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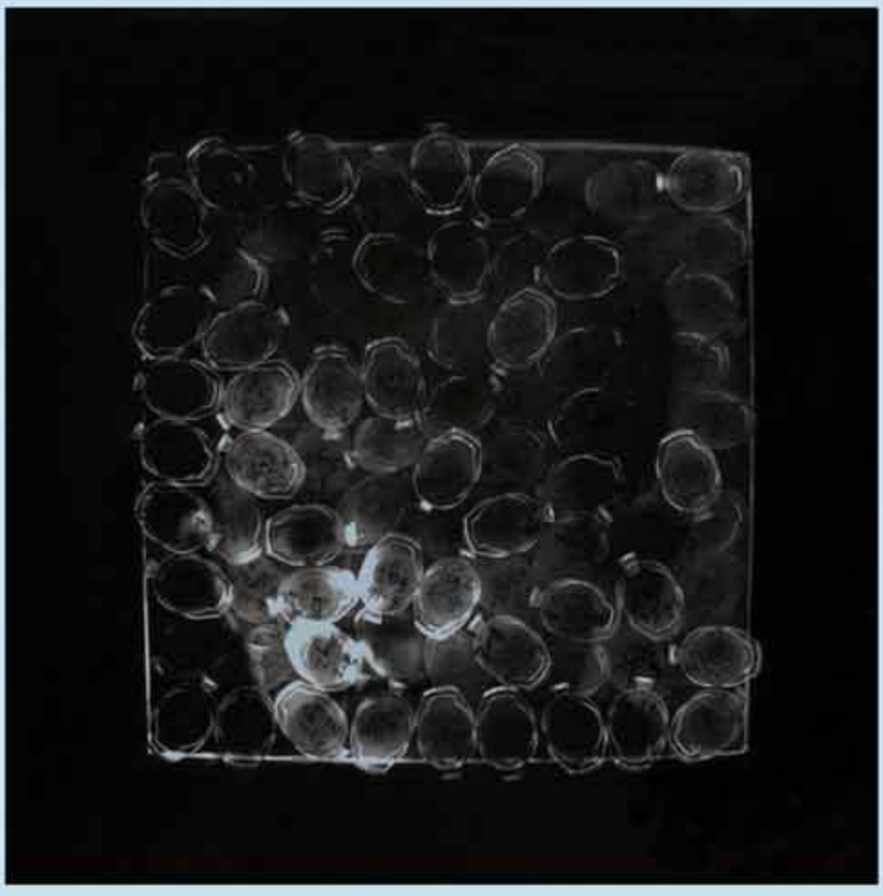
Ariel Ruiz i Altaba

Hedgehog-Gli Signaling in Human Disease



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Hedgehog-Gli Signaling in Human Disease

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HEDGEHOG-GLI SIGNALING IN HUMAN DISEASE

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Dedication

To Robert Gorlin for the formulation of the syndrome that carries his name and for his story of a Gorlin's syndrome patient carrying a snake in a taxi that crashed, who was bitten by the snake and had a broken leg, as told at a recent meeting. To all the past and present members of the Ruiz i Altaba lab, including Nadia Dahmane, Barbara Stecca, Virginie Clément, Verónica Palma, Pilar Sánchez, Christophe Mas and José Mullor. To our collaborators, to the scientists in this exciting field, to the contributors to this book, and to the patients who give their tissues and cells for research. I am grateful to Barbara Stecca, Virginie Clément, Mare Zbinden, Simón Restrepo and Christophe Mas for help in the editing of this volume.

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PREFACE

The last two decades have seen an explosion in the understanding of how specific mechanisms of cellular communication regulate embryonic development, adult homeostasis, stem cell behavior and cancer. One of these systems that cells use to talk to each other and thereby know their position utilizes Hedgehog glycoproteins. Hedgehog signals regulate key transcription factors of the Gli family in the responding cells. The Hedgehog-Gli pathway is used many times in many organs in the life of an individual, including a number of stem cells and derived progenitors. Insufficient signaling in the embryo can lead to cyclopia and other birth defects while enhanced function can lead to tumor formation. Why? What is the context and rationale? What do the Gli proteins regulate? What is the exact link between embryonic development, stem cell lineages and cancer? Can Hedgehog-Gli signaling be used to regulate stem cell lineages in regenerative medicine? These are some of the questions that the chapters in this primer address.

A decade has passed since the discovery of the Hedgehog genes in vertebrates and the establishment of causal links between Hedgehog signaling and cyclopia, Gorlin's syndrome and familial basal cell carcinomas by a number of laboratories. An important step in the understanding of human sporadic cancer came four years ago with the finding that sustained signaling is required for the proliferation of brain tumors, a result from our laboratory that has since been expanded to an increasing number of cancers. Thus, we are now witnessing the birth of a great new hope for the understanding of cancer and other diseases that is taking place hand in hand with the elucidation of the molecular mechanisms orchestrating embryonic development.

It is with this enthusiasm that the present book has been edited, knowing full well that all scientific knowledge is, per necessity, partial and in need of revision and expansion, and that great hopes and expectations are often just that. However, considering the enormous advances made after the discovery of Hedgehog-Gli function in animal development and cancer described in the chapters in this book, it is possible to suggest that we are just entering a long ascending ramp, one that opens new avenues for further scientific exploration. Importantly, the research in this book also raises possibilities for the development of rational, anti-cancer therapies targeting Hedgehog-GLI signaling in the very near future.

Ariel Ruiz i Altaba
Geneva, Switzerland
2005

CHAPTER 1

How the Hedgehog Outfoxed the Crab:
Interference with HEDGEHOG-GLI Signaling as Anti-Cancer Therapy?

Ariel Ruiz i Altaba

The enormous success of the last two decades in molecular embryology and developmental genetics have afforded fresh views on old problems. For instance, from a developmental point of view there is an alternative way to think about solid cancers that contrast with the single cell focus derived from molecular and cell biology and their emphasis on the cell cycle and the concepts of transformation and oncogene. One can consider cancers as diseases of patterning affecting stem cell lineages or properties. Within this framework, the funnel hypothesis[1] suggests that the multiple ways to induce a given type of tumor in the mouse and the many functional mutations identified in a given tumor in humans lead to the activation of a single or few events in the cell that affect its position and identity. A one to one relationship between phenotype and final molecular mechanism with some redundancy is proposed for cancer, much as in embryonic development. Moreover, the ability of pathologists to recognize consistent morphologies in tumors and to give appropriate diagnoses is explained by the action of consistent paradevelopmental programs.[1] Concepts like cell competence, specification, niche, developmental history and positional information thus become central to understand, and hopefully treat, cancer. Indeed, under this light, understanding cancer becomes necessary in order to have a complete view of developmental potential.

We can then consider—with caution as we presently lack the perspective that elapsed time affords—what has been learned and achieved on Hedgehog signaling in cancer in recent years. The Hedgehog story is an unfolding one, one that does not yet have a known end, and is only one of several stories,[2] but so far it is the most promising one.

This story starts with the discovery of the *Hedgehog* (*Hh*) gene in *Drosophila melanogaster* ~25 years ago, well before Hh signaling was implicated in cancer. The identification of the *Hh* gene through its mutation, which causes aberrant embryonic patterning giving the mutant larva a 'hairy' aspect, and thus named hedgehog,[3] was published in 1980. The cloning of the single *Drosophila Hh* gene was published by several groups in the early '90s,[4-6] with the cloning and identification of *Hh* genes in vertebrates (*Sonic hedgehog* (*Shh*), *Indian Hh* and *Desert Hh* in mice and humans) following shortly afterwards.[7-10] The definition of the expression patterns of *Hh* genes in the developing embryo showed that they are often strikingly restricted to cells and areas previously known to have special patterning activities. The exquisite spatio-temporal regulation of *Hh* genes in the developing embryo and adult tissues, together with the ability of Hh proteins to elicit defined cell fate changes in a concentration-dependent manner highlighted a revolution in molecular embryology, providing mechanistic explanations for critical events in

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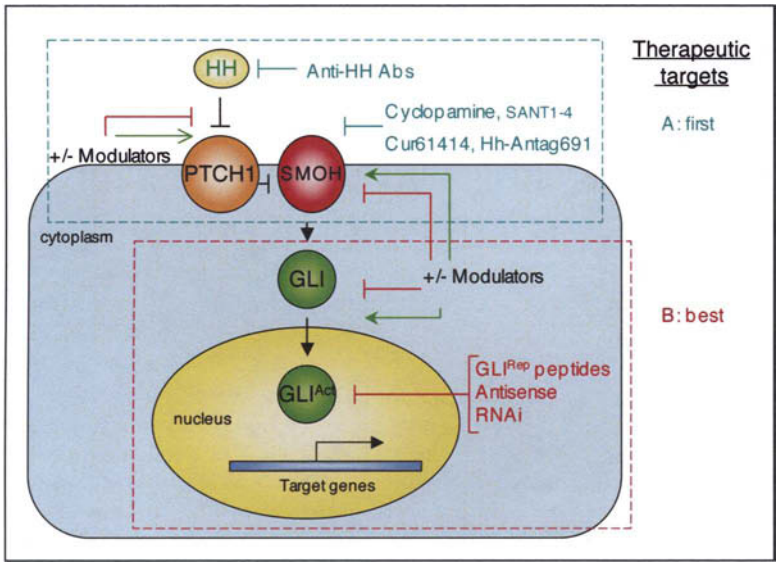

Figure 1. Scheme of the HEDGEHOG-GLI pathway. The action of HH, PTCH1 and SMOH at the membrane level is emphasized, together with the site of action of natural and artificial inhibitors. The former include HIP, GAS and PTCH1 itself, while the latter include cyclopamine and anti-HH antibodies. The action of SMOH leads to the activity of the GLI proteins. Resident GLI proteins in the cytoplasm, likely bound to the cytoskeleton are activated and translocate into the nucleus where they regulate target gene transcription. The function of the GLI proteins depends on the function of several modifiers acting downstream of SMOH. The scheme emphasizes the action of endogenous modifiers as well as artificial blockers of GLI function, including GLI repressor peptides, antisense molecules and RNAi. Inhibitors to the upper component of the pathway (A) have lead the way, but inhibitors of the lower part, inhibiting directly or indirectly GLI function (B), are predicted to be superior as these will inhibit pathway function by any activating event at any level.

the formation of the animal body plan. This includes for instance, the patterning and growth of limb buds and of the brain and spinal cord.[7-13]

The activity of the Hh pathway involves several steps (Fig. 1).[12-14] Secreted Hh glycoproteins act via the transmembrane proteins Patched1 (Ptch1) and Smoothened (Smo). It is thought that Hh binding to Ptch1 inhibits the repression of Smo by Ptch1 so that Smo functions only when Hh is present. Smo in turn transduces the Hh signal intracellularly to activate the zinc-finger Gli transcription factors.[13] The Gli proteins are required and sufficient to mediate Hh activity. A number of modulators, positive or negative, some involving negative feedback such Hip1, Gas1 and Ptch1 itself, also regulate Gli activity so that this is tightly controlled in time and space. Because the Hh-Gli pathway is to a large extent linear, activity can be achieved by loss of function of negative factors, such as Ptch1 or the gain of function of positive factors, such as mutations in Smo that may render it insensitive to Ptch1 repression.

The initial link of *HH* signaling and familial human cancer was published in the mid-1990s[15,16] linking mutations in *PATCHED1* (*PTCH1*, an inhibitor of HH signaling; Fig. 1), with the rare Basal Cell Nevus syndrome or Gorlin's syndrome.[17] Affected individuals can develop numerous basal cell carcinomas (BCCs) of the skin at an early age as well as medulloblastomas and other tumors, and display additional malformations including odontogenic keratocysts.[17] These studies converged on *PTCH1* from the study of its function in flies and mammals[16] and from skin carcinogenesis.[15]

The first demonstration of a functional link of HH-GLI signaling with sporadic human cancer was published in 1997, proposing that all human BCCs—the most common type of

cancer—derive from the activation of the HH-GLI pathway as all examined BCCs expressed *GLI1*[18] (Fig. 1), since its transcription is the best marker of a cell's response to HH signaling.[19] In addition to *GLI1*, *GLI2* and *PTCH1* (another HH regulated gene[20]) have also been found to be expressed consistently in sporadic BCCs.[18,21-25]

GLI1 was discovered as an amplified gene in a human glioma line in 1987.[26] This was followed by the discovery of a related gene in vertebrates (*GLI3*[27]) and independently of a single related gene, *Cubitus interruptus (Ci)*, in *Drosophila*.[28,29] A role for GLI1 in cancer was negated by subsequent findings[30,31] and research on *GLI1* waned for several years. However, *GLI3* was shortly afterwards identified as the gene mutated in Greig's Cephalopolydactily syndrome[32-34] and later implicated in Pallister-Hall syndrome.[35,36] Patients affected with these diseases display a wide range of phenotypes, including additional digits, skull defects, imperforate anus and hypothalamic growths (hamartomas). The misregulation of post-transcriptionally cleaved C-terminally-deleted forms of GLI3 acting as dominant repressors,[37-41] as first shown for Ci,[42] has been implicated in these syndromic phenotypes with debated genotype-phenotype correlations.[43,44,228] Aberrant GLI function has also been implicated in other syndromic phenotypes, including VACTERL,[45,46] a syndrome that includes vertebral anomalies, anal atresia, cardiac defects, tracheoesophageal fistula, and renal and limb defects.

Three *Gli* genes were then cloned from different species, including mice,[47-49] chicks,[50] frogs,[19,51] and zebrafish.[52,53] They were originally shown to affect neural patterning by mimicking Shh.[19,54] In addition, microinjection of human or frog Gli1 lead to sporadic epidermal and CNS tumors or hyperplasias (Fig. 2).[18,55] Interpretation of GLI1-induced epidermal hyperplasias as tumors related to human BCCs awaited the papers on the involvement of loss of *PTCH1* in Gorlin's syndrome[15,16] in which patients develop numerous BCCs.[17]

Following the finding of familial mutations in *PTCH1* in Gorlin's syndrome patients[15,16] and simultaneous with the involvement of HH-GLI signaling in sporadic cancer,[18] mutations in the HH-GLI pathway components *PTCH1* and *SMOH* (Fig. 1) were sought and found in a small fraction of sporadic skin, brain and other tumors,[21,56-67] some of which have been proven to have functional relevance.[62] There are also reported mutations in Suppressor of fused[68] (*SUFUH;* but see ref. 69), a negative modulator of GLI function.[70-72] Mutations in the *GLI* genes have not been found in tumors, with the exception of *beta-actin-GLI1* fusions in a subset of pericytomas.[73] These findings provide possible bases for the activation of the HH pathway in a fraction of tumors although many others might derive from epigenetic events.

Various animal models demonstrating the sufficiency of an active Hh-Gli pathway to induce tumor formation (Table 1, Fig. 2) began to be published in 1997 for skin (*Shh*;[74] *Gli1*;[18,75,76] *Smo*;[62,77] *Ptch1*;[78] *Gli2*[77,79-81]), brain (*Ptch1*;[82,83] *Gli1*,[55] *Shh+c-Myc*;[221] *Shh+IGF2*;[222] *Shh+Akt*[222]) and muscle (*Ptch1*[83]). At the same time, a rational context for the various human tumors now associated with HH-GLI signaling began to be uncovered with the realization that elements of this pathway are expressed in the corresponding normal tissues and that HH-GLI signaling controls growth, patterning and/or homeostasis of developing and adult organs such as the skin[18,74,84,85] and the cerebellum[86-88] (Fig. 3). Indeed, the control of cell proliferation in the late embryonic and perinatal dorsal brain by SHH-GLI signaling may account for its involvement in many types of CNS tumors.[55]

Shh-Gli signaling is not only required for the normal expansion of many types of progenitors but it is also required for the control of neural stem cell behavior in the perinatal and adult rodent brain, including the embryonic neocortex[89] (Fig. 4), the perinatal and adult hippocampus[90,91] and the subventricular zone of the lateral ventricle of the forebrain[91,92] (Fig. 4). Shh also regulates the proliferation of embryonic spinal cord precursors.[93] Similarly, there is evidence that Hh signaling regulates precursor/stem cell lineages and morphogenesis in other tissues and organs, such as the intestine[94-96] and perhaps the skin.[81,97] Together, these findings have raised the possibility that tumors derive from HH-sensitive stem cell lineages, possibly acting at the level of stem cells in some cases or at the level of derived early precursors.

Generally, the best evidence that stem cells exist in tumors come from the transfer of leukemia from a sick mouse to a healthy recipient by the implantation of a few rare cancer stem cells[98-100]

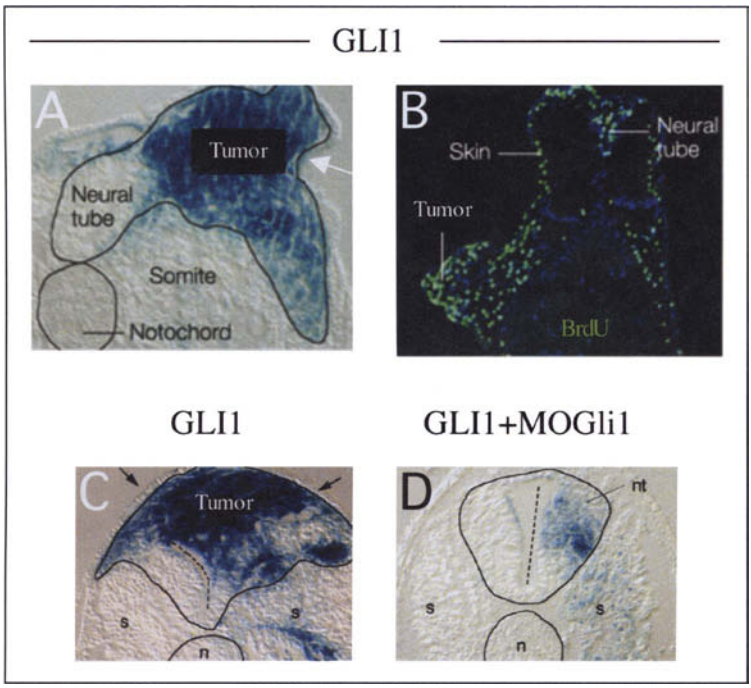

Figure 2. Induction of tumors by misexpressed GLI1. Misexpression of human GLI1 in the developing frog embryo leads to CNS (A,C,D) and skin (B) tumors. Coinjection of *GLI1* RNA plus *LacZ* RNA allows the identification of tumor-forming cells as descendants of the cells that received the injected materials and are blue after X-Gal reaction (A,C,D). Labeling of cycling cells with BrdU (B, green) shows tumor hyperproliferation. Coinjection of human *GLI1* plus a morpholino-modified antisense oligonucleotide specific for the endogenous frog *Gli1* RNA leads to tumor suppression (D), indicating the requirement of sustained endogenous Gli1 function for tumorigenesis. Reprinted with permission from refs. 18 and 55.

and the impressive findings that teratocarcinoma cells can contribute to the development of an entire fertile mouse when transplanted into the inner cell mass of a recipient embryo.[101-103] Here, cancer would appear to be induced by genetic/epigenetic changes that are reversed in the appropriate context or niche. These original experiments demonstrate the stemness of teratocarcinoma cancer cells and their totipotency.

For adult solid tumors, evidence in favor of the existence of cancer stem-like cells derives from the finding that cells with self-renewal properties have been reported in tumor cells from brain and breast.[104-110] Since a small number of glioma cells show clonogenic properties that are regulated by HH signaling (P. Sanchez, V. Clément and ARA, pers. com.) stem cell lineages (stem cells and/or their early derived more restricted progeny) may thus be the target of carcinogens, mutations and epigenetic changes that lead to the initiation of tumorigenesis in the brain and other HH-responsive organs and tissues, such as the epithelial compartment of the prostate, lung or gastrointestinal tract.

The first demonstration that human sporadic cancers require sustained HH-GLI signaling for continued growth derived from the ability to inhibit the proliferation of medulloblastoma cells by blocking HH signaling.[55] Since then, a rapidly increasing number of sporadic human tumors has been found to express *GLI1* and sometimes *SHH* (Fig. 5), and to be dependent on sustained HH-GLI pathway function (Table 2). These include basal cell carcinomas;[18,111,224] medulloblastomas,[55,112] gliomas[55] (P. Sanchez, V. Clément and ARA, pers. com.), pancreatic

Table 1. Human cancers dependent on sustained HH-GLI signaling

Tumor	*Inhibitors*	*Target**	*References*
Medulloblastoma	Cyclopamine	PC, CL	55
	Cyclopamine	PC, MA, CL	112
Glioma	Cyclopamine	PC, CL	55
Basal cell carcinoma	Cyclopamine	patients	169
Oral squamous cell carcinoma	Cyclopamine	CL	125
Small cell lung cancer	Cyclopamine Hh antibody	MA, CL	115
Pancreatic cancer	Cyclopamine	MA, CL	113
	Hh antibody	MA, CL	116
	Cyclopamine	CL	114
Digestive tract cancer	Cyclopamine Hh antibody	PC, MA, CL	116
Prostate cancer	Cyclopamine RNA interference Hh antibody	PC, CL	117
	Cyclopamine Hh antibody	MA, CL	118
	Cyclopamine	CL	19
Breast cancer	Cyclopamine	CL	121
Colorectal cancer	Cyclopamine	CL	129

* PC: primary culture; MA: mouse xenograft; CL: cell line

tumors,[113,114] small cell lung cancer,[115] stomach tumors[116] and prostate tumors.[117-120] Additional studies show that a variety of (benign and malignant) tumor cells express HH-GLI pathway components, that tumors are correlated with mutation in genes that affect the pathway and/or that tumor cell lines are sensitive to inhibition of HH signaling. These include breast tumors,[121] sarcomas,[122] ameloblastomas,[123] a subset of pericytomas,[73] astrocytomas,[55,124] oral squamous cell carcinomas,[125] bone exostoses,[126] neurofibromas,[127] bladder cancer,[128] colorectal tumors[129-132] and plasmacytomas[220] among others. The number of tumors and benign tumor-like growths, such as odontogenic keratocycts,[133] in which HH-GLI signaling may play a critical role is presently growing at an impressive rate. For example, tumors of liver, colon, rectum, lung and stomach are reported to have low levels of Hedgehog-interacting protein[134] (HIP1), a HH signaling antagonist.[135]

The rationale for the initial testing for the requirement of sustained HH-GLI function for tumor maintenance first derived from the timing of appearance of somatic GLI1-induced tumors in tadpoles[18,55] and from the finding that the activity of mutations in SMOH and PTCH1 is inhibited by cyclopamine,[136] although the latter cannot distinguish between an involvement in tumor initiation or tumor maintenance. The appearance of GLI1-induced tadpole tumors (or hyperplasias) occurred well after all the injected material was degraded,[55] suggesting the presence of 'memory'. As the endogenous *Gli1* gene was found to be expressed in the induced tumors, it was hypothesized that the activation of the endogenous pathway leading to *Gli1* expression was involved in tumor development and could account for the delay in tumor appearance. To test this idea, a morpholino-modified antisense oligonucleotide specific for the endogenous *Gli1* mRNA (selectively inhibiting its translation) was coinjected

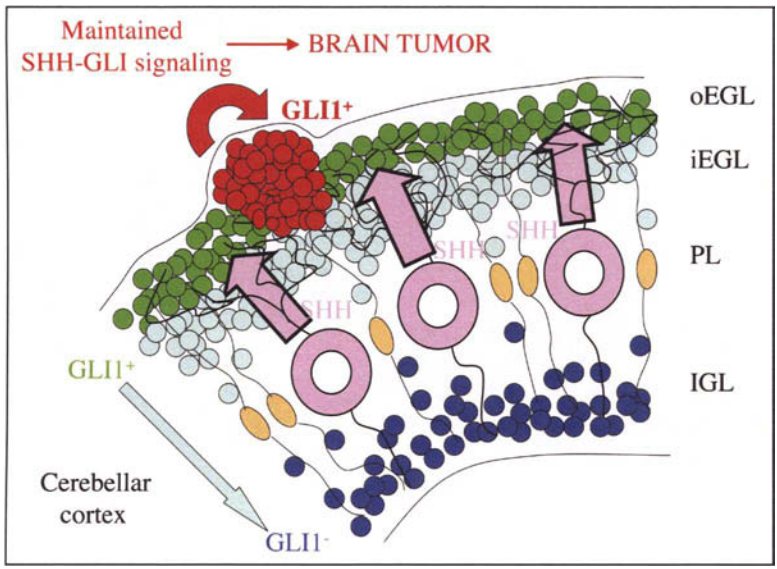

Figure 3. SONIC HEDGEHOG signaling controls cerebellar patterning and growth and its deregulation leads to medulloblastoma. Schematic representation of a section through the developing cerebellar cortex showing the layered disposition of cell types and the action of SHH from Purkinje neurons (pink) in the Purkinje layer (PL) regulating the proliferation of granule neurons precursors (green) in the outer external germinal layer (oEGL). These then become postmitotic (light blue), move inwards to the internal EGL (iEGL) and migrate to form the internal granule layer (deep blue, IGL) where they terminally differentiate displaying an axon and dendrites. SHH signaling is regulated to allow proliferation during a defined time frame to produce the correct number of precursors needed for a given species. Granule neuron progenitors in the EGL normally undergo a $GLI1^+$ to $GLI1^-$ transition concomitant with differentiation and their inward migration (light blue arrow). Inappropriate maintenance of an active SHH-GLI pathway (red arrow) in precursors (red) leads to hyperproliferation of $GLI1^+$ cells and tumor formation. The case of cerebellar medulloblastomas exemplify the derivation of tumors from stem cells/precursors that utilize the SHH-GLI pathway or proliferation, expansion or renewal. Reprinted with permission from ref. 86.

with synthetic human *GLI1* RNA. Coinjected embryos developed normally without tumors, demonstrating the requirement of the endogenous Gli1 protein for tumor development.[55] This result prompted us to originally test the requirement of sustained signaling for tumor maintenance in humans, even if Gli1 was reported to be redundant in mice.[137,138]

The requirement of sustained signaling for tumor maintenance and growth has been also recently demonstrated in mice. Conditional expression of Gli2 in basal skin cells leads to the formation of BCCs, which regress when the expression is turned off after tumor formation.[81] This result is in line with previous data on the dependence of mouse tumors on the maintained expression of the inducing oncogene.[139-141]

Inhibition of HH-GLI function in human tumor cells was first achieved with cyclopamine,[55] is a plant alkaloid with an overall resemblance to cholesterol that is produced by *Veratrum californicum*, a corn lily.[142-145] It was named cyclopamine because it is the active compound, along with jervine, responsible for the production of cyclopic embryos and newborns after ingestion of *V. californicum* by pregnant range and farm animals.[144,146,147] Cyclopamine-induced cyclopia phenocopies the loss of *Shh* in mice[148] and humans[149,150] and it selectively inhibits the HH-GLI pathway,[152] binding and blocking the function of SMOH (Fig. 1).[153]

Inhibition of HH-GLI signaling by noncyclopamine small molecule agents[154,155] that, like cyclopamine, target SMOH has been achieved in mouse basal cell carcinoma punch explants and cell lines.[111,155] Antibodies against SHH[156] and immunization with HIP1 have also been

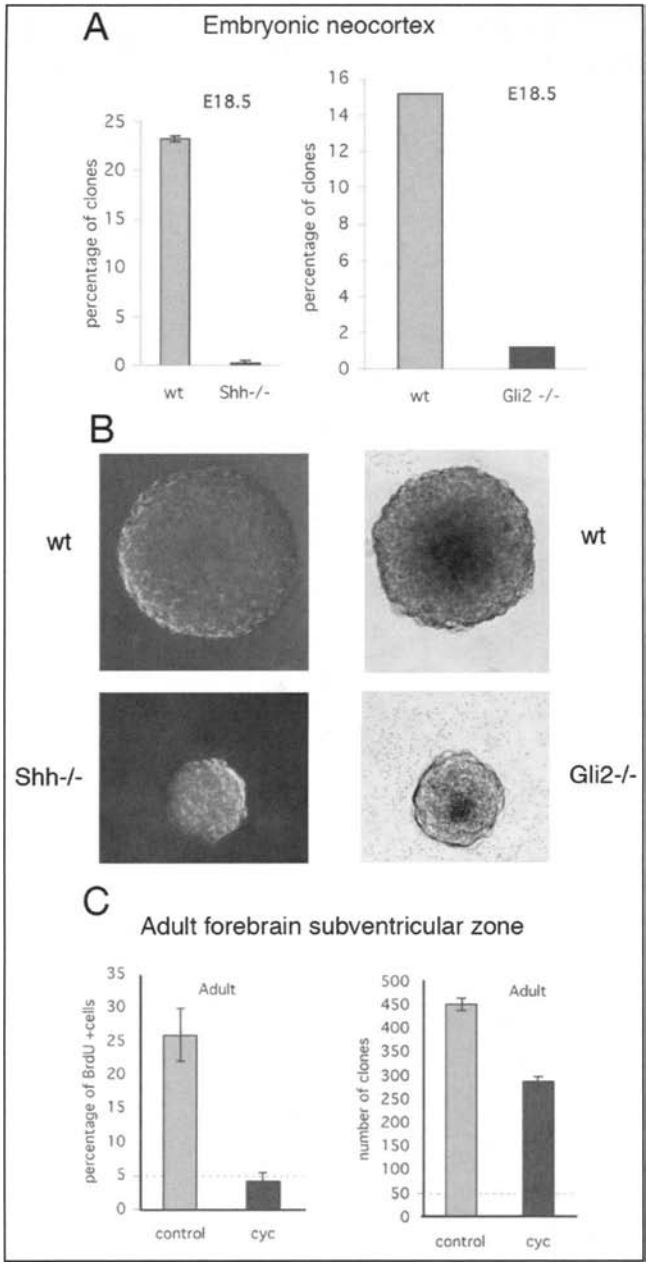

Figure 4. Hedgehog-Gli signaling controls stem cell behavior. A,B) Effects of loss of Shh or Gli2 function in neurosphere cultures derived from mutant mice. E18.5 neocortical neuropsheres produced from *Shh* or *Gli2* null mice show a severe defect in the capacity to self-renew, shown as percentage of clones obtained in clonogenic assays (A), and to grow, shown as neurosphere size (B), C) Similarly, in the adult subventricular zone of the lateral ventricle of the forebrain Shh signaling regulates the behavior of stem cells, as seen by the ability of cyclopamine to reduce the number of $BrdU^+$ cells and the number of clones. Reprinted with permission from refs. 89 and 92.

