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REGULATION OF HEDGEHOG SIGNALLING DURING DEVELOPMENT AND CARCINOGENESIS

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To the loves of my life,
Fredrik, Alexander
and Felicia

ABSTRACT

Hedgehog (Hh) signalling is one of the most important signalling networks in the body and, when miss-regulated, has been implicated in many human tumours, particularly that of skin (Basal Cell Carcinoma, BCC) and brain (medulloblastoma). BCC most commonly arises due to mutations in the gene sequence of the Hh receptor protein PATCHED1 (PTCH1). The *PTCH1* gene is reported to have four first exons and we showed in paper I that *PTCH1* is transcriptionally regulated by three independent promoters. Additionally, we provided evidence of a single functional Gli-consensus site in the *PTCH1* promoter region and revealed that transcriptional activation, mediated via the Hh signalling pathway, is fully dependent on this single Gli-binding site. Earlier studies have revealed that one of the PTCH1 isoforms shows induced levels in BCC, but so far no connection between these isoforms and breast tumourigenesis exists. However, several lines of data implicate a role for Hh in breast carcinogenesis, and in support of this we found that an altered expression of the human Hh effector protein, GLI1, in the mammary epithelial cells of mice could induce the formation of heterogeneous mammary tumours (paper III). GLI1-induced mammary tumour cells showed high expression of the progenitor cell marker, Bmi-1 and cytokeratin 6 as well as proliferative, cell survival and metastatic markers. GLI1-induced mammary tumours did not fully regress after removal of the transgenic expression. However, the mammary tumour appearance shifted towards a more stromal epithelial content. Prior to tumour formation GLI1-induced the formation of hyperplastic lesions and defective terminal end buds in both male and female mammary glands. In addition, transgenic GLI1 mice were unable to feed their pups (Paper II). This failure could molecularly be explained by a reduced alveolar differentiation and milk gene expression during pregnancy. In addition, mammary glands with induced expression of GLI1 showed an increased expression of the GLI1 response gene *Snail*, coupled with reduced expression of E-cadherin and Stat5. Removal of the transgenic expression did not restore the lactation ability of these mice. We further demonstrated that the involution process was impaired in the GLI1 transgenic mammary glands, with cellular residuals residing in the mammary ducts. Interestingly, embryonic mammary and salivary gland development, according to branching morphogenesis share common mechanisms and when GLI1 expression was induced in salivary epithelial cells, hyperplastic lesions with basaloid content appeared in 100% of the transgenic salivary glands (Paper IV). These lesions showed strong homology with the hyperplastic lesions which appeared in the GLI1-induced mammary glands, suggesting that GLI1 targets analogous cells in different tissues. Additionally, we demonstrated that salivary acinar differentiation was blocked, cystic lesions formed and the ductal structures appeared more prominent in the GLI1 transgenic salivary glands when compared to wild type. The lack of salivary acinar differentiation in combination with the extensive morphological alterations, indicated a diminished secretion of salivary fluid in the GLI1 transgenic mice. This is analogous to the mammary gland where induced expression of GLI1 resulted in the appearance of immature and undifferentiated alveolar secreting cells at parturition, which resulted in an inability to secrete milk. The GLI1-induced mammary changes could not be restored after termination of the oncogenic expression, while GLI1-induced salivary gland lesions could.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their roman numerals:

I. **Agren M**, Kogerman P, Kleman M. I, Wessling M and Toftgard R. Expression of the *PTCH1* tumour suppressor gene is regulated by alternative promoters and a single functional Gli-binding site. Gene 2004 Apr 14 , Vol.30: 101-14

II. **Fiaschi M**, Rozell B, Bergström A, Toftgård R and Kleman MI. Targeted expression of GLI1 in the mammary gland disrupts pregnancy-induced maturation and causes lactation failure. J Biol Chem. 2007 Dec 7;282(49):36090-101.

III. **Fiaschi M**, Rozell B, Bergström A and Toftgård R. Development of mammary tumours by conditional expression of GLI1. Cancer Res. 2009 Jun 1;69(11):4810-7

IV. **Fiaschi M**, Nilsson M, Toftgård R and Rozell B. Targeted expression of GLI1 in the salivary glands results in an altered differentiation program and hyperplasia (2009). Manuscript submitted

CONTENTS

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)

BCC	Basal Cell Carcinoma
CK1	Casein Kinase 1
cos2	Costal2
ci	Cubitus interruptus
Dhh	Desert hedgehog
Disp	Dispatched
dox	Doxycycline
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial Mesenchymal Transition
FACS	Fluorescence-activated cell sorting
Fu	Fused
GSK3β	Glycogen Synthase Kinase 3β
GCPS	Greig cephalopolysyndactyly
Hh	Hedgehog
HE	Hematoxylin and eosin staining
HPE	Holoprosencephaly
HC	Hydrocephalus
Ihh	Indian hedgehog
LOH	Loss of heterozygosity
MB	Medulloblastoma
MMTV	Mouse mammary tumour virus
NBCCS	Nevoid Basal Cell Carcinoma Syndrome
PHS	Pallister-Hall syndrome
PTHrP	Parathyroid hormone-related protein
Ptch	Patched
PG	Parotid gland
PKA	Protein Kinase A
RND	Resistance nodulation division
ski	Skinny hedgehog
Smo	Smoothened
SMA	Smooth muscle actin
Shh	Sonic hedgehog
SSD	Sterol sensing domain
SLG	Sublingual gland
SMG	Submandibular gland
Sufu	Suppressor of fused
TEB	Terminal end bud
TSG	Tumour suppressor genes
tv	Tout velu
UV	Ultraviolet radiation
WAP	Whey acidic protein

1 INTRODUCTION

1.1 CANCER

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. In order for a cell to become a cancer cell it has to acquire a number of functional capabilities, including self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustainment of angiogenesis and facilitation of tissue invasion and metastasis [1]. Nearly all cancers are caused by abnormalities in the genetic material of the transformed cells. These genetic abnormalities may occur at different levels. Entire chromosomes can be gained and/or lost or a single DNA nucleotide can be mutated. Mutations can also be induced by carcinogens (e.g. tobacco smoke, radiation, chemicals and infectious agents), acquired through errors in DNA replication or be inherited. In the last-mentioned case, the genetic change is present from birth. Genetic abnormalities found in cancer typically affect two general classes of genes, proto-oncogenes and tumour suppressor genes.

1.1.1 Oncogenes

Oncogenes are altered forms of normally occurring cellular genes properly called proto-oncogenes. Transformation of a proto-oncogene into an oncogene by mutation or enhanced expression helps to turn a normal cell into a cancer cell. Mutational changes in oncogenes act dominantly, that is, mutation in only one of the two alleles is sufficient for cancer growth. Proteins encoded by the proto-oncogenes, generally belong to one of the following five groups: growth factors, growth factor receptors, signal transducers, transcription factors or regulators of cell death [2]. Examples of proto-oncogenes include *RAS*, *MYC* and *GLI1*.

1.1.2 Tumour suppressor genes

Tumour suppressor genes (TSG) normally encode proteins that retain the non-malignant phenotype by dampening or restraining the regulation of the cell cycle or apoptosis. Tumour suppressor genes are recessive genes and follow the Knudson two-hit hypothesis [3]. According to this hypothesis, a TSG needs two inactivating events to promote carcinogenesis. In many cases, the first mutation is an inherited germ-line mutation, while the other mutation, which inactivates the second allele, occurs later in life. Inactivation of the second allele is called loss of heterozygosity (LOH). Germ-line mutations increase the cancer susceptibility for coming generations. Members of these families will have a higher incidence and a shorter latency of tumour formation and the tumour types appearing in these families depend on which TSG that is inactivated. There are some exceptions to the Knudsons two-hit rule and these TSG exhibit haploinsufficiency [4]. $P27^{kip}$ is one example of such an exception where a single mutation in one allele is sufficient to cause increased cancer susceptibility [5]. Other examples of TSG are *p53*, the retinoblastoma gene, *BRCA1*, *BRCA2*, *PTCH1* and *SUFU*.

1.1.3 Metastasis

Most tumours and other neoplasms have the ability to metastasise, i.e. to invade nearby tissues or spread through the blood vessels and the lymphatic system to other parts of the body. These are called malignant tumours while none-invading tumours are called benign tumours. In order to metastasise, tumours shed large numbers of cells, and less than 0.01% of these cells develop into metastases [6]. When tumour cells metastasize, the new tumour is called a secondary or metastatic tumour, and it contains cells that are similar to those in the original tumour. The most common sites of metastases from solid tumours are the lungs, bones, liver, and brain.

1.2 CANCER AND DEVELOPMENT

There has been a steady increase in the cancer incidence in Sweden since 1970, which can be partially explained by the increased age of the general population [7]. More than sixty percent of the people who were affected by cancer during 2007 had reached the age of 65 [7]. The coupling between age and cancer is particularly attributed to the accumulation of DNA damage that occurs during life and also to a reduced ability to repair these damages when the tissue becomes older. All people harbour genetic changes, but only a few of these alterations cause carcinogenesis and it is of utmost interest to elucidate which changes lead to tumour formation and in which settings.

A change in the genetic material (DNA) can lead to the production of a defective protein. In connection with this, it is important to bear in mind that proteins do not function as separate islands in the cell but interact with each other in defined pathways. These pathways can be seen as roads with crossings where different pathways interact with each other and create networks. Correct function and interaction between these networks are vital during embryogenesis and metamorphosis, and when deregulated they will not seldom induce cancer formation. Remarkably, from insects to mammals, there appears to be a set of basic signalling networks upon which development mainly depends. One pathway that plays an essential role in all these events is the Hedgehog (Hh) signalling pathway.

1.3 SIGNAL TRANSDUCTION OF THE HEDGEHOG PATHWAY

The molecular mechanisms of the Hh signalling pathway is best understood and most thoroughly investigated through genetic studies in the fruit fly model organism [8]. In the fly, the Hh signalling process starts with the ligand hh, which requires several posttranslational modifications to be fully active (Fig.1, signalling cell A). First, autocleavage occurs which yields an N- and a C-terminal hh fragment [9]. Next, cholesterol (C) covalently binds to the C-terminal part of N-hh, and Skinny hedgehog (ski), an acetyl transferase, palmitoylates (P) a cystein in the N-terminal part of N-hh [10, 11]. The palmitoylated and cholesterol-coupled hh binds to the plasma membrane through the hydrophobic part of cholesterol in the hh-producing cells. Release of hh from producing to responding cells is mediated by a protein named Dispatched (disp) [12]. For efficient transport, an enzyme named Tout velu (tv), involved in heparan sulphate biosynthesis, is also required [13].

In the absence of hh induction, patched (ptc, Ptch in vertebrates) inhibits the activity of the seven-pass transmembrane protein Smoothened (smo) (Fig.1, responding cell C). At this stage, the full-length form of Cubitus interruptus (ci-A) is hyperphosphorylated by Protein Kinase A (PKA), Glycogen Synthase Kinase 3β (GSK3β) and Casein Kinase 1 (CK1), an action which promotes recognition of ci-A by the ubiquitin E3 ligase Slimb, leading to the generation of a truncated transcriptional repressor form of ci, termed ci-R [14-16]. Ci-R allocates to the nucleus where transcriptional repression occurs [17]. In cells not exposed to hh, full-length ci (ci-A) is found in the cytoplasmic complex which withholds Costal2 (cos2), Suppressor of fused (sufu) and Fused (fu) [18, 19].

