated in the pathophysiology of multiple sclerosis (MS) and Parkinson's disease (PD). Shh-N (N-terminal) levels are reduced in both grey and white matter from MS patients. However, the 45 kDa precursor Shh protein is still present, suggesting a defect in the autocatalytic cleavage reaction (Mastronardi et al., 2003). Intrastriatal injections of Shh-N (in form of purified recombinant protein or delivered by adenoviral vector) resulted in partial protection of dopaminergic nigrostriatal neurons in a rat model of PD (Hurtado-Lorenzo et al., 2004; Tsuboi and Shults, 2002). This protection likely occurs via normal Shh signaling, since transfection of Gli1 encoding DNA in the rat striatum had the similar effect (Hurtado-Lorenzo et al., 2004).

1.10 Hedgehog pathway inhibitors and activators

Hedgehog signaling pathway is attractive target for drug discovery scientists because of its important role in the embryonic patterning, the development of many tissues and somatic structures as well as maintaining and repairing tissues in adults. Its role in tumorigenesis is also an important factor.

Several compounds altering SMO activity have been developed and second generation of SMO antagonists have entered phase I clinical trials (Mahindroo et al., 2009). These drug candidates are claimed to be effective in situations where pathway is stimulated by ectopic Hh ligand production or alterations at the PTCH/SMO level. Cyclopamine, a teratogenic steroidal alkaloid derived from plant *Veratrum californicum*, was associated with holoprosencephaly and other teratogenic effects in lambs. In 1998, it was reported that this compound blocks the Shh signaling pathway (Cooper et al., 1998). Modified cyclopamine (KAAD-cyclopamine) is currently in preclinical development. In some cases, cyclopamine and other SMO antagonists are not likely to be effective, favoring the development of synthetic GLI inhibitors. Recently compounds targeting Gli-mediated transcription have also been reported (Tabl. 3) (Arai et al., 2008; Lauth et al., 2007).

Table 3. Hh pathway inhibitors.

Inhibitors of Shh	**Reference**
Robotnikinin	(Stanton et al., 2009)
Inhibitors of Smo	
Cyclopamine and its derivatives	(Chen et al., 2002a), (Tremblay et al., 2008), (Zhang et al., 2008), (Kumar et al., 2008)
Noncyclopamine-Scaffold compounds (SANT, aminoprolines, quinazolinones and quinazolines, biarylcarboxamide, bisamide, benzimidazole, pyridyl and quinoxaline derivates, triazole derivates)	(Chen et al., 2002b), (Williams et al., 2003), (Brunton et al., 2008; Peukert et al., 2009; Remsberg et al., 2007)
Inhibitors of alkohol dehydrogenase IV	
JK184, JK35	(Lee et al., 2007)
Inhibitors of Gli-mediated transcription	

GANT61, GANT58	(Lauth et al., 2007)
Natural compounds for inhibition of Gli transcription	
Extract from plants *Zinginber zerumbet, Physalis minima, Zizyphus cambodiana*	(Arai et al., 2008; Hosoya et al., 2008)

Cholesterol and other certain oxysterols, naturally occurring products, participate in the activation of the Shh signaling pathway (Corcoran and Scott, 2006; Dwyer et al., 2007). The oxysterols do not bind directly to Smo, but they may indirectly affect Smo, perhaps by stabilizing it in the conformation where it is less sensitive to Ptch1-mediated repression and activate Gli-mediated transcription in a variety of cell types (Corcoran and Scott, 2006; Dwyer et al., 2007).

Purmorphamine is a synthetic Shh pathway agonist, discovered by Schultz and co-workers (Wu et al., 2002). Initially, purmorphamine was found to induce osteoblast formation in C3H10T1/2 cell line. Subsequent gene expression profile studies showed that purmorphamine upregulates *Gli1* and *Ptch1*, but not *Ihh*, *Dhh*, or *Shh*, confirming purmorphamine's role as a Shh pathway agonist (Wu et al., 2002).

Lately, several small molecule activators of the Shh pathway have been identified (Chen et al., 2002b; Frank-Kamenetsky et al., 2002). Screening of 140000 compounds for the ability to activate luciferase expression in the luciferase reporter assay in the absence of Shh protein led to the identification of the leiosamine family of compounds (Fig. 5).