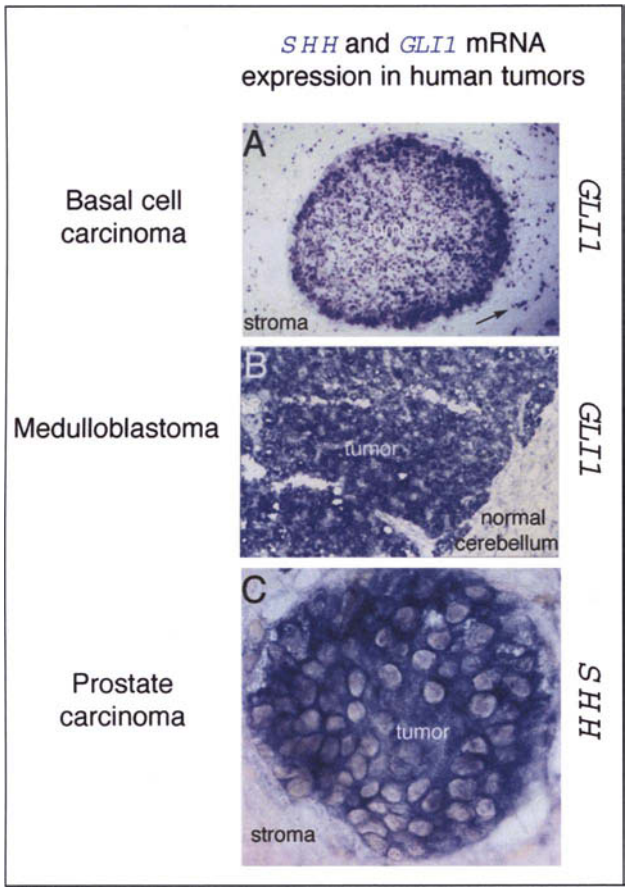

Figure 5. Consistent expression of SHH-GLI pathway components in sporadic human tumors: basal cell carcinoma, medulloblastoma and prostate carcinoma. Many sporadic human tumors display a constitutively active SHH-GLI1 pathway, as seen by *GLI1* mRNA expression, the best marker so far. Expression in tissue sections after in situ hybridization is shown for *GLI1* in a basal cell carcinoma nodule and in single cells, likely invading tumor cells (A), and in a cerebellar primitive neuroectodermal tumor or medulloblastoma (B). Surrounding normal tissue and tumor stroma are not positive. A number of tumors also express *SHH*, indicating the presence of autocrine signaling, as in the case of prostate carcinomas (C). Reprinted with permission from refs. 18, 55 and 117.

used.[116,117,157] At the other end of the signaling pathway, inhibition has been achieved by targeting Gli1 with antisense oligonucleotides in skin and CNS tumors in tadpoles[55] and with siRNAs in human prostate cancer[117] (Fig. 6) and in brain tumor cells (P. Sanchez, V. Clément and ARA, pers. com.). Targeting GLI1 offers that best therapeutic outlook as this would inhibit activation of the pathway by any event acting at any level. For instance, alterations in *SUFUH* have been observed in different tumors[68,119] and tumors arising from such events would be insensitive to inhibitors acting on SMOH, as SUFUH acts downstream of SMOH. Indeed, tumor cells insensitive to cyclopamine but dependent on GLI1, as assessed by RNA interference (RNAi), have been found.[117]

Inhibition of cancer growth through interference with HH-GLI signaling has been shown for established cell lines and for primary cultures from the operating room[55,112,113,115-118] (Table 2) as well as for mouse grafts.[112,113,115,116,118] It is important to note, however, that while

Table 2. Vertebrate animal models of HH-GLI dependent cancers

Animal	*Model*	*Tumor*	*Refs.*	*Inhibitors*	*Refs.*
		BRAIN			
Tadpole	*Somatic misexpression*				
	GLI1	CNS tumors	55	Gli-antisense	55
Mouse	*Germ line transgenesis*				
	Ptch1$^{+/-}$	Medulloblastoma	82,83		
	Ptch1$^{+/-}$ + *X-ray irradiation*	Medulloblastoma	219		
	Ptch1$^{+/-}$; p53$^{-/-}$	Medulloblastoma	160	Hh-Antag691	161
				Cyclopamine	162
	Somatic retroviral infection				
	Shh	Medulloblastoma	138		
	Shh; Shh+cMyc	Medulloblastoma	221		
	Shh; Shh+IGF2 ; Shh+Akt	Medulloblastoma	222		
		SKIN			
Tadpole	*Somatic misexpression*				
	GLI1	Skin tumors	18	Gli-antisense	55
Mouse	*Germ line transgenesis*				
	K14-Shh	Basal cell carcinoma	74		
	K5-SMO-M2	Basal cell carcinoma	62		
	Ptch1$^{+/-}$ + *X-ray irradiation*	Basal cell carcinoma, trichoepitelioma	78,218	Cur61414	111
				Cyclopamine	224
	K5-GLI1	Basal cell carcinoma, trichoepithelioma	75		
	K5-Gli2	Basal cell carcinoma	79		
	K5-N'ΔGli2	Trichoblastoma, cylindrioma, follicular hamartoma	80		
	K5-SMO-M2	Follicular hamartoma	77		
	K5-rTA x tetO-Gli2	Basal cell carcinoma	81	Conditional Gli2 expression OFF	81
		OTHER			
Mouse	*Germ line transgenesis*				
	Ptch1+/-	Rhabdomyosarcoma	83		
	Shh	Pancreas dysplasia	113		
	Ptch1+/- + carcinogen	Bladder cancer	128		

orthografts are valuable, the predictive value of subcutaneous xenografts and allografts is questionable at best and equal or inferior to primary cell cultures.[158,159]

Genetic mouse models of endogenous tumors, however, provide excellent tools for preclinical assessments in a mammal. In this sense, the efficient development of medulloblastomas in all *Ptch1+/-;p53-/-* mice[160] provides a valuable model with a genetically engineered endogenous tumor. Using this model it has been recently shown that systemic interference of HH signaling in *Ptch1+/-;p53-/-* adult mice leads to tumor inhibition and regression using a synthetic small molecule antagonist[161] of SMOH function or using the natural alkaloid cyclopamine[162] (Fig. 7). Similarly, oral cyclopamine treatment was preventive for uv-induced BCC development in *Ptch1+/-* mice.[224] Together, these findings set the stage for the initiation of clinical trials on human patients suffering from incurable terminal cancers such as metastatic prostatic and pancreatic tumors or glioblastoma multiforme.

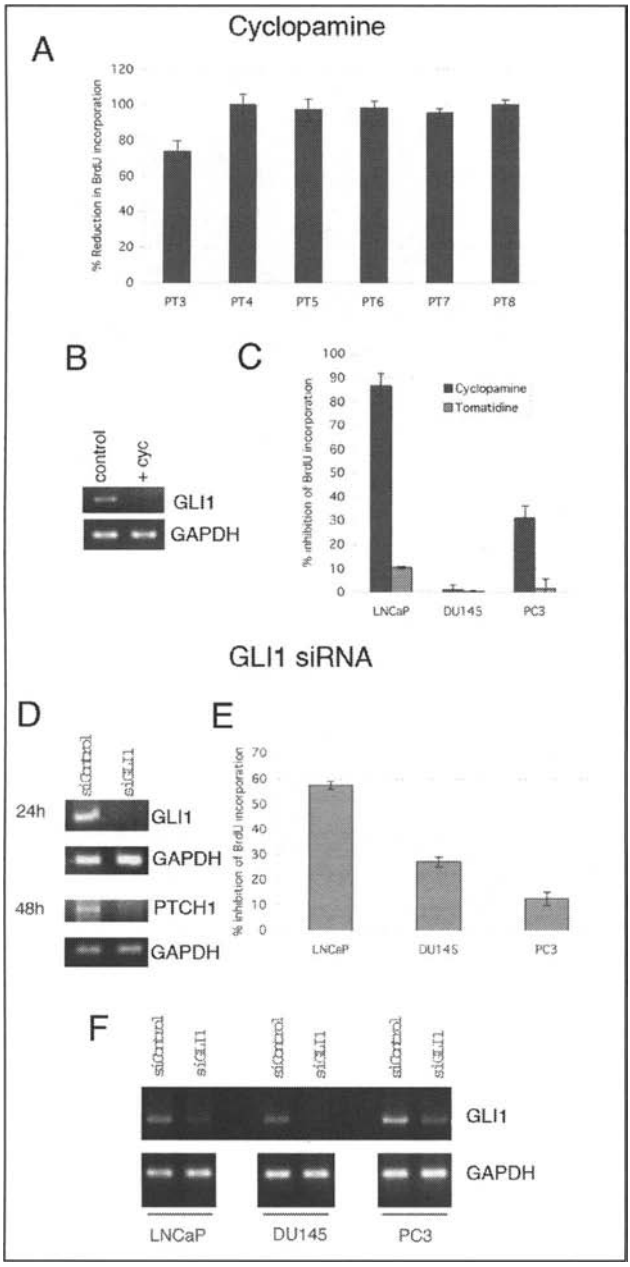

Figure 6. Inhibition of human prostate cancer cell proliferation by cyclopamine and *GLI1* RNA interference. Cyclopamine inhibits the proliferation of primary in situ prostate tumors (A) and of metastatic prostate cancer cell lines (C), as measured by BrdU incorporation, and inhibits expression of *GLI1* (B). Similarly, *GLI1* small interfering RNAs (siRNAs) decrease *GLI1* and *PTCH1* expression (D,F) and inhibit the proliferation of prostate cancer cells (E). The cell line DU145 is not sensitive to cyclopamine while it is sensitive to *GLI1* RNAi, suggesting activation of the pathway downstream of SMOH. The efficiency of *GLI1* siRNAs in PC3 cells is low due to the low siRNA transfection efficiency. Reprinted with permission from ref. 117.

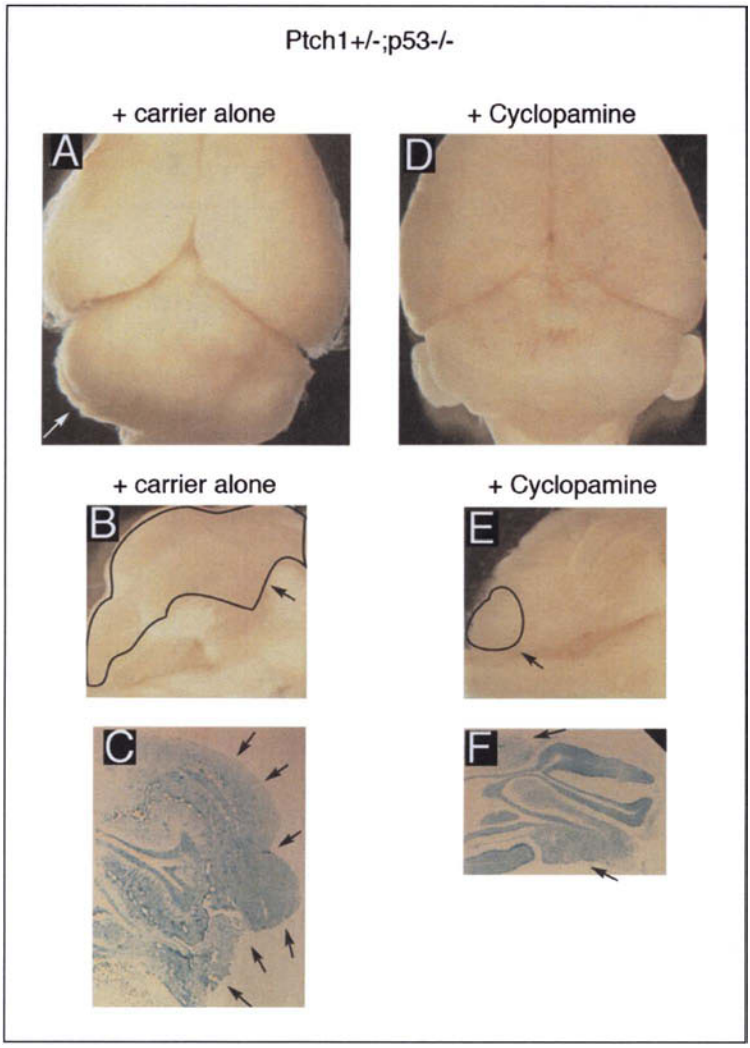

Figure 7. Beneficial anti-cancer effects of systemic in vivo cyclopamine treatment in mice. Ptch1+/-;p53-/- mice develop medulloblastoma and die at 2 months of age. Treatment with cyclopamine through systemic intraperitoneal injections for 30 days starting at 1 month of age results in a great diminution of tumor size, seen from a dorsal view of the whole dissected brain (A,D) and lack of significant tumor-associated behavior anomalies. Hemisections of the brain and histological preparations show massive tumors in Ptch1+/-;p53-/- mice treated with carrier alone (cyclodextrin, B,C), but much reduced tumors in cyclopamine-treated siblings (E,F). A,B,D,E) show unstained tissue. C,F) show cerebellar sections after X-Gal histochemistry. Arrows point to tumors. Reprinted with permission from ref. 162.

Inhibition of HH-GLI function appears to be effective for all tumor grades of all ages and subgroups of a given type that is HH-GLI signaling dependent. For example, primary culture of in situ prostate tumors of different grades and cells lines derived from distant metastatic lesions[117] (Figs. 5, 6), as well as primary culture of fresh prostatic metastatic lesions from postmortem specimens,[118] are sensitive to cyclopamine. These and similar findings in other tumor types is amazing and suggest that the HH-GLI pathway is an essential basis on which a

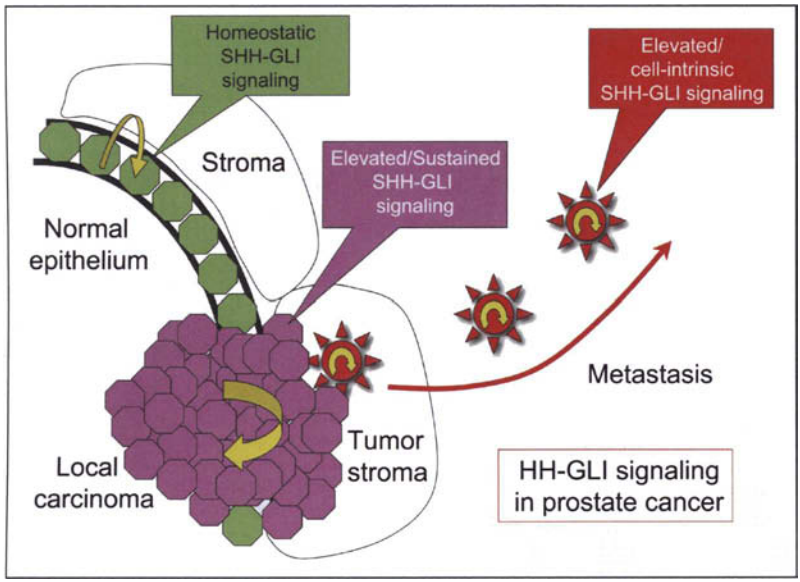

Figure 8. Schematic representation of the proposed causal relationship of the abnormal state of HH-GLI signaling in prostate cancer. Homeostatic HH-GLI signaling participates in the normal maintenance and regeneration of the adult prostatic epithelium. Enhanced levels or inappropriate maintenance lead to tumor initiation in situ. Metastasis may derive from further elevated levels or from the acquisition of cell-intrinsic signaling leading to elevated GLI1 function. Reprinted with permission from ref. 117. The role of the stroma remains to be clarified, as GLI1 is expressed in the epithelium in men[117] and in the stroma in rats.[225]

tumor is built and that no tumor cell can exist without sustained signaling, be it autocrine or paracrine, leading to positive GLI function (Fig. 8). HH-GLI signaling may thus be a critical sensor of the 'fitness' of a cell, with tumor cells requiring a high fitness.

Metastatic lesions may derive from the acquisition of cell-autonomous pathway activation allowing efficient activity independent of the original niche[117] and/or from increased overall signaling levels (Fig. 8).[118] High levels of GLI1 function lead to high metastatic behavior in xenografts.[118] Intrinsic/elevated signaling may then affect genes involved in epithelial-mesenchymal transitions, such as *C-Myc*, *N-Myc*[163] and *Snail*,[164] genes which are responsive to Hh-Gli activity[118,165-167] to start the metastatic voyage and departure from the original tumorigenic niche. C-Myc also cooperates with Shh signaling in tumorigenesis.[221] The context of the site of origin of the tumor might also determine the site of metastatic growth, perhaps not just in a random selective manner but also in a directed way, possibly recapitulating aspects of normal cell migration, such as that of the neural crest during embryogenesis.

Unexpectedly for its protocols and expediency, two publications report beneficial effects of cyclopamine on BCCs and psoriasis in human patients.[168,169] If confirmed and extended, these results are in perfect agreement with the expected outcome of interference with HH signaling as a novel therapeutic approach in humans. Toxicology and side effect issues need to be addressed before general and systemic treatments can be started but cyclopamine could indeed be a perfect leading compound.

Systemic cyclopamine treatment could cause a number of side effects that might include problems with the gastric mucosae, hair growth or renewal and production of new neurons. However, these are not worse than the side effects of present-day chemotherapy and local, topical cyclopamine treatment did not affect hair follicles adversely.[168] Moreover, a minimal treatment period required to induce growth arrest and death of tumor cells may result in

transient secondary effects that can be compensated following cessation of the treatment: mice treated for several weeks with cyclopamine[162,224] or a different SMOH antagonist[161] appear normal.

In principle, however, the best therapies could derive from inhibition of GLI function (Fig. 1), which as it is the last element of the pathway would appear to be the best target. Moreover, GLI proteins are sufficient for tumor induction in animal models[18,55,75,79] and might respond to additional tumorigenic inputs other than HH.[170] Given that the GLI code[13,171,172] is critical for cell fate and cancer, and that it is modulated by several kinases (Fused, PKA, GSK3β, CK1 and GRK2)[173-183] and many cofactors, inhibitors of GLI function may target the GLI proteins themselves or such modifying proteins (Fig. 1). These include DYRK1,[184] intraflagellar transport proteins,[185] ZIC proteins,[186,187] REN,[188] DZIP1 (Iguana),[189,190] RAB23,[191] FKBP8[192] and SUFUH[70-72,193,194] and BEGH.[223] Approaches with antagonist (for positive factors) or agonist (for negative regulators) small molecules as discussed above, peptides mimicking dominant-repressive C-terminus deleted GLI proteins[37,38] and RNA interference[117] (Fig. 7) will very likely lead to the production of efficient, specific and potent inhibitors for patient therapy. Additional beneficial strategies could derive from finding the key targets regulated by HH-GLI signaling the inhibition of which may partially recapitulate loss of activating GLI function. Moreover, large-scale gene profiling, microchip-based diagnoses[195] and individual treatment regiments could also lead to more focused and thus better outcomes.

Given the results summarized here, it is difficult to temper enthusiasm for the development of a rational wide-spectrum tumor therapy derived from research on molecular embryology, plant-induced teratology, cancer biology and developmental genetics. Such a therapy would target cancers from brain, lung, pancreas, prostate, muscle, stomach, skin and other organs and of any grade, including most importantly metastatic cancer.

This story is still ongoing and a conclusion has not been reached. At present, pressing questions include the following. How many different types of human tumors depend on HH-GLI signaling? Do tumors derive from stem cells and/or from de-differentiating cells that acquire stem cell properties? What are the paradevelopmental programs that dictate tumor initiation, growth and metastasis? Do HH proteins act as mitogens and/or morphogens in the control of stem cell behavior? Do they contribute to the definition of a normal and tumorigenic stem cell niche? Is there a specific GLI code for each tumor type or a general one that is modified in a context-dependent manner by local cofactors? Is there a correlation of levels of GLI expression/function with tumor grade or character? What are the targets that execute the programs instructed by GLI proteins? Can specific and effective inhibitors of HH-GLI function be produced for patient treatment? Will these have limited and tolerable side effects? Will HH-GLI inhibitors also affect and kill cancer stem cells, thought to be responsible for recurrence? Can cancer result from repeated injury and the eventual perversion of the behavior of stem cells recruited for repair? Can one develop not only anti-cancer but also preventive therapies?

Other questions for which we begin to have some insights include: Do other oncogenic inputs affect or regulate HH-GLI function or vice versa? What are the mechanisms that guide cancer-causing events towards regeneration in some instances? We know that there are several intercellular communication systems that have been implicated in cancer, including FGF, WNT, NOTCH and TGFβ signaling.[2] How these interact with HH-GLI function is not yet clear although we know, for instance, that Gli proteins regulate batteries of *Wnt* genes,[196] that FGF signaling can regulate *Gli2* and *Gli3*[170] and that *Gli2* can be responsive to Notch signaling.[197,198] And as far as cancer and regeneration is concerned, insights derive from a variety of sources. For example, on the one hand, treatment of a newt with highly tumorigenic compounds after lens extirpation leads to normal regeneration of the lens from the dorsal iris, but tumor formation from the ventral, nonregenerative ventral iris.[199] And on the other hand, some mammalian tumors can be induced to differentiate. For instance, Shh- and Gli2-induced BCCs can differentiate into hair follicle components if placed adjacent to a normal environment,[74,81] and teratocarcinomas participate in the formation of a normal mouse when transplanted into a

blastocyst.[101-103] The developmental history, competence and position of a cell could thus turn tumorigenic into (re)generative information (and vice versa!).

More specifically, these issues on signaling, pattern formation and cancer have lead to renewed interest in homeobox genes. The demonstration of the control of *Xhox3* (*Evx1*) expression by Gli2 and Gli3,[170] the regulation of *HoxD* genes by Gli3,[200,201] a physical and functional interaction between HoxD12 and Gli3[202] and the genetic interactions between Proboscipedia (HoxA2/B2) and Ci,[203] the Gli homologue in *Drosophila*, suggests the possibility that cell number may be organized to lead to pattern through an interaction between Gli and homeobox proteins. The latter being often regulated by extracellular inputs and best known as organizers of body pattern.[204,205] This could explain several unresolved observations, for example, the role of HoxB1 in the control of Shh-mediated D-V pattern in rhombomere 1,[206] the ability of HoxB4 to induce hematopoietic stem cell proliferation,[207,208] and the involvement of Msx1 in Shh signaling during tooth development.[209] The roles of HH-GLI signaling in cancer and stem cell/precursor proliferation (see above), together with the documented participation of homeobox proteins in cancer,[210,211] raise the possibilities that these factors regulate size and shape in embryos and homeostasis and regeneration in adults, and that their regulation underlies a unified principle in cancer and development. This hypothesis could also explain evolutionary constrains in body plan and cancer selection.[212] Patterning targets/cofactors, such as HOX proteins, in addition to the GLI proteins and their modulators, could thus also be interesting targets of anti-cancer, preventive and pro-regenerative therapies.

In contrast with the inappropriate activation and/or maintenance HH-GLI signaling in cancer, its ability to regulate neurogenesis and brain stem cell behavior,[89-92,186,213] raises new and exciting prospects to increase cell number by acting positively on this signaling pathway. This could be critical to increase the pool of cells that is undergoing degeneration, and thus reverse cell loss as in Parkinson's disease,[214] or to provide exogenous stem cells to increase repair. Indeed, the ability of SHH to act as a motor neuron survival factor[215] and of SHH and GLI1 to act as protective factors for dopaminergic neurons[216,217] suggest novel therapies, which may also include the manipulation of homeobox proteins.[226,227] Given the adult expression of components of the HH-GLI signaling pathway in the adult brain it is not inconceivable that its deregulation could be involved in additional mental diseases and disorders. Clearly, much awaits to be discovered although the data gathered up to this point already strongly suggest that manipulation of the SHH-GLI1 pathway will lead to cancer treatment, rationally reducing cell number, and to the treatment of degenerative diseases, rationally increasing cell number. Current results are very encouraging and enthusiasm is high. With caution, the goal of developing rational therapies based on knowledge derived from research on flies, lilies, tadpoles, mice, stem cells and human disease is within reach.

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CHAPTER 2

The Patched Receptor:
Switching On/Off the Hedgehog Signaling Pathway

Luis Quijada,* Ainhoa Callejo, Carlos Torroja and Isabel Guerrero*

Abstract

The activities of the Hedgehog (Hh) protein family are central to the growth and patterning of developing tissues and organs in many different organisms. Hh proteins are secreted ligands synthesized in discrete regions. The receptor of Hh is Patched (Ptc) and it is expressed in the cells close to the source of Hh. Ptc binds the ligand and transduces a signal which is modulated depending on the context and the concentration of Hh received. Hh and several molecular components of the pathway were first identified and characterized in *Drosophila*, providing relevant milestones to our understanding on how the Hh signal is transduced. However, important gaps in the pathway still need to be elucidated. Some of these gaps converge on the Ptc receptor and its intriguing mechanisms of Hh reception and signal transduction. Mutations of Ptc that prevail both in animal and human populations are giving some clues on crucial aspects of its function. Patients bearing mutated forms of Ptc suffer a variety of serious diseases. Molecular and cellular studies in *Drosophila* have given us a clue of the function of Ptc receptor such as the normal topology and/or sorting of the receptor. Thus, a widened knowledge of the function of Ptc might help to design specific therapies for these disorders. This chapter focuses on recent advances that shed some light on how Ptc may operate in the cell.

The Hedgehog Signaling Pathway

Several genes of the Hh pathway were first identified in the fly *Drosophila melanogaster* and later in vertebrates. Many of the names of the genes involved in the Hh signaling pathway were originally descriptive of the phenotype manifested in mutant *Drosophila* larvae. Wild-type larvae show a clearly segmented pattern due to alternate bands of denticles in ventral position, whereas the inter-band space is naked. During a screening for mutations that affected the segmental pattern, Nüslein-Volhard and Wieschaus described a group of mutants with alterations in the patterning of the segments.[1] Instead of the wild-type alternate belts of denticles and naked cuticle, Hh mutants showed a continuous lawn of denticles, which gave the larva a resemblance to a hedgehog (Fig. 1B), and Ptc mutants showed patches of denticles (Fig. 1C).

The active Hh protein is a peptide that has undergone an autocatalytical processing in which the peptide is also modified at its N- and C-termini by palmitoyl and cholesterol adducts, respectively. Hh is secreted by discrete subsets of cells within a developing organ in a process that seems mediated, at least in part, by the transmembrane protein Dispatched (Disp), which has a structure very similar to Ptc. In the wing imaginal disc of *Drosophila*, Hh is

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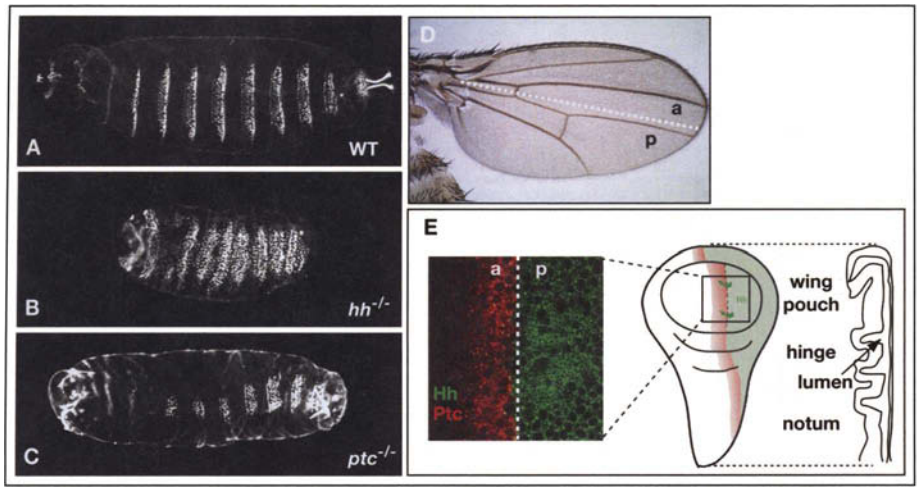

Figure 1. Two model systems in *Drosophila melanogaster*: The larva cuticle and the wing imaginal disc. A) Wild-type ventral larva cuticle showing its regular pattern of denticles. B) Hh mutant ventral cuticle. C) Ptc mutant ventral cuticle. D) Adult wing. The dashed line marks the boundary between the anterior (a) and posterior compartment (p). These compartments are already present in the wing imaginal disc, the wing primordium (E; front and lateral view) in the larva. Hh is produced in the posterior compartment (green) and signals to cells in the anterior compartment. Ptc expression is induced by Hh signaling (red). A color version of this figure is available online at http://www.eurekah.com/chapter.php?chapid=2439&bookid=166&catid=82.

expressed in the cells of the posterior compartment and signals to the cells of the anterior compartment, which in turn express the Ptc receptor (Fig. 1D,E). A graded and short-range response to Hh signaling occurs in the Hh receiving cells (Fig. 1E). Ptc, a 12-transmembrane protein, is a negative receptor because it keeps the Hh pathway silenced in its unliganded state.[2] In the absence of Hh, Ptc suppresses the activity of Smoothened (Smo), a 7-transmembrane protein that is the positive modulator of the pathway. Although Hh does not bind to Smo, without Smo there is no signaling. The target cells bind Hh by Ptc, which results in the activation of Smo, and, subsequently, in the activation of transcription factors: the Cubitus interruptus protein (Ci) in *Drosophila* and the orthologous Gli proteins in mammals (reviewed in ref. 10). One peculiarity of the Hh signaling pathway is that Ptc is up-regulated in response to increasing amounts of Hh.[3-7] Therefore, Hh controls both its own activity and its own spreading expressing high levels of the receptor.[8] By this means, a morphogenetic gradient is formed in the Hh receiving cells. Theoretical analysis of model systems built to explain how a morphogen gradient is formed indicates that this feedback is indeed required to give robustness to the gradient while maintaining its range of action.[9] Thus, according to one model, it is expected that fluctuations in ligand production have minor effects on the slope of the gradient, compared to a model in which the receptor has a constant, or ligand-independent level of expression.[9]

Since this chapter is particularly centered in the Ptc receptor, for a more general view on the Hh response network, the reader is referred to some other recent and exhaustive reviews.[10-14]

Although this sequence of events is well established, many molecular mechanisms connecting these and other components of the pathway remain elusive. Focusing in the case of the Ptc receptor, several fundamental questions remain unanswered: How does the Ptc receptor recognize the Hh ligand? What happens after the receptor-ligand complex is formed? Is it internalized or does it remain in the membrane before the signal is transduced? How does the unliganded Ptc receptor repress the Smo activity? And how does the liganded Ptc derepress Smo activity?

Sequence and Functional Analysis of Ptc

A single *ptc* gene is present in *Drosophila*, whereas two are present in vertebrates (*PTCH1* and *PTCH2* in humans; *Ptc1* and *Ptc2* in mice) (reviewed in ref. 10) and several *ptc*-related genes are found in the nematode worm *Caenorhabditis elegans*.[15] Ptc shows homology to bacterial proton-driven transmembrane molecular transporters,[16,17] and presents a conserved a Sterol-Sensing Domain (SSD) that overlaps between transmembrane segment 4 to 6, and also two extracellular large loops (reviewed in ref. 18). In general, SSDs are thought to function as a regulatory domain involved in linking vesicle trafficking and protein localization with processes such as cholesterol homeostasis and cell signaling.[18] Concretely, the SSD of Ptc seems to mediate the intracellular trafficking of Ptc, a process that might be essential to regulate Smo activity.[19,20] Other functional studies have assigned particular roles to regions of the protein with no obvious similarity to conserved domains. Thus, the two extracellular large loops are required for Hh binding,[21] the cytoplasmic C-terminus has been involved in the transduction of the signal,[22] and an intracellular small loop between the SSD and the following transmembrane domain has been reported to interact with cyclin B1 to regulate cell-cycle progression in vertebrates.[23] However, despite this emerging body of data, we still do not know the full sequence of molecular events that involves the reception of the Hh ligand and how Ptc regulates Smo.

PTCH2 is a 12-transmembrane protein as well and the two large extracellular loops and an SSD are also conserved (reviewed in ref. 24). However, both amino- and carboxy-termini are different from PTCH1. Similar differences are found between Ptc1 and Ptc 2 in mice. PTCH1 and PTCH2 proteins are expressed differentially during the development of the epidermis, suggesting specific roles for each protein,[25] although the function of PTCH2 remains unclear to date. A recent study suggests that PTCH2 may act as a Hh receptor that tunes finely the signaling in various cellular environments.[26]

Hedgehog Lipid Modifications and Morphogen Distribution

The lipid modifications on the Hh protein appear to regulate its activity and distribution. Hedgehog proteins undergo two sequential lipid modifications during their posttranscriptional maturation. Following cleavage of an N-terminus signal sequence upon entry into the secretory pathway, Hh proteins undergo an autoprocessive reaction that results in an internal cleavage inside the 45-KDa precursors, between glycine-cysteine residues from a conserved GCF motif. After this, a cholesterol molecule is covalently added at the newly generated C-terminus of the proteins.[27] In *Drosophila*, a construct of Hh lacking the cholesterol moiety is active in signaling but not appropriately restricted spatially in its signaling activity.[28] Therefore, the cholesteryl moiety restricts the spatial deployment of the signal via insertion into the lipid bilayer of the cell membrane and also functions as an essential molecular handle for a proper intracellular and extracellular trafficking and localization of the signal (reviewed in refs. 29, 30). In addition, Hh proteins are palmitoylated on a highly conserved amino-terminal cysteine residue, the first of a pentapeptide CGPGR.[31] In *Drosophila* this palmitoylation is a total requirement in order to produce a fully active Hh signal. Although in vitro studies in vertebrates indicated that Hh mutant forms lacking the pamitoyl adduct retained significant activity,[32,33] knockout mice deficient in Skn (the murine ortholog of the *Drosophila* Ski, which catalyzes Hh palmitoylation) showed that Hh acylation is absolutely required for Hh long-range signaling.[24]

In summary, dual lipidation of Hh protein promotes membrane affinity and allows its association to sterol-rich membrane microdomains in *Drosophila*, and to lipid rafts in mammalian cells, that function as platforms for intracellular sorting and signal transduction.[34] The close association of Hh proteins to the plasma membrane due to the lipid modifications could provide a mechanism for restricting the range of their activities.