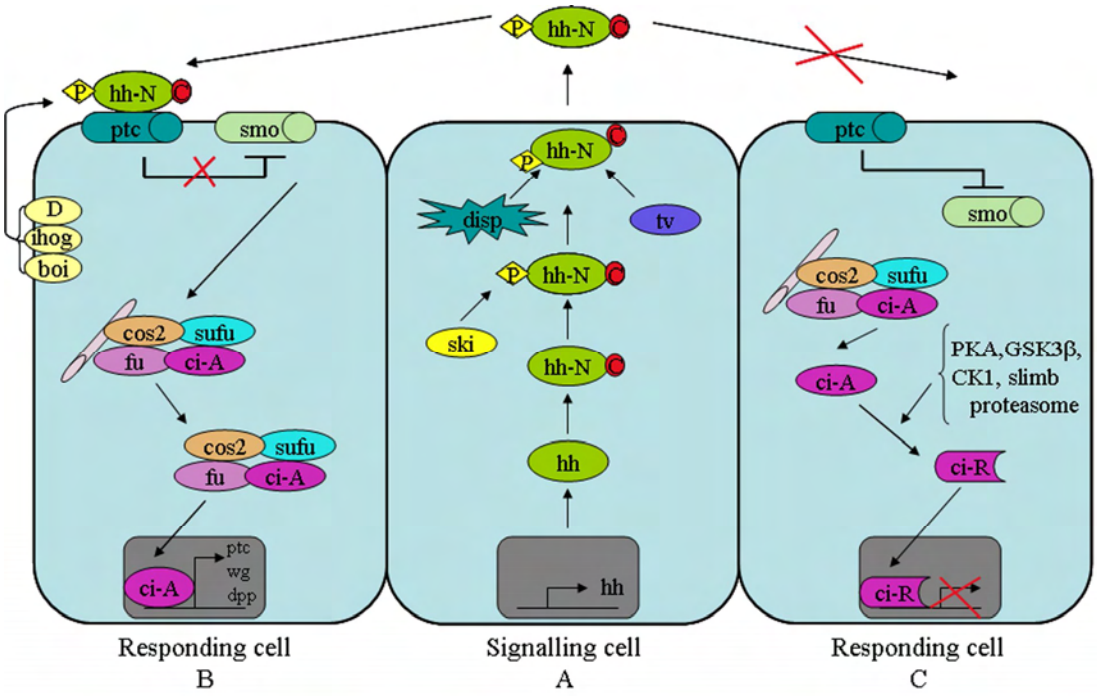

Fig.1 The proposed Hedgehog signalling pathway in Drosophila melanogaster. © Marie Fiaschi

In the presence of hh, hh binds to ptc [20] with the help of the glycan-family of cell surface proteoglycans, dally-like (D) and the transmembrane proteins ihog and boi (Fig.1, responding cell B) [21, 22]. Upon ligand binding, the inhibitory effect exerted by ptc on smo, is relieved and followed by dissociation of the multi-protein complex from microtubules [23]. The full-length ci-A is released and enters the nucleus where it binds to specific sequences [24] in promoter and enhancer regions and controls the transcription of the Hh target gene(s) [25].

1.4 ANALYSIS OF COMPONENTS

Although the Hh signalling network is well conserved through evolution [26] a fraction of the pathway components have undergone duplications (Table I), and divergence of the Hh signalling mechanisms have been reported between flies and mammals [27].

TABLE I Hedgehog components in different model organisms

Drosophila melanogaster:	vertebrates:
hh [28]	Shh, Ihh, Dhh [29]
ski [10]	Gup1 [30]
disp [12]	Disp1[31]
tv [32]	Ext1, Ext2 [13]
ihog, boi [22]	Cdo, Boc [22]
ptc [33]	Ptch1, Ptch2 [34]
smo [35]	Smo [36]
cos2 [37]	Kif7 [38, 39]
fu [40]	Fu [41]
sufu [42]	Sufu [43]
ci [44]	Gli1, Gli2, Gli3 [45]

1.4.1 Hedgehog proteins

Unlike *Drosophila melanogaster*, vertebrates express three different Hh proteins, Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh) [29]. All Hh homologues can bind to Ptch1 and activate the subsequent biological pathway [46]. Shh is the most broadly expressed Hh protein and is essential for the patterning of several organs during development. It is expressed in the developing CNS, limb, gut, lung, teeth and hair follicle [47]. The other two members of this family, Ihh and Dhh, primarily participate in bone and testis development, respectively [48, 49]. Ihh also plays a role in gut [50] and mammary gland development [51]. Knockout of the *Hh* genes (*Shh, Ihh, Dhh*) in mice confirm the difference in expression pattern and the actual sites of action of the different Hh proteins. Shh null mice [52] have severe limb deficiencies and holoprosencephaly, whereas mice null for Ihh [53] show a delay in the formation of the growth plate, and Dhh null mice [54] suffer from male infertility. However, the different Hh ligands sometimes act in the same tissues during development, and can function partially redundantly. For example, Shh and Ihh act together in early embryonic development, and their combined loss phenocopies the loss of the Hh receptor component Smo, leading to early embryonic lethality due to defects in heart morphogenesis and embryonic vasculogenesis [55].

1.4.2 The Hedgehog receptor Patched

Two ptc homologues [34, 56], Ptch1 and Ptch2, have been isolated in vertebrates. They bind Shh, Ihh and Dhh with similar affinity and can both form a complex with Smo [34, 57]. Ptch1 is primarily expressed in Shh-receiving mesenchymal cells, whereas Ptch2 is expressed in Shh-producing epithelial cells [58]. Ptch1 is expressed in all major Shh target tissues, while Ptch2 distribution is detected almost exclusively in the skin and testis [59, 60]. Knockout models of *Ptch1* and *Ptch2* reveal that even though the proteins are similar (PTCH2 has a 57% identity to PTCH1 and a 90% identity to mouse Ptch2 [34]), loss of *Ptch1* leads to embryonic lethality while loss of *Ptch2* only renders skin lesions on male mice [61, 62].

The human *PTCH1* gene (GenBank accession number U43148, U59464) spans 34 kb of DNA, is located on chromosome 9q22.3 and contains 23 exons. *PTCH1* contains four alternative first exons, exon1, exon1A, exon1B and exon1C, followed by 22 exons

common to all four transcripts [59, 63-65]. Functional assays reveal that *PTCH1* alternative first exons affect protein stability, expression pattern as well as their capacity to inhibit the Hh signalling pathway [65-67]. The exon1B and exon1C isoforms harbour a Gli-consensus binding site in their promoter region which upon Hh stimulation affects the expression of these isoforms [65, 66]. Furthermore, the expression of the exon1B isoform is specifically induced in nodular BCC [66].

Ptch1 encodes a transmembrane protein predicted to have twelve transmembrane domains, two major extra cellular loops where Hh ligand binding occurs, and two major intracellular loops [20]. The second to sixth transmembrane regions of *Ptch1* show high similarity to the sterol-sensing domain (SSD) of the Niemann-Pick disease protein and HMG-CoA reductase, which play a role in cholesterol homeostasis and in intracellular trafficking [68]. *Ptch1* is also structurally similar to the resistance-nodulation-division (RND) family of prokaryotic permeases that transport small molecules across membranes [69]. Mutations in conserved residues of either the SSD- or RND-like structure impair Ptch1 function [69-71]. Ptch1 has therefore been proposed to function as a transporter of a small molecule that acts as Smo agonist or antagonist [69]. Several candidates for this small molecule have been proposed, including pro-vitamin D3 [72] and oxysterols [73]. Recently Rothagi *et.al.* demonstrated that addition of oxysterols cause Smo to move into the primary cilia and there activate Shh signalling [74].

PTCH1 is suggested to be a tumour suppressor [75, 76], and germline mutations of *PTCH1* cause Nevoid Basal Cell Carcinoma syndrome (NBCCS, PTCH Mutation Database: www. cybergene.se/PTCH/.) [77]. Mutations in *PTCH1* is also found in BCC, medulloblastoma (MB), rhabdomyosarcoma, rhabdomyoma, meningioma, esophageal carcinoma, squamous cell carcinoma, trichoepithelioma and breast carcinoma [78]. *PTCH2* is also suggested to be a putative tumour suppressor and *Ptch2* was recently shown to cooperate with *Ptch1* to induce a higher incidence and wider range of tumours in mice [79, 80].

1.4.3 Signalling from Smoothened to ci/GLI

Smo relays the Hh signal from the plasma membrane of receiving cells into the cytoplasmic compartment in both vertebrates and invertebrates. Hh binding to Ptch1 stimulates hyper phosphorylation of Smo, an event inducing a conformational switch and dimerization of Smo carboxy-terminal cytoplasmic tail [81-83]. In Drosophila, these changes enable smo to physically bind to cos2, an interaction which in turn allow cos2 to relay a signal from the receptor to the transcription factor ci [84-86]. Cos2 is a kinesin-related protein that regulates the production of both the repressor and activator form of ci in *Drosophila* [37, 87]. Furthermore, cos2 moves along microtubules in an Hh-dependent manner through its kinesin-like motor function and regulates ci localisation [88]. The mammalian homologue of cos2, Kif7 was just recently shown to play a role in vertebrate Hh signalling [38, 39]. Similarly to cos2, Kif7, regulates signalling from Smo to the ci/Gli transcription factors, a process which in vertebrates resides in the cilia [38, 39] and also involves the accumulation of Gli2 and Gli3 at the tip of the cilia [39]. Kif7 is suggested to act as an anterograde IFT motor protein and transports itself and cargo to the ciliary tip [38, 39]. Kif7 further opposes the formation

of the Gli3 repressor form in the presence of Hh [39] and activation of the Gli2 transcription factor in the absence of Hh [38].

Additional cytoplasmic components include sufu and fu. Sufu helps to tether the activator form of ci in the cytoplasm, an event that keeps the pathway switched off in the absence of hh ligand [89], but sufu also appears to play a role in the inhibition of ci-activator within the nucleus [90, 91]. Noteworthy, the role of sufu in the Hh-signalling pathway is not evolutionary conserved. Whereas *sufu* null mutant flies are viable and fertile, *Sufu* null mutant mouse embryos die at E9.5 with severe cephalic and neural tube defects similar to those seen in *Ptch1* knockout embryos [92-94]. Moreover, *Sufu* heterozygous mice develop a skin phenotype with basaloid appearance and jaw keratocysts, which are characteristic features of the Gorlin syndrome [94].

While Sufu has acquired an essential function in mammals, mice lacking a functional Fu, a seronine-threonine kinase essential for positively regulating Hh signalling in *Drosophila* [18], show no obvious phenotype connected to a defective Hh signalling pathway during mouse embryonic development [95, 96]. Postnatally, they show growth defects and lethality due to progressive hydrocephalus (HC). HC is also observed in *Ptch1* [97] and the $Gli3^{Xtj}$ [98, 99] heterozygous mice (abnormal alleles in *Gli3* are subsumed under the category "extra toes" $Gli3^{Xtj}$, the Xtj allele contains a 51.5 kb deletion [100]). In *Drosophila*, fu physically interacts with cos2 and is phosphorylated in response to active Hh signalling, an event which blocks the negative influence of sufu on ci [25, 101].

1.4.4 The ci/Gli transcription factors

The *Drosophila* ci protein has three vertebrate homologues, termed Gli1, Gli2 and Gli3 [45]. The *Gli* gene family was identified originally by the amplification of human *GLI1* in glioblastoma [102]. Although ci and the Gli proteins are highly similar, there are important differences in their regulation. Gli1 lacks an N-terminal repressor domain and cannot be processed into a repressor protein, whereas Gli2 and Gli3 are bifunctional and can be processed into both activators and repressors [103]. The Gli2 role as a repressor is questionable, however, since Gli1 can replace Gli2 *in vivo* [104]. Gli3 primarily functions as a negative regulator of Hh signalling, and processing of Gli2 and Gli3 is conducted in a similar manner as *Drosophila* ci [15].

In the absence of Hh signalling, Gli1 is transcriptionally silent since it is a canonical target gene and activated in response to positive Hh signalling [104, 105]. Gli1 is therefore commonly used as readout of pathway activation. Gli2 and Gli3 can be expressed without active Hh signalling and are then present mostly as cleaved repressor forms, silencing Hh-Gli targets [103]. In the presence of Hh, Gli repressor forms are lost and activators are formed. It has been proposed that Gli2 and Gli3 are the primary mediators of Hh signalling, and when converted to their active forms they activate Gli1 at the transcriptional level [104, 106]. The expression and post-translational stabilisation of the various Gli family members create a distinct combination of Hh transcriptional activators and repressors that results in a specific biological readout [107].