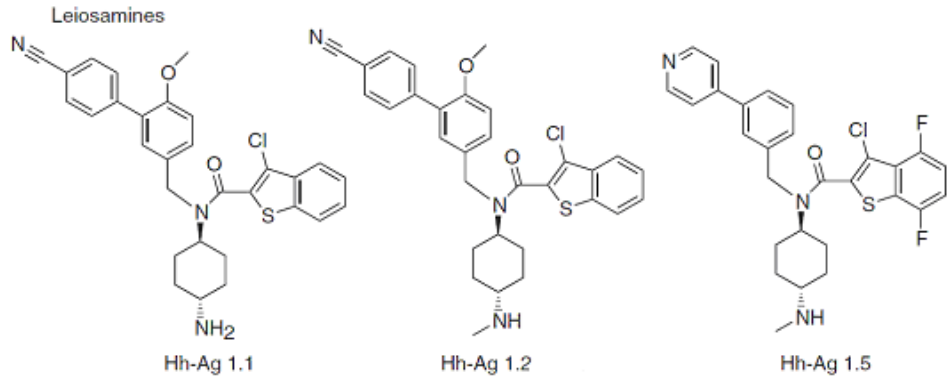

Figure 5. The structures of small molecules that activate Hh signaling.

The leiosamine family of compounds can activate Hh signaling by binding to Smo heptahelical domain (Fig. 6). Hh-Ag 1.1 was the orginal compound identified in the high-throughput screen by Frank-Kamenetsky et al. (Frank-Kamenetsky et al., 2002) with an EC50 of 3 μM in their luciferase reporter assay.

Hh-Ag 1.2, a more potent derivative, was characterized by Chen et al. (Chen et al., 2002b). Hh-Ag 1.5, refered as SAG, is the most potent Hh agonist reported (Frank-Kamenetsky et al., 2002), with an EC50 of 1 nM. Moreover, SAG and cyclopamine activities are mutually antagonistic, consistent with opposing actions on a common target (Chen et al., 2002b).

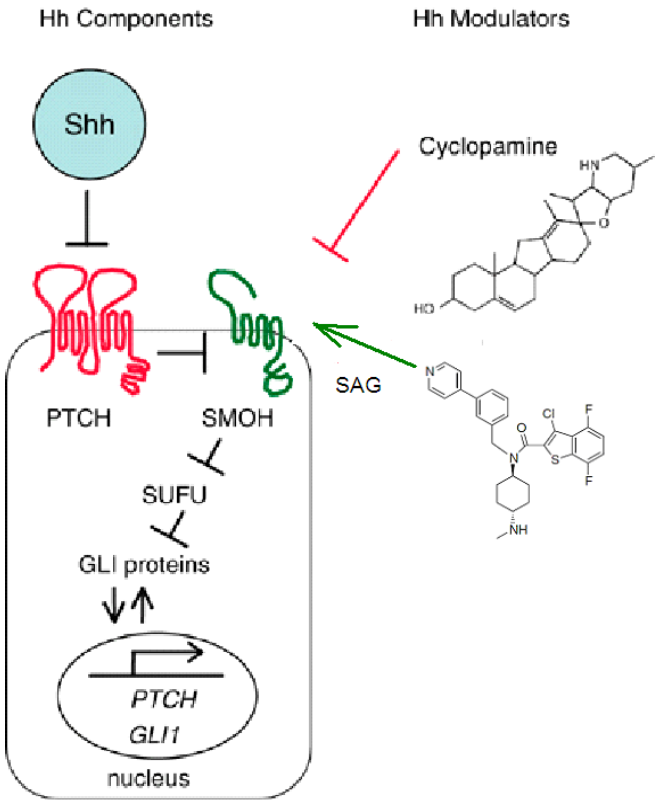

Figure 6. Schematic representation of the pharmacological modulators' action target in the Hh pathway. Depicted are major Hh pathway components and gene targets *PTCH* and *GLI1*. Small molecule antagonist (cyclopamine) and agonist (SAG) that modulate SMOH activity (modified from (Ehtesham et al., 2007)) are shown.