Reception of Hh

Biochemical studies have shown that Hh monomers can form multimeric complexes in which the hydrophobic moieties have been proposed to be sequestered in the interior of the multimer, making the complex soluble and diffusible.[35,36] This could be relevant to the movement of Hh through the extracellular matrix[37] and to the reception of Hh by Ptc. On one hand, the restricted diffusion of Hh through the extracellular matrix might require the formation of these multimeric complexes of lipidated Hh. On the other hand, a multimeric Hh complex might have either a higher intrinsic affinity for Ptc, or maybe is capable of eliciting greater biological responses, for instance through receptor oligomerization, or binding to coreceptor proteins. In this context, structural studies on transmembrane transporters related to Ptc determined that these proteins operate as homotrimers.[38] Interestingly, a mutant version of Ptc that internalizes inefficiently is, however, localized to intracellular vesicles when coexpressed with a dominant negative Ptc.[39] This ptc inter-allelic complementation strongly suggests that Ptc has an oligomeric structure.

Several other proteins have been identified as capable of binding to Hh, raising the question of whether they are coreceptors or adjuvants involved in the reception of Hh. Especially, recent analysis in *Drosophila* have illustrated the critical roles of heparan sulfate proteoglycans (HSPGs) in developmental signaling pathways (reviewed in ref. 40). These large macromolecules are found at the cell surface and form part of the extracellular matrix. A major feature of these proteins is the attachment of long unbranched chains of repeating and sulfated disaccharides to specific serine residues in their protein core. Dally and Dally-like (Dly), two *Drosophila* glypican members of the HSPG family, has received great attention. A specific role in Hh signaling in the embryonic epidermis has been ascribed to Dly,[41] while both Dally and Dly seem to be functionally redundant in the wing disc,[42] indicating distinct activities of these two glypicans in the embryo and the wing disc. Hh signaling might also be regulated by other HSPGs in different tissues or developmental processes. For example, Trol, the *Drosophila* ortholog of Perlecan, seems to form a complex with Hh, and mutations in the gene encoding it cause neuroblasts to undergo cell cycle arrest in the larval brain.[43,44] In addition, *trol* is required for the neuroblast division induced by Hh.[43]

Two data favour a model in which the polyanionic branches of the HSPGs would act mainly as a molecular trap to keep the morphogens in touch with epithelium surfaces. First, the strongest loss-of-function phenotypes are achieved with mutants affecting the synthesis of sugar chains of the HSPGs;[42] and second, the fact that the sugar chains seem to be directly involved in the movement of other morphogens.[45,46] Hence, the HSPGs might restrict the morphogen diffusion to the environment of the extracellular matrix, and thus preventing a massive spreading to cavities such as the imaginal disc lumen. This lateral distribution of Hh would facilitate the encounter with Ptc. This scenario does not rule out the possibility of specific regulations involving Dally or Dly. In fact, in tissue culture experiments, Dally-like protein (Dlp), but not Dally was required for Hh signaling.[47] In addition, it has been shown that Dlp is specifically required for Hh signaling in the embryonic epidermis.[41] A Dlp-mediated regulation of Hh signaling might involve Dlp acting as a coreceptor, perhaps by transferring Hh to Ptc, or by forming a Hh-Dlp-Ptc interaction or stabilizing a Hh-Ptc complex (reviewed in ref. 48). In the case of the Wingless pathway (another morphogenetic signal), it has been elegantly shown that Dlp is cleaved specifically from the cell surface by Notum, a secreted enzyme, resulting in an adequate regulation of the pathway.[49]

Hh Internalization and Signal Transduction

After receiving Hh, the receptor-ligand complex is internalized and the morphogen is thus depleted from the extracellular milieu. It was proposed that this internalization has a role shaping the Hh gradient.[8,50] This hypothesis has been reinforced by experiments that uncoupled the Hh sequestration and the Hh signaling using a Ptc mutant that is unable to sequester Hh and yet retains a normal capacity to mediate Hh signaling.[39] Furthermore, the

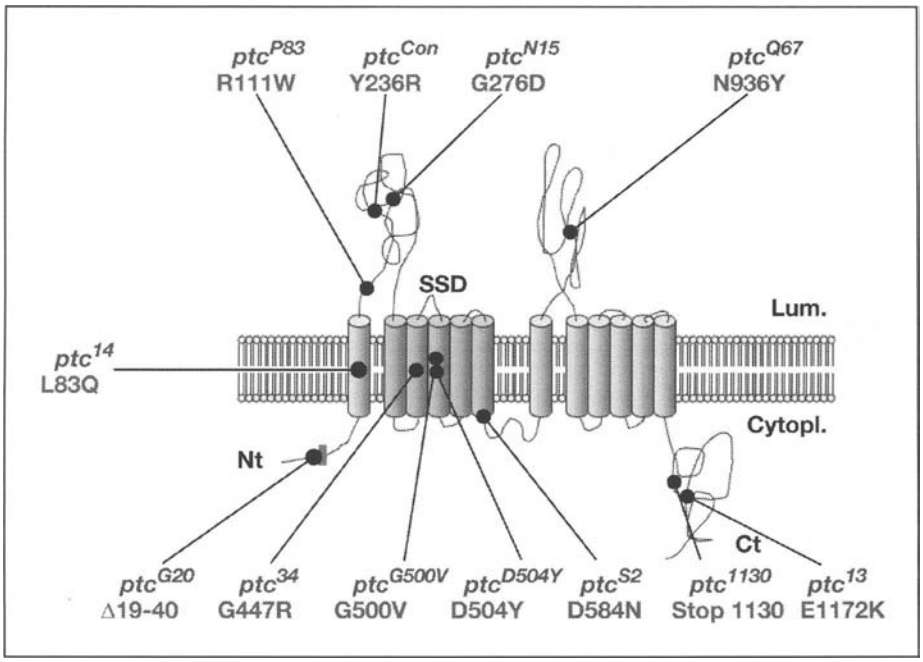

Figure 2. Ptc mutations. Ptc is a 12-transmembrane protein. The transmembrane domains highlighted in green correspond to the sterol-sensing domain (SSD). Mutations *ptc*^{P83}, *ptc*^{N15}, *ptc*^{Q67} (see ref. 51) and *ptc*^{Con} (see ref. 72) affect both Hh sequestration and signaling. Mutations *ptc*^{13}, *ptc*^{34} (see ref. 20), *ptc*^{S2} (see ref. 19, 20), *ptc*^{1130} (See ref. 20, 22), *ptc*^{G500V}, *ptc*^{D504Y} (see ref. 52) and *ptc*^{G20} (unpublished data and ref. 73) affect only signaling. In the *ptc*^{14} mutant only the sequestration is affected.[39]

ability to uncouple these functions suggests that Ptc-mediated internalization of Hh does not play a major role in the transduction of the signal but serves mainly to limit and control the Hh gradient.[39]

The analysis of different ptc mutants (Fig. 2) in *Drosophila* have demonstrated that the two functions of Ptc, sequestration and signalling, can be genetically separated.[8,19,20,22,39,51] Ptc^{S2}, Ptcm^{13}, Ptc^{34} and Ptc^{1130} are defective for Hh signal transduction but sequestration properties are normal,[19,20,22] while the Ptc^{14} mutant protein is defective for Hh sequestration, but normal in terms of Hh signal transduction.[39]

One of the most intriguing aspects of the Hh pathway is how Ptc regulates Smo activity. The mechanism is most probably indirect, since Ptc and Smo do not need to bind or to colocalize to control signaling and, in addition, Ptc acts substoichometrically to inhibit Smo, possibly through changes in the distribution or concentration of an as yet uncharacterized small molecule (Fig. 3).[52] A recent study[53] suggests a mechanism by which the ratio of unliganded to liganded Ptc determines the cellular response (reviewed in ref. 54). In this context, several alternative models are possible. For example, unliganded Ptc might import a Smo antagonist, while liganded Ptc might promote its export. Alternatively, the functional Hh receptor may comprise a multimer of Ptc proteins and the binding of Hh to one Ptc subunit in a receptor complex may block the ability of the multimer to inhibit Smo activitity.[53,54]

Smo Regulation by Ptc

When Hh binds Ptc, Smo is somehow liberated to start the process of activating target gene transcription. The mechanism in which Ptc inhibits and Hh activates Smo protein remains

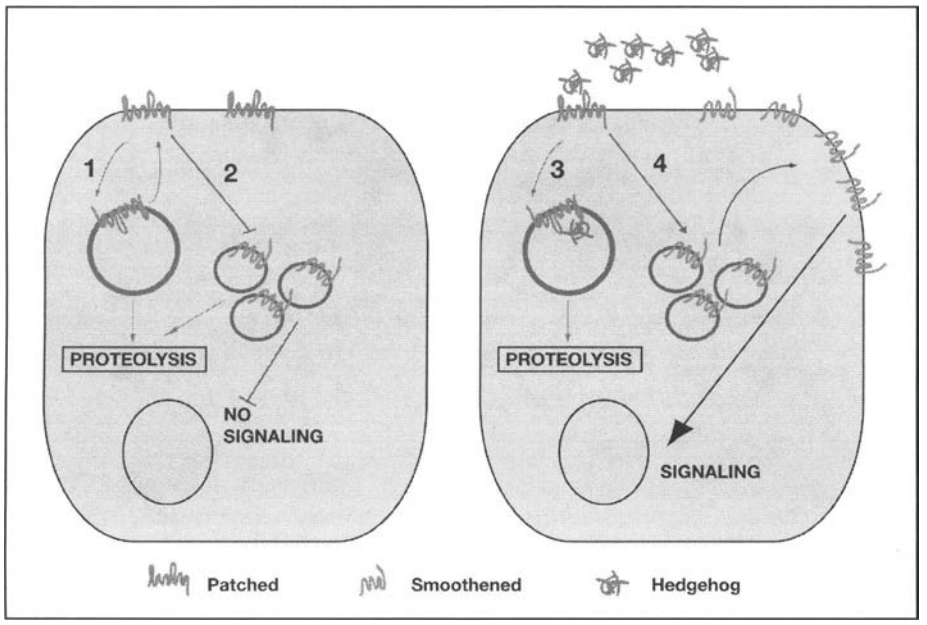

Figure 3. Model of Hh signal activation. 1) In the absence of Hh (left panel), Ptc is constantly recycling from the plasma membrane to vesicles inside the cells. Occasionally, these vesicles enter the proteolytic pathway. 2) At the same time, Smo is kept inactive in an intracellular compartment, unable to signal. Smo enters as well the degradative pathway under this situation. 3) Upon the reception of Hh (right panel), the receptor-ligand complex is internalized, contributing to the slope of the gradient of Hh. 4) At the same time, Ptc no longer represses Smo, which is stabilized and traffics to the plasma membrane preferentially. This plasma membrane stabilization of Smo correlates with signal activation.

unknown. *Smo* is transcribed in a generalized pattern not transcriptionally regulated by the Hh signal. Smo protein is posttranscriptionally regulated by phosphorylation, becoming more stable, and by moving to the cell surface after a Hh signal is received.[55-60] A detailed study has shown that Smo accumulates in the plasma membrane of cells in which Ptc activity is abrogated by Hh but is targeted to the degradative pathway in cells where Ptc is active.[61] Thus, Ptc could regulate Smo activity through inhibition of its accumulation in the plasma membrane, targeting instead to lysosomal compartments. In this view, Ptc might regulate Smo activity simply by modulating the levels of protein present in the cell. Alternatively, it may be that sub-cellular location of Smo is critical for its activation, the plasma membrane providing an environment for Smo to be accessible to an intracellular agonist (see Fig. 3 for details).[61]

Ptc and Human Disease

Genetic studies in various model organisms are beginning to elucidate the factors that are likely candidates for the causes of early embryonic defects in humans (reviewed in refs. 62, 63). Thus, detailed knowledge of the Hh signaling pathway is fundamental to an understanding of vertebrate development as well as several birth defects in humans.

Since the unliganded Ptc receptor exerts a repression on the Hh pathway, mutations affecting Ptc are frequently associated to a constitutive activation of the Hh signaling pathway. This is in agreement with the fact that most of the mutations characterized to date in *PTCH1* result in protein truncation.[24] The spectrum of human *PTCH1* mutations also includes deletions, insertions, splice site alterations, and nonsense and missense mutations distributed throughout the gene (for an example, see Table 1).[64-66]

Table 1. Mutations of the* PTCH1 *gene detected in patients with Gorlin syndrome and BCC (see refs. 64, 65, 74)

Mutation	Exon	Nucleotide Change	Effect on Protein (When Reported)	Disease Associated	Ref.
1	2	260del5TinsAA	L87del, stop at 87	GS	64
2	2	280insA	94 frameshift, stop at 138	GS	64
3	2	269insT		GS	65
4	2	277AA→C		GS	65
5	2	238		BCC	74
6	3	531del ACA	Q177del	GS	64
7	3	429		BCC	74
8	5	C672A	Y224X	GS	64
9	5	742delCTinsGGAG	248 frameshift, stop at 249	GS	64
10	intron 5	IVS5+3 del AA		GS	64
11	6	853insC		GS	65
12	8	1194delCinsATATG	398 frameshift, stop at 436	GS	64
13	8	1208delAT	403 frameshift, stop at 435	GS	64
14	8	C1081T	Q365X	GS	65
15	intron 9	1336-135		BCC	74
16	10	G1450A	G484R	GS	64
17	11	C1511T	P504L	GS	64
18	11	G1513	G509R	GS	65
19	11	G1514T	G509V	GS	65
20	11	1552		BCC	74
21	12	1665		BCC	74
22	12	1686		BCC	74
23	13	2000insC		GS	65
24	13	2047insCT		GS	65
25	14	C1941A	S647R	GS	64
26	14	2178insC	721 frameshift, stop at 1048	GS	64
27	14	2434del3	Q815del	GS	65
28	14	2199		BCC	74
29	15	T2465C	L822P	GS	64
30	intron 15	2560+9		BCC	74
31	16	C2619A	Y873X	GS	64
32	16	2875+1G→C		GS	65
33	18	3042delC	1014 frameshift, stop at 1048	GS	64
34	19	C3383A	S1132Y	GS	65
35	17	T2776C	W926R	GS	64
36	21	A3583T	T1195S	GS	64
37	23	3944		BCC	74

PTCH1 deregulation in the epidermis is sufficient to induce Basal Cell Carcinomas (BCCs) of the skin. 30-40% of Gorlin syndrome patients (GS; also known as Nevoid basal cell carcinoma syndrome) have familial loss-of-function mutations in the *PTCH1* gene. GS is an autosomal dominant disease with nearly complete penetrance and variable expressivity because mutations in *PTCH1* seem to be haploinsuficient in humans (a mutation in only one of the two alleles is enough to show a phenotype). Clinically, GS patients present congenital abnormalities that includes skeletal defects (polydactyly, fused or bifid ribs), early onset of multiple BCCs and an increased rate to develop other tumors, including medulloblastomas of the cerebellum (reviewed in refs. 67-69). A limited number of mutations in *PTCH1* have been linked to Holoprosencephaly (HPE).[70] HPE affects the forebrain and face to various degrees, from the most extreme lethal alobar type to milder microforms that include small midline facial defects. However, since HPE arises from loss of SHH signaling, these mutations might rather reflect an impaired ability of PTCH1 to interact with SHH, thus permanently shutting down the pathway. In fact, two out of four rare *PTCH1* missense mutations that have been reported to be associated to HPE, were localized in the extracellular loops of PTCH1 required for SHH binding.[70]

There is a limited number of missense mutations described for *PTCH1*. Missense mutations do not occur as frequently as frameshift or nonsense mutations. In a clinical study on French patients, missense mutations span the second group of six transmembrane domains of the protein.[64] Unfortunately, no data are available to interpret the pathologic value of the missense mutations described in humans.[64] So far, only the molecular and cellular studies of *Drosophila ptc* mutations indicate an alteration of the correct topology and/or sorting of the Ptc receptor. We propose *Drosophila* as an ideal model system to analyze the functional alterations of the missense mutations found in the PTCH1 receptor. In fact, a recent study analyses in the fly the function of an artificially mutated form of Ptc analog to a mutated PTCH1 previously characterized in GS patients.[71] Therefore, understanding the molecular mechanisms of Hh signal reception will allow identifying potential drug targets in order to devise strategies for the treatment of BCC.

Concluding Remarks

More genes of the Hh pathway are going to be uncovered in the near future and *Drosophila* genetics will undoubtedly help in to resolve this issue. Hence, concerning the function of Ptc receptor, an unresolved question is how Ptc is able to repress the activity of Smo, and whether there are intermediate elements. Although other molecules have been found as possible candidates in the reception of Hh in vertebrates such as Megalin, Hip or Gas1, mutagenesis screens need to be done in *Drosophila* to find more proteins implicated in Hh reception. The study of their function will help to understand how several cellular and molecular processes drive and regulate the pathway. Another important unresolved issue in the Hh pathway is the role of cell polarity in the transduction of the signal, or the endocytic and exocytic routes involved. Also questions remain regarding how a morphogen gradient is formed, how the cytoskeleton affects the pathway, or how the transcription of target genes is regulated. New model systems in vertebrate genetics, cellular biology and biochemistry will help to elucidate some of these questions. Definitely, a huge biochemical and cell biology effort needs to be done in order to solve the role of this kind of proteins in Hh signaling pathway. This knowledge will undoubtedly help to design specific therapies to serious human diseases caused by mutations on genes of this signaling pathway.

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CHAPTER 3

Making a Morphogenetic Gradient

Henk Roelink*

The characteristics of Sonic Hedgehog (Shh) represent a challenge to the simple model in which a morphogen establishes a gradient via diffusion through the extracellular space in a developing tissue. The lipophilic nature of appended molecules, renders Shh virtually insoluble. Large, soluble multimers have been proposed to be the form in which Shh traverses the space between the source and the responding tissue. However, such large multimers would be expected to diffuse poorly. In addition, away from the source, Shh is mainly localized in endocytic vesicles, and not in the extracellular space as would be predicted if Shh diffused mainly in the extracellular space. A model in which Shh is handed over from cell to cell in a receptor-dependent manner could reconcile these disparate observations.

Sonic Hedgehog (Shh) is a morphogen. The principle of morphogen signaling is straightforward; an inducer is released from a localized source, moves away from the source by diffusion, thus establishing a gradient away from the source. Cells in this gradient somehow read the local concentration of the inducer and differentiate appropriately, resulting in a stereotypic local patterning. For morphogens to work, it is necessary that they travel across many cell diameters. Although diffusion may account solely for their movement in some cases, in particular for small molecules that can pass through membranes, for larger molecules strict reliance on diffusion might not be effective. In particular in epithelia, the extracellular space is a small, crowded space, filled with molecules that bind many morphogens quite well.[12] Not surprisingly, a more active mechanism of transport trough tissues has been described for some morphogens.

This caveat is even more significant for Shh, since it has undergone two modifications that involve the addition of lipophilic adducts, cholesterol at the C-terminus in conjunction with cleaving off a large carboxyterminal fragment[21] and the addition of an acyl group at the N-terminus.[24] The cholesterol adduct alone anchors the protein to the membrane with significant strength and a high half-time far too long for spontaneous transfer.[18] The available structural data of the hedgehog signaling domain do not indicate that Shh has a hydrophobic binding pocket that could bury the cholesterol anchor.[6] Given the fact that the protein carries a palmitic acid as a second strong membrane anchor, spontaneous release of the protein to generate a signaling gradient can be ruled out on the basis of biophysical considerations alone. Several explanations have been put forward the most widely accepted involves the generation of a soluble multimer. The mature, active form of Shh has been proposed to mediate long-range signaling as a soluble multimeric complex,[28] but given its size, it might be an unlikely candidate to be distributed strictly via diffusion trough the extracellular space. In addition, early experiments demonstrated that contact between the Shh source and responsive tissue is required to elicit a response, inconsistent with the presence of a freely diffusible form of Shh. There appear to be two fundamental models how the long-range signal is generated. Besides diffusions of a soluble form of Shh mediating long range signaling, I will discuss a model that

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can reconcile the apparent contradictory observation about long-range signaling by Shh, by describing a "lipophilic pathway" along which Shh can travel. This models involves the movement of Shh trough the responding cells, utilizing endocytosis and excretions mechanisms, coordinated such that Shh is 'handed over' from cell to cell. I will refer to this movement trough cells as transcytosis, although it should be noted that I use the term transcytosis in the loose definition, i.e., passage of materials or across a cell by endocytosis at one side, transport in vesicles and release at the other side. In its narrow definition transcytosis refers to Apical-basal transport trough epithelia.

Disp1, A Molecule Dedicated to the Export of Shh

Transcytosis requires the existence of a regulated mechanism of endocytosis, coupled to a regulated mechanism of export, while still allowing the Shh response to occur in the cells transporting Shh. Using a variety of systems, convincing evidence has been provided that both the uptake and export of Hedgehog proteins requires dedicated accessory proteins. It remains to be established, however, whether the uptake/export mechanisms are functionally linked, to enable directed transcytosis.

Intriguingly, the cholesterol moiety on Shh is required for long-range transport. Truncation of Shh at the autoproteolytic processing site results in an active form of the protein not coupled to cholesterol (ShhN*).[20,22] In contrast to the wildtype form of processed Shh, the ShhN* is soluble, and is thus expected to easily diffuse long distances. However, mice homozygous for *Shh* alleles coding for ShhN* only exhibit short range Shh signaling capabilities,[3,13] indicating that the cholesterol moiety is what allows long-range transport of Shh. Additionally, exporting the sterol-associated form of Hh from the cells where it is synthesized requires the activity of a dedicated protein. In *Drosophila* and vertebrates, the sterol-sensing domain-containing transmembrane protein Dispatched (Disp) is required in cells synthesizing the sterol-modified Hh, but is not necessary for the excretion of a form of Hh lacking the sterol conjugation.[2,9,14] *Disp1* null mice resemble *Smo* null mice,[9] indicating that Disp1 is required for the proper functioning of all Hedgehog family members

Together these observations are in apparent contradiction; the form of Shh that is poorly soluble is the form that mediates the long-range signal, while a freely diffusible form of Shh only mediates short-range signaling. To reconcile this, we hypothesize that Disp1 functions in cells not expressing Shh to transport the ligand in an epithelium of cells not expressing Shh.

First, a dedicated mechanism is required for the release of Shh from the source cells, since mature Shh unable to disassociate itself from the membrane.[18] Disp1, a membrane-bound molecule is absolutely required for the generation of an active Shh signal. In embryos lacking Disp1 no Shh, nor Ihh or Dhh response can be detected, consistent with the failure of mature Hedgehog proteins to leave the source cells.[9,14] A well-documented activity of Disp1 is the generation of a hydrophilic complex consisting of hedgehog multimers and an unknown amount of lipids. These multimeric forms of Shh are highly active in a signaling, although the largest multimers are unable to elicit a response in telencephalic explants.[5] These results support the hypothesis that a Disp1 mediates the conversion of a membrane bound form of Shh to a soluble multimeric form, which mediates the long-range effects, possibly by diffusion through the extracellular space. This idea is supported by the observation that forms Shh that miss either the Acyl or Cholesterol modification are severely impaired in mediating long-range signaling, which correlates with their ability to form large multimeric structures.[3] Still, it leaves the puzzling observation that a small, nonsoluble form of Shh is unable to mediate long-range signaling, while a large multimeric form seemingly diffuses over long distances, and no good explanation has been offered to date.

The biochemical analysis of the multimeric forms of Shh has largely been performed in vitro, and the actual form of Shh found in tissues away from the source is still unknown. Still, if these large multimeric forms of Shh are the principal mode of long-range signaling, it might be predicted that a significant fraction of Shh outside the source is located in the extracellular

space. However, this model in which large structures are actually the forms of Shh that diffuse over long distances is not supported by the localization of Shh in tissues outside the source. Outside it's site of production, Shh is largely localized in endocytic vesicles[8] and is not detectible in the extracellular space. So the question arises if the multimeric form of Shh diffusing through the extracellular space is largely undetectable, or if Shh localized in endosomes is actually on a leg of its itinerary along the 'lipophilic highway'. In this scenario an active mechanism involved in moving Shh away from its source would be important in the generation of the long-range signal.

Long-Range Signaling by Shh; Diffusion in the Extracellular Space?

Tout velu (*ttv*), a homolog of the mammalian *EXT* tumor suppressor gene family, is required for movement of Hh. *ttv* is involved in heparan sulfate proteoglycan (HSPG) biosynthesis, suggesting that HSPGs influence Hh distribution.[25] HSPGs have been implicated in the binding and presentation of several growth factors to their receptors, thereby regulating cellular growth and differentiation. Addition of chlorate, a competitive inhibitor of glycosaminoglycan sulphation, to embryos demonstrates that in the neural tube, HSPGs appear to play a role in the transport of Shh. *ptc1* transcripts are abnormally abundant in the notochord, and diminished in the overlying neuroepithelium, suggesting that Shh signaling from the notochord may be perturbed by inhibition of heparin sulphation.[27] The HSPG binding site of Shh is distinct from the domain that binds to Ptc. Interestingly, not all Shh responses are dependent on HSPGs. While Shh interactions with HSPGs promote maximal proliferation of postnatal day six granule cells, proliferation of less mature granule cells is not affected by Shh-proteoglycan interactions.[23] What remains to be determined is whether HSPGs assist with the presentation of Shh to Ptc1, or are actively involved in the transport of Shh in the intercellular space. In our model, we suggest that part of the long-range signaling occurs via a mechanism in which Shh is actively moved from cell to cell. Although it is possible that the lateral movement occurs while Shh is associated with the cell surface, Shh in responding cells is located in endocytic vesicles.

Shh Is Internalized by Neural Plate Cells during Signaling

Naïve neural plate cells are first exposed to endogenous cholesterol-modified Shh from the notochord, which induces midline neural plate cells to become Shh-expressing floor plate cells. Punctate accumulations of Shh in neural plate cells are present in a gradient extending more than 10 cell diameters away from Shh source cells in the floor plate. The level of Pax6 repression is proportional to the density of Shh-containing endocytic vesicles. The endocytosis of Shh is mediated by Ptc1. The Ptc1-mediated endocytosis of Shh is independent of the lipid modifications. Remarkably, the Shh-CD4 fusion protein is internalized intact, perhaps suggesting that Shh associated with lipid vesicles is taken up by the Ptc1 cells.[8] This would suggest that Ptc1 is able to internalize forms of Shh associated with lipids, and perhaps even extract Shh-containing lipid vesicle from neighboring cells. This would completely obviate the need for free diffusion of Shh to move from the source cell to the responding cell, consistent with the localization of Shh. It would also necessitate the need for contact between the Shh presenting cells.

Ptc1 and Smo display comparable cellular distributions, consistent with Ptc1 function repressing Smo activity. Ptc1HA and Smo appear to have some level of interaction; the location of Ptc1HA and SmoFlag is distinctly different in cells expressing only Ptc1HA or Smo. In such cells, Smo distribution resembles that of many other 7-transmembrane receptors; it is visible at the cell surface, and in early endosomal compartments. In contrast, Ptc1 resides in late endosomal compartments and the trans-Golgi network.

Treatment of cells with pharmacological agents that interfere with trafficking provides further insight into the dynamic interplay between Ptc1 and Smo. The protease-inhibitor leupeptin prevents lysosomal degradation of Ptc1. When Ptc/Smo expressing cells cells were incubated in leupeptin, but without ShhN*, Ptc1 and Smo retained similar distributions, indicating that Ptc1 and Smo do not enter lysosomal compartments quickly.

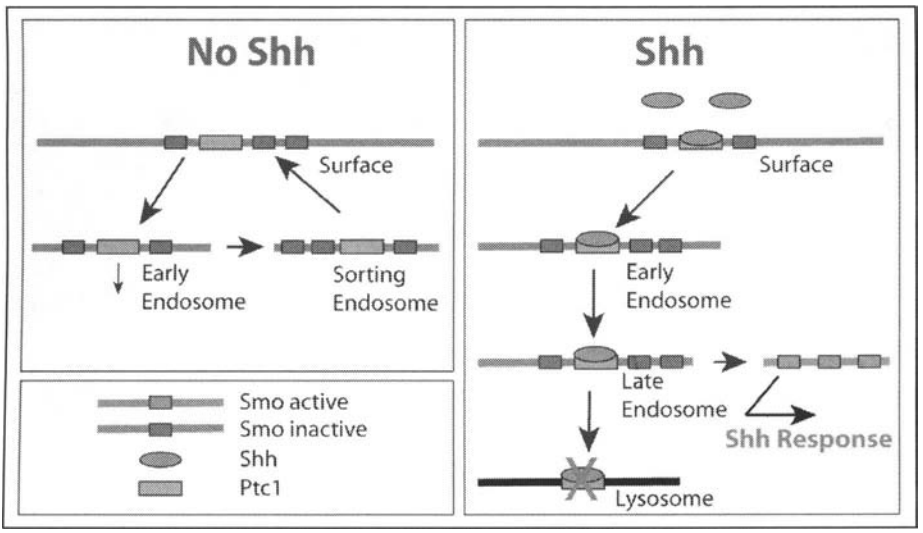

Figure 1. Colocalization of Ptc1 and Smo inhibits the activity of Smo (Left panel). In the presence of Shh, Ptc1/Shh and Smo transit to late endosomes, where Smo is able to traffic away from the Ptc1 inhibition (Right Panel). Separation of Ptc1 and Smo allows Smo to move into a separate compartment and initiate the Shh response.

Chloroquine, a drug that prevents acidification of endosomes, and thus prevents the maturation of early endosomes into late endosomes, blocks normal trafficking of proteins into late endosomes. Binding of Shh dramatically changes the intracellular trafficking pattern of Ptc1 and Smo, directing them towards late endosomes. Chloroquine also inhibits the Shh response,[4] which is consistent with our observation that late endosomal activity is required for signal transduction. In cells expressing Ptc1 but not Smo, chloroquine treatment causes an accumulation of Ptc1 in endosomal vesicles, indicating that the normal itinerary of Ptc1 includes a chloroquine-sensitive compartment. In cells expressing both Ptc1 and Smo, both proteins appear to enter late endosomes slowly. Interestingly, after addition of ShhN* to Ptc1/Smo hybrid cells incubated in chloroquine, both Ptc1 and Smo, and presumably Shh, accumulate quickly in chloroquine-sensitive vesicles. This indicates that Shh alters the trafficking of the Ptc1-Smo complex, directing it toward late endosomes.

Once Ptc1 and Smo enter the late endosome, something remarkable happens. In Ptc1/Smo cell fusions exposed to ShhN*, Ptc1 immunoreactivity accumulated in lysosomes, but showed no colocalization with Smo. In these same cells, Smo appeared in juxtanuclear and small, peripheral punctate structures. Thus, when endocytic transport and sorting is allowed to proceed normally (i.e., in the absence of chloroquine), Smo does not accumulate in late endosomes with Ptc1/ShhN* complexes, but instead it is sorted to a distinct compartment in the cell, and is no longer associated with Ptc1 (Fig. 1).