1.4.5 Target genes

All Gli proteins bind to DNA through five zinc-finger domains that recognize the consensus Gli-selective sequence 5'-TGGGTGGTC-3' and thereby activate or repress specific target genes [24]. Primary target genes for the Hh network include the *Ptch1*, *Ptch2* [108] and *Gli1* [104, 105] genes, which in response to network activation show an elevated mRNA and protein level [104, 109]. Thus, increased expression of *Ptch1/Ptch2* and *Gli1* are highly reliable indicators of network activation and provide negative and positive feedback regulation of Hh signalling, respectively.

Other target genes include e.g. the Hh-interacting protein, *Hip* [110], regulators of the cell cycle (*CyclinD2*, *CyclinE*) [111] and apoptosis (*Bcl2*) [112], the *N-myc1* proto-oncogene [113] and *Snail* [114], a transcription factor with a critical role in the transition from epithelial to mesenchymal character, which is associated with increased invasiveness and metastasis. In addition, Gli1 transcriptionally induces *FoxE1* [115] and *FoxM1* [116] in BCC. Activation and deactivation of these genes, are mechanisms by which Hh can contribute to normal tissue/organ development but also to tumour formation.

1.5 HEDGEHOG'S INVOLVEMENT IN DEVELOPMENT

The developmental processes regulated by Hh in *Drosophila* appear remarkably conserved through evolution [8]. In vertebrates, Hh signalling network components are critical regulators of cellular identity, patterning and tissue interactions during embryogenesis and organogenesis and have been shown to be required for normal development of virtually every tissue and organ in the body (Fig.2) [117, 118].

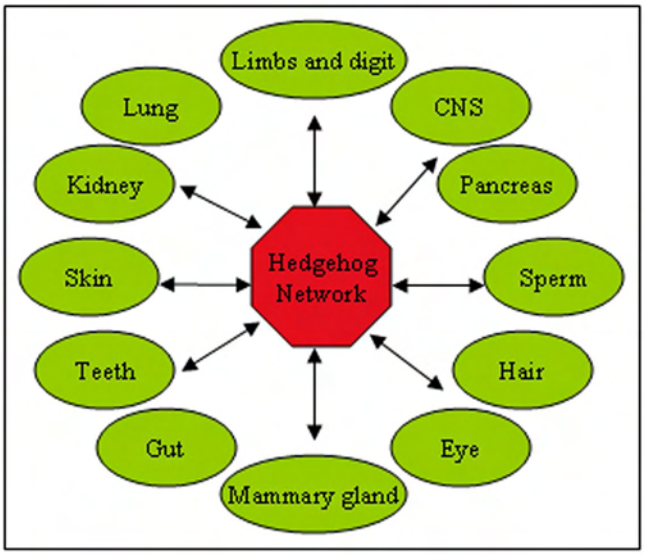

Fig. 2 Organs and structures that are developmentally regulated by Hh signalling. © Marie Fiaschi

At the cellular level, the effects of Hh range from growth and self-renewal to cell survival, differentiation and/or migration [8, 119]. In perhaps its best-known role, in tissues such as the neural tube [120] and the limbs [121], the Hh ligand functions as a morphogen, with different concentrations of the ligand directing the adoption of different cell fates. In other contexts, Hh signalling drives the proliferation of precursor

cells in tissues, such as the skin [122] and cerebellum [123], or mediates interactions between epithelial and mesenchymal compartments that sculpt organs such as the lung [124].

How long-range morphogenetic Hh gradients are created is not well understood, but Hh distribution and movement is strongly influenced by post-translational modifications [11, 125]. Furthermore, Hh secretion requires the transmembrane protein Disp, which like Ptch1 belongs to the bacterial RND family of transport proteins [12]. Loss of Disp leads to accumulation of Hh in producing cells and failure of long-range signalling [12]. Other studies point to the heparan sulphate transferase EXT1, the vertebrate homologue of *Drosophila* tv, as being involved in proteoglycan-dependent aggregation and target-cell diffusion of Hh [13, 126]. Recent genetic data also suggest that long-range signalling requires assembly of Hh with lipids, forming large lipid/protein particles [127]. The biological readout of the Hh signalling is not only dependent on the range that the Hh ligand travels but also on modification and expression of intermediate factors and especially the combination of Hh transcriptional activators and repressors that are expressed. Finally, the cellular response mediated by Hh also depends on the induction of different sets of target genes.

1.6 HEDGEHOG'S INVOLVEMENT IN SYNDROMES AND CANCERS

Since Hh is such an important player during development of several tissues and organs, it is not surprising that aberrations in the Hh pathway are associated with various disorders, like Nevoid Basal Cell Carcinoma Syndrome (NBCCS), Holoprosencephaly (HPE), Greig cephalopolysyndactyly (GCPS) and Pallister-Hall syndrome (PHS) (Table II) [78].

TABLE II Germline and/or somatic mutations in the Hh signalling components coupled to genetic disorders and cancers [78]

Conditions:	Mutations reported in:
Nevoid basal cell carcinoma syndrome	*PTCH1, SUFU*
Greig cephalopolysyndactyly	*GLI3*
Pallister-Hall syndrome	*GLI3*
Holoprosencephaly	*SHH, PTCH1, GLI2*
Basal cell carcinoma	*PTCH1, PTCH2, SMO*
Medulloblastoma	*PTCH1, PTCH2, SMO, SUFU, GLI3, SHH*
Rhabdomyosarcoma	*PTCH1*
Breast cancer	*SHH, PTCH1*
Bladder carcinoma	*PTCH1*
Digestive tract tumours	*PTCH1*
Esophageal squamous cell carcinoma	*PTCH1*
Fetal rhabdomyoma	*PTCH1*
Meningioma	*PTCH1*
Pancreatic cancer	*PTCH1*
Primitive neuroectodermal tumour (PNET)	*PTCH1*
Small cell lung cancer	*PTCH1*
Trichoepithelioma	*PTCH1*

HPE is a developmental defect, where the embryonic prosencephalon fails to form cerebral hemispheres. The most severe form of HPE, alobar HPE, is embryonic lethal. Mutations in *SHH* [128], *PTCH1* [129] and *GLI2* [130] have been reported to generate HPE, and the involvement of Hh in these genetic disorders has also been confirmed by the development of HPE in *Shh* [52], *Smo* [55] and *Disp* [31] knockout mice. Mutations in *GLI3* is involved in the formation of two different syndromes, namely the Greig cephalopolysyndactyly [131] and the Pallister-Hall syndrome, both of which manifest polydactyly [132]. PHS patients also show hypothalamic hamartoma and GCPS patients abnormal craniofacial features [131, 132]. Even though both syndromes arise from mutations in the same gene, they are clinically distinct. $Gli3^{Xtj}$ mice develop limb defects as well as anterior and posterior digit duplications similar to those found in human Greig cephalopolysyndactyly syndrome patients [98, 99].

1.6.1 Nevoid Basal Cell Carcinoma Syndrome (NBCCS)

The first connection between Hh and cancer was made thirteen years ago when an inactivating mutation of *PTCH1* was found to be the underlying genetic event of NBCCS formation [63, 64]. NBCCS was systematically described and defined by RJ Gorlin in 1960, and is today widely known as the Gorlin syndrome [133]. The minimal prevalence of Gorlin syndrome is suggested to be 1 per 56 000 [134]. Gorlin syndrome is an inherited autosomal dominant disorder characterized by a predisposition to different neoplasms and a wide range of developmental anomalies [77]. The major feature is the formation of BCC, which arises in almost 100% of the reported cases [77]. Other tumours like medulloblastoma, meningioma, cardiac fibroma, fetal rhabdomyoma and odontogenic keratocysts can also be found in Gorlin syndrome patients but appear less frequently (Fig.3) [77]. Developmental defects characteristic for NBCCS include calcification of falx cerebri, polydactyly, syndactyly and cleft lip and palate (Fig.3) [77].

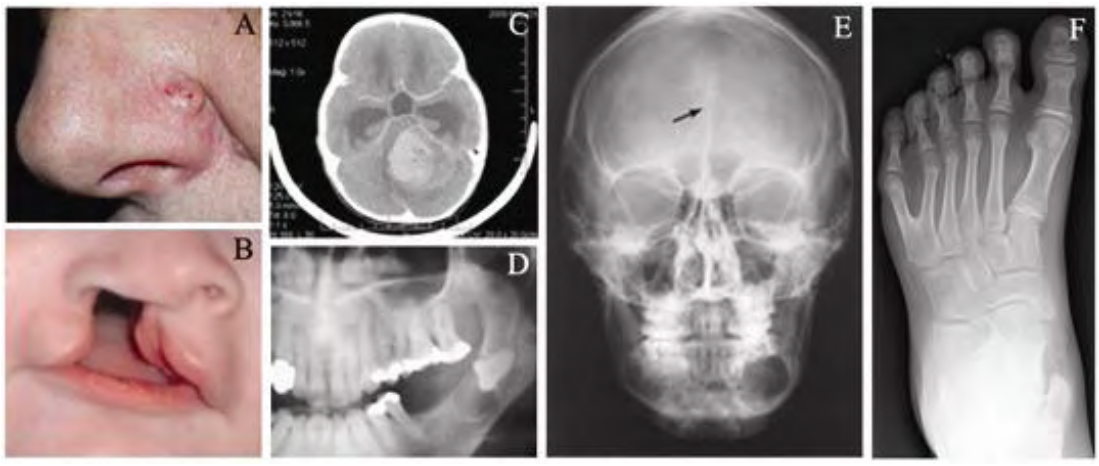

Fig.3 The major features of Gorlin Syndrome: BCCs (A), cleft lip and palate (B) medulloblastoma (C), odontogenic keratocysts (D), calcification of falx cerebri (E, arrow) and polydactyly of left foot (F).
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Although Gorlin syndrome is an inherited syndrome, about one-half represents new mutations [135, 136]. Analysis of gene mutations in the syndrome reveals that about 70% of germline *PTCH1* mutations are rearrangements, and of these 80% result in

truncation of the PTCH1 protein [137]. Mutations in *PTCH1* generating NBCCS have been reported in almost all of the 23 exons comprising *PTCH1* except for exon1 (www.Cybergene.se/PTCH/). Although inactivation of *PTCH1* is suggested to be the main disease-causing event, it was recently reported that a family with Gorlin syndrome was negative for *PTCH1* mutations but showed a *SUFU* germline mutation [138].

1.6.2 Basal Cell Carcinoma

BCC was first described in 1824 [139] and is the most commonly diagnosed human cancer, at least among persons of European ancestry [140]. Statistically, approximately one out of five Swedes develop BCC within their lifetime [7]. Despite its high prevalence, BCC rarely metastasizes and seldom causes death, but has a high potential for local invasiveness and disruption. Race, age, gender, and decreased DNA repair capacity, ultraviolet (UV) exposure, ionizing radiation and arsenic exposure are factors that clearly correlate with BCC development [141].

Mutations in *PTCH1* play a major role in BCC development, with frequent LOH of the *PTCH1* locus both in sporadic BCCs and hereditary BCCs in Gorlin Syndrome [142, 143]. Currently, it is thought that enhanced activity of Hh signalling is the pivotal abnormality in all BCCs, and disease-causing mutations apart from *PTCH1* have been described in *SMO* [144], *PTCH2* [56] and *SUFU* [144]. Additional evidence for Hh signalling involvement in BCC formation comes from transgenic-mouse models. Over-expression of SHH [145], SMO [146], GLI1 [147] and Gli2 [148] in keratinocytes leads to spontaneous formation of BCC while *Ptch1* heterozygous mice [149] need to be exposed to UV or ionizing radiation to develop BCC. In addition to mutations in genes coupled to the Hh signaling pathway, p53 gene mutations also occur frequently in BCC [150].