Importantly, the Hh pathway agonists can activate Shh signaling pathway in a wide variety of *in vitro* and *in vivo* assays (Frank-Kamenetsky et al., 2002; Harper et al., 2004; Paladini et al., 2005; Wichterle et al., 2002). The SAG, Smo agonist, is a small molecule that directly binds Smo, causes its accumulation in cilia, and potently activates target gene transcription (Chen et al., 2002b; Frank-Kamenetsky et al., 2002; Rohatgi et al., 2007).

These molecules feature many properties that make them attractive as potential therapeutic agents including their low-nanomolar potencies and favorable pharmacokinetic profiles in targeted tissues. Also, a great advantage of these compounds is that the molecules remain active after oral administration and are able to cross the blood-brain and placental barriers in humans.

2. AIMS OF THE STUDY

The general aim of this thesis was to investigate the role of Shh pathway in neuro- and cancerogenesis in adulthood.

Specific aims were:

1. To determine the mRNA structure of the human transcription factor GLI2. To analyse the alternative mRNA splicing forms of human GLI2. To clarify the expression pattern of full length and spliced isoforms of the human transcription factor GLI2 in normal adult tissues and cancer cell lines (Paper I).
2. To generate antibody against human transcription factor GLI3 for the further analyses and usage (Paper II).
3. To determine the role of Shh pathway in prostate cancer (Paper III).
4. To determine the effect of Shh and Smoothened agonist (SAG) on proliferation, survival and differentiation of hippocampal *de novo* produced cells *in vitro* and *in vivo* (Paper IV).

3. METHODS

All molecular biology procedures were performed according to the standard practice (Sambrook and Russell, 2001) or according or according to the manufacturers' instructions. The following methods were used (refer for the detailed description to the original papers in the end of the thesis):

Paper I

- Bioinformatic analyses of gene and mRNA structure;
- Reverse transcription, DNA amplification by PCR and cloning;
- *In vitro* translation;
- Cell culture and transfection;
- Luciferase assay.

Paper II

- Expression and purification of recombinant protein;
- ELISA;
- SDS-PAGE and Western blot analysis;
- RNA isolation and RT-PCR;
- Immunocytochemistry and immunohistochemistry.

Paper III

- Immunohistochemistry;
- RNA isolation and RT-PCR.

Paper IV

- Cell line culture and primary cell culture;
- Double immunocytochemistry;
- RNA isolation and qRT-PCR;
- Luciferase assay;
- Animals surgery;
- Double immunohistochemistry.

4. RESULTS

Paper I

A potential role of alternative splicing in the regulation of the transcriptional activity of human GLI2 in gonadal tissues

In this study we characterized the exon-intron organization of human *GLI2*. The alignment of mouse *Gli2* mRNA (GenBank: X99104), human *GLI2* mRNA (GenBank: NM_030379) to the human genomic contig (GenBank: NT_022135) showed that human *GLI2* consist of the 14 exons, similarly to mouse *Gli2* (Paper I Fig. 1).

Unlike the mouse *Gli2*, human *GLI2* contains two alternative 5' noncoding exons (exon 1a and 1b). RT-PCR analysis revealed that both exons are expressed in defferent tested cell lines and in tissues.

Comparing the published 3' UTR of human *GLI2* (Tanimura et al., 1998) with the 3' UTR of mouse *Gli2* revealed the absence of two thirds of the 14th exon of human *GLI2*. We identified the missing part of 3' UTR of human *GLI2* and showed that it contains a noncanonical polyadenylation signal ATTAAA.

We next analysed the expression of *GLI2* mRNA in different human adult tissues and cell lines. *GLI2* mRNA was strongly expressed in the ovary, testis, pancreas, liver, small intestine and thymus (Paper I Fig. 5), while low level of expression was observed in placenta, prostate and colon. Almost no *GLI2* mRNA expression was detected in heart, brain and peripheral blood leukocytes.