Anti-LBPA antibodies (αLBPA) provide a very specific tool to test the requirement for late endosomal sorting in Shh signaling. When internalized by bulk endocytosis, αLBPA antibodies bind the antigen in late endosomes, perturb the morphology of the internal membranes, and disrupt protein and lipid sorting within this compartment.[10,11] Antibodies directed against a glycoprotein enriched in the limiting membrane of late endosomes, such as LAMP-1, do not produce these effects. Thus, αLBPA antibodies have a very specific effect on late endosome function. Treatment of Shh responsive cells or explants with αLBPA, prevents the response to Shh, but not to other signaling molecules, indicating that normal endosomal function is required to respond to Shh. Under these conditions, endosomal segregation of Ptc and Smo does

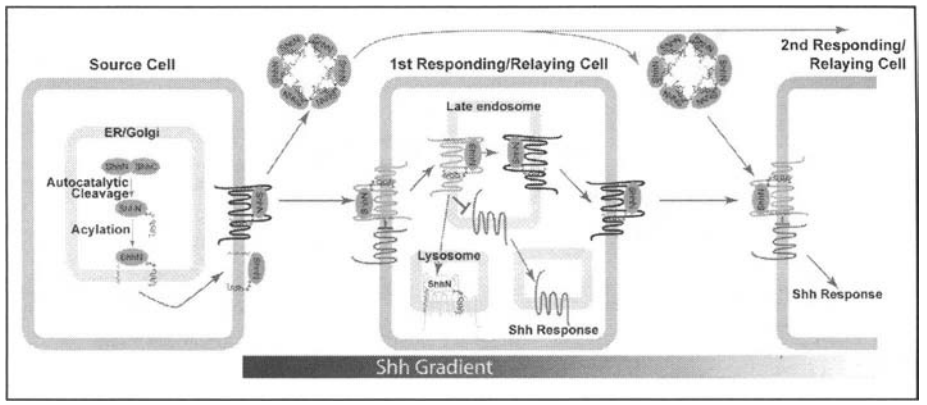

Figure 2. Models of the establishment of a Shh morphogen field. The two fundamental pathways are indicated. The pathways have in common that Shh is released form the source by Disp1. The first pathway would involve the diffusion of a multimeric, soluble form of Shh across the morphogenetic field. The second pathway evokes the activity of Ptc1 and Disp1 to create a "bucket brigade", effectively handing over internalized Shh to neighboring cells.

not occur, demonstrating that the inability to respond correlates with the failure of Ptc1 and Smo to segregate in endosomes. Since Ptc1 blocks the signaling activity of Smo, this Shh-induced segregation of Ptc1 and Smo appears a critical step in initiating the Shh response. The observation that SmoM2 does not colocalize with Ptc1 emphasizes this point.[7]

Is Shh Handed Over from Cell to Cell Using an Active Mechanism?

Ptc1-mediated uptake of Shh, independent of its membrane anchor while Disp1-mediated export of Shh requires the cholesterol moiety. The cholesterol moiety is also necessary for long-range signaling. Thus, one model that would reconcile these observations would evoke the activity of a Disp-like molecule to move Shh from the endosomes in the responding cells back to the surface for presentation to a neighboring cell. The itinerary of Shh would be as follows (Fig. 2). In the source cells, Disp1 is involved in mediating the export of cholesterol-associated Shh, and possibly generating the multimeric form. Subsequently Shh binds to Ptc1 in the primary receiving cell, and Ptc1/Shh and Smo move trough late endosomes, from which Smo enters a Ptc1-free compartment thus initiating the Shh response. This part of the model is well substantiated. The following part is still speculative. Disp1, the itinerary of which, based on it's similarity to Ptc and NPC1,[17] is predicted to include late endosomes, could facilitate the trafficking of Shh from endocytic compartments back to the cell surface, where Shh is presented to Ptc1 on a neighboring cell. The near ubiquitous expression of Disp1 is consistent with this role.[9,14] Disp1 is not the only candidate to mediate the return of Shh back to the surface, other members of the Ptc/Disp family might fulfill this role. Megalin might play an important role in this process, it mediates Shh internalization as well, although it does not appear to play a primary role in Shh signaling.[15,16] The mechanism of uptake, activation of the Shh response, and export could then repeat itself, resulting in the formation of a Shh gradient (Fig. 2). An attractive part of this model is that the intracellular trafficking required for the Shh response is correlated to the movements required for transcytosis, allowing the ligand to be 'recycled' after it evokes a response. This models also provides an explanation why the Shh detected outside the source is located in intracellular vesicles, rather than in the extracellular space.

In the developing neural tube, the presentation of Shh is to the basolateral side to the overlying neural tissue from the notochord and the prechordal plate, which express Shh.

Interestingly, contact is required between these Shh sources and responding tissue,[19,26] strongly arguing against an important role for free, long-range diffusion of Shh. In addition, it means that Ptc1 must be present on the basolateral membrane of the neuropepithelial cells. It remains to be determined if Ptc1 can traffic to the apical side of the neuroepithelium as well. Resolution of this issue might be relevant, since it is possible that Shh is excreted from the apical side of the Shh expressing floor plate cells. It would in principle be possible that Shh in the lumen of the neural tube is transported over a longer distance, since it's movement here would be much less impeded than in the extracellular space. Although this mode of transportation might have some importance, it cannot be elicited by expressing Shh unilaterally in the neural tube. Under these conditions no effects on the Shh response on the contralateral side are observed, indication that diffusion of Shh across the lumen of the neural tube does not occur, or that Ptc1 does not traffic to the apical side of the neural epithelium (our unpublished data). It should be noted also that long-range Shh signaling also occurs in tissues made of mesenchymal cells, showing that neither cellular polarization nor luminal transport appears to be required to transport Shh across several cell diameters.

Conclusions

Although the proposed model is more proficient reconciling the observations of the activities and distribution of Shh with that of a simple diffusion model, many aspects remain unresolved. Whereas the binding of Shh to Ptc1 is well documented, Ptc1 subsequently delivers Shh to the lysosome for degradation. The required transfer of Shh from Ptc1 in endosomes back to the cell surface is speculation, but trafficking of membrane associated molecules from late endosomes to the surface is well documented in particular of the Ptc1/Disp1 homolog NPC1.[1] The model in which Shh is "handed over" from cell to cell provides a better explanation of the Shh signaling characteristics. It reconciles the requirement for contact between the Shh source and the responsive tissue with the ability of Shh to mediate long-range signaling. It also provides a more satisfying explanation why Shh away from its source resides in endocytic vesicles rather than in the extracellular space. Lastly it reconciles the discrepancy between the requirement for lipophilic modification to establish the long range signal.

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CHAPTER 4

Spatial and Temporal Regulation of Hair Follicle Progenitors by Hedgehog Signaling

Anthony E. Oro*

Abstract

Epithelial organs such as the vertebrate hair undergo programmed self-renewal in part by controlling the growth of progenitor cells. The hedgehog signaling pathway regulates progenitor growth in a variety of tissues. This chapter discusses evidence for how the skin regulates the production of Shh ligand, the reception of the Shh signal, and maintenance of the signal to promote hair morphogenesis and prevent tumor formation.

Introduction

Despite their architectural and functional diversity, epithelial organs or adnexa, such as the vertebrate hair, pancreas, intestine, or prostate share common developmental strategies. Each of them derives from a sheet of cells, whether ectoderm or endoderm, and then interacts with the underlying mesenchyme to undergo a characteristic pattern of differential proliferation, differentiation and migration to form the mature organ.[1-3]

An intriguing property of many epithelial organs such as the hair is periodic self-renewal through the regulation of multipotent progenitors.[4-6] Hidden in the midst of the tissue are multipotent cells capable of regenerating the mature adnexal organ. These progenitor cells constitute a small proportion of the cells in the tissue, are in a characteristic location, or niche, within the tissue, and divide rarely. In some tissues such as the skin and oral epithelium, hair and tooth renewal occurs at predetermined intervals, while in others such as the lung epithelium, lung alveolar branching and growth is associated with injury and repair.

The timing for growth and proliferation of the progenitors depends on the activity of a group of conserved signaling molecules or morphogens. In perpetually renewing tissues, proper growth and patterning requires repeated signaling of molecules such as Sonic hedgehog (Shh), Wnt, Fibroblast growth factors (FGFs), Bone Morphogenetic Protein (BMP), Notch, and Epidermal growth factor at the right times and places. Insufficient signaling results in failure of the mature tissue to emerge, inappropriate signaling yields excess progenitors and susceptibility to cancer.

One of the key signals involved in the growth and differentiation of many epithelial organs is the Shh signaling pathway.[7-9] As is evident from other chapters in this book, Shh signaling plays a key role in the growth of many organs throughout metazoan development. The numerous human diseases attributable to defects in hedgehog signaling include failure to expand progenitors such as those of the limb, skin, and nervous system. Mutations resulting in loss of pathway activity have been associated with holoprosencephaly (*Shh*,[10] *Gli2*[11]) or Grieg or Pallister-Hall polydactyly syndromes (*Gli3*[12,13]). By contrast, activating mutations have been

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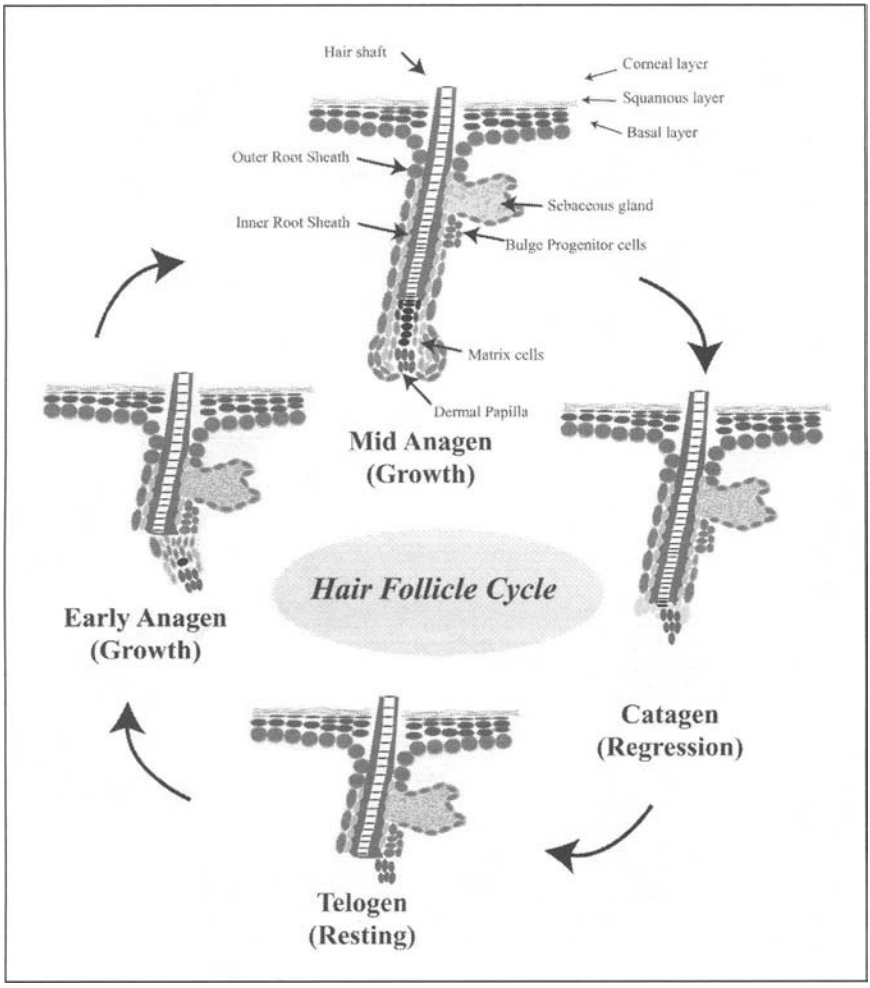

Figure 1. The hair follicle cycle. Diagram of programmed tissue renewal. During anagen hair follicle progenitors derived from the bulge (red) interact with the mesenchymal dermal papilla cells (blue) to generate the lineages of the hair follicle. During catagen, the lower portion of the hair regresses, leaving the dermal papilla cells attached to the remaining hair and, ultimately, adjacent to the bulge progenitor cells during telogen. When the next cycle begins the progenitor cells derived from the bulge interact again with the dermal papilla cells and proliferate. A color version of this figure is available at http://www.Eurekah.com.

strongly associated with tumor susceptibility of basal cell carcinomas (BCCs) (*ptch1*,[14,15] *smoh*[16]), medulloblastomas (*ptch1*,[17,18] *sufu*[19]), and rhabdomyosarcomas(*ptch1* [20]).

Consistent with these ideas, induction of Shh expression is seen during repair of lung epithelium, with persistent constitutive Shh expression associated with small cell lung carcinoma.[21]

One of the best systems to study the cyclic requirement for morphogens comes from studying the Sonic hedgehog effects on progenitor cells in the skin. Previous work has established the location[22] and identifying markers[23,24] associated with the multipotent progenitors and have begun to elaborate how they contribute to the hair and interfollicular epithelium. Many hundreds of hair follicles regenerate themselves with great frequency (Fig. 1), about every two

weeks in the mouse, providing easy access to a developing organ and ease in visualizing subtle abnormalities in the process. Renewal occurring in animals with a high density of hair follicles within the skin illustrates the critical need for precise spatial and temporal control of Shh signaling. This chapter discusses evidence for how the skin regulates the production of ligand, the reception of the Shh signal, and maintenance of the signal to tightly control the expansion and differentiation of hair follicle progenitor cells.

The Hair Follicle Cycle

Much work has been done over the past forty years understanding the hair follicle cycle (Fig. 1). During embryogenesis, a hair follicle niche is created by reciprocal signaling between the epithelium and aggregates of mesenchymal cells that eventually become the dermal papilla (DP) of the postnatal hair (blue cells, Fig. 1).[25] The inductive signals from the mesenchymal DP cells segregate portions of the epithelium into hair and nonhair epithelium and then cause the former to grow. Histologically, the growth of postnatal hair epithelium resembles embryonic hair growth. The postnatal hair follicle originates from multipotent progenitors believed to be located in a specialized epithelial compartment of the permanent hair follicle referred to as the bulge (red cells, Fig. 1).[22] The cells within the bulge give rise to progenitors that migrate in one of two directions (Fig. 2a).[26,27] The progenitor cells that migrate superficially away from the hair follicle populate the sebaceous gland and the basal layer of the interfollicular epidermis that forms stratified epithelium. Alternatively, progenitors that migrate inward toward the dermis in response to DP signals populate the growing hair epithelium and form the hair follicle. As the hair follicle progenitors (matrix cells) divide and move further away from the DP, they stop proliferating and begin a process of hair follicle differentiation, giving rise to the concentric epithelial layers of the growing hair follicle.[28] Growth and differentiation of the progenitor cells continues throughout the growth or anagen phase (Fig. 1), after which proliferation ceases and the distal hair follicle regresses (catagen) to reach the quiescent hair follicle stage (telogen). The start of a new growth cycle (early anagen), a fascinating step whose regulation is still poorly understood, stimulates proliferating epithelial cells from the bulge progenitors and completes hair follicle renewal.[29] In the mouse, the first anagen phase extends from mid-gestation to approximately postnatal day 12. The first postnatal hair cycle anagen phase begins at approximately day 25 and lasts through day 40.

Shh expression and target gene induction occurs only in the anagen or growth phase of the hair cycle in the cells within the hair follicle niche and not in interfollicular cells.[30] *Shh* is transcribed asymmetrically in the growing hair follicle in the distal most cells of the follicle, with *ptch1* expression induced in adjacent cells (Fig. 2b,c). As the skin begins early anagen, the epithelium grows out from reactivated hair follicles. *ptch1*, as judged by in situ hybridization and X-gal staining of *ptch1*-lacZ knock-in mice, is transiently expressed in epithelial and mesoderm cells surrounding the bulge (Fig. 2e). As the new follicular epithelium grows, *ptch1* expression is visualized at the distal tip of the growing hair follicle (Fig. 2f). High-level *ptch1* transcription continues in mid anagen as the hair epithelium increases in size. During this time *ptch1* staining is seen in dermal papilla cells and the outer root sheath (Fig. 2g). At the start of catagen when apoptosis occurs, expression of *Shh* and many of its target genes ceases. Expression is undetectable in quiescent (telogen) hairs or anywhere in the interfollicular skin (Fig. 2d). The expression of Shh and the reception of signaling are tightly controlled in time and space within the skin.

Interfollicular Epithelial Cells Are Competent to Induce Shh Target Genes

Shh signaling is normally restricted to the cells of the hair follicle/sebaceous gland lineage. Restricted signaling could occur because reception of the Shh signal is prevented in epidermal progenitor cells or merely because Shh is not expressed there. Important to understanding the

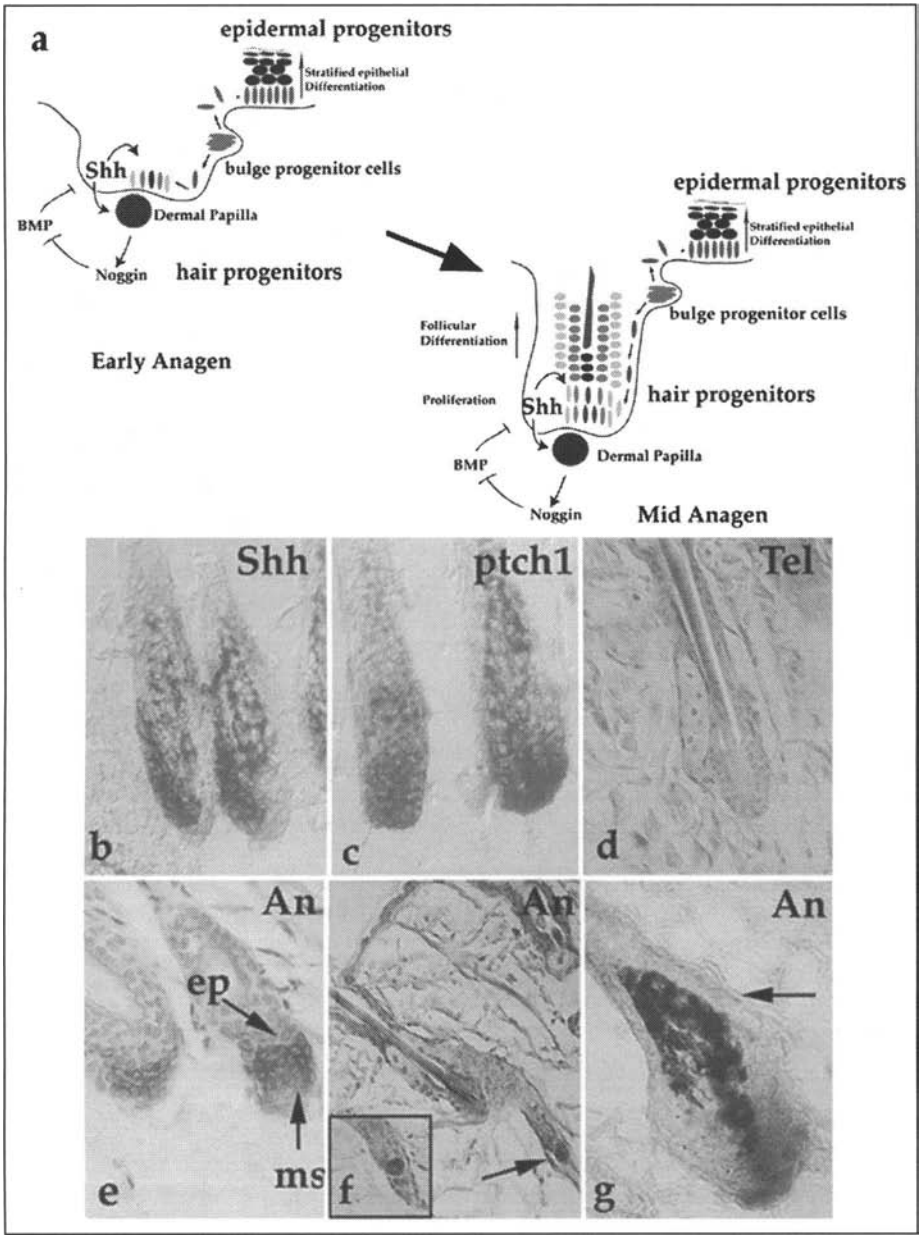

Figure 2. Shh signaling is spatially and temporally restricted. a) Diagram of the ability of the bulge progenitors to contribute to interfollicular epidermal progenitors and hair follicle progenitors. Shh acts on hair follicle progenitors in the hair follicle niche to induce proliferation. From reference 75. b) in situ hybridization of anagen hair follicle showing the expression of Shh and c) *ptch1* at the tip of the growing hair epithelium. d) X-gal staining of *ptch1*-lacZ mice at telogen showing no Shh target gene induction in the resting hair. e) X-gal staining of early anagen hair epithelium showing Shh target induction in progenitors (blue) f) X-gal staining of early anagen hair as it grows out from previously resting hair. Much of the staining is also in the dermal papillae cells. g) X-gal staining of mid anagen hair showing Shh target gene induction in dermal papilla and outer root sheath staining. Reprinted with permission from ref. 30.

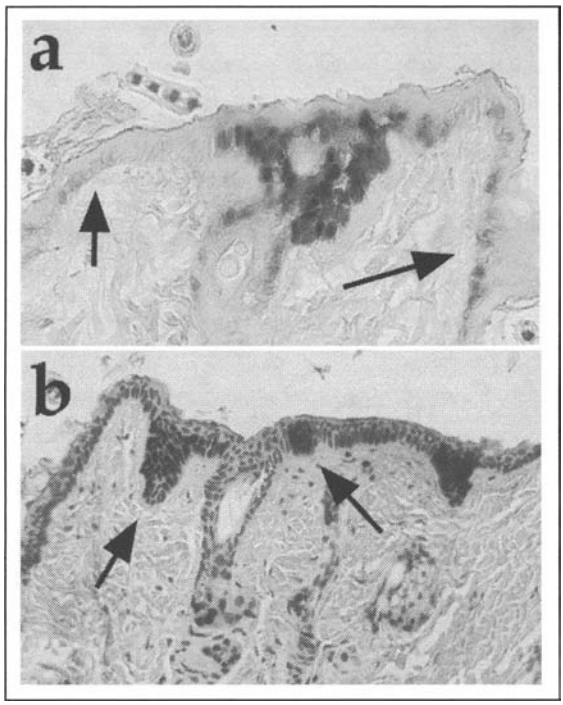

Figure 3. Many interfollicular cells are capable of inducing Shh target genes. a) X-gal staining from Keratin14-Gli1; *ptch1*-lacZ mice at anagen shows most of the basal cells of the interfollicular epidermis (arrows) express Shh target genes. b) Different section showing X-gal stained epithelial buds deriving from interfollicular epithelium (arrows). Reprinted with permission from ref. 30.

role of Shh signaling in disease processes is the effects of ectopic signaling in ectopic locations outside the hair follicle niche.

Recent data suggests that interfollicular cells are also capable of inducing canonical Shh targets such as *ptch1* and *gli1*. The competence for epidermal progenitor cells to respond to Shh signaling was first suggested by experiments exposing interfollicular epidermis to dermal papilla cells and isolated morphogens normally found within the hair follicle niche. Recombination experiments using hairless human foreskin and the dermal papillae cells within the niche resulted in sebaceous glands and rudimentary hair follicles, processes which require Shh signaling, while recombination with regular dermal fibroblasts yielded just interfollicular epithelium.[31,32] Further, forced expression in the interfollicular cells of an activated β-catenin, a integral component of the Wnt pathway normally expressed during anagen, induced ectopic hair follicle morphogenesis in interfollicular epithelium with concominant Shh and Shh target gene expression.[33-35]

Two additional experiments demonstrate that the epidermal progenitor cells are competent to directly induce Shh target genes. In adult *ptch1*/+, small tumor buds expressing Shh target gene induction arise from the interfollicular epidermis (Fig. 3). While this suggests that there may be some cells that can respond to Shh signaling, it did not test which of the cells could respond. Using a transgenic promoter driving the *gli1* gene in the background of *ptch1*-lacZ, cells capable of responding to Gli1 could be visualized by X-gal staining. In anagen animals, the staining occurred throughout the epithelium, both follicular and interfollicular. This demonstrates that during anagen the entire basal epithelium appears to be capable of inducing Shh target genes if stimulated. These data further suggest that the difference between cells destined

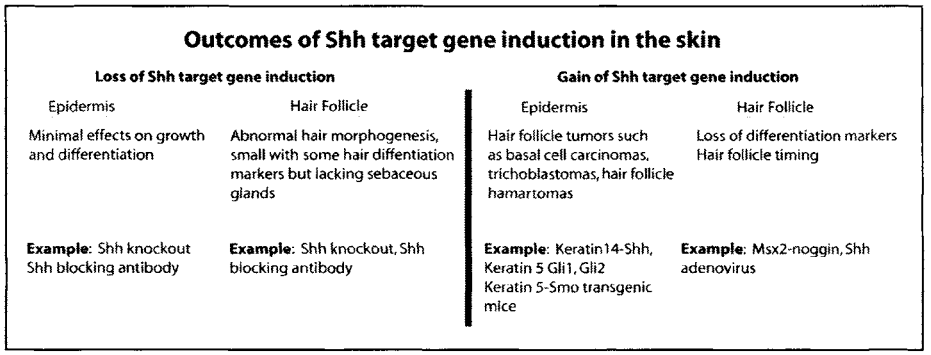

Figure 4. Outcomes of Shh target gene induction depending on where expression occurs.

to become hair follicle and stratified epidermis is not lineage, but the restricted exposure and/or response to powerful morphogens from the niche.

Outcomes of Shh Target Gene Induction in the Skin

While it is apparent that many cells are competent to induce Shh target genes, alterations in Shh signaling have different effects on the different compartments in which they occur and may be helpful in understanding pathway-dependent diseases (Fig. 4). Complete lack of Shh in the skin in Shh-null mice demonstrates the essential role in hair progenitor growth and morphogenesis. Shh mutant mice develop normally spaced hair placodes, which are precursors to mature hairs, but fail to undergo normal hair morphogenesis.[36,37] Differentiation markers demonstrate that much of normal hair differentiation occurs, but the outgrowth of the progenitor cells is blunted. *Shh* mutants also fail to express sebaceous glands, a differentiation pathway from the hair follicle, suggesting hedgehog signaling (likely involving cooperation from another hedgehog, Indian hedgehog) signaling is involved in sebaceous differentiation.[38,39] Similarly, in adult hair follicle, antibodies that block Shh function arrest hair morphogenesis, indicating not a single requirement, but continued requirements for Shh function throughout anagen.[40]

While epithelial Shh target gene induction regulates the growth of hair progenitor cells, the Shh pathway appears to have a more subtle requirement in the proliferation of progenitor cells that give rise to the stratified epithelium. Initial studies suggest that Shh is not required for differentiation of epidermal progenitor cells because *shh* mutants and animals injected with anti-Shh antibodies display a wild-type pattern of stratified epithelial differentiation.[36,37,40] Moreover, tumors of epithelial differentiation such as squamous cell carcinomas (SCCs) do not express high levels of the canonical Shh target gene *ptch1*.[41,42] However, Shh signaling may play a minor role in the growth of SCCs. SCCs are found at slightly higher frequency in irradiated *ptch1*/+ mice, and mutations in the human *ptch1* gene are detected at low frequency in sporadic SCCs.[42] Additionally, studies in human keratinocytes suggest Shh, in the absence of *gli1* induction, can oppose cell cycle arrest,[43] and restoration of *ptch1* function in an SCC cell lines can suppress cell growth in vitro.[44] These data suggest that Shh signaling may contribute to differential cell growth during tumor formation.

While loss of Shh target gene induction has little effect on the development of epithelial progenitors, ectopic expresssion can have profound effects. Inappropriate activation of Shh target genes causes formation of hair follicle-derived tumors, of which the most clinically significant are BCCs. Beside BCC, a variety of other hair follicle and sebaceous tumors[30,45] have been shown to derive from Shh pathway activation. Many of these tumors have been shown to be associated with Shh target gene induction at interfollicular locations. In transgenic animals overexpressing activating components of the Shh pathway in both the outer root sheath and

interfollicular epidermis, epithelial buds come from the interfollicular epithelium.[30,45] Similar findings are seen in UV irradiated *ptch1*/+ mice, with trichoblastomas and BCC-like lesions arise from epidermis not adjacent to a hair (Fig. 3b).[42]

By contrast, the effects of increased Shh signaling in the context of the developing hair follicle appear to have quite different effects. Shh expressed from the bovine keratin 6 promoter that directs Shh expression to the inner root sheath failed to induce a distinct hair phenotype (A. Oro, unpublished). Furthermore, forced induction of Shh via transgenic mice expressing noggin in the hair is associated with abnormal differentiation, but lacks hair follicle tumors even in older mice.[46] While additional experiments using hair-specific promoters may discover particular conditions that would suggest otherwise, the available data point to a restriction of the Shh effects on progenitor proliferation when it occurs within the context of the hair follicle niche.

Regulation of Shh Induction in a Multipotent Epithelium

The ability of ectopic Shh target gene induction to redirect cell fates in cells outside the hair follicle underscores the critical need for spatial and temporal restriction of Shh target gene induction for controlled hair follicle growth and the prevention of follicular tumorigenesis. How then does the adult hair normally regulate Shh target gene induction in progenitor cells? The hair follicle niche, created by inductive signals during embryogenesis, continues to control the spatial and temporal expression of Shh in the adult. Key regulators of the developing niche include the FGFs, which promote Shh expression in the hair placode and the bone morphogenetic proteins (BMPs), which antagonize Shh expression.[47-49] As in embryonic hair development, the domain of epithelial Shh expression in the postnatal hair appears to be limited by BMP activity in the differentiating hair progenitor cells. Noggin, an inhibitor of BMPs, is secreted from the underlying DP cells to antagonize BMP activity, allowing Shh expression to promote hair follicle growth. Loss of noggin function results in increased BMP activity and the decreased number and size of hair follicles,[50] while application of noggin-soaked beads can induce anagen and Shh expression.[51] Noggin overexpression in the hair matrix cells, using the Msx2 promoter, results in a broadening of Shh expression which is associated with an increased domain of proliferating hair follicle progenitor cells and decreased markers of differentiation.[46]

Recent results suggest that along with noggin to repress BMP, the induction of Wnt target genes is also required for Shh induction in the embryonic hair follicle niche.[52] Canonical Wnt signaling requires the presence of a member of the TCF family of transcription factors and the persistence of its cofactor β-catenin. In the developing hair follicle niche, noggin expressed from the mesenchyme induces the expression of LEF-1, a founding member of the TCF transcription factor family. In turn Wnt expression stabilizes β-catenin expression allowing Wnt target gene induction to occur. With such signaling that includes the decreased expression of the adhesion molecule E-cadherin, the hair niche is formed and Shh expression occurs. Among the evidence that this occurs upstream of Shh expression is that E-cadherin repression occurs prior to Shh expression and occurs even in Shh mutants.

Regulation of Shh Signal Reception

In addition to spatial and temporal regulation of Shh production in the niche, controlling Shh signal reception also plays an important role. Since many epidermal progenitor cells are capable of responding to Shh signaling, proper restriction of target gene induction acts to limit hair progenitor growth in a multipotent sheet of cells. The first example of such restriction was demonstrated in the developing Xenopus epithelium.[53] Ectopic floor plate differentiation, a hallmark of Shh effects on the early nervous system, was not detected in response to injected Shh in the neural plate stage. With closure of the neural tube came floor plate differentiation, but it was still restricted predominantly to the dorsal region of the neural tube.