1.6.3 Hedgehog and Cancer

Activation of the Hh pathway by mutations has been associated with the development of BCCs and MBs and also with the development of other tumours, such as pancreatic, small cell lung, digestive tract and breast cancer (Table II). In addition, several studies have suggested that even in the absence of mutations in the Hh pathway components, tumour cells produce and respond to Hh ligand in an autocrine/paracrine fashion and thereby facilitate growth and survival of the tumour cells [151]. The mechanism behind the formation of these tumours is unclear. However, ligand-dependent activation of the Hh-signalling pathway has been reported in human malignant tissues, such as lung [152, 153], pancreatic [154], digestive tract [155], bone/cartilage [156], prostate [157], melanoma [158], colorectal [159, 160] and ovarian [160] tissues. All tissues and organs that become tumourigenic in response to ligand-dependent activation of the Hh-signalling pathway have probably not been elucidated yet. One tissue where Hh seems to play a role both during development and tumourigenesis is the breast, and ligand-dependent activation could be one mechanism by which Hh induces breast carcinogenesis.

1.7 MAMMARY GLAND DEVELOPMENT AND CARCINOGENESIS

1.7.1 Mammary carcinogenesis

Breast cancer is the most common malignancy in women with 1 million new cases per year worldwide [161], and about one in nine women will develop breast cancer during their lifetime [162]. In Sweden, 7049 new cases of breast cancer were diagnosed in 2007 and this represented an incidence of 29.2% of all female cancer cases during this particular year [7]. The primary risk factors that have been identified include race, age, gender and exposure to endogenous or exogenous estrogens. Breast cancer history in the family is another established risk factor [161, 163]. Key genes involved in the familial forms of breast cancers include tumour suppressors *BRCA1, BRCA2, p53* and *PTEN*. Familial breast cancer cases only account for about 5-10% of total breast cancer cases, which makes it likely that high- and/or low-penetrance breast predisposing factors will be found in the future.

During the last decades there has been a significant decline in breast cancer mortality, and this has primarily been attributed to screening, early detection and particularly the introduction of specialized treatment such as Tamoxifen in estrogen positive tumours and Trastuzumab directed against human epidermal growth factor receptor gene 2 (Her2) overexpressing tumours. Another key to the increased survival rate is the specification and determination of clinicopathological parameters that serve as a guide for the use of systemic therapy and prognostication. These include tumour size, lymph node stage and histological grade, vascular invasion, histological type, patient age and menopausal status. In addition, to this, the expression of the estrogen, progesterone and the HER2 receptor is determined. However, there have been a pressing need for further refinement of these subgroups, and recently new breast cancer subgroups were proposed. These classify the tumours according to their molecular portraits, and breast cancers were grouped into luminal epithelial-like, basal epithelial-like, HER2 amplified and normal breast-like phenotype [164]. The luminal epithelial–like tumours express luminal specific genes such as estrogen, the basal–like subtype displays high expression of markers characteristic of mammary duct basal myoepithelial cells (such as cytokeratin 5). The HER2-amplified cancers express high levels of HER2, and the normal breast–like phenotype showed the highest expression of genes which are normally expressed by adipose tissue and none-epithelial cells. Of these estrogen-negative tumours, basal-like cancers and HER2 cancers are associated with a particularly poor prognosis [164, 165]. Identification of new therapeutic targets in the poor prognosis groups will be critical for further improvements in breast cancer treatment.

1.7.2 Mammary gland development

Development of the mammary gland occurs in defined stages that are connected with sexual development and reproduction [166]. These stages are embryonic, prepubertal, pubertal, pregnancy, lactation and involution (Fig.4). The mammary gland, unlike other branched organs, undergoes most of its branching postnatally rather than during foetal development.

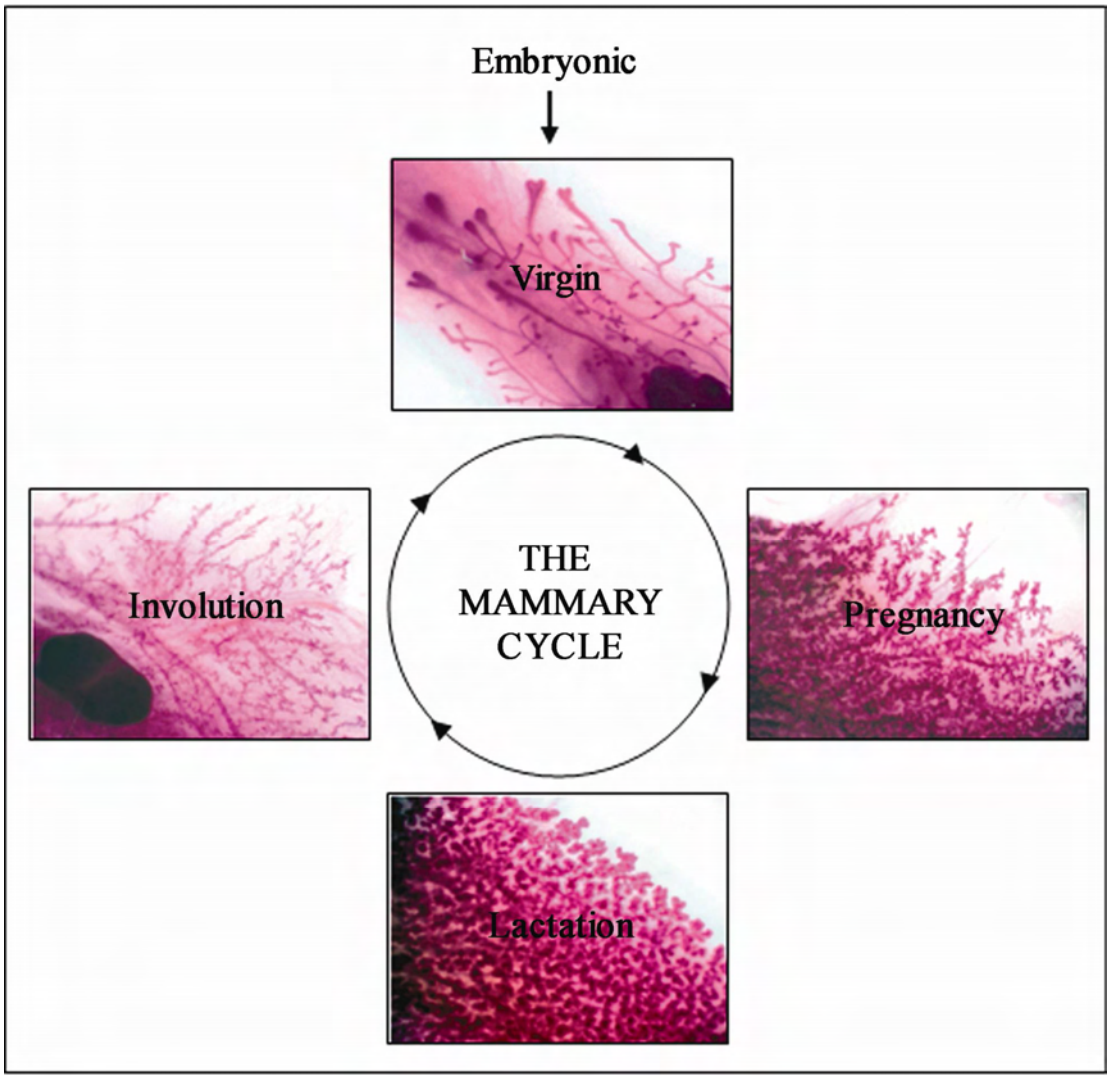

Fig.4 Major phases of mammary gland development. The morphology of the mouse mammary gland at different developmental stages is represented by whole-mount pictures. Virgin phase (5-10 week, growth active), pregnancy (alveolar growth and secretory differentiation), lactation (milk secretion) and involution (apoptosis, regression and remodelling). © Marie Fiaschi

Mammary gland development is initiated around embryonic day 10 (E10) with the formation of an epithelial bulb. In this stage, mammary gland development is hormone independent but under the regulation of paracrine communication between neighbouring epithelial and mesenchymal cells. At puberty (about 4 weeks of age) ovarian hormones stimulate rapid proliferation and ductal elongation of the terminal end bud (TEB). The TEB consists of body and cap cells, which differentiate into luminal epithelial and myoepithelial cells (Fig.5A). The TEB cells invade and communicate with the fat pad stroma, and ductal elongation ceases when the limit of the mammary fat pad is reached, leaving a branched system of differentiated ducts. During pregnancy the mammary tree changes from a ductal to a lobualveolar appearance (Fig.5B). Progenitor cells in the ducts proliferate and form alveolar buds, which further differentiate to form alveoli. After parturition, upon suckling, milk secretion is induced as a consequence of altered hormone levels.

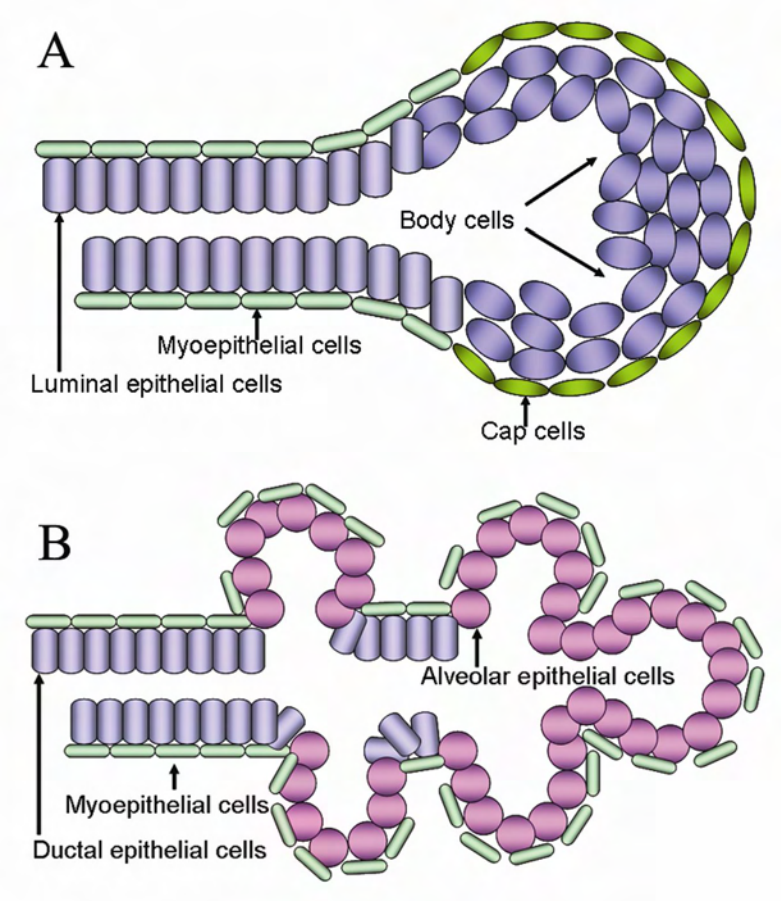

Fig.5 The terminal end bud (TEB). (A) Schematic view of the TEB. The body cells are surrounded by a single layer of cap cells. Differentiated myoepithelial and luminal epithelial cells line the neck of the duct and the subtending duct. (B) Schematic view of the ductal end alveolar cells during mid-pregnancy. The ducts are surrounded by a basal layer of overlapping myoepithelial cells, whereas the alveoli cells are surrounded by a basket-like layer of myoepithelial cells. © Marie Fiaschi

After lactation, decreased hormone levels and the discontinuance of suckling cause mammary involution. During involution, all alveoli and secretory duct structures collapse by programmed cell death (apoptosis). The remaining gland is extensively remodelled to resemble the pre-pregnant state. The transition between these phases is tightly regulated by factors known to play a major role in the development of the mammary gland. These factors are both systemic hormones (estrogen, progesterone and prolactin), growth factors as well as different proteins and pathways [167].