We identified novel alternatively spliced forms of human *GLI2* mRNA (Paper I Fig. 6). These transcripts were present exclusively in ovary, testis and several cell lines (SH-SY5Y, 293, NTera2D1, SK-N-SH, MDA-231). We cloned the identified spliced forms (*GLI2*Δ3 and *GLI2*Δ4-5) and analyzed their activation or repression potential in the luciferase reporter assay. We found that GLI2Δ4-5 increased the reporter activity about 10-fold, whereas GLI2Δ3 activity was comparable to that of the GLI2fl.

Paper II

Generation and characterization of mouse monoclonal antibody 5E1 against human transcription factor GLI3

In this study we produced a monoclonal antibody against transcription factor GLI3 for the further characterization of the Gli3.

Human His-tagged GLI3 protein was expressed in *E. coli*, purified and used for immunization of Balb/c mice. Hybridoma screening revealed a panel of monoclonal antibodies. After specificity analysis by ELISA, one antibody clone - 5E1 - was chosen and characterized further in different immunological assays (western blotting, immunohistochemistry and immunocytochemistry).

RT-PCR analysis showed the presence of *Gli3* mRNA in human NTera2D1 (teratocarcinoma) and mouse TM3 (Leydig-like) and TM4 (Sertoli-like) cell lines. The endogenous GLI3 protein was detected in the cytoplasm of NTera2D1 cells by immunocytochemistry. Application of cyclopamine to this cell line changed the localization of GLI3 from cytoplasmic to nuclear (Paper II, Fig. 4A, C).

Although Gli2 and Gli3 share homology in repressor domain region, the novel antibody does not cross-react with Gli2. Anti-GLI2 antibody showed mainly nuclear localization of GLI2 protein in NTera2D1 cells (Paper II, Fig. 5A, B).

Paper III

Sonic Hedgehog pathway activity in prostate cancer

In this study we identified age-related dependence of prostate cancer development on activation of Shh signaling pathway in transgenic adenocarcinoma mouse prostate (TRAMP) mice.

The expression of the following components of Shh signaling pathway was investigated: Shh, Gli1, Gli2 and Gli3 (Paper III, Fig. 2). We examined changes in the number of positive cells in prostate by immunohistochemistry at three time points - 12, 17 and 21 weeks of age.

We found that the number of Shh-positive cells was increased 5-fold during cancer progression in TRAMP mice compared to wild type (WT) mice. Older TRAMP (17 and 21 weeks of age) had increased number of Gli1 and Gli3 positive cells compared to WT. Detected increase in the Gli1 positive and Gli3 positive cell number as well as decrease in the number of Gli2 cells was age-dependent in the TRAMP mice. Interestingly, the number of Shh-positive cells significantly decreased in WT mice in age-dependent manner.

Increase of *Shh*, *Gli1* and *Gli3* and decrease of *Gli2* mRNA was confirmed by RT-PCR.

We also examined changes in the number of FoxA1- and Notch1-positive cells, two important regulators of cell proliferation and differentiation (Paper III, Fig. 3, panel II). The number of FoxA1-positive cells was increased three-fold in older TRAMP mice compared to WT mice.

We did not detect any significant difference in Notch1-positive cells between TRAMP and WT mice at any time points.

Paper IV

Smoothened agonist augments proliferation and survival of neural cells

In this study we detected SAG induced Gli-dependent luciferase activity in Shh-LIGHT2 cells. qRT-PCR showed SAG concentration dependent increase of Gli1 mRNA (Paper IV, Fig. 1).

We also detected direct effect of SAG application on cortical/hippocampal progenitor cells *in vitro*. The number of newly produced BrdU-labeled cells was increased in cell culture and the highest effect was reached with SAG concentration of 1 nM.

Double-immunocytochemistry with anti-BrdU and anti-GFAP (glial marker) or anti-TUJ1 (neuronal marker) antibodies revealed no effect of SAG administration on the differentiation of the precursors derived from neuronal culture (Paper IV, Fig. 2B and C).

Next we tested the neurotoxicity of Shh and SAG on the primary cerebellar granular cells. We detected increased neuronal death induced by application of Shh compared to SAG at the concentration of 50 nM (Paper IV, Fig.3).