Additional studies in chick and mouse confirm these original studies. Injection of Shh-expressing retroviruses into developing feather bud epithelium reveal a select developmental

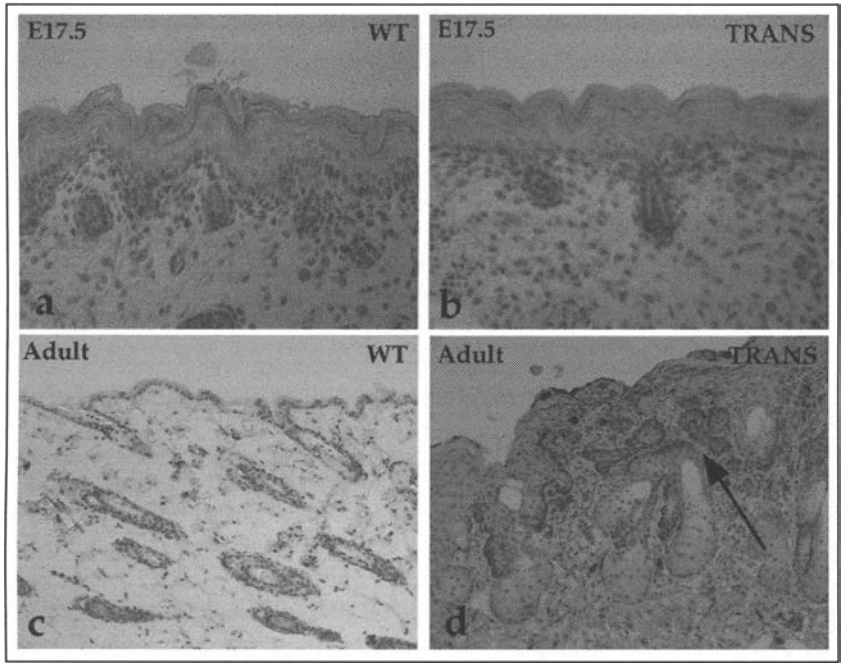

Figure 5. Post-transcriptional regulation of gli1 during the hair cycle. a, b) Keratin-14-Gli1 animals (TRANS) appears identical with wild-type (WT) littermates until anagen. With the phenotype comes accumulation of Gli1 immunoreactivity, suggesting a post-transcriptional regulation during anagen. Reprinted with permission from ref. 30.

window where target gene and follicular bud formation is possible.[54] In *ptch* -/+ mice, small ectopic tumor buds form in interfollicular epithelium as measured by X-gal staining coming from the *ptch1*-lacZ mice.[30,55] Interestingly, the buds are only detected during anagen and not other stages of the hair cycle, suggesting that the epithelium is restricted to target gene induction during the stage when Shh signaling normally occurs.

Additional experiments point to the regulation of the *gli* transcription factor as part of the basis for the signaling restriction. Transgenic *gli1*[30,56] and *gli2*[57] animals are generally indistinguishable from their wild-type littermates at birth, a striking difference from those overexpressing Shh in the skin, which induces drastic morphological changes at the time it is expressed.[58] Like the *ptch1*/+ mice, the Keratin14-*Gli1* mice induced target genes and developed epithelial proliferation in interfollicular epithelium with the onset of anagen, supporting an anagen signal that activates *gli1*. The activation is posttranscriptional in that *gli1* RNA is present, but Gli protein is only detected during anagen (Fig. 5).[30] How protein accumulation is regulated over the hair cycle is unknown, but is likely to occur at many levels. *gli1* translation has been shown to be regulated through alternative 5' UTR splicing.[59] Moreover, stability of Gli family members have been linked to proteosome-dependent degradation via phosphorylation by protein kinase A, casein Kinase and glycogen synthase kinase β.[60-62] A combination of alternative splicing and phosphorylation may contribute to reception of the Shh signal.

An open question is the nature of the signal that regulates Gli accumulation. One possibility is that Shh itself, which is present at anagen, functions to regulate Gli. Shh is known to allow preformed Gli to become nuclear and could also be responsible for Gli accumulation. This hypothesis is appealing in that ectopic Shh can regulate the timing of anagen. Injection of Shh-expressing adenoviruses into mouse skin prematurely triggers the onset of anagen,[63] and thus can influence hair cycle staging. However, Shh is expressed in the invaginating matrix cells

a great distance from the interfollicular epidermis (Fig. 2b) making it less likely to be able to influence the epidermal cells directly. Shh involvement would have to be part of an indirect or long range signaling mechanism.

Maintenance of Shh Signaling during Tissue Morphogenesis

In order to precisely pattern developing tissues, morphogens must induce Shh ligand production and the presence of pathway members in receiving cells. However, additional regulators are required to both maintain Shh signaling once started and terminate it appropriately. In the developing limb, Shh expression and limb outgrowth is maintained via a positive feedback loop with FGF4 that is mediated by the BMP antagonist gremlin.[64,65] Shh induces gremlin in the adjacent mesenchyme which relieves BMP-mediated repression of FGF in the overlying ectoderm. In the hair follicle, little is known about what maintains Shh signaling. The juxtaposition and functional relationship between Shh and the dermal papilla-derived BMP antagonist noggin (Fig. 2a) would suggest a similar positive feedback loop. Indeed, ectopic noggin expression in the hair matrix induces Shh levels[46] and the presence of Shh target gene induction in noggin-expressing cells supports this hypothesis. However, expression of the activator form of Gli2 in the epithelium rescues the skin phenotype of a Gli2 mutant, suggesting that Shh signal reception is not necessary in the dermal papilla for hair morphogenesis.[66] This argues that any Shh regulation of dermal papilla function must occur indirectly through additional, as yet unidentified factors. Candidate proteins include the Wnt family, which are known to be targets of Shh in the hair[67,68] and function to maintain dermal papilla competence.[69]

Along with factors that maintain signaling, factors that precisely shut off signaling are important in tissue morphogenesis. In the hair follicle, proteins that regulate the anagen-to-catagen transition fit into this class. Perhaps the best studied example is Fibroblast growth factor 5 (FGF5), a protein expressed in the outer root sheath of growing hairs.[70] Mutations in FGF5 significantly extend the period of anagen and fail to shut off Shh expression. Homozyous angora mice lack FGF5 protein and have extremely long hair shafts, indicative of continued anagen. Interestingly, while Shh expression persists, angora mice do not get hair follicle tumors or BCCs, indicating that other factors are still present in the hair matrix to regulate the outcome of Shh signaling.

Additional evidence comes from studies of plakoglobin's effects on hair cycling. Plakoglobin is a β catenin-like protein that also binds to members of the TCF transcription factor family. In contrast to β-catenin, which facilitates Shh induction, overexpression of plakoglobin represses epithelial growth, proliferation, and results in an early transition to catagen with the resultant shortened hair follicles.[71] Given the known functions of β-catenin in regulating the hair cycle, it is tempting to speculate that plakoglobin acts through competition with β-catenin, but the data is also consistent with β-catenin independent mechanisms.[72]

Future Perspectives

The precise delivery and reception of morphogenic signals such as those from Shh underlies the ability for the body to regenerate tissues as part of normal homeostasis or during wound repair. Summation of signals that regulate the initiation, reception and maintenance of these growth factors allow precise regeneration each time.

This chapter attempts to separate different aspects of signaling to illustrate the importance of each one during morphogenesis. However, in reality a single factor is likely to contribute to one or more process. For example, Shh ligand expression is clearly dependent on Wnt signaling during early anagen, but β-catenin and members of the TCF transcription factors are also present in the receiving cells. Xgal staining using a Wnt target gene β-galactosidase readout indicates that Wnt signaling is also occurring, suggesting that Wnt may be regulating aspects of Shh reception and signal maintenance.[73] Because current genetic techniques in mice or chick lack single cell resolution, the ability to remove or add gene function in donor or receiving cells at different times during hair follicle morphogenesis must be developed. Recent advances using

estrogen receptor-regulated cre recombinase[74] could allow such temporal regulation and the identification of stage-specific promoters could provide means to address the issue.

The data summarized in this chapter point out the continued need to study the differences in outcomes of cells exposed to powerful morphogenic signals like Shh both inside and outside the hair follicle niche. The present data suggests that many of the cells in the interfollicular epidermis are capable of responding to the signals and that induction of Shh target genes in cells outside the hair follicle matrix where they normally exist can lead to ectopic hair follicle progenitor proliferation and follicular tumors. In particular, understanding the mechanisms of restricting Shh reception may be useful both for patterning and for the development of anti-tumor therapies. This will be a particularly exciting area of research given the growing number of tumors in which the Shh signal pathway has been implicated.

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CHAPTER 5

Mode of *PTCH1/Ptch1*-Associated Tumor Formation:
Insights from Mutant *Ptch1* Mice

Heidi Hahn*

Abstract

Patched1 (*PTCH1/Ptch1*) is a member of the *SHH/Shh* signaling pathway, where it serves as a receptor for SHH/Shh. Inactivating mutations in the *PTCH1/Ptch1* gene result in a pathological activation of the pathway, which may lead to several forms of familial and sporadic cancers. Although *PTCH1/Ptch1* is generally supposed to be a tumor suppressor gene, mutational inactivation of one allele seems to be sufficient for tumor formation. In this chapter the current knowledge about the mode of tumor formation caused by *PTCH1/Ptch1* mutations is presented. Most of the insights in these processes are derived from *Ptch1* mutant mice. The impact of the genetic background on tumor susceptibility, the involvement of additional molecular pathways and epigenetic mechanisms leading to tumor formation, are discussed.

Introduction

PTCH1/Ptch1 is a key modulator of signaling in the Sonic hedgehog (SHH/Shh) pathway. It is predicted to contain 12 transmembrane-spanning domains and two large extracellular loops.[1] Within the plasma membrane PTCH1/Ptch1 forms a receptor complex with its signaling partner Smoothened (SMO/Smo), which transduces SHH/Shh signaling[2] (Fig. 1). The signaling pathway controls cell differentiation and proliferation and plays a prominent role in patterning of several organs during embryogenesis. The physiological activation is induced by SHH/Shh. Binding of SHH/Shh to PTCH1/Ptch1 suspends the inhibition of SMO/Smo, which leads to signal transduction resulting in induction of target genes such as *GLI1/Gli1* and *PTCH1/Ptch1* itself. Pathologically, the pathway can be switched on by activating mutations in *SHH/Shh* or *SMO/Smo* or by mutational inactivation of *PTCH1/Ptch1*[2] (Figs. 1 and 2).

In humans, inherited mutations in *PTCH1* result in the nevoid basal cell carcinoma syndrome (NBCCS), which is an autosomal dominant disorder characterized by a combination of developmental defects with a predisposition to tumor formation.[3,4] The clinical findings in NBCCS include a generalized overgrowth of the body, closure defects of the neural tube, and skeletal abnormalities such as bifid or missing ribs and polydactyly.[5] The most frequent tumor in NBCCS is basalioma (BCC), which is usually located in sun-exposed skin areas and may be very pleioformic (Fig. 3). Less frequent, but clinically more problematic are medulloblastoma (MB), which develop in approximately 5% of the patients, and other tumors, including meningeoma, fibroma, and rhabdomyosarcoma (RMS).[6]

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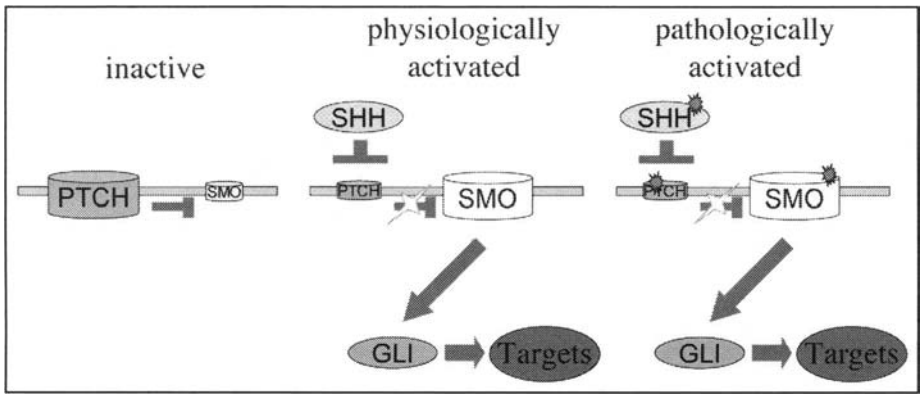

Figure 1. SHH/Shh signaling pathway. Left: in the absence of SHH/Shh, PTCH1/Ptch1 blocks the activity of SMO/Smo. Middle: during embryogenesis activation of the pathway can be triggered by binding of SHH/Shh to PTCH1/Ptch1. Right: activation of the pathway by activating mutations in SHH/Shh or SMO/Smo or by loss of function PTCH1/Ptch1 mutations (mutations are indicated as stars). Mutations in PTCH1/Ptch1 are the most frequent cause of tumorigenesis.

The two available strains of mice heterozygous for *Ptch1*[7,8] have proved a valuable tool to investigate the role of *Ptch1* in the control of normal cell proliferation and in neoplastic transformation. In the *Ptch1*$^{neo12/+}$ strain,[7] exons 1 and 2 of *Ptch1* have been replaced by the LacZ gene, whereas in *Ptch1*$^{neo67/+}$ mice exon 6 and 7 are missing.[8] As in human tumors associated with *PTCH1* mutations, mutational inactivation of *Ptch1* results in a pathological activation of its signaling pathway with consecutive expression of high levels of *Gli1* and *Ptch1* mRNA[7-9] (Fig. 2). Tumors that arise in these animals are the same as in patients with NBCCS and include MB and RMS (Fig. 4). Although BCC do not develop spontaneously in these mice, they can be induced by irradiation.[10,11]

Since the initial discovery of inherited *PTCH1/Ptch1* mutations, the role of this gene in the pathogenesis of a range of sporadic tumors has been the subject of intensive studies. Mutations in *PTCH1* have been detected in sporadic BCC, which is the most common sporadic tumor in humans with more than 600 000 cases per year alone in the United States.[12] BCC are semimalignant and they rarely metastasize, but they can lead to extensive morbidity due to local tissue destruction. The majority of BCC, both sporadic and hereditary, have been linked to the *PTCH1* locus[13] and biallelic inactivation of *PTCH1* has been detected in a subset of these tumors.[14] Inactivating mutations in *PTCH1* have also been found in sporadic MB, which is a very malignant primitive neuroectodermal tumor of the cerebellum and accounts for one-quarter of all intracranial tumors in children.[15,16] The list of sporadic tumors with mutations in *PTCH1* today includes also trichoepitheliomas, esophageal carcinoma, bladder cancer and other cancers, although *PTCH1* mutations account for only a fraction of these tumors.[17]

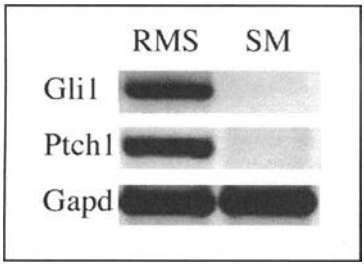

Figure 2. Activating mutations in *SHH/Shh* or *SMO/Smo* or mutational inactivation of *PTCH1/Ptch1* result in increased levels of *GLI1/Gli1* and *PTCH1/Ptch1* mRNA in tumor tissue. RT-PCR shown was performed on RNA isolated from murine normal skeletal muscle (SM) and rhabdomyosarcoma (RMS).

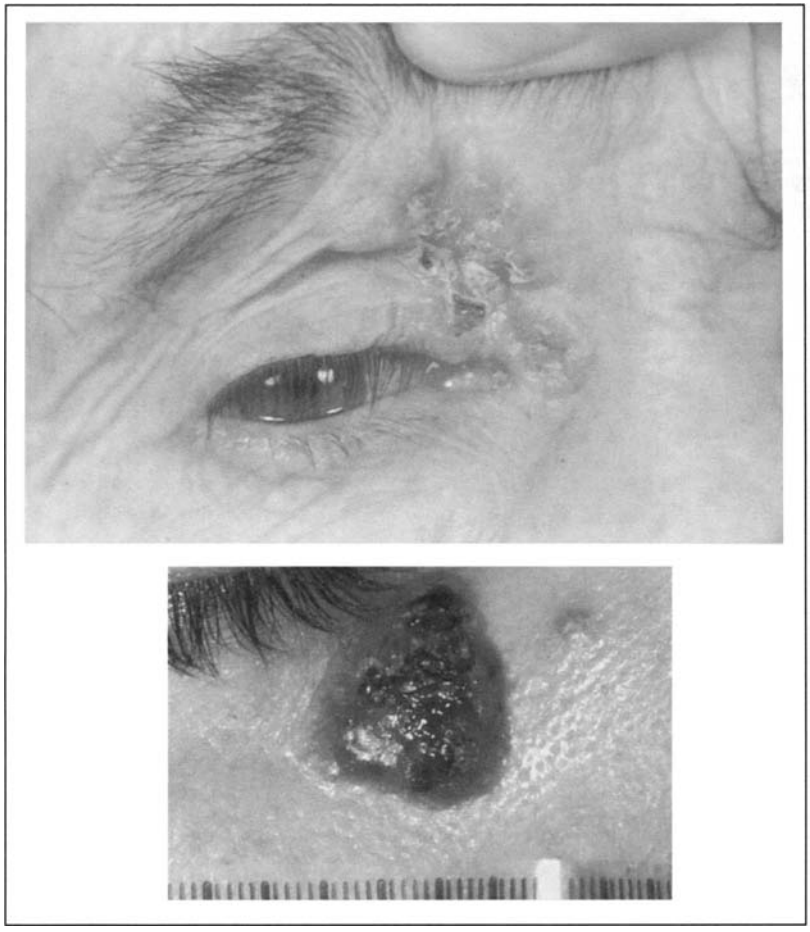

Figure 3. Basal cell carcinomas of the eye lid.

Tumor formation can be also caused by mutations in other members of the SHH signalling pathway including *SHH* and *SMO*, or by a deregulation of the signaling cascade due to other mechanisms. Thus activating *SMO* mutations have been detected in up to 20% of sporadic BCC[18] and recently, *SHH* mutations have been reported in BCC from patients with Xeroderma pigmentosum.[19] Abnormal expression of SHH seems to be involved in the formation of tumors such as pancreatic cancer, digestive tract tumors and in a subset of small-cell lung cancers.[20-22] The focus of this chapter is on tumorigenesis associated with *PTCH1/Ptch1* mutations and on the genetic modifiers of this process.

Is Loss of Both *PTCH1/Ptch1* Alleles Required for Tumor Formation?

PTCH1 is widely assumed to be a tumor suppressor gene.[23] Tumor suppressor genes normally exert a negative control on cell growth and the accepted paradigm is that inactivation of both alleles is required for tumor formation.[24] For BCC, it has been proposed that mutations in *PTCH1* are necessary for passing the genetic "threshold" of the neoplastic process and that PTCH1 plays a gatekeeper function in BCC development.[23] Following inactivation of both

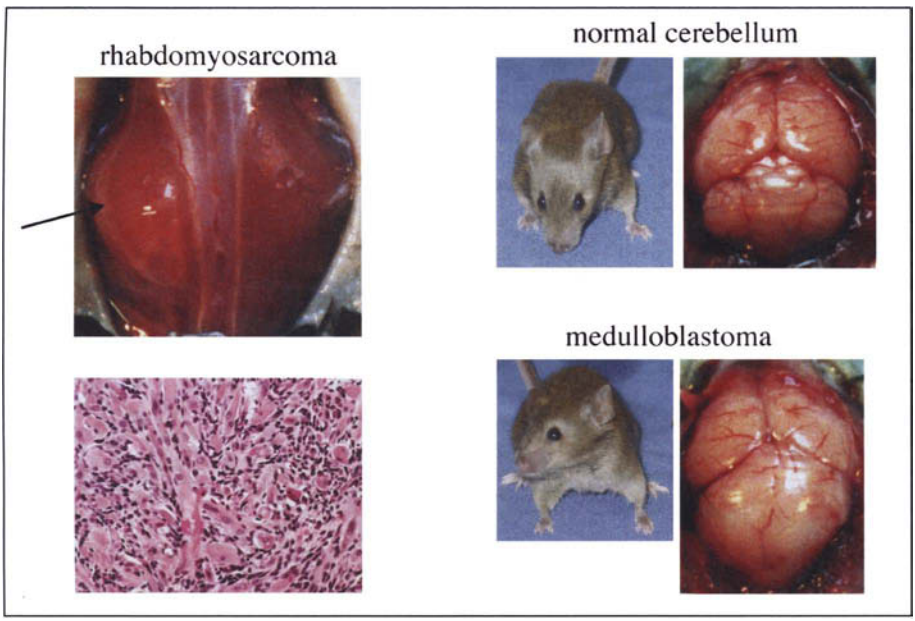

Figure 4. Tumors in *Ptch1*$^{neo67/+}$ mice. Left top: macroscopic view of a rhabdomyosarcoma of the back/hindleg (arrow indicates the tumor); left bottom: microscopic (H&E staining) view of the same tumor. Right top: healthy mouse and its normal brain; right bottom: characteristic bearing of the head of an animal with medulloblastoma; gross appearance of the tumor.

alleles in one cell, clonal expansion occurs, accompanied by the accumulation of further, genetic events, which likely include mutations in genes such as *TP53* or *RAS*.[25]

However, only 60% of sporadic BCC show LOH at the *PTCH1* locus,[26] for half of which loss of both *PTCH1* alleles has been demonstrated.[14]The same applies to MB, where 12% tumors show LOH at the *PTCH1* locus but only 7% have additional inactivating mutations in the coding region of *PTCH1*.[27-30] Thus, not all tumors with LOH at the *PTCH1* locus show mutations in the remaining wild type allele, although it should be noted that most screens hitherto have covered only the coding region of *PTCH1*, leaving out possible *PTCH1* mutations in the regulatory elements of the *PTCH1* gene.

Similar observations have been made in tumors found in *Ptch1*$^{neo12/+}$ or *Ptch1*$^{neo67/+}$ heterozygous mice. As in many human *PTCH1*-associated tumors, the wild type *Ptch1* allele is retained in MB and RMS of these mice. Also in nodular BCC, which can be induced in *Ptch1*$^{neo67/+}$ mice by ionizing radiation, retention of the normal *Ptch1* allele is detected in all tumors examined.[11] Only the infiltrative BCC in these mice show loss of wild type alleles. Similarly to most *PTCH1*-associated tumors in humans, all tumors of *Ptch1* mutant mice overexpress *Gli1* and *Ptch1* transcripts.[7,8]

On the other hand, loss of one *PTCH1* allele is not automatically sufficient for tumorigenic transformation of every cell accompanied by overexpression of *GLI1* and *PTCH1* in patients with NBCCS (i.e., *PTCH1* heterozygous).[31] What is the explanation for this discrepancy?

The answer may be emerging from recent *Ptch1* analyses of tumors in *Ptch1* heterozygous mice. The mechanism of overexpression of *GLI1/Gli1* and *PTCH1/Ptch1* transcripts in tumors with *both PTCH1/Ptch1* alleles inactivated is well established. *PTCH1/Ptch1* regulates itself via a negative feedback mechanism. Loss of PTCH1/Ptch1 function results in a transcriptional activation of *GLI1/Gli1*, protein product of which binds to GLI1/Gli1 binding sites in the *PTCH1/Ptch1* promoter. This results in a transcriptional activation of the *PTCH1/Ptch1* gene and an overexpression of *PTCH1/Ptch1* transcripts[32,33] (Fig. 2).

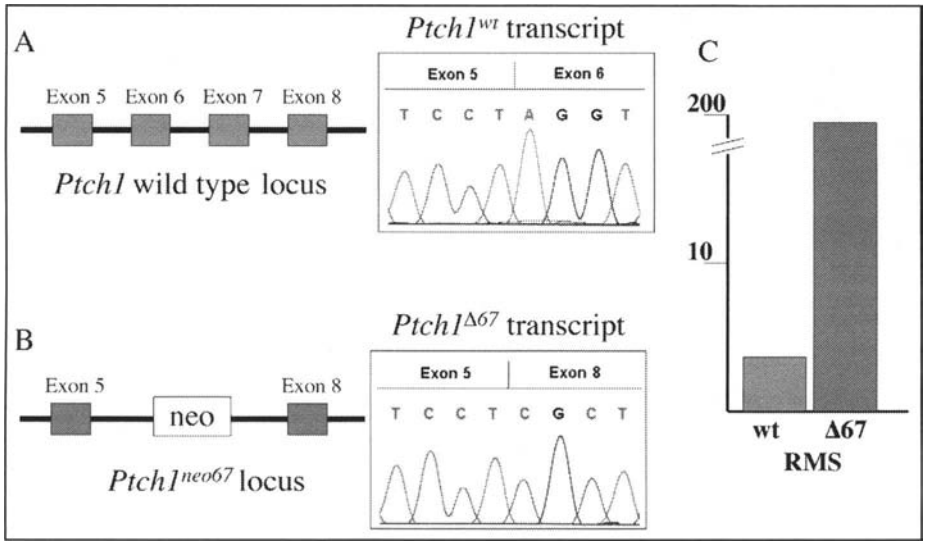

Figure 5. (A Wild type and B) mutant *Ptch1* locus of heterozygous *Ptch1*$^{neo67/+}$ knock-out mice. The neo-cassette of the targeted *Ptch1*^{neo67} allele is excised and exon 5 is spliced together in frame with exon 8 of the murine *Ptch1* gene. The resulting *Ptch1*$^{\Delta 67}$ transcript is highly overexpressed in RMS of *Ptch1*$^{neo67/+}$ mice as demonstrated in C).

In RMS of *Ptch1*$^{neo67/+}$ mice the observed overexpression of *Ptch1* is restricted to *Ptch1*$^{\Delta 67}$-transcripts, i.e to transcripts derived from the allele mutated by homologous recombination.[34] In these transcripts, the neo cassette is spliced out and exon 5 is spliced into exon 8 (Fig. 5). The corresponding data from tumors derived from *Ptch1*$^{neo12/+}$ mice, in which exons 1 and 2 have been replaced by a lacZneo cassette,[7] are more equivocal. One report showed no expression of wild type *Ptch1* in cell lines derived from medulloblastoma of these mice.[35] In contrast, earlier reports about the same tumors showed Northern blot analysis and in situ hybridization which indicated that wild type *Ptch1* transcripts are expressed in medulloblastoma.[36,37] One way to explain this discrepancy is that Northern and in situ hybridization techniques are semi-quantitative and a repression of *Ptch1* wild-type transcripts in these tumors in comparison to normal cerebellum may have been overlooked. The existence of similar asymmetry of allelic usage in human tumors has not been investigated.

Why is the overexpression of *Ptch1* restricted to only one *Ptch1* allele? One possible explanation is a selective epigenetic block of the wild type *Ptch1* allele in tumor tissue, a mechanism that has been described in other tumor suppressor genes.[38] Examples include the genes *VHL*, *MLH1* or *p16*^{INK4A} and the predominant underlying mechanism appears to be methylation-triggered transcriptional suppression.[39] Similar mechanism could result in a complete loss or in a repression of the wild type *Ptch1* allele. In support of a methylation involvement, Berman and colleagues reported that treatment with the DNA demethylating agent 5-azacytidine (5-azaC) increased wild type *Ptch1* mRNA expression in cell lines derived from medulloblastoma in murine *Ptch1*$^{neo12/+}$ heterozygotes.[35] The location of the responsible regulatory elements in the *PTCH1/Ptch1* gene is at present unknown, as is the mechanism restricting the silencing to the wild-type allele. In any case, in consequence of transcriptional repression, the levels of functional Ptch1 protein most likely drop below a distinct threshold, finally resulting in tumor formation. These recent observations suggest the therapeutically exciting possibility of restoring *PTCH1* expression by demethylating agents.

In conclusion, mutational inactivation of both *PTCH1/Ptch* alleles apparently is not required for tumor formation, although it occurs in a subset of human and murine tumors. In

BCC induced by irradiation of *Ptch1*$^{neo67/+}$ mice loss of both alleles rather seems to be characteristic for later stages of the tumor.[11] Repression of the remaining wild type allele, possibly by methylation, may be involved in the formation of RMS and MB in *Ptch1*$^{neo67/+}$ mice. Both mechanisms—mutational inactivation of both alleles or mutation of one *PTCH1/Ptch1* allele and repression of the remaining wild type *PTCH1/Ptch1* allele—are expected to have the same effect, i.e., a deregulation of the SHH/Shh signaling pathway.

Alternatively, haploinsufficiency in *PTCH1/Ptch1* may be sufficient to trigger tumor formation in combination with mutations in other genes of the SHH/Shh pathway and other pathways, although experimental evidence is lacking. There is, however, increasing knowledge that inter-pathway interactions modify the penetrance and expressivity of tumors associated with *PTCH1/Ptch1* mutations.

What Are the Modifiers of *PTCH1/Ptch1*-Associated Tumorigenesis?

Evidence from Mouse-Strain Comparisons

Although inherited inactivating mutations in *PTCH1* are highly penetrant,[5] their expressivity is highly variable.[40] Thus, patients with the same molecular lesion can exhibit very different symptoms, suggesting either a strong impact of environmental factors or the existence of genetic modifiers.

The detection of cancer susceptibility modifiers in humans is a difficult and challenging task,[41,42] and these modifiers remain unknown for *PTCH1*-associated tumors. In contrast, there is emerging evidence for genetic modifiers of tumorigenesis in *Ptch1* mutant mice. Both the incidence and type of tumors in *Ptch1* mutants vary considerably with their genetic background. The C57BL/6 (B6) strain is susceptible to MB, which develop in 45% of *Ptch1*$^{neo67/+}$ mice, but is completely resistant to development of RMS. In contrast, RMS develop in more than 50% of *Ptch1*$^{neo67/+}$ A/J x B6 or Balb/c x B6 F1 mice. MB is less frequent in these crosses than on the B6 background (21% and 8%, respectively; unpublished data). This implies the existence of either dominant A/J or Balb/c alleles that confer MB resistance and RMS susceptibility, or recessive B6 alleles that confer MB susceptibility and RMS resistance. In addition, initiation as well as progression of BCC in *Ptch1*$^{neo67/+}$ mutant mice is influenced by genetic background. Crosses between *Ptch1*$^{neo67/+}$ mice and Car-S (F1S) or Car-R mice (F1R), which are susceptible and resistant to skin carcinogenesis, respectively, showed that F1S*Ptch1*$^{neo67/+}$ mice were highly susceptible to IR-induced BCC, whereas F1R*Ptch1*$^{neo67/+}$ mice were completely resistant. The development of microscopic and macroscopic BCC lesions was also influenced by Car-S and Car-R genotypes, indicating that tumor penetrance as well as initiation and progression of BCC can be modulated by genetic background (Pazzaglia et al, Cancer Research, *in press*).

These results raise the prospect of mapping the genomic loci that are responsible for the tumor susceptibility or tumor resistance. That can be done by QTL (quantitative trait loci) analysis either by intercrossing the F1 mice or by backcrossing them to one of the parental strains. The mapping of the loci is achieved by typing of strain-specific microsatellite markers, which are available for the strains mentioned above.

Identification of these modifying genes is relevant to humans, in whom BCC is the commonest malignancy, and MB and RMS are the most common brain tumor and soft tissue sarcoma in children, respectively. Especially for sporadic BCC, a genetic predisposition has been demonstrated in the general population and efforts to identify the susceptibility loci are in progress.[43,44] As mentioned above, the identification of tumor-modifying loci in humans is difficult. The hope is that QTL studies in *Ptch1* mutant mice will help to identify genes modifying *PTCH1/Ptch1*-associated tumor formation.