1.7.3 Hedgehog in mammary gland development

Although all three Hh homologues are expressed in the mouse mammary gland, the inactivation of, any one of *Shh*, *Ihh* or *Dhh* does not result in a defective mammary gland phenotype [168-171]. Most likely, this reflects redundant functions of these proteins during mammary embryogenesis. Ihh, however, is suggested to be the main Hh regulator in the mammary gland as it is regulated by progesterone, one of the key steroid hormones which control ductal and alveolar mammary gland development [172, 173]. During embryonic and pubertal mammary gland development, Hh signalling is suggested to be actively repressed as no Gli1 or Ptch1 expression is found within the mammary tree or stromal cells [174, 175]. This repression is suggested to be mediated by Gli3. In support of this, bud pairs three and five fail to develop in the majority of embryos that lack functional Gli3 [174]. Similarly, targeted replacement of *Gli2* by

Gli1 within *Gli3* heterozygous (Gli3 $^{xt/+}$) mice results in loss of the same mammary buds [174]. The ability of the constitutive active Gli1 activator to antagonize Gli3 clearly reveals that Gli3 functions as a repressor in this developmental context. In addition, embryonically induced expression of Shh within the epidermis leads to the absence of several mammary buds [176]. Disruption of the Hh signalling, by heterozygous loss of *Ptch1* [177] or constitutive activation of Smo (SmoM2) [178] within the mammary epithelium, leads to abnormalities in the terminal end buds and/or ducts of virgin mice. Neither of these mice faile to develop a correct number of mammary buds during embryogenesis. In contrast, transplantation analyses of mice carrying the mesenchymal dysplasia allele of *Ptch1,* led to either complete failure of gland development, failure of post-pubertal ductal elongation, or delayed growth with ductal dysplasia [179]. Interestingly, pituitary isografts can to some extent rescue the ductal elongation phenotype in these mice, suggesting that Ptch1 functions in the pituitary to regulate ductal elongation and ovarian hormone responsiveness [179].

1.7.4 Hedgehog in mammary carcinogenesis

There is emerging evidence that aberrant Hh signalling may be important in breast carcinogenesis [180]. Mutations in Hh pathway genes (*SHH* [181], *PTCH1* [182] and *GLI1* [183]) have been identified at low frequency in breast-cancer samples and/or cell lines. A functional role for any of these identified mutations in breast cancer has sofar not been proven. However, a biallelic Pro1315Leu (C3944T) polymorphism of *PTCH1* is significantly associated with breast cancer. This polymorphism in *PTCH1* significantly lowered the risk of breast cancer in premenopausal women taking oral contraceptives [184], suggesting a relationship between hormones and PTCH1. The major mechanism of Hh pathway activation in breast tumourigenesis may not be due to mutations in Hh genes, but rather depend on ligand activation. In line with this hypothesis, several research groups have reported ectopical expression (GLI1 [185-187], SMO [178, 187], SHH [186, 187]) and/or reduced expression (PTCH1[178, 187, 188]) of Hh components in breast-cancer cell lines and samples. Furthermore, growth of a number of breast-cancer cell lines, which show elevated SHH, DHH and GLI1 protein levels, could be inhibited by the Smo antagonist, cyclopamine [187, 188]. Additional evidence supporting a role for Hh signalling in breast tumourigenesis comes from high-resolution comparative genomic hybridization analysis performed on breast-cancer samples and breast-cancer cell lines which revealed frequent loss of the *PTCH1* and amplification of the *GLI1* chromosomal regions [189, 190]. Epigenetic events are also suggested to play a role in activation of the Hh pathway in breast cancer [188]. Furthermore, *in vivo* analysis with mouse models supports a role for Hh in mammary tumourigenesis. Mice heterozygous for targeted disruption of *Ptch1* display ductal hyperplasia of the mammary gland, which is reminiscent of human ductal carcinoma *in situ* [177]. These proliferations disappear during pregnancy and reapper during involution, suggesting a direct link between Ptch1-regulated cellular signalling and hormonal status of the organism [177].

Constitutively active human SMO (SmoM2) in the mammary epithelium as well as over-expression of GLI2 in mammosphere initiating cells transplanted into cleared fatpads of immuno-suppressed female mice both result in mammary hyperplasia [178, 191].

1.8 SALIVARY GLAND DEVELOPMENT

Salivary glands in humans are exocrine glands that secrete more than half a litre of saliva daily. Saliva has many functions in maintaining the normal homeostasis of the oral cavity, and when the salivary glands are damaged, the equilibrium is disturbed, resulting in a risk of considerable oral morbidity. Impairment in salivary secretion leads to rampant dental caries, frequent mucosal infection, and difficulties in swallowing, chewing and speaking. Salivary gland carcinoma is a relatively rare malignancy, and it is estimated that the global incidence varies from 0.4 to 0.65 new cases per 100,000 population [192]. However, the major cause of salivary dysfunction is rather iatrogenic, immunogenic and metabolic. Furthermore, a large group of patients suffer radiation damage to their salivary glands during their course of radiation therapy for head and neck cancers [193].

In the human body, saliva is secreted predominantly from three pairs of major salivary glands, submandibular (SMG), sublingual (SLG) and parotid (PG) glands (Fig.6).

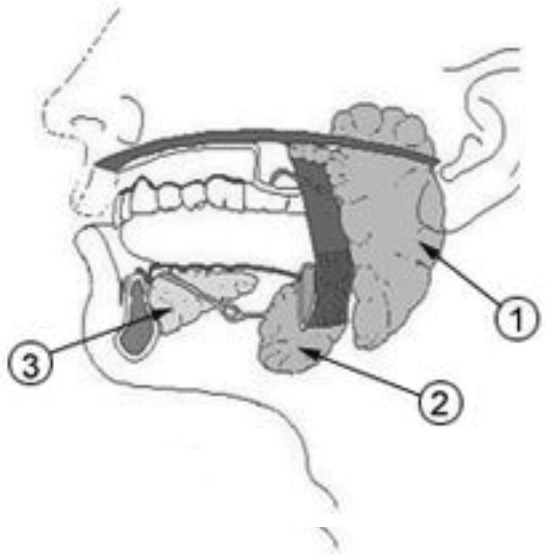

Fig.6 Salivary glands: parotid gland (1), submandibular gland (2) and sublingual gland (3).

In addition to this, hundreds of minor salivary glands are spread throughout the oral cavity, but they only account for 10% of salivary secretion. Salivary gland development is initiated around embryonic day 11.5 and starts as a thickening of the oral epithelium adjacent to the developing tongue. The primitive oral cavity epithelia then grow down into compact mesenchyme and form a solid, elongated epithelial stalk terminating in a bud. The epithelial cord grows by repeated end-bud branching, giving rise to multiple ductal cords and buds. The majority of the ducts develop lumen in a process which occurs due to apoptosis of the centrally located epithelial cells. The luminal containing branches and terminal buds form the presumptive ducts and acini, and secretory proteins start to be produced by terminal bud epithelia. Differentiation of the salivary gland continues postnatally and is finalized during puberty. Salivary gland morphogenesis requires a complex interplay between cell proliferation, apoptosis and histo-differentiation, mediated by transcription factors at specific times and places [194].

1.8.1 Hedgehog in salivary gland development

The first indication of a role for Hh in salivary gland development came from *Shh* null mice which showed absence of mandibular derivatives from which salivary gland development protrudes [52]. Surprisingly, *Shh* knockout mice develop an SMG primordial, which fails to develop beyond embryonic day 14 [52]. Shh has been implicated in SMG branching and shown to promote lumen and cell polarization of the developing SMG epithelium [195, 196].

1.9 TREATMENT OF MALIGNANCY

It has been estimated that aberrant Hh signalling contributes to the development of up to one third of all human malignancies [197] and gives added weight to the importance of finding therapeutic agents specifically targeting Hh signalling. Today there is no clinically available drug but there are a small number of Hh inhibitors in clinical trials (both in phase I and II) [198].

The first identified inhibitor of the Hh pathway was a small molecule inhibitor of Smo, derived from the corn lily (*Veratum californicum*) and named cyclopamine [199]. Cyclopamine was discovered through epidemiological investigations of malformed lambs whose mothers grazed on wild corn lily. These lambs were born with the congenital disorder cyclopia, which is a rare form of holoprosencephaly, a malformation closely connected to the Hh signalling pathway [199]. Preclinical studies with cyclopamine show, promising results in reducing MB and BCC formation in mice without affecting the overall survival of the animals [200]. Of all the Hh pathway components, Smo appears to be an especially susceptible target of small molecules since several compounds acting at the level of Smo have been described [151]. Genentech in collaboration with Curis have performed significant research around Smo inhibitors culminating in GDC-0449, which has now entered phase II clinical trials [160].

Whereas Smo is a clinically validated target on this pathway, there is considerable interest in exploring other targets that may open up opportunities for treating tumours with Hh pathway activation occurring downstream of Smo. Several lines of evidence show that mutations in *SUFU*, *GLI1* gene amplification or GLI2 protein stabilization quite frequently lead to tumour formation [102, 201, 202]. Development of compounds blocking Gli activity has just begun, and recently two molecules, Gant61 and Gant58, which inhibit, GLI1-mediated transactivation, were identified [203].

2 AIMS OF THIS THESIS

The general aim of this study was to investigate the regulation of the Hh receptor gene, *Ptch1*, as well as Hh's involvement in mammary and salivary gland development and disease.

Specific aims:

Paper I: To map the basic promoter region(s) in the human *PTCH1* gene and investigate whether *PTCH1* is regulated by more than one promoter. To delineate regions in the *PTCH1* promoter where the proteins involved in the Hh-signalling pathway act directly or indirectly on the *PTCH1* promoter.

Paper II: To study Hh's involvement in mammary gland development by creating transgenic GLI1 mice.

Paper III: To explore the hypothesis that transgenic expression of GLI1 in the mouse mammary gland will induce mammary carcinogenesis

Paper IV: To study the role of Hh in mouse salivary gland development and carcinogenesis.

3 RESULTS

3.1 PAPER I

Expression of the *PTCH1* tumour suppressor gene is regulated by alternative promoters and a single functional Gli-binding site

PTCH1 functions as a gatekeeper. In its unbound form it inhibits downstream signalling, and in its HH-bound form it releases its restrainment on SMO that carries the signalling further in the cell. The *PTCH1* gene contains several alternative first exons (1, 1A, 1B, 1C), all of which can interact with the SHH protein and to some extent restrain SMO activity [59, 63-66].

In this study we were interested in finding out if the *PTCH1* variants 1, 1A and 1B were transcribed from the same promoter or if transcripts were produced from three individual promoters. Putative elements, which may control the expression of the *PTCH1* gene, were found through bioinformatic analysis in the promoter region in front of exon1 (a TATA-box, a pyrimidine-rich initiator (inr) element and one E-box element), exon1A (an inr element) and exon1B (an E-box element). In addition, several possible promoters, *cis*-acting elements like binding sites for SP-1, Adf distal factor-1 and activator protein-2 and GC-rich areas were predicted in the proximal *PTCH1* sequence. By using the luciferase assay on parts of the *PTCH1* promoter region subcloned into the promoter and enhancer less pGL3-basic reporter vector we could conclude that *PTCH1* was transcriptionally regulated by three independent promoters. Prior to luciferase analysis the *PTCH1* variant content was confirmed in 293-cells by RT-PCR using RNA extracted from this cell line and forward primers specific for each one of the different first exons and a reverse primer located in exon2. The promoter activity in the 293-cells varied, with high expression of the promoter construct containing the sequence region in front of exon1B and exon1 and lower expression of the promoter construct containing the sequence region in front of exon1A.

Transcriptional activation of *PTCH1* is primarily suggested to be regulated by different GLI proteins binding to the Gli-consensus sites (5´-TGGGTGGTC-3) located in the 5´UTR of *PTCH1*. Two Gli-consensus sites (one perfect and one with two mutations) are present and positioned in front of exon1B. From the *PTCH1* promoter construct containing the region in front of exon1B and the Gli-binding sites ($P1_A$), a shorter promoter construct was made excluding the Gli-binding sites ($P1_C$) (Fig.7). When analysed through the luciferase assay, a dramatically decreased promoter activity in the $P1_C$ promoter construct compared to the $P1_A$ promoter construct was revealed. This discrepancy is probably due to the missing Gli-binding sites in the $P1_C$ construct. However, another feasible explanation could be the presence of a yet undiscovered activator in the area upstream of the $P1_C$ region.

To confirm binding of GLI proteins to the Gli-binding sites residing in the *PTCH1* promoter region, electrophoretic mobility shift assay (EMSA) was performed.