In vivo study showed that intracerebroventricul administration of Shh or SAG at doses 2.5 nmol or 2.5 µmol respectively, intracerebroventriculary, significantly increases the number of newly produced cells in adult rat hippocampus (Paper IV, Fig. 4A, B). BrdU-labeled cells, often found in clusters, were distributed in the inner layer of the granular cell layer and in the hilus of the dentate gyrus. Detected increased number of *de novo* produced neural cells three weeks after treatment indicated drug-induced prolonged *in vivo* survival of newly produced cells.

The phenotype of *de novo* produced, BrdU-labeled cells in adult rat hippocampus, was determined by double immunohistochemistry with antibodies against BrdU and the glial marker, GFAP, or the neuronal marker, TUJ1 (Paper IV, Suppl. Fig. 2 and 3). Neither Shh nor SAG administration affected the proportion of cell differentiation into neurons or glial cells (Paper IV, Fig. 4).

5. GENERAL DISCUSSION AND PERSPECTIVES

Shh pathway acts on gene expression through the activity of the Gli transcription factors family – Gli1, Gli2 and Gli3. It has been proposed that Gli2 and Gli3 are the primary mediators of Hh signaling. The expression and posttranslational modification of the various Gli family members create a distinct combination of Hh transcriptional activators and repressors that results in a specific biological readout (Ruiz i Altaba et al., 2007). One of these ways is the processing of Gli2 and Gli3 (Sasaki et al., 1999). Processing is phosphorylation- and proteasome- dependent (Pan et al., 2006; Pan et al., 2009). Another possibility of creating proteins with different activities is mRNA alternative splicing.

Previous studies suggested that human *GLI2* mRNA may exist in at least four different isoforms, which can be detected in tumor cell lines or tissues (Tanimura et al., 1998; Tojo et al., 2003). We analyzed the expression of human GLI2 spliced forms (skipping exon 3 and exons 4-5) (Paper I) and determined enhanced expression of GLI2Δ4-5 in Gli-dependent luciferase reporter assay. The detected enhancement was most likely due to the loss of repressor activity, i.e. excision of the repressor domain (or part of it) by alternative splicing. These results showed that alternative splicing is involved in the deletion of the repressor domain encoded by exons 4 and 5 and may be responsible for the enhanced activation of GLI2 protein. Moreover, we identified the tissue-specific pattern of GLI2 spliced forms' expression in normal tissues. Thus, GLI2Δ3 and GLI2Δ4-5 spliced proteins were uniquely expressed in human ovary and testis. Particular expression pattern suggests a specific role of GLI2 as activator in normal adult human gonadal tissues. The determination of the factors causing alternative splicing was not in the scope of our studies but certainly warrants further investigation. Changes in such factors may cause increase in proportion of active GLI2, which in turn leads to the overexpression of GLI2 target genes and subsequent tumorigenesis.

Gli3 is indispensable part of the Hh pathway and its analysis is absolutely required to fully understand the mechanisms of Hh pathway activation/inhibition. The lack of commercially available antibodies against transcription factor GLI3 incited us to develop this reagent (Paper II). Cell immunocytochemistry indicated that GLI3 is located in the cytoplasm in human teratocarcinoma cell line cells, where the Shh signaling pathway is known to be activated (Satoh and Kuroda, 2000). Application of cyclopamine to the cells blocks Shh pathway transduction and as a result, GLI3R form enters to the cell nucleus. Obtained results indicated that monoclonal antibody against GLI3 recognizes endogenous GLI3 in GLI3R and also in GLI3FL form. Calculated by NCBI BLAST algorithm similarity between human GLI3 repressor domain and mouse Gli3 repressor domain was 97, 4%. Immunohistochemistry in mouse embryo samples (10.5 days post-coitum) showed that antibody recognizes also mouse Gli3 repressor motif. Immunocytochemistry using antibodies against human GLI3 and human GLI2 showed specificity of

created antibody without any cross-reactivity against human GLI2. Thus, developed monoclonal antibody 5E1 against human transcription factor GLI3 repressor motif was highly specific and was used in subsequent studies.