Evidence from Gene Expression and from Biochemical Studies

Besides QTL, other approaches can be used to identify interacting signaling molecules that might contribute to the malignant transformation of a heterozygous *PTCH1/Ptch1*+/- cell. Several labs have performed gene expression analyses of *PTCH1/Ptch1* associated tumors or of cells overexpressing *GLI1/Gli1* or treated with SHH/Shh. It has been shown that the expression of the proto-oncogene *Nmyc* can be stimulated by Shh in cerebellar granule neuron precursors and that Nmyc is required for the full Shh proliferative response of these cells.[45] Nmyc is involved in cell cycle progression and is implicated in repression of genes that promote cell cycle exit. Interestingly, Nmyc is expressed at high levels in MB.[46]

It also has been demonstrated that the expression of *PDGFRα* is activated by Gli1.[47] The expression of PDGFRα is accompanied by the activation of the Ras/Raf/Mapk pathway, which regulates cell proliferation. The induction of PDGFRα by GLI1/Gli1 is likely to occur in vivo, since PDGFRα is overexpressed in human and murine BCC.[47]

Furthermore, *PTCH1* binds to, and sequesters Cyclin B1 and thus inhibits cell cycle progression. It has been speculated that some *PTCH1/Ptch1* mutations increases cell proliferation by releasing Cyclin B.[48]

Shh also promotes the transcription of *Cyclin E* and *Cyclin D*, two inhibitors of Rb and principal regulators of the cell cycle.[49,50] The upregulation of Cyclin E expression was accomplished through binding of Gli1 to the *Cyclin E* promoter, which mediated the ability of Shh to induce DNA replication. Upregulation of cyclin D expression by Shh may be responsible for the distinct ability of Shh to promote cellular growth. Altogether, it is likely that tumor formation is contributed to by a proliferative advantage resulting from the induction of Nmyc, Cyclin B, Cyclin D, Cyclin E and/or PDGFRα expression.

Gene expression analyses also revealed an interaction between the tumor-promoting Igf2 and Shh signaling.[51]Tumors in *Ptch1* heterozygotes consistently overexpress Igf2. In addition, $Ptch1^{neo67/+}$ mice deficient for *Igf2* did not develop MB and RMS, which demonstrates that *Igf2* is indispensable for tumor formation. Since the overexpression of *Igf2* in tumors of $Ptch1^{neo67/+}$ mice seemed to be independent from genetic or epigenetic changes, it has been proposed that *Igf2* is a direct target of Shh signaling pathway.

The following observations suggest that an important function of Igf2 in RMS formation in $Ptch1^{neo67/+}$ mice is to activate the PI3K/Akt/PKB signaling pathway (Fig. 6): *Igf2* usually activates receptor tyrosine kinases such as the Igf-1 receptor or the insulin receptor.[52] Activation of these receptors both increases mitogenesis and inhibits apoptosis, which is attributed primarily to the activation of the Ras/Raf/Mapk pathway and of the PI3K/Akt/PKB kinase pathway, respectively.[53] However, the relative importance and contribution of each pathway to tumor growth is unknown. The phosphorylation status of the growth-stimulating Mapk pathway was unchanged in tumors in *Ptch1* heterozygous mice, whereas the cell-survival-promoting PI3K/Akt/PKB signaling pathway was highly activated.[54] Furthermore the cell cycle inhibitor $p27^{kip1}$ and the growth inhibitor Gadd45α were upregulated in these tumors. In agreement with this result, a low proliferation rate of RMS cells was demonstrated using the cell proliferation marker Ki67. In addition, the anti-apoptotic Bcl-2 protein was overexpressed in tumor cells whereas the expression of the pro-apoptotic Bax was low. These results show that besides induction of proliferative signals (see above) deregulation of the SHH/Shh pathway also seems to induce anti-apoptotic signals. In agreement with these observations, *Bcl-2* and *Gadd45α* have been recently shown to be direct targets of Gli1[55](Kappler et al, *Int. J. of Oncology*, in press). A model is conceivable in which elevated levels of Gli1 increase the expression of the anti-apoptotic Bcl-2, of the growth inhibitor Gadd45α and of the tumor-promoting factor Igf2. That, together with the activation of the anti-apoptotic PI3-kinase signaling pathway, may decrease apoptosis and enhance tumor growth (Fig. 6).

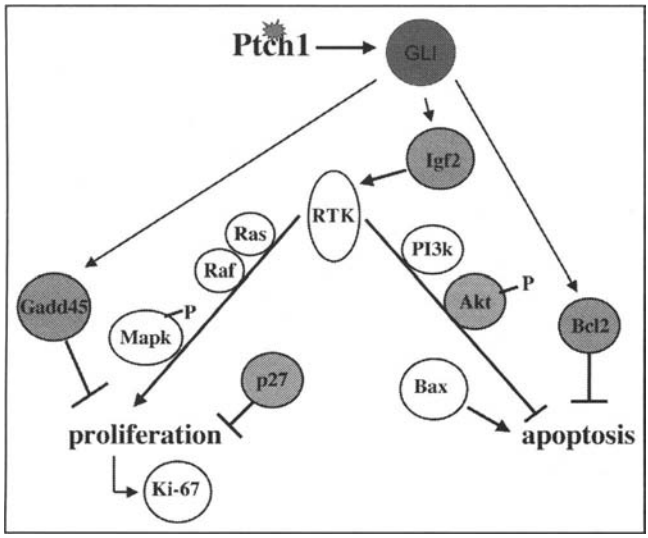

Figure 6. Delineation of the molecular events in RMS of *Ptch1*$^{neo67/+}$ mice. Mutation in *Ptch1* is indicated by a star. Our data indicate that anti-apoptotic signals predominate and that proliferative signals in these tumors are only weak.

PTCH1/Ptch1 also induces apoptotic cell death via increased caspase activity unless its ligand SHH/Shh is present to block the signal.[56] Therefore PTCH1/Ptch1 may be a pro-apoptotic dependence receptor. Dependence receptors transduce two different intracellular signals: in the presence of a ligand, they transduce a positive signal leading to cell survival, differentiation or migration. In the absence of a ligand they initiate or amplify signals for programmed cell death.[57] The finding that PTCH1/Ptch1 is a dependence receptor may have important implications in the understanding of *PTCH1/Ptch1*-associated tumor formation. It is possible that null mutations in both or one *PTCH1/Ptch1*-allele may lead to the absence of caspase-mediated cell death. This may allow certain cells to survive and initiate tumor formation.

Conclusion

Aberrant SHH/Shh signaling initiated by inactivating *PTCH1/Ptch1* mutations contributes to the neoplastic transformation of cells arising from different cell types and tissues. Mice with inactivated *Ptch1* alleles provide an excellent system to study the genetic pathways that determine the malignant transformation. Tumors arising in heterozygous *PTCH1/Ptch1* individuals probably result from multiple mechanisms. A first possibility to consider is a *PTCH1/Ptch1* first hit mutation followed by a point mutation or deletion of the other *PTCH1/Ptch1* allele.

A second possibility is that one *PTCH1/Ptch1* allele is mutated or lost, whereas the inactivation of the remaining normal allele is brought about by epigenetic silencing by a mechanism such as methylation. If true, this latter possibility would have a huge impact on therapies of both inherited and sporadic *PTCH1*-associated human tumors, since epigenetic gene silencing is potentially reversible.

It is also conceivable that haploinsufficiency in *PTCH1/Ptch1* is sufficient to initiate the process of transformation. In this case, the tumorigenesis may be triggered by genetic changes in other members of the SHH/Shh pathway and/or pathways such as the PI3-kinase, the Ras/Raf/Mapk, or the Igf2 signaling pathway. The search for these alterations is subject of many

ongoing studies. The recent observations that *PTCH1/Ptch1*-associated tumorigenesis is linked to a blockade of cell death points way to new therapeutic approaches against *PTCH1*-associated tumors.

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CHAPTER 6

Basal Cell Carcinomas, Hedgehog Signaling, and the Ptch1+/- Mouse

Ervin Epstein, Jr.*

Abstract

Progress in understanding the molecular underpinnings of basal cell carcinomas (BCCs) was hindered severely until recently by the difficulty of growing human BCCs in vitro or in nude mice and by the lack of a mouse model for their development. With the identification of PTCH1 mutations as the basis of the basal cell nevus syndrome and their identification in sporadic BCCs,[1a] it became possible to produce several mouse models in which BCCs develop either spontaneously or after exposure of the mouse to environmental insults that cause human BCCs. In particular, Ptch1+/- mice are quite useful for study since they develop BCCs in the same site and with the same molecular defects and following the same environmental insults as in man. The first burst of studies have identified effects of genetic background, immune status, and environmental insults. In addition, these mice appear to provide a very promising resource for preclinical testing of the effects of anti-BCC chemopreventive and chemotherapeutic agents.

Progress in understanding basal cell carcinomas (BCCs) has long been hampered by technical difficulties—fundamentally no system has been devised to allow robust growth of human BCCs in vitro or in nude mice, and mice subjected to carcinogenic insults—ultraviolet or ionizing radiation, chemical carcinogens, or engineered activation/inhibition of candidate genes/pathways—uniformly have developed papillomas and carcinomas of the squamous rather than basal cell lineage. Of note, however, older reports indicate that ionizing radiation can produce BCCs in the skin of rats,[1] and the underlying mechanisms contributing to these differences in susceptibility among rodents remain unknown.

To be more specific, several papers have reported methodologies for establishing cultured human BCCs. Techniques proposed have touted the benefits of focusing on poorly-adherent cells—replating the tumor cells that fail initially to adhere to tissue culture plastic[2]—and of eliminating "contaminating" normal keratinocytes by raising the media calcium concentration temporarily to a level that induces differentiation of the normal keratinocytes but to which the BCC cells are resistant.[3] Other authors have used explant culture techniques, e.g., on feeder layers.[4] Unfortunately, the published approaches generally have not been replicated by others, have not produced immortalized cell lines, and have not become standard methodologies. One exception is the report of the establishment of a long-term in vitro cell line from a BCC[5] but at least some stocks of these cells have been contaminated with HeLa cells.[6] In addition, none of these (older, pre1996) reports of the establishment of BCC cultures have been characterized for

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what would seem now to be for BCCs the "gold standard" characteristic—abnormality of hedgehog (HH) signaling—i.e., none have had genetic analyses of PTCH1 mutations or of possible aberrant HH signaling.

Several authors also have tried to transplant BCCs to immunodeficient mice. Thus transplantation of human BCCs to nude mice produced long-term maintenance but, alas, little to no growth of these transplanted tumors.[7] Transplantation of human BCCs to SCID-beige mice can produce tumors that are more anaplastic and metastatic and which can be serially passaged in vivo transferable but which do not have BCC morphology and are not capable of growth in vitro.[8] In often cited/discussed experiments Van Scott and Reinertson transplanted BCCs to the back of the patients from whom the tumors had been removed.[9] Intact tumors grew whether transplanted to dermis or to the subcutaneous space, and the histology resembled that of the original tumor. However if tumor nodules were dissected away from the stroma, they generally failed to grow, although one tumor transplanted to the dermal-subcutaneous junction had some cells that appeared to be growing but with a more undifferentiated morphology. The conclusion from these experiments, which was consistent with earlier suggestions by Pinkus,[10] is one that has lasted now for four decades – that the growth of BCC tumor cells requires a highly specialized stroma and that it is this requirement that may contribute to the essentially uniform lack of metastasis of BCCs. The postulated permissive stroma is to be found only at the site of the tumor. This concept fits well with increasing recent emphasis on "cancer as an organ" with crucial, essential stromal components rather than on cancer simply as a collection of mutant epithelial cells.[11] However, this putative abnormal BCC stroma has been studied minimally, and it is not clear whether any such unique character of the stroma is inherent in the dermis in which the BCC grows and/or is produced by the remodeling of the surrounding stroma by the expanding tumor cells.

Therefore the identification of HH signaling pathway activation in BCCs was seized upon by mouse modelers to develop mice susceptible to the development of BCCs. Their efforts have taken two forms. The first is the production of mice in which activators of HH signaling are over-expressed in the epidermis of transgenic mice. Such models include epidermal over-expression of hedgehog ligand, of mutant Smo (i.e., a transgenic Smo with an activating mutation that was identified initially in several BCCs), or of the downstream HH transcription factors Gli1 or Gli 2.[12-15] All of these mice develop BCC-like lesions of varying fidelity without further environmental insult. In general the mice are not healthy, and establishment of colonies of these mice has been difficult. The exception has been the long-term survival of transgenic Gli2 mice. Alternatively, two groups have produced mice whose genotype mimics that of patients with BCNS—they inherit one functioning Ptch1 allele. The first is that produced by Hahn and colleagues at the National Cancer Institute, U.S.A.—the defective allele has lost exons 6 and 7 - $Ptch1^{+/-exon6-7}$.[16] The second was developed by Goodrich and colleagues at Stanford—the defective allele lacks part of exon 1 and all of exon 2, and these sequences have been replaced by foreign sequences including those encoding the bacterial lacZ gene - $Ptch1^{+/exon1-2}$.[17] Ptch1 is a target of activated HH signaling, which under normal circumstances increases Ptch1 message and protein and hence inhibits activated HH signaling, thus providing a negative feedback for activated HH signaling. Therefore the engineered lacZ gene is over-expressed in cells with activated HH signaling, and such cells can be identified histologically by their β-galactosidase catalytic activity. In both models, the homozygous knockout genotype causes embryonic lethality; the heterozygous knockout mice are born in normal numbers, have normal fertility, and survive well (colonies of these mice can be established readily), and environmental insults that produce human BCCs also enhance BCC carcinogenesis in these mice.[18,19]

These Ptch1+/- mice recapitulate many aspects of the developmental and neoplastic abnormalities that have been reported in patients with the PTCH1+/- genotype – patients with BCNS. Since many of the abnormalities in the two mouse models are similar, if not identical, it appears that they are due indeed to disruption of Ptch1 function rather than to some nonspecific abnormality introduced as an unexpected consequence of the genetic engineering. What

follows is a summary of the abnormalities reported in these two models as well as comments on some unpublished observations we have made in our lab using the Ptch1$^{+/-exon1-2}$ mouse. Unfortunately no laboratory appears to have compared the two models directly; fortunately, as noted above, the abnormalities reported in the two are very similar. Like BCNS patients, the Ptch1$^{+/exon1-2}$ mice are larger than their Ptch1 wildtype siblings.[20] Pits occur in the soles of the mice and resemble the pits in the stratum corneum of the palms and soles of BCNS patients.[18] One report identified odontogenic keratocysts of the jaws of Ptch1$^{+/exon\ 6-7}$ mice, a problem which plagues BCNS patients.[21,22] In addition, Ptch1$^{+/-exon1-2}$ mice have breast epithelial dysplasia resembling ductal carcinoma in situ but these changes regress during pregnancy, and the mice do not seem to develop breast cancers.[23] Of interest, analogous to the behavior of BCCs transplanted autologously in humans (see above), these mouse breast epithelial abnormalities normalize when transplanted free of stroma. Ptch1+/- mice have some heightened sensitivity to carcinogen-induced transitional cell cancer of the bladder, although the status of HH pathway activation in human bladder tumors is unclear.[24,25] Responses of these mice to other carcinogens have not been reported. However it is especially the rhabdomyosarcomas, medulloblastomas, and basal cell carcinomas that these mice develop that have been the object of the most study.

Rhabdomyosarcomas

Mice of both models spontaneously develop rhabdomyosarcomas arising in the muscles of the limbs. These are histologically similar to the rhabdomyosarcomas that develop rarely in BCNS patients. The murine tumors have up-regulated HH signaling—they have enhanced β-galactosidase activity in the Ptch1$^{+/exon1-2}$ mice and have increased expression of the HH target genes Gli1 and Ptch1 in the Ptch1$^{+/exon\ 6-7}$ mice.[26] Typically the tumors become large enough to necessitate euthansia of the mouse; they have not been found to metastasize. Their incidence is approximately 0-10% in the Ptch1$^{+/exon\ 1-2}$ mice depending on strain background (unpublished data). In the Ptch1$^{+/exon\ 6-7}$ mice they occur in approximately 15% of mice in which the Ptch1 knockout allele is carried on a CD1 background but in no mice with that allele on a C57BL/6 background.[27] When the Ptch1 exon67 allele is expressed on a background lacking Igf2, rhabdomyosarcoma expression is suppressed completely, arguing for its essential role for development of these tumors.[28] Expression microarray studies of RNA from mouse rhabdomyosarcomas identified up-regulation of Akt signaling as well as enhanced regulation of the anti-apoptotic protein Bcl-2.[26] Rhabdomyosarcomas in Ptch1$^{+/exon6-7}$ mice have been reported to lack detectable mutations of the wild type Ptch1 allele but nonetheless to have selective over-expression of the mutant allele, suggesting inhibition of expression of the wildtype allele by an unknown mechanism.[27]

Medulloblastomas

Mice of both models also spontaneously develop medulloblastomas. Often the development of the tumor is heralded by a day or two of continuous turning due, no doubt, to cerebellar dysfunction, followed by death of the mouse. The incidence of these tumors is approximately 8% in the Ptch1$^{+/exon6-7}$ mice on a CD1 background.[29] When the Ptch1$^{exon1-2}$ allele is combined with a p53-/- genotype, the incidence of medulloblastomas rises to 100%, and all mice have died by age 12 weeks.[30] Treatment with ionizing radiation in the newborn period increases the medulloblastoma incidence (exon 6-7 model, CD1 background) from 8% to 51%. Administration of the same dose of ionizing radiation at age 3 months causes no increase in the incidence of medulloblastomas.[29] Medulloblastomas appear to arise in cells of the external granular layer of the cerebellum, which normally respond to Shh released from the underlying Purkinje cells. These cells normally then migrate past the Purkinje cells to form the internal granular cell layer where they are unresponsive to Shh and are terminally differentiated.[31] Thus the susceptibility to ionizing radiation-induced medulloblastomas and the up-regulation of hedgehog signaling both wane with aging, and it reasonable to hypothesize that it is the waning of hedgehog signaling that confers insensitivity to IR treatment. However, Ptch1+/-

Figure 1. Macroscopic BCC arising in the skin of the chest of a Ptch1+/- mouse treated with 5 Gy ionizing radiation at age 5 weeks.

mice appear to have a subset of external granular layer cell "rests", only some of which progress to medulloblastomas, suggesting they represent one stage of a multi-hit tumorigenic process.[32] The cells of the medulloblastomas have strong up-regulation of hedgehog signaling, as indicated by the intense blue staining of the β-galactosidase product on gross or microscopic examination. As in rhabdomyosarcomas, generally the wild type allele of Ptch1 may not be lost.[33,34] Thus the mechanism underlying the up-regulation of HH signaling in these tumors remains uncertain. One possibility for such mechanism is indicated by a report of human medulloblastomas with sporadic and perhaps constitutional mutations in SU(FU).[35] The exact function of this intracellular downstream component of the HH signaling pathway is uncertain but it does act as an inhibitor of HH signaling, and its loss is one of several possible genetic and/or epigenetic changes which might induce the aberrant HH signaling that appears to underlie the malignant behavior of these tumors. Downstream targets of HH signaling apparently crucial to medulloblastoma development include Notch[35a] and N-myc, the expression of which has been reported by several laboratories to be up-regulated in medulloblastomas and in turn to drive increased expression of cyclin D.[36,37] This increased expression of N-myc appears to be MAPK-independent, as it is not affected by the MAPK-inhibitor PD-98,059.[38] Consistent with the above-mentioned upregulated AKT signaling and dependence on Igf2 of rhabdomyosarcomas, the incidence of medulloblastomas induced by targeted expression of Shh in cerebellar neural precursor cells is increased markedly by coexpression of AKT or Igf2—from 15% to 48% or 39% respectively.[39]

BCCs

Mice of both models have grossly and microscopically normal-appearing skin, and no abnormalities of hair growth have been described, despite evidence for tightly—regulated HH signaling as crucial to hair morphogenesis and perhaps to hair follicle cycling.[40] Eventually, small budding microscopically-appreciable lesions consistent with BCCs do appear—in low numbers and of small size—e.g., at age 18 months. However, the situation is very different when environmental insults are added. Thus treatment with UV, given at 240 mJ three times

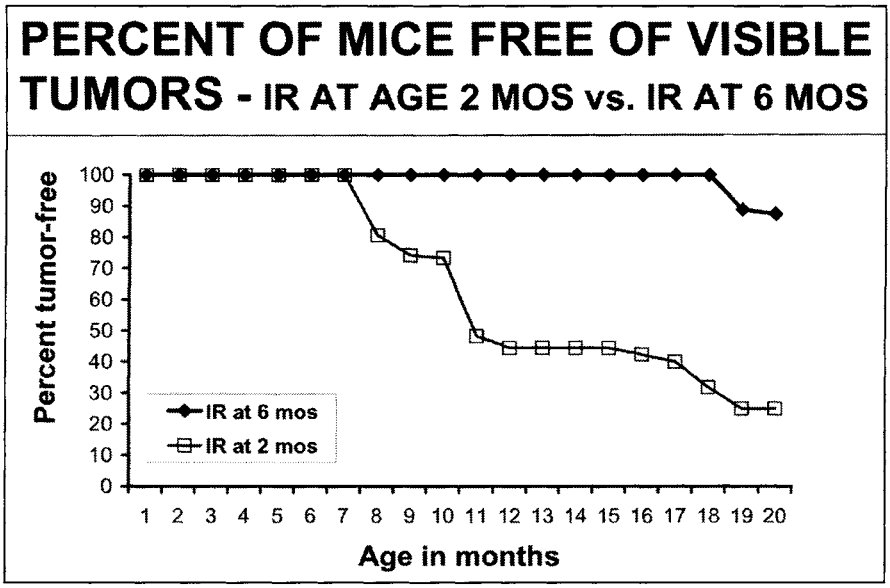

Figure 2. Older mice are less susceptible to ionizing radiatioin-induced BCCs.

each week (a dose three times that necessary to produce a barely perceptible redness – sunburn) starting at age 6 weeks, a single exposure to Cs137 ionizing radiation at 8 weeks, or a single painting with the tumor initiator dimethylbezanthracene (DMBA) at 8 weeks reproducibly produce microscopic BCCs by age 7 months[18] (unpublished data). The Ptch1$^{+/exon1-2}$ mice treated with Cs137 develop macroscopic BCCs starting at age 8-12 months, and of those surviving to 15 months, approximately half have developed these tumors (Fig. 1). When given at age 6 months instead of at the routine 8 weeks of age, the number of microscopic BCCs is the same but the size is less – the microscopic BCC are smaller, and the lag time until the appearance of macroscopic BCCs is prolonged. Thus by 12 months post-IR, none of the mice treated at age 6 months have developed visible BCCs as opposed to 55% by 12 months post-IR of those treated at age 8 weeks (Fig. 2). The UV-treated mice uniformly develop macroscopic tumors, of which at least half are spindle cell tumors ("fibrosarcomas," SpCTs), and approximately 25% each are squamous cell carcinomas and BCCs.[18] SpCTs also develop in Ptch1 wild type mice treated with UV but appear to do so in considerably lower incidence and later in life. These SpCTs in the Ptch1$^{+/exon1-2}$ mice have strong up-regulation of hedgehog signaling as indicated by their intense blue staining when β-galactosidase is assessed using frozen sections (by contrast when intact skin biopsies are incubated with β-galactosidase substrate, the blue staining of SpCTs frequently is limited to the periphery, presumably reflecting poor penetration of the substrate into the interior of these densely-fibrotic tumors). The incidence of UV-induced SCCs also appears to be higher in Ptch1+/- mice than that in Ptch1 wild type mice, but the SCCs do not have up-regulation of HH target gene expression, as judged by the lack of β-galactosidase catalytic activity. Treatment with a single dose of DMBA also produces microscopic BCCs (but no SpCTs or SCCs), and we have not seen these progress to macroscopic tumors.

The histology of these BCCs is somewhat variable. Some do resemble human BCCs but others histologically resemble BCC-related human tumors that appear more closely to mimic various cells of the hair follicle. Thus many are consistent with the diagnosis of trichoblastoma.[18] One piece of data that may be relevant to this variability is the finding of Dlugosz and colleagues that histologic differences can be correlated with the strength of the Gli2 promoter.[41]

Thus, promoter constructs driving Gli2 expression will produce BCC-like tumors, and the stronger the promoter and the higher the degree of expression of downstream target genes, the more closely the histology resembles that of the usual human BCC. Of further interest, Oro and Higgins have found that the degree of HH signaling up-regulation in microscopic BCCs of Ptch1$^{+/exon1-2}$ mice correlates with the phases of the hair cycle – high in anagen and low in catagen/telogen,[40] and Mancuso et al have identified the same variability in IR-induced tumors in the Ptch1$^{+/exon6-7}$ mice.[19] This correlation disappears as the tumors become more advanced, at which time HH target gene expression remains high irrespective of the phase of nearby hair follicles. Thus it is possible that there are real "phase transitions" between microscopic and macroscopic tumors, one of which is the acquisition of hedgehog signaling that is independent of surrounding cues. Furthermore it suggests that the explanation for the essentially complete limitation of BCCs to hairy areas may be more complicated than the idea that this limitation is due to BCCs arising from hair follicles - rather that early BCCs may depend on hair follicle-derived cytokines for growth stimulation to expand to a number of cells more likely to develop further genetic/epigenetic changes. Of interest, skin biopsies of visibly normal skin of some BCNS patients have widespread budding of pre-BCC like tumors from the epidermis, and perhaps this is the human equivalent of the microscopic BCCs that are present in the Ptch1+/- mice.

Further studies in our lab in San Francisco (and in that of our collaborators in New York) can be grouped into several categories. The first of these is an effort to utilize the predictability of BCC tumor development in the UV- or IR-treated Ptch1$^{+/exon1-2}$ mice to screen various agents for chemopreventive efficacy vs. these tumors. This effort is directed initially at identifying agents that might reduce the enormous toll of BCCs in BCNS patients but since the molecular abnormalities in BCCs appear to be the same irrespective of whether they arise sporadically or in BCNS patients, it seems reasonable to hope that agents effective in chemoprevention of BCCs in BCNS patients also might have chemopreventive efficacy vs. BCCs in nonhereditary cases. The latter is of particular importance since it is estimated that 44% of patients with one BCC will develop a second BCC within the ensuing three years.[42] We focused initially on tea – green tea, black tea, and partially-purified tea extracts containing the putatively chemopreventive compounds. None of these had any chemopreventive efficacy whatsoever vs. the development of BCCs in Ptch1+/- mice treated with UV despite their chemopreventive efficacy vs. murine SCCs of these same preparations.[43] In addition we have studied extensively the BCC–chemopreventive efficacy of nonsteroidal anti-inflammatory agents (NSAIDs), which have been shown to have good chemopreventive activity not only against colon cancer in humans and mice but also against experimental cutaneous SCC photocarcinogenesis.[44] We have studied the broad-spectrum Cox-1/2 inhibitor sulindac (Clinoril®) as well as the selective Cox-2 inhibitors celecoxib (Celebrex®) and MF-tricyclic, a compound quite similar to rofecoxib (Vioxx®). Overall these agents in some of our trials appear to have had some anti-BCC effect but the magnitude of this effect, like that of oral tea, is considerably less than is that of the same agents in preventing SCC-photocarcinogenesis. The interpretation of these data is complicated somewhat by preliminary data we have accrued in our lab indicating that Ptch1$^{+/exon1-2}$ mice genetically-deficient in either Cox-1 or Cox-2 have BCCs that are of smaller size, albeit normal numbers, than do Ptch1+/- mice with wild type Cox1/2. Therefore there remains the possibility that the minimal activity of the NSAIDs in our mice may be a quantitative phenomenon rather than an indication of the lack of dependence of BCCs on Cox enzyme activity for their growth. In BCCs in our mice, Cox2 enzyme localizes primarily to the stroma rather than to the tumor cells, the opposite of its localization in SCCs.[45] We also have completed enrollment for a trial of the chemopreventive efficacy of celecoxib, 200 mg twice daily, vs. the development of BCCs in human BCNS patients. This is a double-blind randomized, placebo controlled study with 30 patients on placebo and 30 on celecoxib, and we are counting BCC tumor numbers over two years of treatment as well as during the ensuing year off therapy. Results will not be available for several years, but at least so far there is no evidence (from unblinded analysis by the Data Safety Monitoring Board) of

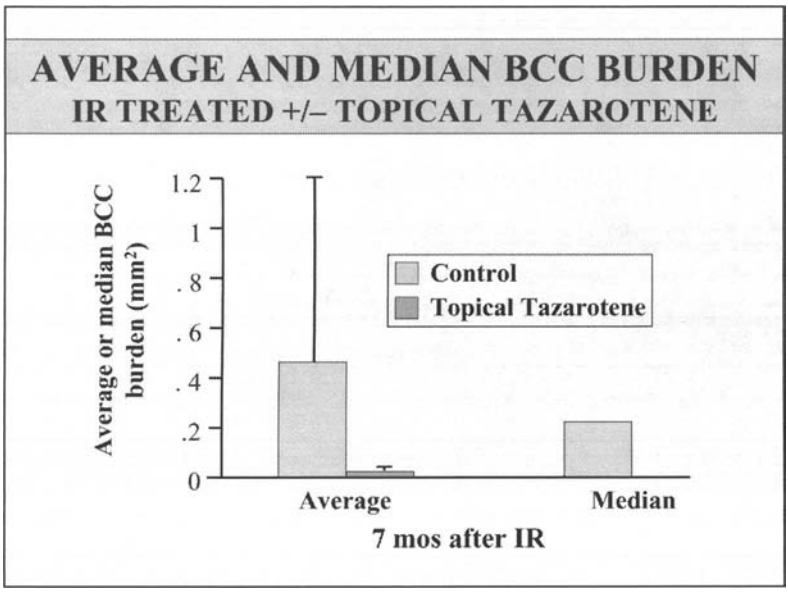

Figure 3. Topical applications of the retinoid tazarotene prevent BCC development following ionizing radiation. Shown is the burden (number of tumors x cross sectional area) of microscopic BCCs in standard skin biopsies taken at age 9 months.

unexpected side-effects of this medication in this population. Due to concerns regarding possible cardiovascular risks this trial was suspended in December, 2004, pending the results of a futility analysis.

More impressive have been the chemopreventive effects in mice of two other agents—difluoromethyl ornithine (DFMO) and the retinoid tazarotene, a RAR-β/γ ligand. DFMO is a suicide inhibitor of ornithine decarboxylase (ODC), which is responsible for the conversion of ornithine to putrescine, which then in turn is converted to the pro-prolferative higher polyamines. Our collaborators at Columbia found that oral DFMO reduces UV-induced BCC formation by 40-80% in Ptch1$^{+/exon1-2}$ mice, and genetically engineered mice with inhibition or overexpression of ODC have reduced or enhanced BCC carcinogenesis respectively.[46] These findings, unlike those with tea and NSAIDs, are similar to the ability of DFMO to inhibit experimental skin SCC carcinogenesis and argue for a contributing role of polyamine cell cycle stimulation in formation and growth of both BCCs and SCCs. DFMO has been used orally in humans, where its major side effect appears to be ototoxicity.

Tazarotene is available topically in Europe as well as in the United States, where it is FDA-approved for treatment of acne in the young, photodamage (i.e., wrinkles in particular) in the mature, and psoriasis. Retinoids first were reported two decades ago to have significant anti-skin cancer efficacy; in particular oral retinoids are quite effective in reducing the skin cancer burden in patients with BCNS or xeroderma pigmentosum, unfortunately often at doses that also produce poorly-tolerated retinoid side effects.[47] In addition, topical tazarotene has been reported to cure approximately 50% of sporadic human BCCs to which it is applied for prolonged periods (e.g., up to 8 months).[48] This curative effect appears to be associated with reduced proliferation and increased apoptosis.[49] One paper argues that all-trans retinoic acid (ATRA) can interfere directly with Gli signaling,[50] and it is clear that inhibition of hedgehog signaling pathway activation in hedgehog-driven tumors can lead to apoptosis.[51,61] Topical tazarotene applied 5x/week starting at age 6 weeks can reduce by approximately 85% the BCC number and size in Ptch1$^{+/exon1-2}$ mice treated starting at age 8 weeks either with a single dose

of Cs-137 ionizing radiation or with thrice weekly UV[52] (Fig. 3). Of note, this result is quite different from that found for the chemopreventive effects of ATRA on skin SCC photocarcinogenesis, in which modest changes in tumor formation were noted (e.g., ref. 53). In parallel with that difference in efficacy, we, like others, have found that murine BCCs retain expression of the retinoid receptors normally found in skin – RARα, RARγ, and RXRα (unpublished data).[54] This is in contrast to their typical loss in skin SCCs[55] (our unpublished data), as also has been described in several other tumors. We are now organizing a blinded clinical trial of topical tazarotene, assessing its efficacy against BCCs in BCNS patients – both its chemopreventive and its chemotherapeutic efficacy.