Probes corresponding to each of the separate Gli-binding sites, as well as a mutated Gli-binding site harbouring six mutations were used for EMSA analysis. EMSA was performed on NIH-3T3 and 293-cells which prior to use were investigated for the presence of endogenous GLI mRNA by RT-PCR (293-cells) and endogenous and transfected GLI protein content by western blot (293- and NIH-3T3 cells). EMSA confirmed that the GLI proteins were unable to bind the Gli-binding site harbouring six mutations as well as to the Gli-binding site harbouring two mutations which were analogous to the Gli-binding site located in the *PTCH1* promoter sequence. However, the perfect GLI1 consensus site was able to successfully bind the GLI proteins (GLI1, GLI2 and GLI3) and thereby alter the transcriptional efficiency of the *PTCH1* gene.

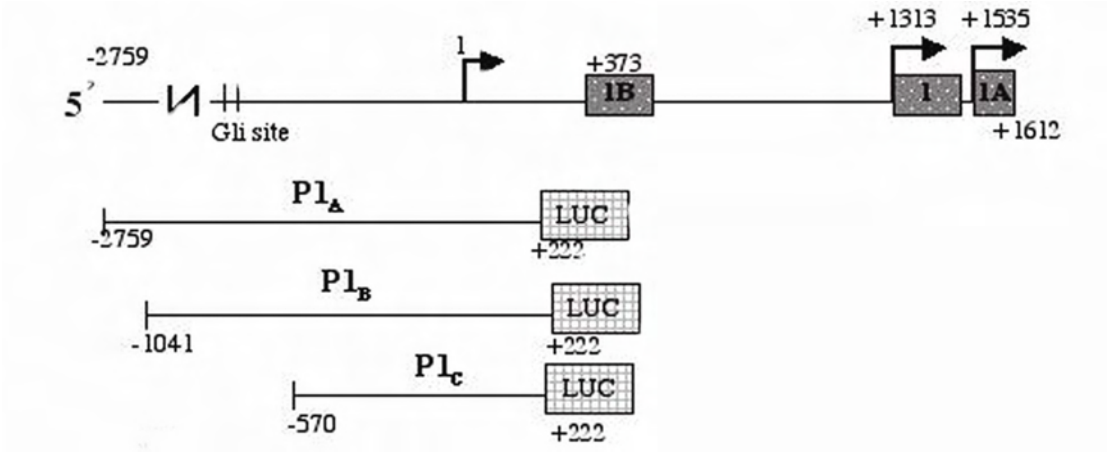

Fig.7 The 5' end of the PTCH1 gene and its three alternative exons (1, 1A and 1B) designated with grey spotted rectangles, three reported transcriptional start sites and two potential binding sites for Gli transcription factors. Constructs containing various parts of the 5' region were coupled to a luciferase vector designated with a grey chequered rectangle. © Marie Fiaschi

Next, we explored the Hh activator proteins (GLI1, GLI2, GLI3, SHH and SMO) and repressor variant [GLI3(N674)] transcriptional regulation of the *PTCH1* promoter. These proteins were coexpressed with the *PTCH1* promoter constructs in 293- and NIH3T3 cells. Luciferase analysis revealed an increased luciferase activity of the $P1_A$ driven promoter activity by all GLI proteins, SHH and SMO, most pronounced by GLI1. We also demonstrated a significant repression of the *PTCH1* transcriptional regulation by GLI3(N674). When performing the same transcriptional analysis with *PTCH1* promoter constructs not harbouring the Gli-binding site no activation or repression was obtained. Finally, the perfect Gli-binding site was mutated to a replicate of the Gli-binding site containing two mutations in the *PTCH1* promoter region that had previously been shown to be non-functional. Luciferase assay performed on this promoter construct harbouring two non-functional Gli-binding sites with Hh proteins almost totally abolished the transcriptional regulation of *PTCH1*. In combination, this suggests that regulation of the *PTCH1* expression by various components of the Hh signalling pathway is critically dependent on the single functional Gli-binding site.

3.2 PAPER II

Targeted expression of GLI1 in the mammary gland disrupts pregnancy-induced maturation and causes lactation failure

Breast cancer is one of the most common cancer forms occurring in women of the Western world. However, the role of Hh in breast tumourigenesis is elusive so we set out to clearly establish what happens if the Hh signalling pathway is miss-regulated in the mammary gland. To achieve this, we transgenically expressed the Hh effector protein, GLI1, under the mouse mammary tumour virus (MMTV) promoter [204].

First, we crossed TRE*GLI1* mice with MMTV*rtTA* mice, and doxycycline (dox) was supplemented during pregnancy or three weeks after the pups' birth. Dox supplementation resulted in target gene activation, which was confirmed by RT-PCR for GLI1 mRNA detection and immunohistochemistry for GLI1 protein detection. GLI1 expression levels were analysed at different time-points during pregnancy [6.5 days past pregnancy (dpc), 18.5 dpc and L1 (lactation day 1 or parturition day)]. Due to the auto-regulatory properties of the Hh signalling pathway, Ptch1 expression was enhanced in the GLI1 transgenic mammary glands at the same time-points as GLI1 [63]. Furthermore, RT-PCR analysis performed on the GLI1 transgenic mammary glands revealed that the time of dox supplementation did not affect the levels of GLI1 during pregnancy.

Strikingly, the first phenotype we observed in the GLI1 transgenic female mice was a failure to nurse their offspring. This defect was consistent and appeared only when dox was supplemented to the GLI1 transgenic mice. Interestingly, dox supplementation during embryogenesis or to a virgin or a multiparous dox untreated female rendered the same phenotype inability to lactate and feed their offspring. Whole mounts and hematoxylin and eosin (HE) stainings of mammary glands dissected from pregnant GLI1 transgenic mice at different time-points (6.5 dpc, 18.5 dpc and L1) revealed a mammary tree with a reduced lobuloalveolar network and minimal side branching and ductal sprouting. Immunohistochemical detection of the mammary stage specific proteins, Aquaporin 5 (AQP5), Na-K-Cl co-transporter 1 (Nkcc1 or Slc12a2) and Na-Pi type IIb co-transporter (Npt2b or Slc34a2) demonstrated an incomplete maturation and differentiation of the GLI1-transgenic mammary secretory epithelium during pregnancy. This finding was further supported by Northern blot analysis performed on mRNA from GLI1 transgenic mammary glands, which revealed a dramatic reduction in expression of milk protein genes such as whey acidic protein (WAP), β-casein and a-lactalbumin. Together, these results points towards a defect in the Jak2-STAT5 pathway.

The Jak2-STAT5 pathway is closely connected to mammary gland development, and disruption of signalling through this pathway during pregnancy results in impaired lobuloalveolar development [167]. We provide evidence that increased expression of GLI1 in the mouse mammary gland affects the expression of Stat5. Western Blot performed with a Stat5 antibody and an antibody specific for Stat5

phosphorylated at Y694 on protein extracts dissected from mammary fat pads revealed a reduced expression of Stat5 and a reduced amount of tyrosine phosphorylation of the Stat5 protein in the GLI1 transgenic mammary gland when compared to wt. Conditional gene inactivation of E-cadherin in differentiating mammary lobuloalveolar epithelial cells has been shown to result in diminished Stat5 expression, reduced milk protein expression and affected terminal differentiation [205]. Interestingly, western blot, immunofluorescence and immunohistochemistry with an anti-E-cadherin antibody revealed a dramatically reduced expression of E-cadherin in the GLI1 transgenic mammary glands. Moreover, GLI1 can regulate the transcriptional expression of E-cadherin via Snail and immunohistochemistry performed with an anti-Snail antibody demonstrated a nuclear increase of Snail expression in the GLI1 transgenic mammary glands [206-208]. The results described above depict a signalling cascade comprising GLI1, Snail, E-cadherin and Stat5 that, when altered, culminates in impaired lobuloalveolar development.

Unexpectedly, GLI1 transgenic females were never able to lactate again even if the oncogenic expression was withdrawn. RT-PCR and immunohistochemistry confirmed the low GLI1 expression in the bigenic females that had gone through one pregnancy under the influence of dox and another pregnancy without GLI1 induction [termed GLI1(+dox,-dox)]. These females showed similar morphological defects and Slc34a2 expression pattern as observed in GLI1 transgenic mice with permanent dox exposure. However, GLI1(+dox,-dox) mammary glands did not show an increased expression of Snail or a reduced expression of E-cadherin suggesting that the signalling cascade depicted above is not responsible for the impaired lobuloalveolar development detected in the GLI1(+dox,-dox) mammary glands. Interestingly, it was recently shown that impaired mammary remodelling could cause impaired lactation during following pregnancies [209]. HE and whole mounts revealed remaining epithelial clusters, enlarged ducts filled with cellular debris and inflammation in the GLI1(+dox,-dox) mammary glands one day after parturition and in GLI1 transgenic mammary glands after a full involution period (I14). In addition to this, staining with an anti-F4/80 antibody demonstrated an increase of macrophages in the GLI1 transgenic mammary glands taken at I14, which supports the hypothesis that an inflammatory response is triggered in the GLI1-induced mammary glands. The data presented above suggests, that the GLI1 transgenic mammary glands taken at I14 have a defect in mammary ductal clearance and epithelial involution, which may explain the irreversible changes detected in the GLI1 transgenic mammary glands.

Finally, we revealed that an altered GLI1 expression in the mammary gland affects mammary proliferation as measured by BrdU incorporation and that GLI1 further promotes cell cycle progression through enhanced and nuclear expression of Cyclin D1. Interestingly, morphological defects, comprising hyperplasia, appear in the GLI1 transgenic mammary gland at the same point in time when the level of BrdU and CyclinD1 was altered.

3.3 PAPER III

Development of mammary tumours by conditional expression of GLI1

Considering the mammary hyperplasia observed in our previous study and the fact that one of the features of neoplastic progression is inappropriate and increased frequency of proliferating cells, we decided to examine if mammary tumours would form in the GLI1 transgenic mice. In line with this, we showed that increased expression of GLI1 predisposes the mammary gland to carcinogenesis. The earliest tumours were detected after treating the animals with dox for 18 weeks, starting when the animals were three weeks old. All animals that developed mammary tumours had gone through at least one cycle of pregnancy (between 2 and 10). The tumours showed a heterogeneous epithelial cell content and were histologically classified as solid, ductal and squamous differentiated tumours (Fig.8).

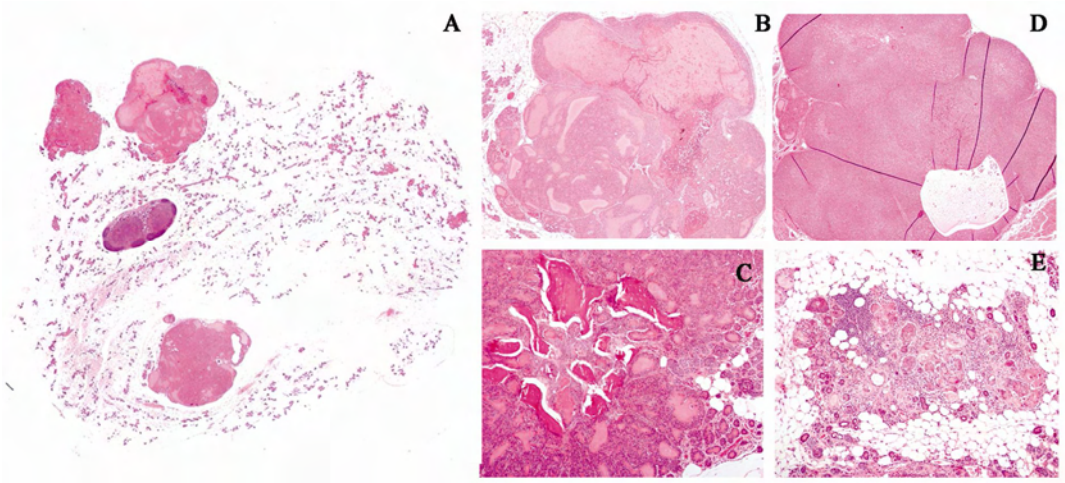

Fig.8 Mammary tumourigenesis in mice with targeted GLI1 expression. Sections of GLI1-induced mammary glands stained with HE. Mammary glands from GLI1-transgenic mice showing multiple heterogeneous tumours (A). Ductal (B, C), solid (D) and squamous (E) differentiated tumours present in mammary glands from GLI1-transgenic mice. Ossification of GLI1-induced ductal tumour (C, dark red areas). Necrotic area found in GLI1-induced solid tumour (D). © Marie Fiaschi

The solid tumours showed a cell-dense growth pattern and immunohistochemistry with epithelial markers [cytokeratin 5 (K5), cytokeratin 14 (K14), cytokeratin (K17), cytokeratin 8 (K8), Smooth muscle actin (SMA) and p63] revealed that the solid tumours almost exclusively consisted of basal epithelial cells. The ductal tumours presented a ductal like growth pattern, with K5 expression on the basal epithelial cells, SMA on the myoepithelial cells and K8 on the inner luminal epithelial cells. Ductal tumours showed a massive expansion of luminal epithelial cells and occasionally they were ossified. Squamous differentiated tumours contained K5 and K8 positive cells but no SMA positive cells were present. Immunohistological analysis revealed that all GLI1-induced mammary tumours expressed GLI1. Histological analysis further demonstrated the appearance of hyperplastic lesions and defective TEBs as early as 6.5 dpc in the GLI1 transgenic mammary glands. These lesions consisted of a disorganized epithelium and an invasion of epithelial cells in the mammary ducts and buds, respectively. Immunohistochemistry

performed on these hyperplastic lesions revealed an epithelial content, similar to that of the solid tumours with high expression of the basal markers (K5, K14, K17 and p63) and low absent expression of K8 and SMA. Mammary glands taken from GLI1 transgenic male mice showed hyperplastic lesions but no neoplasms during the experimental time-period (1 year).