The role of Hh pathway in prostate cancer development is not clearly established. SHH pathway components, for example GLI, are detected in adult human prostate cancer with enhanced levels as compared to those in the healthy conditions (Karhadkar et al., 2004; Sanchez et al., 2004; Sheng et al., 2004). Although many recently identified genes have been implicated in the progression of prostate cancer, relatively few were suggested to initiate prostate tumorigenesis. It was demonstrated that Hedgehog over-expression (via introducing a Hedgehog expressing vector by intra-prostate injection) caused prostate tumorigenesis and such transformation involved morphological changes within both the epithelial and the stromal prostate compartments (Chen et al., 2006). On the other hand, in LADY prostate cancer mouse model Shh pathway was inactive and did not influence tumor formation (Gipp et al., 2007).

We identified that TRAMP prostate cancer mouse model is the first prostate cancer mouse model where tumor formation is correlated with Shh pathway activation (Paper III). According to our data, Shh pathway activity increased at the 21st week of age in TRAMP mice. We found that Shh, Gli1 and Gli3 expression was enhanced in TRAMP mice compared to WT mice at the same week of age. To our surprise, the transcription factor Gli2 was decreased at both protein and mRNA level in TRAMP mice. The possible explanation may be that Gli2 mRNA spliced to produce protein modification with higher activity and even small amounts of active-spliced Gli2 was able to induce expression of the target genes. As we found in Paper I, Gli2 spliced forms are present in tumor cell lines, like NTera2D1 (teratocarcinoma), SH-SY5Y (neuroblastoma) and G168P44 (glioma). Many groups have documented abnormal or alternative mRNA splicing in cancer cells. Thus, alternative splicing of *DNMT3B, BRCA1, KLF6, Ron, Gemin5* genes, has been associated with cancer formation (Bonatti et al., 2006; DiFeo et al., 2008; Fabbri et al., 2007; Klinck et al., 2008). Moreover, alternative splicing (alternative 5' and 3' splice site selection and intron retention) was found to be elevated in cancerous compared to normal tissues (Kim et al., 2008). Our findings suggested that the alternative splicing of Gli2 may be an important factor in tumor initiation; however, additional studies are required to clarify this matter.

It was found earlier that Hedgehog signaling modulated metastatic potential of rodent prostate cancer cell lines (Karhadkar et al., 2004). Based on the breeder description, TRAMP mice develop prostate adenocarcinoma by the 24th week and metastasis by the 30th week of age (www.jax.org). Age-dependent activation of the Shh pathway in TRAMP transgenic mice detected in our studies points out the possible role of Shh pathway in metastasis spreading. Invasion and tumor metastasis are closely related and both occur within tumor-host microecology, where stroma and tumor cells exchange enzymes and cytokines that modify the local extracellular matrix, stimulate cell migration, and promote cell

proliferation and tumor cell survival. The most important changes occur in genes which regulate cell cycle progression, extracellular matrix homeostasis and cell migration. In a variety of epithelial cancers aberrant Hh signaling was recently detected and it has been shown that the Hh target gene *Gli1* induces expression of Snail, which represses E-cadherin and induces epithelial–mesenchymal transition, a process also exploited by invasive cancer cells (Fendrich et al., 2007). Moreover, it was reported that the SHH signaling pathway, acting through the TGF-β/ALK5 pathway, may selectively contribute to tumor cell motility and invasion in gastric cancer (Yoo et al., 2008). Despite the critical role that Hh signaling plays in the promotion of tumorigenesis, the molecular and cellular mechanisms behind Hh regulation in prostate tumor metastatic behavior are unknown. Recent study showed that combined use of both the selective inhibitors of EGFR and Hh signaling cascades, may represent a promising strategy for improving the current standard antihormonal and radiotherapeutic treatments used in the early stages against localized prostate cancers (Mimeault et al., 2007). Further studies are necessary to define the exact role of Shh pathway in prostate cancer metastasis, particularly its influence on the expression of metastasis-associated genes.