A second initiative has been to assess the effects on BCC formation of background modifying genes. Therefore we have expended considerable effort in transferring the Ptch1$^{exon1\text{-}2}$ allele onto several inbred backgrounds. Although none of these backcrosses has been completed to "inbred" status (i.e., 20 backcrosses), it is clear that differences in BCC burden are emerging. Thus, Ptch1+/- mice of the C3H/HeOuJ background have 6-fold greater BCC burden than do Ptch1 mice of the FVBN/J background (a strain which is especially sensitive to skin SCC carcinogenesis), and the mouse-to-mouse variability of these comparatively homogeneous mice is considerably less than is that of mice of the B6.D2 50-50 mixed background. Of considerable interest, we also have crossed the FVBN/J Ptch1$^{+/exon1\text{-}2}$ mice with Spretus mice. The Ptch1$^{+/exon1\text{-}2}$ F1 IR-treated offspring have a **greater** BCC burden than do the parental Mus mice, indicating that the Spr mice bring an overall balance of susceptibility alleles to the cross. This is in very marked contrast to the usual **resistance** alleles contributed by Spr parents to F1 crosses; e.g., F1 Spr-Mus mice are completely resistant to DMBA/TPA two stage chemical carcinogenesis.[56] Thus these data, as well as the differing response of BCCs and SCCs to chemopreventive agents, argue for fundamental differences in skin carcinogenesis for BCCs vs. SCCs. At odds with this conclusion are recently published studies in which CD1 mice with the exon 6-7 Ptch1 KO allele were crossed to mice bred selectively for resistance or susceptibility to two-stage chemical carcinogenesis, and the F1 Ptch1+/- mice were tested for IR-induced BCCs. The mice with the SCC-resistant parent also were quite resistant to BCC carcinogenesis; those with the SCC-susceptible parent also were highly susceptible to BCC carcinogenesis.[57]

A third initiative has been to assess the potential effects of the immune system on the development of BCCs. This work was stimulated (i) by the high predictability of the over-expression of hedgehog target genes in BCCs, thereby providing a set of known tumor-associated antigens for immunoprevention/immunotherapy and (ii) by the reported approximately 10-fold increase in BCCs in organ transplant recipients. Of note, the latter patients also develop large numbers of SCCs, out of proportion to their increased relative risk of BCCs. We have completed one trial of immunoprevention using two different immunization schedules with peptides derived from the hedgehog target gene Hip and have identified an approximately 40% reduction in BCCs in mice immunized with peptide vs. those immunized with adjuvant alone.[58] It should be noted that immunization was accompanied by readily-detectable cell-based and humoral immune response to these peptides, despite the high level of Hip expression during development. Thus it might be possible to immunize fair-skinned humans moving e.g., to Tucson and reduce their likelihood of developing BCCs. We also have completed one trial in which we treated Ptch1$^{+/exon1\text{-}2}$ mice with the anti-rejection drug combination of prednisolone plus cyclosporine A at doses sufficient to block acquisition of new immune responses (both cellular and humoral) and found a two-fold enhancement of BCC carcinogenesis as compared to vehicle-treated mice.[60] Taken together, these studies indicate that the immune system normally may have a role in conferring resistance to BCC carcinogenesis and that its up-regulation through active immunization may be a previously-unexplored approach to prevention and perhaps even to therapy.

We have established a single cell line (ASZ001) from a murine BCC that had been induced in a Ptch1+/- mouse by UV treatment. This cell line appears to be immortal (has been in culture since 1998), grows in calcium concentrations too high to permit proliferation of normal mouse epidermal keratinocytes, fails to undergo differentiation, has lost the wild type

allele of Ptch1, and has mutations in p53.[18] Collaborators have identified enhanced expression of PDGFRα and subsequent Ras/Erk activation as crucial to BCC carcinogenesis, and the proliferation of AZ001 cells can be inhibited by antibodies to PDGF or (unlike medulloblastomas) by the MAPK-inhibitor PD98,059.[59] Inhibition of hedgehog signaling – e.g., by cyclopamine—causes apoptotic cell death both in vitro in the ASZ001 cell line and in BCCs in vivo.[61]

Summary

It seems likely that the studies summarized above will be only the start of more extensive work utilizing mouse models, in particular the Ptch1+/- mice, of BCC carcinogenesis. Clearly the study of experimental BCC carcinogenesis lags far behind the study of experimental SCC carcinogenesis, which is not surprising given that these BCC models became available less than a half decade ago. Already studies have suggested interesting differences between BCCs and SCCs, despite their arising at the same site, and understandings of those differences is likely to have broader implications for the general process of carcinogenesis. Hence there is much work yet to be done to understand BCCs better and perhaps even eventually to replace the time honored techniques of physical destruction with curative chemical agents and thus to treat BCCs with a more elegant and less destructive approach.

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CHAPTER 7

GLI Genes and Their Targets in Epidermal Development and Disease

Fritz Aberger* and Anna-Maria Frischauf

Introduction

During the last decade an enormous wealth of data has been generated by many different laboratories showing that the Hedgehog (HH)/GLI signaling pathway plays key roles not only in the control of vertebrate embryonic development but also in adult organisms by regulating multiple biological processes such as cell differentiation, proliferation or programmed cell death. Interest in this signaling pathway further increased by the discovery that inappropriate activation of the pathway can be associated with the development of different cancer types, ranging from relatively harmless semi-malignant tumors of the skin such as basal cell carcinoma (BCC) to highly aggressive and lethal malignancies of the brain, lung or gastrointestinal tract.[1-3]

In this chapter we focus on the role of the GLI zinc finger transcription factors as nuclear mediators of Hedgehog signaling in the epidermis and BCC. During epidermal development HH/GLI signal transduction has been implicated in the formation of hair follicles by controlling proliferation and morphogenesis. The pivotal role of this pathway in regulating cell division has been underlined by the finding that mutations causing constitutive activation of HH/GLI signaling can have fatal consequences and lead to the formation of BCC, the most common tumor in the western world.

First insight into how aberrant activation of HH/GLI signal transduction can trigger tumor formation came from genetic analysis of Gorlin (or NBCCS) syndrome, an autosomal dominant hereditary disease that predisposes patients to the early development of multiple BCCs. It was shown that BCCs of Gorlin patients as well as the majority of sporadic BCCs have inactivating mutations in Patched (PTCH), a twelve-pass transmembrane protein whose normal function is to suppress the pathway in the absence of HH ligand. As a result, the HH/GLI pathway becomes constitutively active in epidermal cells regardless of the presence of ligand.[4-6] Furthermore, in a small fraction of sporadic BCC activating mutations have been identified in SMOH, a seven-pass transmembrane protein that acts downstream of PTCH and is essential for transduction of the HH-signal.[7,8]

The pivotal role of the pathway in BCC was further supported by a series of experiments carried out in different laboratories showing that sustained activation of HH-signaling in the epidermis and hair follicles by transgenic expression of either Sonic Hedgehog protein or downstream effectors such as an oncogenic form of SMOH or members of the GLI family of zinc finger transcription factors leads to the formation of BCC-like tumors in mice.[7,9-12]

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Despite this clear understanding of the role of HH/GLI signaling in the initiation of BCC, numerous questions relating to the complex downstream mechanisms triggered by aberrant pathway activation remain to be answered. In particular, the oncogenic function of the zinc finger transcription factors and mediators of HH-signaling, GLI1 and GLI2, is not entirely understood. Both transcriptional regulators are highly expressed in BCCs and are able to induce epidermal tumorigenesis when overexpressed in the basal layer of mouse epidermis.[10,12-14] But what transcriptional changes controlled by GLI1 and GLI2 underlie Hedgehog-induced carcinogenesis and what is their respective role in and relative contribution to tumor formation? Do they share redundant functions or do they control distinct biological properties by regulating different sets of target genes? How is their own expression regulated?

Although this chapter will not be able to answer these questions, the aim of the following sections will be to reconcile results from recent experiments on Hedgehog/GLI signaling in various biological contexts, to infer models of GLI function in epidermal development and disease and to outline future studies that will need to be done to understand in detail the molecular basis of HH-induced tumorigenesis.

GLI Genes and Epidermal Development

The role of HH/GLI signaling in vertebrate skin has been extensively studied during embryonic hair follicle formation. One of the first morphological signs of hair follicle development is the appearance of epidermal placodes induced by signals originating from specialized mesodermal cells underneath the placode and from adjacent epidermal cells. As a result, cells of the epidermal placode start to grow down into the dermal compartment of the skin, eventually forming the mature hair follicle, a process that requires reciprocal communication between epidermal and mesodermal cells (Fig. 1A) (for review see ref. 15).

Genetic analysis of mice lacking functional Sonic Hedgehog (Shh) protein revealed that Hedgehog/GLI signaling is essential for hair follicle morphogenesis. Epidermis of Shh -/- mice is still able to form placodes, which, however, do not develop significantly beyond this stage but arrest due to defects in proliferation and morphogenesis.[16,17] Expression of Shh is first detected in the placode itself, while the Shh receptor Patched (Ptc) as well as the downstream effectors Gli1 and Gli2 are expressed in the placode and underlying mesodermal cells, suggesting autocrine as well as paracrine signaling in Shh-regulated hair follicle development.[16]

Recent experiments taking advantage of sophisticated transgenic and gene-targeting approaches have shed more light on the respective roles of Gli transcription factors in mediating Shh-signaling during hair follicle morphogenesis in mice. While Gli1 and Gli3 loss of function mutations do not affect hair development, Gli2 homozygous mutants, similar to Shh -/- mice, fail to grow hair follicles beyond the placode stage, suggesting that Gli2 is the major player in transducing the Shh-signal in the nucleus of responding cells (Fig. 1B). Transgenic expression of wild-type Gli2 in the epidermis of Gli2 mutant mouse embryos could overcome the early arrest in hair follicle development and restored Shh-target gene expression and proliferation, showing that Gli2 function is required in the epidermis rather than in the underlying mesoderm. Strikingly, the ability of wild-type Gli2 to rescue the Gli2 -/- epidermal phenotype depended on the presence of functional Shh protein, which would be consistent with the conversion of murine Gli2 from an inactive to an active form by upstream signals initiated by Shh. Activation of mouse Gli2 protein may involve modification of the N-terminal region, since an N-terminal Gli2 deletion variant is constitutively active and does not depend on Shh-signaling.[18,19] Whether a similar regulatory mechanism also applies to the human homologue is unclear at present, since the N-terminus of human GLI2 is substantially shorter than that of mouse Gli2. Furthermore, full-length human GLI2 is already a strong transcriptional activator comparable to GLI1, while mouse Gli2 is a much weaker activator unless the putative N-terminal repression domain is removed.[14,19] A more detailed comparison of the activities of human and murine Gli2 proteins will therefore be important for understanding their functional properties in both normal and diseased tissues.

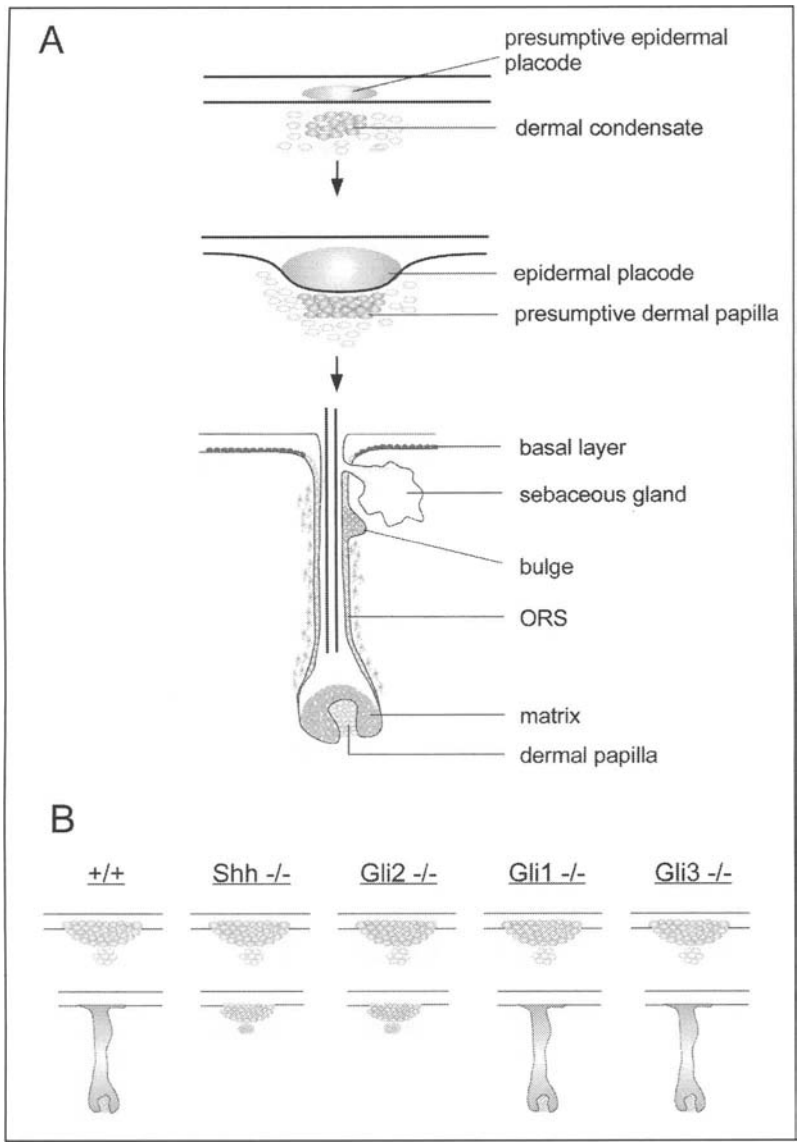

Figure 1. Role of GLI proteins in embryonic hair follicle development. A) Hair follicle development is initiated by reciprocal signaling between epidermal and underlying dermal cells leading to the formation of the epidermal placode that expresses Shh. Paracrine as well as autocrine signaling by Shh is thought to be required for the elongation, down-growth and morphogenesis of the hair follicle. In the mature hair follicle SHH expression is found in the matrix region, while GLI1, GLI2 and the GLI target genes PTCH, BCL2 and FOXE1 are restricted to the outer root sheath (ORS), which is contiguous with the basal layer of the epidermis. GLI2 and FOXE1 mRNA is also expressed in the basal layer of interfollicular epidermis.[24,53] B) Hair follicle development is disturbed in Shh -/- and Gli2 -/- mice but normal in Gli1 and Gli3 homozygous mutants, suggesting that Gli2 rather than Gli1 or Gli3 acts as mediator of Shh-signaling during embryonic hair development.

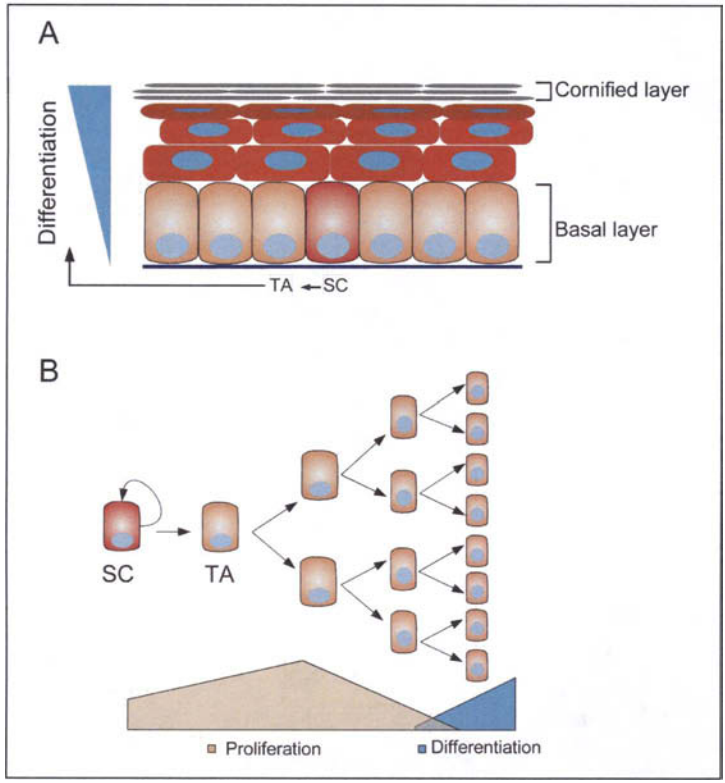

Figure 2. Tissue homeostasis in stratified human epidermis. A) Loss of cells from the cornified layer is compensated by rapid proliferation of transit amplifying cells (TA) in the basal layer. TAs are the progeny of stem cells (SC, red cell), destined to undergo terminal differentiation after they withdraw from the cell cycle. When TAs enter the differentiation pathway they detach from the basement membrane and start moving towards the surface of the skin. During transit, they produce large amounts of keratin proteins, which become cross-linked to strengthen the cells. Eventually they begin to synthesize and secrete lipids resulting in the formation of the cornified layer, which fulfills the barrier function of the skin (for review see ref. 65. B) Schematic illustration of the proliferative activity and differentiation of stem cells and transit amplifying cells in human epidermis. Stem cells divide slowly, have the capacity to self-renew and produce rapidly proliferating TAs. After a few rounds of divisions TAs exit the cell cycle and begin to differentiate to build the protective layers.

Human Epidermis and the Origin of BCC

One of the major functions of the multilayered epidermis is to protect the body against physical traumas and environmental damage caused by UV-irradiation or toxic substances. In addition to protective stratified epidermis, epidermal cells give rise to sebaceous glands, sweat glands and hair follicles that cyclically undergo phases of regression and regrowth. Epidermal cells have a relatively short lifetime. The continuous loss of cells from stratified epidermis and the cyclic growth and degeneration of hair follicles requires that these epidermal structures be maintained by a precise balance between cell proliferation, differentiation and cell loss or death, a process referred to as tissue homeostasis. Maintenance of the epidermis and its appendages throughout life is ensured by stem cells that are able to self-renew as well as to produce progeny termed transit amplifying cells that undergo several rounds of proliferation before differentiation[20,21] (Fig. 2A,B).

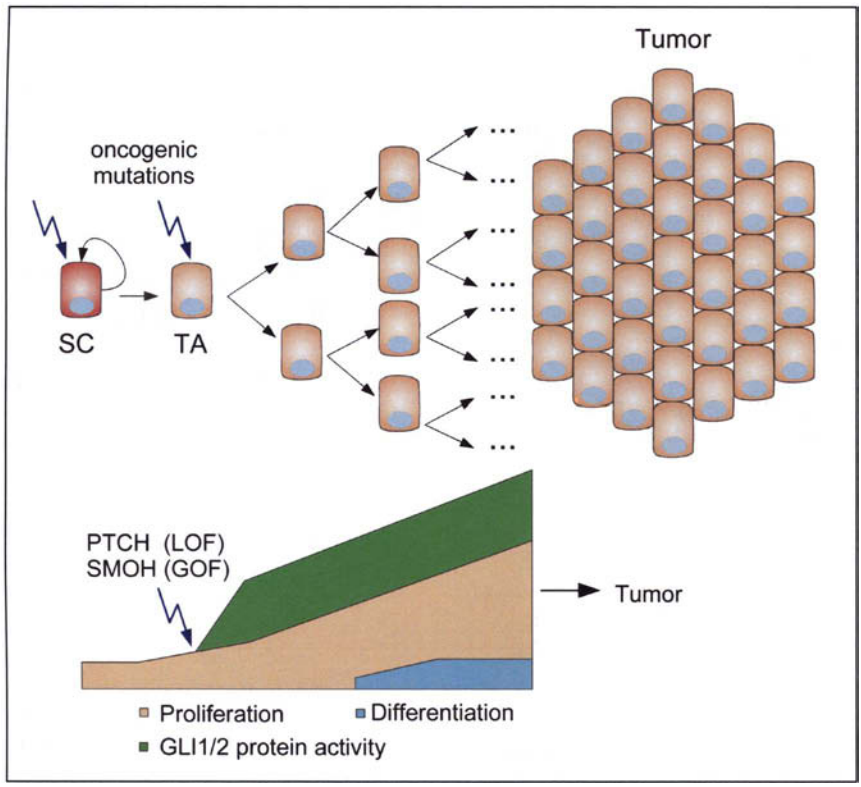

Figure 3. Hierarchical model of oncogenic mutations leading to BCC development. Mutations in tumor suppressors or oncogenes are likely to occur in transit amplifying (TA) or long-lived epidermal stem cells (SC), resulting in tumor expansion by increased proliferation and a gain of self-renewal capacity at the expense of cell differentiation. The semi-malignant character of BCC may indicate that oncogenic events occur in cells that have been committed to differentiation such as TAs rather than in stem cells.[23] According to this model, BCC development may be initiated by loss of PTCH or gain of SMOH function in TA cells, resulting in constitutively increased levels of GLI1 and GLI2, which activate oncogenic transcriptional programs and concomitantly repress differentiation signals. Further mutations such as loss of TP53 function may promote BCC development, though ligand-independent activation of HH-signaling may suffice for tumorigenesis. LOF = Loss of function, GOF = Gain of function.

Epidermal stem cells have been shown to reside in the basal layer of the epidermis and in the bulge region of hair follicles. Consistent with their multipotent nature, stem cells are thought to contribute to all epidermal lineages. Given the relatively short lifetime of differentiated epidermal cells, long-lived stem cells and transit amplifying cells are considered likely targets of oncogenic mutations (Fig. 3). Accumulation of mutations in these cells can lead to aberrant activation of signaling pathways that normally control proliferation and differentiation, eventually resulting in tumor formation.[20,22,23]

Whether HH/GLI signaling is inappropriately activated in epidermal stem cells or in committed cells is unclear at present. BCCs present as poorly differentiated tumors that resemble in some aspects undifferentiated aberrant hair follicle-like structures, suggesting that BCCs could represent hair follicle derived tumors. Expression analysis of Hedgehog pathway components in normal hair follicles showed that Hedgehog-responsive genes including PTCH, GLI1 and

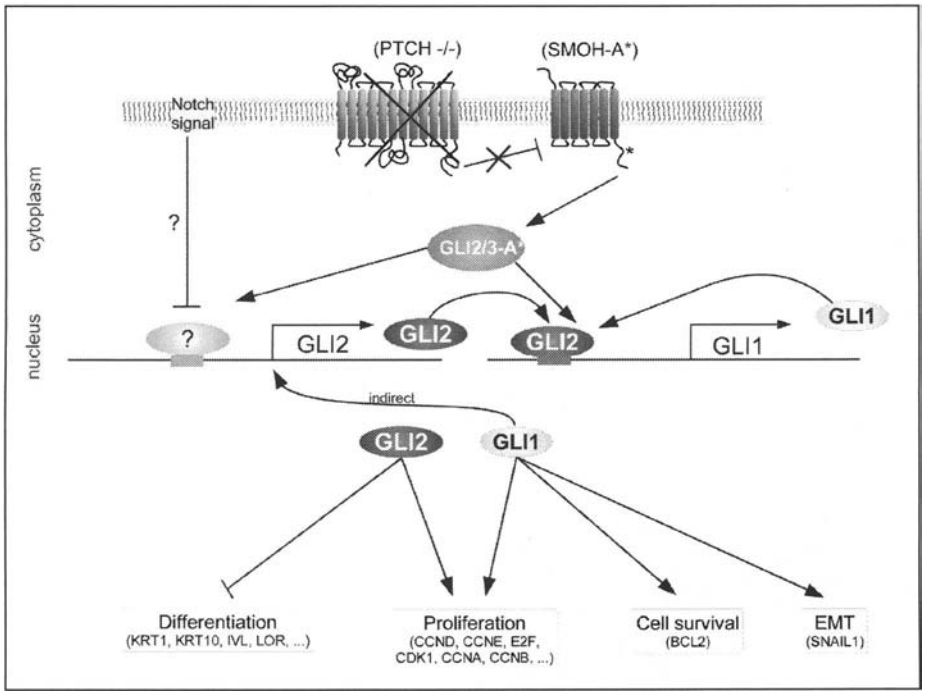

Figure 4. Model of GLI activation in BCC development. Mutations resulting in loss of PTCH or gain of SMOH function (SMOH-A*) convert a latent inactive transcription factor - possibly GLI2 or GLI3 - into a strong activator form. Activated GLI2/3 directly induces transcription of GLI1 and possibly GLI2, although the GLI2 promoter has not yet been analyzed for the presence of functional GLI-binding sites. In wild-type cells, Notch-signaling negatively controls expression of GLI2. High-level expression of GLI1 and GLI2 in BCC is maintained by a positive feedback mechanism involving GLI1 and GLI2. In this scenario, GLI2 stimulates GLI1 expression by direct binding to the GLI1 promoter, while GLI1 in turn induces its own expression as well as GLI2 transcription probably via activation of an intermediate transcription factor. As a result, constitutive expression of GLI1 and GLI2 contributes to tumor formation by repressing differentiation signals and activating cell cycle progression genes, anti-apoptotic factors and genes promoting EMT and invasive growth. EMT: Epithelial-Mesenchymal Transition;

GLI2 as well as the GLI targets FOXE1 and BCL2 are expressed in the outer root sheath (ORS) of hair follicles, while SHH expression is restricted to the matrix region, which comprises proliferating cells.[24-27] It therefore appears possible that BCCs derive from the ORS or possibly from the bulge region, where ligand-independent HH-signal transduction may cause increased proliferation at the expense of differentiation (Fig. 3), a scenario that would be consistent with the biological activities of GLI genes.

Alternatively, BCC may also originate from the basal layer of interfollicular epidermis or even from cells that have already entered the differentiation pathway. Support for the latter comes from experiments showing that activation of oncogenes can reprogram post-mitotic, differentiated keratinocytes to undergo proliferation.[28] In this context it is noteworthy that forced expression of the GLI2 oncogene in human epidermal cells in vitro is able to oppose differentiation signals and induce reentry into the cell cycle,[29] suggesting that sustained HH-signaling is able to reactivate proliferation in differentiated cells of human epidermis (see Fig. 4).

GLI1 and GLI2 in Basal Cell Carcinoma

Signal transduction pathways such as Wnt/wingless or Hedgehog have long been known to be important regulators of embryonic development. Over the last few years, however, it has become evident that grave consequences for the organism ensue from unscheduled activation of these pathways in later life. While in normal cells and tissues the activity of these pathways is restrained closely in space and time by the deployment of negative regulators, cells carrying mutations in these repressor genes may readily escape these control mechanisms and undergo uncontrolled proliferation.[30]

In a series of experiments carried out in different laboratories it has been shown that loss of the HH-pathway repressor PTCH results in constitutive, ligand-independent pathway activation, thereby triggering BCC development.[4-6] As a result of ligand-independent HH-signaling in BCC, tumor cells express highly elevated levels of GLI1 and to a lesser extent also of GLI2, while GLI3 expression appears to be unchanged.[13,14,31] Activation of GLI1 and GLI2 is thought to be responsible for the mediation of aberrant HH-signaling in the nucleus of epidermal cells and the activation of the oncogenic transcriptional program underlying BCC development. The oncogenicity of these transcription factors (TFs) has been demonstrated in transgenic mouse models, where the expression of both human GLI1 and murine Gli2 was directed to the basal layer of the epidermis by using a keratin5-promoter for transgene expression. Both TFs were able to induce epidermal tumors, though expression of full-length Gli2 mainly induced BCC-like structures, while GLI1 expression led to multiple types of epidermal cancer including trichoblastoma, trichoepithelioma and BCC-like tumors.[10,12] The distinct phenotypes of the transgenic mice might be explained by the fact that murine Gli2 on its own is a relatively weak transcriptional activator that appears to require upstream HH-signaling in order to be converted into a strong inducer of Hh-targets genes.[18,32,33] Given that in murine skin expression of Sonic Hedgehog is restricted to the hair follicle region, Gli2 would only become activated in the K5-transgenics in a sub-population of cells of the hair follicle (those that receive the Hh-signal), where it could promote the growth of BCC-like tumors with similarities to aberrant hair follicles. Consistent with this model, mice expressing a constitutively active form of Gli2 under control of the K5-promoter develop a broader spectrum of tumors similar to mice expressing GLI1.[34]

Although these experiments have provided valuable information on the identity of the nuclear transducers of oncogenic HH-signaling, the results also raise a number of questions. It is still unclear whether GLI1 and GLI2 have redundant functions or whether they accomplish distinct tasks in response to oncogenic HH-signaling, and if the latter is the case, how they regulate distinct sets of target genes. Are both GLI1 and GLI2 functions required for transducing oncogenic HH-signaling in the nucleus of responsive cells? If so, what is the relative contribution of these proteins to tumorigenesis? Answering these questions will be an important step towards defining the critical molecular events at the distal end of HH-signaling in tumor development.

The Oncogenic Nature of GLI Transcription Factors

Much of our current knowledge about how GLI transcription factors promote cellular transformation and tumorigenesis are based on studies of the GLI1 oncogene, which was first identified as a gene highly amplified in glioblastoma.[35] GLI1 has been implicated in HH-associated cancer formation and/or maintenance by its ability to transform cells in combination with E1A, its tumor-inducing activity in mice and frogs when overexpressed in the epidermis and its high-level expression in tumors with increased HH-signaling.[12,13,35-39] Furthermore, forced expression of GLI1 promotes proliferation of human epidermal cells in a cell-autonomous manner, probably by the direct regulation of genes involved in cell cycle progression.[14,32,40]

More recent genetic data, however, support a pivotal role for GLI2 in mediating HH-signaling in the epidermis and possibly in tumorigenesis. Like Shh, Gli2 function is essential for proper hair follicle formation, while Gli1 and Gli3 are dispensable for normal hair development.[18]

Gli1 is also not required for Shh-induced medulloblastoma formation, since brain tumors do develop in Gli1-/- mice in response to overexpression of Shh.[41,42] Together with the finding that overexpression of Gli2 in mouse epidermis is sufficient to induce BCC-like tumors, these data raise the possibility that GLI2 rather than GLI1 may encode the critical factor, involved in executing oncogenic HH-signaling in response to constitutive pathway activation. Finding out whether loss of Gli2 function abrogates the ability of aberrant HH-signaling to induce tumors will allow greatly refined and clearer models of Hh-induced tumorigenesis to be constructed.