The GLI1-induced mammary tumours showed a mixed luminal/basal or pure basal epithelial content, and human tumours with this epithelial content have been shown to have a poorer prognosis than breast tumours with a pure luminal phenotype [210]. GLI1-induced mammary tumours also showed an aggressive profile with necrotic and mitotic areas and a dramatic increase of mast cells. Mast cells are important for angiogenesis, and they were located in the vicinity of the tumours and infiltrating the hyperplastic areas. Stainings with an anti-estrogen-α antibody gave no positive signals in the GLI1 transgenic hyperplastic changes or tumours, which indicates poor histological differentiation and a high risk for metastasis [211, 212]. Immunohistological stainings performed on GLI1-induced tumours and hyperplastic changes with an E-cadherin antibody revealed low or even absent expression of E-cadherin. Reduced expression of the transmembrane protein E-cadherin is an important step in epithelial to mesenchymal transition (EMT), a process implicated in the progression of primary tumours towards metastases. In addition, imunofluorescence analysis on mammary glands removed from multiparous transgenic GLI1 mice with an anti-laminin and an anti-SMA antibody revealed a none-continuous staining of both these markers on the basement membrane on the GLI1 induced tumours. This indicates that the basement membrane is disrupted and that there is a possibility for tumour epithelial cells to invade neighbouring cells and become metastatic. Together these data clearly indicate that the GLI1-induced mammary tumours are aggressive and that they may metastasize.

A clear connection between Hh signalling and cell proliferation exists, and immunohistochemistry with anti-BrdU, anti-cyclin D1 and an anti-histone H3 antibodies revealed that the hyperplastic lesions and tumours formed in the GLI1 transgenic mammary glands, were highly proliferative [213, 214]. Dual immunofluorescence stainings for GLI1 with BrdU and histone H3 revealed that tumour cells coexpressed GLI1 with both BrdU and histone H3, indicating that GLI1 transgenic cells enter the cell cycle and divide. Hh signalling has been implicated in the regulation of stem and/or progenitor cells [215]. K6 and Bmi-1 are suggested mammary progenitor markers and immunohistological analysis revealed an increased staining in the GLI1 transgenic hyperplastic lesions and tumours when compared to wt mammary glands [191, 216, 217]. We quantified the progenitor marker K6 in the GLI1-induced hyperplastic lesions and found that 12.6 % (±1.5%) of all epithelial cells expressed K6. Dual immunofluorescence stainings with K6 and BrdU revealed that 0.7% (±0.1%) of these suggested progenitor cells were proliferative. The data described above, together with the fact that GLI1 transgenic mice generated heterogeneous mammary tumours, suggest that an altered expression of GLI1 in the mammary gland can affect the proliferation or differentiation capacity of the progenitor cells.

Recently it has been suggested that removal of the oncogene expression is not enough for reversal of the tumourigenic process. Unexpectedly, this proposal holds true for the GLI1-induced tumours. We induced the GLI1 expression by supplementation of dox during two pregnancies (15-21 weeks) followed by six additional pregnancies in the absence of induced transgenic expression [40-45 weeks, termed GLI1(+dox,-dox)]. RT-PCR and immunohistological analysis demonstrated that the GLI1 transgene expression was absent in the remaining GLI1(+dox,-dox) mammary tumours. The remaining mammary tumours showed high resemblance to the GLI1-induced ductal tumours, and they were ossificated and contained glycogenic fluids and cholesterol. The tumours that remained after dox removal showed a high content of rapidly dividing S-phase cells when staining with an anti-Cyclin D1 antibody and an increased expression of the progenitor marker K6 when they were stained with an anti-K6 antibody. Finally, the GLI1(+dox,-dox) tumours contained more stroma when compared to mammary tumours with continued transgenic expression.

In summary, we provide evidence that induced expression of the Hh effector protein, GLI1, in the mammary gland can give rise to heterogeneous mammary neoplasm's, which show aggressive characteristics and are not dependent on continued oncogenic expression. We also show that GLI1-induced mammary tumours are highly proliferative and possibly arise from progenitor cells.

3.4 PAPER IV

Targeted expression of GLI1 in the salivary glands results in an altered differentiation program and hyperplasia

Whereas exocrine organs such as mammary glands, pancreas and prostate, have received immense interest from developmental as well as tumourigenic points of view, less focus has been put on these processes in the major salivary glands (submandibular, sublingual and parotid gland). To study the importance of Hh signalling in salivary gland development and carcinogenesis, we transgenically expressed GLI1 in the salivary epithelial cells of mice using the mouse mammary tumour virus (MMTV) and the bovine cytokeratin 5 (K5) promoters. The MMTV promoter preferentially directs the expression to the epithelial cells in mammary glands but also to epithelial cells residing in the salivary glands and seminal vesicles [204]. The K5 promoter directs the expression to the basal cells of most, if not all, stratified epithelia [218].

First, we crossed TRE*GLI1* mice with MMTV*rtTA* mice and dox was supplemented during pregnancy or three weeks after pups' birth. Gene activation was confirmed by RT-PCR and immunofluorescence. Strikingly, histological analysis of the MMTV-GLI1 salivary glands revealed two major changes, areas comprised of small aggregates of basaloid cells, and ectatic ductal changes. The ductal cells in the cystic lesions seemed to be thinner and elongated, and these lesions emerged very early

and at a high rate in the GLI1 transgenic salivary glands. In addition, ectopic ossification and metaplastic elements appeared frequently in the MMTV-GLI1 salivary glands. The GLI1-induced hyperplastic salivary lesions showed a high proliferative rate as measured through BrdU incorporation as well as immunohistochemistry performed with an antibody directed against the cell cycle regulator CyclinD1. Furthermore, co-localisation studies with BrdU and GLI1 revealed that a fraction of the GLI1-transgenic cells were also actively dividing. Immunohistochemistry with epithelial markers K5, K14, K17, K8, SMA and p63 revealed an almost homogeneous content of basal transgenic cells in the GLI1-induced hyperplastic lesions. Interestingly, GLI1-induced salivary glands induced by the K5 promoter, generated defects similar if not identical to the MMTV-GLI1-induced salivary gland. This suggests that the MMTV-LTR promoter and the K5 promoter are active in analogous cellular compartments and strengthens the notion that the changes we observe develop from basal epithelial cells. No tumours were formed during the experimental time period (one year). However, basaloid aggregates and ductal lesions appeared in all of the parous GLI1 transgenic females as well as in the long-term doxycycline treated male salivary glands. The GLI1 transgenic salivary glands revealed a phenotypic variation which was dependent on the time and duration of dox induction and the parous state of the female mice. Prenatal induction of GLI1 in salivary epithelial cells rendered extensive changes, especially in the SLG. In contrast, postnatal induction of GLI1 in salivary epithelial cells renders similar defects, but these defects were not as widespread and, developed at a more moderate rate. HE staining never revealed any changes in salivary glands dissected from virgin females (5-10 weeks).

Salivary gland development occurs both pre- and postnatally and salivary acinar differentiation is completed around puberty. Unexpectedly, HE staining revealed a dramatically reduced amount of secretory acinar structures in the GLI1 transgenic salivary glands during pregnancy. In verification of this, immunohistological stainings with the differentiation and salivation markers AQP5 and Nkcc1 were shown to be dramatically reduced in the salivary glands with induced GLI1 expression. This further implicates that the GLI1 transgenic mice have a defect in salivary acinar differentiation and salivary gland salivation. No or very low expression of AQP5 and Nkcc1 was revealed in the GLI1-induced hyperplastic lesions. The morphology of the GLI1 transgenic salivary glands are highly similar with the salivary gland morphology observed in patients who have gone through radiation therapy or mice with obstructed salivary ducts [219]. Interestingly, salivary glands with severe atrophy as a consequence of duct ligation can regenerate after duct reopening [220]. Similarly, HE stainings revealed a regenerative capacity in salivary glands dissected from mice 8 weeks post termination. Salivary glands dissected from these mice where histologically reminiscent of wt salivary glands, and they revealed an increase in acinar structures as well as a normalisation of the ducts. However, some minor changes were still present in the ductal region of these salivary glands. Taken together, our data reveal an unexpected role for GLI1 in salivary acinar differentiation and show that GLI1 can induce lesions consistent with a role for GLI1 in salivary gland epithelial cell proliferation.

4 GENERAL DISCUSSION AND FUTURE ASPECTS

Paper I

Alternative splicing and alternative promoter usage are effective mechanisms for generating diverse gene products from the same genomic region. Recent analyses have demonstrated that over 74% of human genes are characterized by alternative splicing variations, and at high frequency these events occur in the 5´ untranslated regions [221, 222]. This suggests that a major pathway for the complexity of expression of the human proteome may depend on the utilization of alternative first exons and consequently promoter regions. Furthermore, alternative splicing is also implicated in pathophysiological processes, and it has been estimated that at least 15% of point mutations that cause human genetic diseases affect splicing [223].

Splice variations of several key components of the Hh signalling pathway exist and recent genome-wide RNAi screening indicated that splicing has a significant role in Hh signalling regulation [224]. The *PTCH2* gene can be transcribed into mRNAs with variable 3'ends [108], the *GLI1* gene can generate isoforms with different 5'UTRs [225] and the *GLI2* gene can generate isoforms with variable internal exons [226]. Furthermore, the *PTCH1* gene is reported to have four first exons (1, 1A, 1B and 1C) and additional mRNA isoforms with changes downstream of exon2 have been identified [59, 63-66, 227, 228]. The biological function of the different PTCH1 isoforms with unique 5'ends is still an open question. One possible way to elucidate the role of the PTCH1 variants would be to do knockout studies in mice where the *PTCH1* 5' regions are targeted one by one. Today, we know that all of the PTCH1 isoforms with unique 5'ends can interact with SHH and partially inhibit SMO, but only the PTCH1 exon1B isoform is able to fully inhibit SMO [65, 66]. In the yolk sac and optic placode, PTCH1 exon1C is exclusively expressed and is suggested to function as the Hh receptor in these tissues [67]. Furthermore, only the PTCH1 exon1B isoform show induced levels in BCC, which is thought to be due to the autoregulatory properties of the Hh signalling pathway [66]. Similarly, one of the GLI1 and one of the GLI2 isoforms show increased expression in BCC [225, 226]. As more and more information about the biological function and gene diversity of the different genes involved in Hh signalling is gathered, chances of identifying the roles of the different splice variants will also increase. In this context it will be important to consider the possible complexity of interactions between different splice variants at different steps along the Hh pathway. Furthermore, it will be important to fully elucidate the role of the different splice variants in development and cancer formation.