Another group of diseases where Shh pathway represents an attractive target for therapy are adulthood disorders associated with diminished neurogenesis (depression, Parkinson's disease, etc). Previous studies have shown that Shh is involved in the regulation of the proliferation of neuronal progenitors in adult brain (Lai et al., 2003). In our studies (Paper IV), *in vitro* SAG, similarly to Shh, promoted proliferation of the cortical/hippocampal cells without significant effect on their differentiation pattern. In addition to *in vitro* experiments, we also demonstrated the effects of Shh and SAG on the survival of newly born cells in the DG of adult rats. While majority of newly proliferated cells in the DG die shortly after birth, some of them survive and differentiate (Cameron et al., 1993). Presence of persistently increased number of BrdU-labeled cells three weeks after Shh or SAG administration in rat hippocampus suggests that these molecules serve as a survival factors for hippocampal neural cells. Our *in vivo* results from cell proliferation, differentiation and survival studies in rats suggest that Shh or SAG-stimulated hippocampal neurogenesis is likely to be the consequence of increased cell proliferation and survival rate rather than modification of the commitment of *de novo* produced cells. A similar neuroproliferative effect with unchanged cell differentiation pattern was observed *in vitro* in adult cortical/hippocampal progenitor cells. Importantly, *in vitro* studies have shown that unlike Shh, SAG does not exhibit sustained neurotoxic effect. Thus, our studies suggest the use of nonpeptidyl Smo agonists as a potential therapeutic approach for neurodegenerative diseases.

CONCLUSIONS

1. The mRNA structure of human transcription factor *GLI2* is clarified.

2. Two alternative spliced forms of human transcription factor *GLI2* are characterized.

3. Alternatively spliced forms Δ3 and Δ4-5 *GLI2* act as activator in normal adult human gonadal tissues.

4. Monoclonal antibody 5E1 against human transcription factor GLI3 is generated, characterized and successfully used in experimental settings.

5. Prostate cancer age-dependent development and possibly its metastasizing are associated with the activation of Shh signaling pathway.

6. Shh and Smoothened agonist SAG enhance the proliferation of neural progenitors *in vitro*.

7. Shh and Smoothened agonist SAG increase survival of neural newly born neural cells *in vivo*.

8. Neither Shh nor Smoothened agonist SAG affects the pattern of differentiation of the neuronal progenitors *in vitro* and *in vivo*.

9. Nonpeptidyl Smoothened agonist SAG bears the therapeutic potential in treatment of neurodegenerative diseases.

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ABSTRACT

Hedgehog (Hh) signaling is crucially important during embryonic development and in adulthood.

Hh signaling pathway is initiated by the binding of the secreted morphogen, Hh, to its receptor, Patched 1 (Ptch1). As a result of this interaction, the inhibition of another receptor Smoothened (Smo) is relieved and activation of the glioma-associated (Gli) family of zinc finger transcription factors is initiated. In vertebrates, there are three Gli proteins, where Gli1 and Gli2 are activating Hh target genes, whereas Gli3 is thought to act mainly as a repressor.

In the first set of experiments performed within the scope of this thesis, we produced monoclonal antibody 5E1 against human transcription factor GLI3. The specificity of 5E1 antibody was confirmed by different immunological methods: immunocytochemistry, ELISA and mouse embryo immunohistochemistry.

In the scope of this work, we identified the exact mRNA structure of human transcription factor *GLI2* and described its expression pattern in normal human tissues. Furthermore, we identified two alternatively spliced forms of GLI2 and their unique expression in human normal gonadal tissue. Interestingly all tested human cancer cell lines expressed *GLI2* alternatively spliced forms. Moreover, these spliced GLI2 proteins activated Gli-dependent reporter with higher efficacy compared to full length GLI2 protein.

Mutations and other regulatory errors in the Hh pathway are associated with a number of birth defects and certain cancers. Recent data indicate that Hh signaling is activated in majority of metastatic prostate tumors and subsets of locally metastasized tumors. In this thesis we have shown age-dependent activation of Shh pathway in the transgenic prostate adenocarcinoma mice model (TRAMP). Importantly, these TRAMP mice are the first transgenic model reported to have activated Shh pathway.