Paper II

The Hh signalling pathway needs to be tightly repressed during embryogenesis and puberty in order for the mammary gland to develop correctly [174]. During these stages no expression of either Ptch1 or Gli1 is evident in the mammary gland [174]. However, during the mammary cycle, comprising pregnancy, lactation and involution, Ptch1 starts to be expressed and becomes elevated throughout pregnancy and lactation and down regulated during involution [177]. In paper II we show that an elevated level of GLI1 severely alters the morphology of the mammary gland during the cyclic phase of

mammary gland development. Pups who nursed from GLI1 transgenic mothers did not survive and histological analysis revealed an undifferentiated lobualveolar network during pregnancy. Conversely and in support of our data, it has been revealed that inhibition of the Hh signalling pathway induces mammary cell differentiation in a mammosphere assay [191]. We suggest that the lactation failure of the GLI1 transgenic mice is a consequence of GLI1 action on the expression levels of Snail, E-cadherin and Stat5. Interestingly, it was recently shown that Stat5a is necessary and sufficient for the establishment of luminal progenitors and loss of Stat5a/5b impaires development of alveolar precursor cells as well as maintenance of mature alveolar cells [229-231]. Furthermore, Stat5 acts as a survival factor during pregnancy, and depletion of Stat5 during this stage results in premature cell death [232]. This is analogous to what we observe in the GLI1 transgenic mammary glands where premature apoptosis at parturition is detected.

We also revealed that an altered level of GLI1 during one pregnancy is enough to elicit sustained damage to the mammary gland and suggests that this failure is due to an incomplete involution process. This failure can partly be explained by the apoptotic cells and milk proteins that remain in the lumen of the ducts. Why these cells and proteins are not removed is an open question. However, it would be interesting to elucidate if these cells express apoptotic markers on the surface, like the phosphatidylserine, which are important for the engulfment of apoptotic cells or if the levels of factors like the milk fat globule EGF factor 8 (MFG-E8) which recognize these apoptotic markers and promote engulfment of apoptotic cells, are altered [209]. Also, we reveal that transgenic GLI1 mammary glands after involution contain an increased amount of epithelial cells. Why these cells can escape apoptosis is not clear. However, it would be interesting to check the levels of the Hh target gene *Bcl2* [112], which should be down regulated during involution in order for apoptosis to occur. Interestingly, the epithelial cells that remain are actively dividing and associated with an increased expression of the Hh target gene *CyclinD1* [111]. This indicates that GLI1, when deregulated in the mammary gland, facilitates two important hallmarks of cancer, viz. evasion from apoptosis and self-sufficiency in growth signals.

Paper III

Tumours appeared in GLI1 induced parous females during the experimental time period (1 year). However, we now know that histologically similar tumours emerge in GLI1-induced mammary glands of virgin mice if the experimental time period is prolonged. The GLI1-induced tumours showed an aggressive profile but did not metastasize. Since the human aggressive basal-like carcinomas usually lack expression of hormones (estrogens and progesterone) and HER2, future plans are to investigate the status of these receptors in the GLI1-induced tumours. Tumours with basal, basal/luminal or squmous epithelial differentiation pattern appear in the GLI1 transgenic mammary glands, which suggests that GLI1 targets stem or progenitor cells.
In support of this, it has been shown that PTCH1, GLI1 and GLI2 are expressed in mammospheres derived from normal human breast tissue, and that activation of the Hh pathway, by addition of SHH protein or overexpression of GLI1, GLI2 or a mutated variant of Smo, results in increased mammosphere formation [191]. Conversely, inhibition of the Hh pathway with cyclopamine was reported to decrease mammosphere formation-efficiency of human mammary cells [178]. The hypothesis is

that Hh increases the proliferation of the progenitors rather than increases the stem cell pool. In line with this, Li et.al. showed that dams dissected from pregnant heterozygous *Ptch1* mice contained an increased progenitor population, whereas the stem cell fraction was constant [233]. This suggests that Hh signalling plays a particular role in pregnancy-associated mammary gland expansion. We reveal an increased expression of the progenitor cell markers K6 and Bmi-1 in the epithelial cells in the GLI1-induced tumours, which also support a role for Hh in the regulation of mammary progenitors. However, to clearly elucidate the impact GLI1 has on mammary stem/progenitor cells it would be interesting to sort, by way of FACS (Fluorescence-activated cell sorting) the mammary stem and progenitor cells from the GLI1-induced tumours and further try to grow them as mammospheres. Furthermore, it would be compelling to use GANT61, a potent small molecule GLI1 inhibitor [203], on GLI1-induced mammary tumour cells either in the GLI1 transgenic mice or, if the cancer cells were transplanted, into nude mice. The hypothesised reduction or suppression of the tumours by GANT61 may not hold true since GLI1-induced tumours remain without induced GLI1 expression.

Paper IV

The role of Hh in salivary gland morphogenesis is not clearly established. However, in paper IV we provide evidence that a deregulated expression of Hh, at the GLI1 level, in salivary epithelial cells severely alters the morphology of all the major salivary glands. Hyperplastic elements appear at high frequency in the GLI1-induced salivary glands, but no tumours are formed, which may imply that the salivary epithelial cells need additional changes to become tumourigenic. One event which occurs in about 50% of all cancer cases and is also prevalent in human salivary carcinomas, is mutation of the *p53* gene [234]. According to this, it would perhaps be possible to induce salivary gland tumours in this mouse model if they were crossed with a p53 mutant strain. The GLI1-induced hyperplastic lesions are highly proliferative and appear as protrusions from the salivary intercalated ductal region. There are speculations that intercalated ducts harbour the salivary stems cells, and to reveal if GLI1 acts on salivary stem cells one might analyze the GLI1 transgenic salivary glands with the salivary stem cell markers [Sca-1(+)/c-Kit(+)] both with FACS and/or immunohistochemistry and further try to grow these cells as salispheres.

In addition to hyperplasia these glands reveal ossifications and cystic lesions. Hh signalling is closely coupled to chondrocyte proliferation and differentiation via parathyroid hormone-related protein (PTHrP) and it would be interesting to study the levels of PTHrP in these glands [235]. The appearance of cystic lesions suggests an expansion of the secreting ducts, an event which has been described previously [195, 196]. Finally, we also reveal that salivary glands with an induced expression of GLI1 have a reduced number of secreting acinar cells. This finding is analogous to what we revealed in the mammary gland when the levels of GLI1 were induced, abrogation of alveolar differentiation (Paper II). The morphology of the GLI1 transgenic salivary glands, with blocked acinar cell differentiation, ductal cell hyperplasia and duct expansion, shows some similarities with the salivary gland morphology observed in patients who have undergone radiotherapy or in mice where the terminal excretory duct has been ligated to prevent outflow of saliva [219]. Due to this similarity and to the fact that injured/stressed salivary glands have been suggested to induce proliferation of stem/progenitor cells, it would be interesting to study the levels of GLI1 in the

obstructed salivary glands of mice and/or in damaged salivary glands from patients who have undergone radiotherapy in the head and neck region. Interestingly, the salivary gland phenotype is reversed if the ligated duct is reopened [220], and similarly GLI1-induced salivary gland changes are reversible upon termination of dox. If it were revealed that radiation- induced salivary glands expressed high levels of GLI1, and that GLI1 acts on salivary stem cells, therapeutic targets for the development of future strategies to manage salivary gland diseases and radiation-induced damages would perhaps be found in the Hh signalling pathway.

5 SAMMANFATTNING PÅ SVENSKA

Utvecklingen från embryo till fullvuxen organism kräver att celler kan signalera till varandra och därmed informera om vilken typ av vävnad som skall bildas. Det är först under de senaste årtiondena som man har förstått att det är proteiner som sköter denna signalering. Proteinerna signalerar till varandra i specifika mönster, så kallade signal-kedjor, och det är ytterst viktigt att korrekt interaktion sker mellan proteinerna i dessa signalkedjor. Evolutionärt, om man studerar embryoutveckling i organismer från bananfluga (*Drosophila*) till människa, har det visat sig vara ett fåtal signalvägar som är essentiella och en av dessa signalvägar är Hedgehog (Hh). Hh signalvägen är viktig under den embryonala utvecklingen, men även viktig för fortsatt utveckling efter födseln.

Hh signaleringen startar med att proteinet Hh bildas i en cell och signalerar vidare till en annan cell genom att binda till det membranbunda receptorproteinet Patched1 (PTCH1). När PTCH1 binds upp av Hh medför detta att ett annat membranbundet protein, Smoothened (SMO), kan skicka en signal vidare in i cellen. Denna signal leder i slutändan till att GLI1 proteinet frigörs och transporteras in i cellkärnan. Inne i cell-kärnan aktiverar GLI1 en process kallad transcription. Under transcriptionsprocessen kopieras det genetiska materialet och därefter translateras/omarbetas det för att till slut resultera i framställandet av ett specifikt protein. Beroende på vilket protein som framställts kan olika processer påverkas t.ex. celldelning, cellspecificering eller celldöd. Förändringar i Hh signalvägen kan leda till uppkomst av olika cancerformer bland annat hudcancerformen BCC som i västvärlden är den vanligast förekommande cancerformen.

Denna avhandling handlar om det Hh-relaterade proteinet PTCH1 vars function är att hindra celldelning i frånvaro av Hh. Avhandlingen handlar även om vilken roll Hh signalvägen spelar i bröst och salivkörtelutveckling samt om förändringar i Hh signalvägen kan orsaka uppkomst av cancer i dessa organ.

I **arbete I** ville vi undersöka hur *PTCH1* uttrycket reglerades. Tidigare studier har visat att det finns tre olika varianter av *PTCH1*, kallade 1, 1A och 1B. Vi fann att uttrycket av de tre *PTCH1* varianterna kunde regleras oberoende av varandra. Vi visade även att uttrycket av *PTCH1* både kunde aktiveras och reduceras av komponenter i Hh signalvägen. Dessutom fastställde vi att Hh signaleringen aktiverar/reducerar uttrycket av *PTCH1* via en specifik DNA-sekvens som GLI1 kan binda till och när denna sekvens är förändrad (muterad) kan Hh signaleringen inte längre påverka uttrycket av *PTCH1*.

I **arbete II** studerade vi vilken roll Hh signalvägen spelar i bröstutveckling. Transgena möss som överuttrycker GLI1 i bröstvävnaden framställdes. Möss med ökat uttryck av GLI1 i bröstvävnaden kunde inte dia sina ungar och de visade en kraftig minskning av mjölkprotein produktionen. Dessuton återbildades bröstvävnanden inte på ett korrekt sätt i dessa möss efter graviditeten och vi kunde detektera en ökning av celler som delade sig, vilket kan vara ett tecken på dysplasi, det vill säga ett förstadie till cancer.

I **arbete III** studerade vi mössen från arbete II under en längre tidsperiod och såg att dessa möss utvecklade brösttumörer. Tumörerna som bildades var inte homogena, utan tre olika typer av tumörer med olika differentieringsmönster och cellinnehåll utvecklades. Tumörerna delade sig aktivt och visade en aggressiv överlevnadsprofil. Dessutom uttryckte tumörcellerna stamcellsmarkörer, d.v.s. markörer som detekterar omogna celler med förmåga att bilda ett stort urval av kroppens celler. Detta arbete är det första som klart påvisar att förändringar i Hh signalvägen kan leda till uppkomst av bröstcancer.

I **arbete IV** studerade vi hur ett ökat uttryck av GLI1 påverkar utvecklingen av salivkörteln hos möss. GLI1 inducerade salivkörtlar uppvisar kraftiga morfologiska förändringar. Vissa av dessa förändringar visar stora likheter med de förändringar som uppträder i bröstvävnad som har inducerats med GLI1. Dessutom såg vi att de salivproducerande cellerna, i likhet med de mjölkproducerande cellerna i bröstvävnaden, inte var fullt utvecklade. Dessa data indikerar att möss som överuttrycker GLI1 i salivkörtlarna förmodligen inte utsöndrar någon saliv samt att förändringar i Hh signalvägen kraftigt kan påverka salivkörtelutvecklingen.

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