Chronic neurodegenerative diseases such as Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis and multiple sclerosis are associated with degeneration of discrete populations of neuronal elements. In this study we characterized the influence of Smoothened agonist SAG on the neural cells *in vitro* and *in vivo*. We found that SAG induced *de novo* production of neural cells without affecting their further commitment rate. Furthermore, tested SAG compound did not have pronounced neurotoxic effects *in vitro*. Based upon our results SAG compound represents a promising drug candidate for the treatment of disorders associated with excessive neuronal death and warrants further investigation.

KOKKUVÕTE

Hedgehogi (Hh) signaalirada mängib olulist rolli organismi embrüonaalses arengus, ku igi ta jääb a ktiivseks k a t äiskasvanueas. Signaali e dastamine algab sekreteeritud morfogeeni, Hh, seondumisega oma retseptorile, Patched1-le (Ptch1). Selle t ulemusel va baneb S moothened (Smo) j a kä ivitatakse signaalikaskaad, m is viib G li pe rekonna t sink-sõrm tr anskriptsioonifaktorite a ktivatsioonini. Se lgroog-setes esineb kolm Gli valku, mis erinevad oma funktsiooni poolest. Gli1 ja Gli2 on Hh raja sihtmärkgeenide aktiveerijad, Gli3 aga käitub peamiselt repressorina.

Esmalt töötasime välja m onoklonaalse an tikeha i nimese t ranskriptsioonifaktori GL I3 v astu. T õestasime 5 E1 an tikeha sp etsiifilisust er inevate i mmunoloogiliste m eetoditega: i mmunotsütokeemiaga rakukultuuris, ELI SA-ga j a i mmunohistokeemiaga hiire embrüo lõikudel.

Peale selle t uvastasime a ntud töös inimese t ranskriptsioonifaktori *GLI2* täpse mRNA st ruktuuri ja k irjeldasime sel le ek spressioonimustrit inimkudedes. Veelgi en am – identifitseerisime *GLI2* kaks alternatiivselt splaissitud v ormi ja määrasime n ende spetsiifilist ek spressiooni i nimese suguorganite k udedes. Normaalsete k udede k õrval an alüüsisime sa muti k asvajarakuliine, mis l isaks täispikale transkriptile ekspresseerisid ka kahte alternatiivselt splaissitud mRNA-d. Leidsime, et alternatiivselt sp laissitud GL I2 v alkudel on su urem märklaudgeenide aktivatsioonivõime võrreldes täispika GLI2-ga.

Hh r aja mutatsioone j a r egulatsiooni vigu seo statakse mitmete sü nnidefektide ja teatud vähi tüüpidega. Hh signaali aktivatsioon on tuvastatud enamikul metastaatiliste eesn äärme kasvajate ja m itmete lo kaalselt metastaseeruvate t uumorite puhul. Antud väitekirjas oleme näidanud ajast sõltuvat Sh h raja aktivatsiooni tr ansgeense p rostata a denokartsinoomi h iire mudelil (TRAMP). Olulise aspektina märgime, et eelmainitud TRAMP hiir on esimene eesnäärmevähi transgeenne mudel, kus Shh rada on aktiivne.

Kroonilisi ne urodegeneratiivseid ha igusi, na gu P arkinsoni j a H untingtoni tõbi, amülotroofne lateraalne skleroos ning hulgiskleroos, on s eostatud neuronaalsete elementide er inevate p opulatsioonide d egeneratsiooniga. Antud uu ringus oleme iseloomustanud Smo agonisti (SAG) mõju neuraalsetele rakkudele *in vitro* ja *in vivo*. L eidsime, et S AG indutseeris n euraalsete raakkude *de novo* produktsiooni nende edasist diferentseerumise suunda mõjutamata. Veelgi enam – kasutatud SAG ühend e i avaldanud ne urotoksilist mõju *in vitro*. T uginedes saad ud t ulemustele, võib j äreldada, e t S AG ühe nd on potentsiaalne r avimikandidaat neuraalsete rakkude ülemäärase surmaga s eotud h aiguste t ulevikuravis n ing v ajaks seet õttu edasist uurimist.

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DISSERTATIONS DEFENDED AT TALLINN UNIVERSITY OF TECHNOLOGY ON *NATURAL AND EXACT SCIENCES*

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