How might GLI genes promote epidermal cancer formation in response to aberrant HH-signaling? Like GLI1, GLI2 promotes proliferation of epidermal cells in the absence of dermal cells, suggesting that both transcription factors act cell-autonomously rather than by stimulating paracrine signals that in vivo may trigger the release of mitogenic stimuli from dermal cells.[14,18] Insight into the molecular changes induced by an increase in GLI2 activity in human epidermal cells has come from DNA-array based gene expression studies. Expression of GLI2 in human keratinocytes induces a number of genes involved in G1-S phase and G2-M phase progression such as E2F, D-type cyclins, Cyclin A2, CDK1, and Cyclin B1.[29] GLI genes may act directly at the heart of the cell cycle machinery. Analysis of the Cyclin D2 promoter revealed the presence of at least one Gli-binding site and experiments using cycloheximide to block protein synthesis in Shh-treated cells corroborated the notion that Cyclin D2 is a direct GLI target gene.[32,40] Also, the rate of E2F1 mRNA accumulation in response to GLI2 is comparable to that of the direct GLI target PTCH, which suggests that E2F1—a transcription factor involved in the activation of S-phase genes in response to mitogenic stimuli—may be a direct GLI target gene like Cyclin D2.[29] Studies in the fruit fly have identified Cyclin E as direct transcriptional target of the Drosophila homologue of vertebrate GLI genes, Cubitus interruptus, but it is not yet clear whether this also applies in mammalian systems.[43]

Not only can human GLI2 act as potent inducer of cell cycle progression genes, GLI2 has also been shown to antagonize epidermal differentiation signals. Expression of GLI2 in human keratinocytes led to repression of epidermal differentiation-associated genes under conditions that normally promote epidermal differentiation.[29] Repression of differentiation-associated genes by GLI2 may point to a role as transcriptional repressor as has been proposed for Gli2 in vertebrate somite patterning.[32] Alternatively, suppression of epidermal differentiation may be accomplished by activation of downstream repressors in response to GLI2. In this context it is noteworthy that GLI2 directly induces the expression of the forkhead transcription factor FOXE1, which has been shown to have repressor function and may therefore be a candidate mediator of at least part of the antagonistic effect of GLI2 on epidermal differentiation.[44]

A further mechanism likely to contribute to the oncogenic activity of GLI factors is the fact that transcription of the key anti-apoptotic factor BCL2 is directly controlled by GLI1 and GLI2.[25,25a] GLI1 has also been shown to directly stimulate the transcription of Snail1, a transcription factor promoting epithelial-mesenchymal transition (EMT) and invasive tumor growth.[45] Another interesting connection has been established in Xenopus, where certain Wnt-signaling molecules are under the control of Gli factors and are required for the ventro-posteriorizing effect induced by Gli2 and Gli3. Consistent with a possible downstream role of Wnt factors in HH-induced carcinogenesis, Gli1-induced BCC-like tumors in frog and human BCCs contain elevated mRNA levels of various Wnt genes.[46] By screening for signaling pathways that act downstream of HH/GLI it could be shown that proliferation of mouse BCC-like tumors involves activation of the ras-ERK pathway, possibly stimulated by activation of the PDGFR-alpha signaling cascade.[47] Given the critical role of Wnt signaling and ras-ERK pathway activation in a variety of malignancies it is, therefore, likely that these signaling cascades mediate at least some aspects of BCC development downstream of HH-signaling.

In summary, the oncogenic activity of GLI1 and GLI2 in BCC is likely to rely on the manifold activities of GLI proteins: promoting cell cycle progression, activating growth-promoting signaling pathways, inhibiting epidermal proliferation, promoting cell survival by direct activation of BCL2 and inducing genes involved in tumor invasion (see Fig. 4).

Regulation of GLI Expression

The regulation of the activity of vertebrate Gli transcription factors occurs at multiple levels and is still not completely understood.

Unlike the Drosophila Gli homologue Cubitus interruptus, which is primarily regulated at the post-translational level by proteolytic processing, vertebrate Gli genes are (also) regulated at the transcriptional level, where Gli1 and Gli2 are activated while Gli3 appears to be repressed in response to Hh-signaling. Similar to Drosophila Ci, Gli3 has been shown to encode a transcriptional repressor generated by proteolytic processing of the full-length protein in the absence of Hedgehog-signaling.[33,48-51] To what extent Gli1 and/or Gli2 are regulated by proteolysis in vivo is not yet fully clear.

Transcriptional control of the GLI1 oncogene has recently been addressed by the analysis of cells mutant for different combinations of Gli transcription factors and by functional promoter studies. It was shown that the activation of Gli1 in response to Shh does not occur in the absence of Gli2.[32] Consistently, mice lacking functional Gli2 protein display significantly reduced levels of Gli1, while overexpression of GLI2 in human keratinocytes rapidly induces GLI1 transcription.[14,52] Analysis of the human GLI1 promoter identified a single Gli-binding site that is essential for activation by GLI2, showing that GLI1 transcription is directly regulated by GLI2.[53] How then is transcriptional activation of Gli1 and also of Gli2 initiated in response to Hedgehog? Experiments using cycloheximide have shown that induction of Gli1 transcription in response to Shh does not depend on protein synthesis, indicating that the activating protein(s) for Gli1 transcription must already be present in the cell prior to activation of the Hh-pathway.[32] Since Gli2 activator function appears to be dependent on Hedgehog-signaling,[18] Gli2 protein itself and/or Gli3 could be the latent activator that induces Gli1 transcription as soon as the Hh-pathway is turned on, either by ligand binding or as in the case of BCC, by inactivation of PTCH (Fig. 4).[32]

To clarify the regulation of GLI genes, it will be necessary to elucidate the molecular mechanism responsible for the conversion of the full-length Gli2 protein into a strong transcriptional activator in response to Hh-signaling and to address whether a similar mechanism applies to human GLI2, which lacks most of the N-terminal domain supposed to be required for keeping mouse Gli2 in a hypo-active state.

Induction of GLI2 transcription in response to Hh-signaling is only poorly understood at present. In particular, it is unclear whether the proposed latent Gli activator also directly induces Gli2 transcription in response to pathway activation. A possible mechanism by which high levels of GLI2 mRNA might be achieved in response to HH-signaling would be by activation of GLI1 itself. Expression of GLI1 in human epidermal cells has been shown to induce the expression of GLI2, indicating the existence of a positive feedback mechanism that may also operate in carcinogenesis and contribute to maintenance of high levels of both GLI1 and GLI2 (Fig. 4).[14] However, since loss of Gli1 function in mice does not appear to have a significant impact on the expression of Gli2, the in vivo relevance of the GLI1-GLI2 feedback remains to be addressed in future experiments.

Another mechanism involved in the regulation of Gli2 expression has been revealed by the analysis of mice homozygous mutant for Notch. Intriguingly, loss of Notch function in the epidermis led to an increase in Gli2 mRNA levels and to the development of BCC-like tumors, possibly as a consequence of elevated levels of Gli2.[54] Expression of Gli2 therefore appears to be negatively regulated by Notch signaling (Fig. 4). Detailed analysis of the Gli2 promoter will be an important prerequisite for the elucidation of the regulatory mechanisms involved in the control of Gli2 transcription.

Unresolved Problems and Future Perspectives

During the past years substantial progress has been made in understanding the involvement of the HH/GLI-signal transduction pathway in cancer and the details underlying the oncogenic activity of GLI genes are emerging but a number of unresolved issues remain. The target

gene specificities of GLI genes are still not well defined. GLI proteins have a highly conserved DNA-binding domain consisting of 5 C2-H2 zinc fingers, which bind to the consensus Gli-binding site GACCACCCA.[55] Despite the high conservation of the DNA-binding domain of GLI proteins, there is evidence that they regulate different target genes in similar biological contexts. In frogs, for instance, Gli2 but not Gli1 is involved in ventro-posterior mesodermal development downstream of FGF-signaling and only Gli2 but not Gli1 is able to directly control the expression of Xbra and Xhox3.[56] Distinct activator specificities of Gli1 and Gli2 have also been observed by heterologous expression in Drosophila imaginal discs and by overexpression in murine presomitic mesodermal cells.[32,33] It will be interesting to see whether all Gli genes bind their target sequences with comparable affinities or whether variations of the consensus Gli-binding site found in promoters of target genes result in subtle alterations of DNA-protein binding properties, which may account for the distinct specificities observed. Another question that needs to be addressed is whether distinct domain organization of GLI proteins contributes to distinct transcriptional read-out. For instance, a CBP-interacting domain was identified in Ci, GLI3 and based on sequence analysis, possibly also in GLI2 but not in GLI1[57-59] (and data not shown), suggesting that interactions with coactivators may change the transcriptional regulator activities of GLI proteins. In this context, a screen for factors that interact with Gli proteins would help understanding the different biological activities of these proteins. With respect to the oncogenic properties of GLI proteins, the identification of a larger number of GLI targets in different Hedgehog-associated tumors such as tumors of the gastrointestinal tract, the lung or the brain will be essential to elucidate the relative contribution of GLI proteins to Hedgehog-induced tumorigenesis. These experiments will also allow addressing the question of whether there exists a common theme at the transcriptional level in GLI-induced tumorigenesis or whether the response of a cell to oncogenic HH/GLI signaling is mainly dependent on the cell type and the biological context.

Given the pivotal role of GLI transcription factors in executing aberrant Hedgehog signaling in cancer, targeted inactivation of these factors may hold promise for future therapeutic approaches, complementary to current approaches that involve systemic and sustained administration of specific inhibitors of the Hedgehog signal transducer Smoothened.[38,39,60,61] Such approaches may rely on stable repression of GLI expression by RNA interference[62] or on the administration of decoy oligonucleotides, which have been designed to specifically block the activity of a given transcription factor (for reviews see refs. 63,64). A more detailed understanding of the regulation and the activity of GLI proteins in disease should accelerate the development of novel anti-cancer strategies in the future.

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CHAPTER 8

Splitting Hairs:
Dissecting the Roles of Gli Activator and Repressor Functions during Epidermal Development and Disease

Pleasantine Mill and Chi-Chung Hui*

Introduction

Constantly turning over throughout adult life, the mammalian epidermis is renewed by the controlled expansion of progeny from resident multipotent stem cells. This fine balance of proliferation and differentiation of epidermal progenitors with sustained cell loss is required to maintain tissue integrity and is disturbed in many human conditions, including alopecia and skin cancer. The continuous turnover of adult skin and its appendages employs many of the same molecular and morphological programs used during embryonic development. One key player is Sonic Hedgehog (Shh) signaling, which is required for growth of embryonic and adult hair follicles,[1-4] while its hyperactivation is intrinsically linked to hair follicle derived tumors in humans and mice.[5-9] In both developmental and disease conditions, Shh-dependent responses are determined by the balance of activator and repressor functions of the Gli transcriptional mediators. Developments in the understanding of how this balance of Gli activities is set within target cells and what transcriptional program they execute in the skin will be the focus of this review.

The Gli Family of Transcription Factors in Hh Signaling

Hh Signal Transduction Cascade

The Hedgehog (Hh) family of secreted signaling molecules can act as dose-dependent morphogens, mitogens or factors directing specific developmental programs.[10] How a target cell responds to Hh signals is determined by the dual activity of its nuclear effectors, the Gli/Ci family of zinc finger transcription factors.[11-14] While Hh signal transduction within target cells is best understood in *Drosophila*, homologues for most pathway components have been identified in other species, suggesting that the Hh signaling cascade is highly conserved. In the absence of Hh, the ligand binding receptor Patched (*Ptc*) represses activity of the signaling coreceptor Smoothened (*Smo*).[15] Expression of target genes is inhibited, as the transcriptional mediator Cubitus interruptus (Ci) is sequestered in the cytoplasm in a multimeric protein complex containing the kinesin-related protein Costal2 (Cos2), the serine/threonine kinase Fused (Fu) and the novel protein Suppressor of Fused (SuFu).[16-18] In the absence of Hh, complex bound full-length Ci is targeted for degradation.[19-21] This processing event removes the transcriptional activation domain generating a truncated Ci, Ci^{REP}, that retains the zinc

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finger DNA binding domain and an N-terminal repressor domain.[22,23] Ci^{REP} translocates to the nucleus and represses expression of target genes. The binding of Hh to Ptc releases inhibition on Smo, leading to stabilization of full-length Ci with subsequent loss of Ci^{REP}. This derepression alone may be sufficient for expression of some target genes. However, the expression of other Hh targets requires additional events, such as conversion of full length Ci to the potent transcriptional activator, Ci^{ACT}, and its nuclear translocation.[24-27]

Until recently, it remained unclear how the same components of the Hh signaling cascade could be used to translate subtle differences in Hh concentrations into different responses in the nucleus. One key means of distinguishing low and high levels of Hh signals intracellularly appears to hinge on different activities of Smo and their influence on Ci fate.[28] Moreover, localization of Smo appears to be Hh-dependent. In the absence of Hh, Smo resides predominantly in cytoplasmic vesicles where it is complexed through Cos2 to the multimeric protein complex containing Ci.[29-32] On Hh stimulation, this Smo-containing complex translocates to the plasma membrane and triggers phosphorylation of its components, preventing formation of Ci^{REP}. While full length Ci may be stabilized through sequestration in these membrane associated complexes, additional activation events, such as the inactivation of Su(Fu), are required for conversion to Ci^{ACT} and its nuclear translocation.[17,18,33] Thus, multiple regulatory mechanisms exist in target cells that are differentially influenced by the level of Hh signals, and together they determine the appropriate transcriptional output by Gli/Ci transcription factors.

Diversity of Mammalian Hh Signaling Pathway

In contrast to flies, certain elements of the vertebrate Hh signaling cascade have been duplicated and may act in a combinatorial manner to increase developmental potential. In mammals, there are three Hh homologues: *Desert hedgehog* (*Dhh*); *Indian hedgehog* (*Ihh*) and *Sonic hedgehog* (*Shh*). All three Hh homologues can bind Ptc receptors with equivalent affinities.[34] Whether there are biochemical differences between members, in addition to differences in temporal and spatial patterns of expression, has yet to be established. There are two Ptc proteins, Ptc homolog1 (Ptc1) and Ptc homolog2 (Ptc2), which can bind Hh ligands with similar affinities and inhibit Smo activities.[35-39] Only one homologue of Smo has been reported,[40] and it remains to be seen whether the multi-tiered regulation of invertebrate Smo is evolutionarily conserved. However, mammalian homologues of the multimeric Cos2/Fu/Su(Fu) complex have been identified and appear to regulate Gli activity in a similar manner.[41-43] (H.C., S.M. and C.c.H., unpublished results) Initially identified as genes amplified in glioblastoma tumors, there are three Gli zinc finger transcription factors, Gli1, Gli2 and Gli3, which have distinct and overlapping functions in transducing vertebrate Hh responses[13,44,45] (Tables 1, 2). While there are strong elements of conservation between the invertebrate and vertebrate Hh signaling pathways, there are likely divergent branches, such as those observed among the Gli proteins.

Gli2 and Gli3 Function as Bipotential Regulators

Although Ci and Gli proteins are highly similar, there are noteworthy differences in their regulation and processing. Unlike *Drosophila* Ci, mouse Gli genes appear to be both mediators and targets of Hh signaling. *Gli1* is a canonical target gene activated in response to Hh signals, while expression of *Gli3* is upregulated in the absence of Hh.[46,47] Structurally, the highest homology between Ci and Gli transcription factors exists the N-terminal repression domain, absent in Gli1, and in the zinc finger DNA binding domain.[48,49] Similarly to Ci^{REP}, C-terminally truncated constructs of Gli2 and Gli3 repress transcription of Hh targets in vitro and in vivo.[50-52] Conversely, deleting the N-terminal negative regulatory regions of Gli2 and Gli3 converts these proteins to potent transcriptional activators.[49,50,53,54] When overexpressed in *Drosophila* imaginal discs, Gli3 was processed to a repressor form in the absence of Hh signals, while Gli2 was constitutively cleaved in a Hh-independent manner.[55,56] Similar to Ci, there is genetic evidence suggesting that both Gli2 and Gli3 can act as repressors in vivo,[57-60] however proteolytic processing has only be demonstrated for endogenous Gli3.[27,42,61] It remains to be seen

Table 1. Functional analysis of the vertebrate Gli proteins in vivo

Gene	Organism	Mutation	Survival	Tissue	Effect	Refs.
Gli1	Mouse	*Gli1*$^{zfd/zfd}$ (Gli1 nul)	Viable		No phenotype	41,64
		Gli1$^{lacZ/lacZ}$ (Gli1 nul)	Viable		No phenotype	63
	Zebrafish	*detour* (Gli1 nul)		Neural tube	Ventral CNS patterning defects	212,226
				Forebrain	Reduced ventral markers of diencephalon	
				Pituitary	Reduced expression of adenohypophysis markers	
Gli2	Human	various (?)		Forebrain	Spectrum of holoprosencephaly features	218
				Pituitary	Abnormal pituitary formation and function	
	Mouse	*Gli2*$^{zfd/zfd}$ (Gli2 nul)	Perinatal lethal	Neural tube	Lack of floor plate, reduced V3 interneurons	41,64,65
					Oligodendrocyte specification	216
				Forebrain	Mild holoprosencephaly	66,190
					Thinner telencephalic vesicles, smaller tectum and cerebellum	190
				Lung	Lung hypoplasia and lobulation defects	67
				Foregut	Tracheo-oesophageal fistula	67
				Hindgut	Imperforate anus with recto-urethral fistula	199
				Skeleton	Absence of vertebrae and intervertebral discs	66
					Cleft palate, delayed ossification of skull	
					Reduced length of limbs and sternum	
				Skin	Hair follicle arrest	99
				Teeth	Abnormal maxillary incisors	66,119
				Mammary	Ductal hyperplasia in explants	124
		Gli2$^{gli1/-}$ (GliACT med)	Viable	Neural tube	Rescue ventral cell types and notochord regression defects of *Gli2*$^{-/-}$	57
				Lungs	Accessory lobe rescued and increased proliferation	
				Skin	Progressive alopecia >3 mos	
		Gli2$^{gli1/gli1}$ (GliACT high)	Postnatal lethal	Neural tube	Similar to *Gli2*$^{gli1/-}$rescue; no expansion of ventral cell types	57
				CNS	Stiffness of movement and loss of hindlimb coordination	
				Skin	Early and severe alopecia and more severe	

Table continued on next page

Table 1. Continued

Gene	Organism	Mutation	Survival	Tissue	Effect	Refs.
Gli2	Mouse	*Gli2*$^{gli3/-}$ (GliACT low)	Perinatal lethal	Neural tube	Identical to *Gli2*$^{-/-}$; lack of floor plate, reduced V3 interneurons	58
		Gli2$^{gli3/gli3}$ (GliACT med)	Perinatal lethal	Neural tube	Partial rescue of FP cells, V3 interneurons and motor neurons (requires Gli1 induction: no rescue in *Gli2*$^{gli3/gli3}$*Gli1*$^{lacz/lacz}$)	58
	Zebrafish	*you-too* (Gli2ΔC)		Neural tube	Midline and axonal guidance defects	212,222,225, 226
				Forebrain	Ventral differentiation defects	226
				Pituitary	Loss of adenohypophysis & ectopic lens formation	214,219,226
				Somites	Lack of horizontal myoseptum; defects in slow muscle formation	208,226
Gli3	Human	GCPS (GLI3 heterozygous)		Skeleton	Post- and pre-axial polydactyly, syndactyly Craniofacial defects and hypertelomerism Macrocephaly	210,223,224
		PHS (Gli3ΔC)		Forebrain	Hypothalamic hamartoma and pituitary disfunction	52,211
				Foregut	Laryngotracheal cleft and malformed epiglottis	
				Kidney	Renal anomalies	
				Hindgut	Imperforate anus	
				Skeleton	Post-axial and central polydactyly	
				Appendage	Hypoplastic nails	
		PAP-A (GLI3 REP mild)		Skeleton	Post-axial polydactyly	217
	Mouse	*Gli3*$^{xtj/xtj}$ (Gli3 nul)	Perinatal lethal	Neural tube	Dorsal expansion of V0-V2 interneurons	73,103
					Neural tube closure defects & edema	68
				Forebrain	Exencephaly and choroid plexus defects	190,209
					Ventralized telecephalon	215,220
				Lung	Altered shape and size	67,178
				Hindgut	Anal stenosis and ectopic anus	199,213
			Perinatal lethal	Skeleton	Polydactyly, syndactyly	66,68
					Craniofacial defects and unfused sternum	66,68
					Shortening and thickening of limbs	66

Table continued on next page

Table 1. Continued

Gene	Organism	Mutation	Survival	Tissue	Effect	Refs.
Gli3	Mouse	*Gli3*$^{xtj/xtj}$ (Gli3 nul)	Perinatal lethal	Skin	Supernumerary vibrissae and pelage follicles	99
				Eyes	Poorly developed eyes	71
		Gli3$^{phs/phs}$ (Gli3ΔC)	Perinatal lethal	Foregut	Epiglottis and larynx defects	207
				Kidney	Hypoplastic and dysmorphic defects and lack of adrenal glands	
				Hindgut	Gastrointestinal defects and imperforate anus	
				Skeleton	Polydactyly, syndactyly	
					Proximal-distal shortening of limbs	
					Premature skeletal mineralization	
	Zebrafish	Gli3 morpholino		CNS	Patterning defects of neural tube and brain	221
				Eye	Reduced retinal ganglion cell differentiation	
Gli1/Gli2	Mouse	*Gli1*$^{zfd/zfd}$ *Gli2*$^{zfd/+}$	Postnatal lethality	CNS	Reduction of floor plate and V3 interneurons	64
					Behavioral and neurological defects	
				Lungs	Hypoplastic and lobular defects	
				Genitals	Males: undescended testes and incomplete external development	
		Gli1$^{zfd/zfd}$ *Gli2*$^{zfd/zfd}$	Embryonic lethality	CNS	Lack all V3 interneurons and floor plate	64
				Lungs	Delayed morphogenesis and severe hypoplasia	
				Pituitary	Absent	
				Skeleton	Post-axial nubbin present on all limbs	
Gli1/Gli3	Mouse	*Gli1*$^{zfd/zfd}$ *Gli3*$^{xtj/-}$	Viable	Skeleton	Post-axial polydactyly, but phenotype identical to *Gli3*$^{xtj/+}$	64
Gli2/Gli3	Mouse	*Gli2*$^{zfd/zfd}$ *Gli3*$^{xtj/-}$	Perinatal lethality	Neural tube	Expanded motor neuron progenitor domain, normal MN develop	53,58
				Skeleton	More severe reduction in stylopod and zeugopod length	66
					More severe pre-axial polydactyly phenotype, post-axial nubbin	
					More severe defects in vertebral column and sternum	
Gli2/Gli3	Mouse	*Gli2*$^{zfd/zfd}$ *Gli3*$^{xtj/-}$	Perinatal lethality	Teeth	Mandible defects, maxillary incisors arrested and small molars	66,119
				Foregut	esophageal atresia with tracheo-oesophageal fistula	67
				Lung	Severely hypoplastic lung with lobulation defects	

Table continued on next page

Table 1. Continued

Gene	Organism	Mutation	Survival	Tissue	Effect	Refs.
Gli2/Gli3	Mouse	*Gli2*$^{zfd/zfd}$ *Gli3*$^{xtj/-}$	Perinatal lethality	Hindgut	Persistent cloaca	199
				Hair follicles	More severe arrest of hair follicle development	99
		Gli2$^{zfd/zfd}$ *Gli3*$^{xtj/xtj}$	Embryonic lethality E10.5	Neural tube	Loss of ventral cell types (required for proliferation and positioning) and extension of dorsal identity, hyperproliferation	58
				Somites	Defects in sclerotome and myotome development	59
				Foregut	No trachea or esophagus form	67
				Lungs	No lung formation	
Ptc1/Gli1	Mouse	*Ptc1-/-Gli1*$^{zfd/zfd}$	E9.5	Neural tube	No rescue of ventralized *Ptc1*$^{-/-}$ phenotype	63
Ptc1/Gli2	Mouse	*Ptc1-/-Gli2*$^{zfd/zfd}$	>E9.5	Neural tube	Ventralized *Ptc1*$^{-/-}$ phenotype suppressed	60,63
				Mesoderm	Reduction of Hh responses	63
Ptc1/Gli3	Mouse	*Ptc1-/-Gli3*$^{xtj/xtj}$	E9.5	Neural tube	No rescue of ventralized *Ptc1*$^{-/-}$ phenotype	60
Shh/Gli2	Mouse	*Shh-/-Gli2*$^{zfd/zd}$	Perinatal lethal	Neural tube	No rescue of *Shh*$^{-/-}$ phenotype: cyclopia, loss of ventral cell types	57
Shh/Gli3	Mouse	*Shh-/-Gli3*$^{xtj/+}$	Perinatal lethal	Neural tube	Some rescue of ventral cell types	69,73,103
				CNS	Telencephalon partially rescued, increased progenitor proliferation	C.c.H. and P.D., unpubl.
				Craniofacial	Two eyes and more defined frontonasal mass	71; P.M. and C.c.H. unpubl.
				Skeletal	Improved distal limb development and autopod number	71,72,74
				Hair follicle	Vibrissae and pelage follicle pattern improvements	P.M. and C.c.H. unpubl.
		Shh-/-Gli3$^{xtj/xtjs}$	Perinatal lethal	Neural tube	Rescue of all ventral cell types, patterning defects remain	69,73,103
				CNS	Exencephaly, high progenitor proliferation	C.c.H. and P.D., unpubl.
				Craniofacial	Two eyelids with hypomorphic eyes and large frontonasal mass	71; P.M. and C.c.H. unpubl.
				Skeletal	Distally complete limb and polydactylous (duplications)	71,72,74
				Hair follicle	Partial rescue of vibrissae and pelage follicle development	P.M.andC.c.H. unpubl.

Table 2. Overexpression functional analysis of vertebrate Gli proteins

Gene	Organism	Tissue	Region	Gli Protein	Promoter	Effect	Refs.
Gli1	Mouse	Skin	Basal epithelia	human GLI1	bovine K5	Spontaneous BCC-like tumors	147
			Basal epithelia		human K14	HF tumors during anagen	100
		Brain	Dorsal midbrain, hindbrain	human GLI1	*En2*	Dorsal brain outgrowth, abnormal head morphology	227
			Dorsal CNS	mouse Gli1	*Wnt1*	Caudal forebrain and rostral midbrain overgrowth	64
				mouse Gli1^{zfd}	*Wnt1*	No effect on dorsal brain growth	
		Gut	Zinc-induced, global	human GLI1	mouse MT-1	Hirschsprung's-like phenotype: dilation of the colon & lack of ganglia	228
	Frog	Epidermis	2 cell embryo ventral pole	frog Gli1	Injection	BCC-like tumors	7
		CNS	2 cell embryo dorsal pole	frog Gli1	Injection	Brain tumors	134
			Neural plate	frog/human Gli1	Injection	Ectopic induction of ventral genes (N-tubulin, HNF3β) and cell types	46,81
Gli2	Mouse	Skin	Basal epithelia	mouse Gli2	bovine K5	Spontaneous BCC-like tumors; slower, milder tumor phenotype	145, P.M and C.c.H. unpubl.
				mouse ΔNGli2	bovine K5	Spectrum of HF-derived tumors; rapid, aggressive tumor phenotype	146, P.M and C.c.H. unpubl.
				mouse Gli2ΔC	bovine K5	Arrest of hair follicle development and block of sebocyte differentiation	101
		Brain	Dorsal midbrain, hindbrain	mouse Gli2	*En2*	No effect on dorsal brain development	49
				mouse ΔNGli2	*En2*	Ventral cell types induced in dorsal neural tube; overgrowth	49

Table continued on next page

Table 2. Continued

Gene	Organism	Tissue	Region	Gli Protein	Promoter	Effect	Refs.
Gli2	Chick	Neural tube	Dorsal neural tube	mouse ΔNGli2	Electroporation	Ectopic target gene expression, hyperproliferative neuroepithelium	53
	Frog	Ectoderm	Neural plate	frog Gli2	Injection	Induced ectopic ventral genes and cell types	51,81
				frog Gli2ΔC	Injection	Block floor plate induction and ventral neuronal differentiation	51
Gli3	Mouse	Brain	Dorsal midbrain, hindbrain	mouse Gli3	*En2*	No effect on dorsal brain development	49
				mouse ΔNGli3	*En2*	Ventral cell types induced in dorsal neural tube; overgrowth	
	Chick	Neural tube	Ventral neural tube	quail $Gli3^{phs}$	Electroporation	Ventral expansion of dorsal progenitors and loss of ventral fates	104
			Ventral neural tube	human Gli3ΔC	Electroporation	Ventral expransion of dorsal progenitors and loss of ventral fates	73
			Ventral neural tube	mouse/human ΔNGli3	Electroporation	Ventral progenitor fates induced in dorsal neural tube	53,54
	Frog	CNS	Neural plate	human/frog GLI3	Injection	No effect on neural tube expression	46
				human Gli3ΔC	Injection	Block floor plate induction and ventral neuronal differentiation	51,81,229

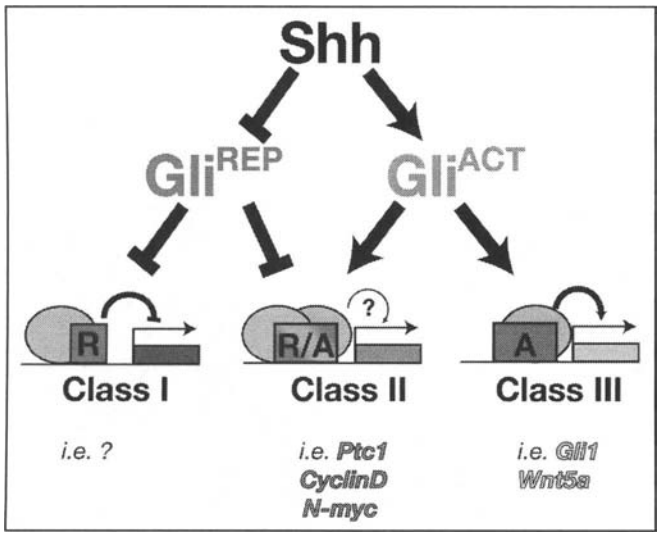

Figure 1. Model of Gli-mediated transcriptional control of Hh target genes. Despite common Gli consensus sequence, three classes of Hh target genes may be differentially regulated by Gli functions. The level of Hh signaling received by a target cell will determine the balance of Gli activator and repressor functions.[24] The balance of Gli functions will influence expression of target genes differently, where specificity of the Gli consensus site may be modulated by other *cis*-elements or transcriptional cofactors. Expression of Class I genes will be controlled solely by Gli3 repressor function. Class II promoters will be influenced by both Gli activator and repressor functions; basal expression can be derepressed in low Hh signals (no Gli^{REP}), while high level induction requires high Hh-dependent Gli^{ACT} function. Class III genes will only be induced by Gli activator functions.

what modifications are required to generate $Gli2^{REP}$, as well as whether Gli/Ci activators and repressors regulate expression of the same set of target genes.[53,59] While both activator and repressor forms bind the same consensus sequence in vitro and in vivo, it appears that the sensitivity of particular target promoters to regulation by either activator or repressor functions is determined by *cis*-regulatory elements (Fig. 1).[53,59,62]

Gli Genes Have Unique and Overlapping Functions in Vivo

Genetic studies suggest that Gli2 and Gli3 are the primary mediators of Hh responses in mammals, where Gli2 functions as the principal activator and Gli3 performs most repressor functions during normal development. Despite its potent activator function and its induction by Hh signaling, Gli1 is likely not involved in initial Hh responses.[63] *Gli1*$^{-/-}$ mice show no apparent developmental defects or alterations in Hh responses, unless one copy of *Gli2* is removed.[64] In contrast, *Gli2*$^{-/-}$ mice die in late gestation due to severe defects in spinal cord development, as well as developmental abnormalities of lung, vertebrae and bones, with global down-regulation of Hh target gene expression.[58,65-67] The importance of Gli2-dependent activator function is further revealed in *Ptc1*$^{-/-}$; *Gli2*$^{-/-}$ double mutants, where *Ptc1*$^{-/-}$ posterior neural tube defects due to hyperactive Hh signaling are rescued by removing Gli2.[60] The importance of Gli2 repressor function during normal development is unclear, as Gli1 can fully substitute for Gli2 activities embryonically. However, $Gli2^{Gli1/Gli1}$ adults do develop progressive skin defects consistent with deregulated Hh responses.[57] In contrast, loss of Gli3 repressor results in ectopic activation of Hh responses contributing to profound skeletal and nervous system defects found in *Gli3*$^{-/-}$ embryos, which die perinatally.[68-70] Controlling Gli3 repressor activities is a key function of Shh signaling. Genetic reduction of Gli3 repressor function in

ShhGli3 compound mutants displays a dose-dependent rescue of the severe growth arrest, ventral spinal cord patterning defects and distal limb defects observed in *Shh*$^{-/-}$ mutants.[69,71-74]

While Gli proteins have distinct activities, their overlapping functions are revealed in compound mutants. Defects in neuronal specification in *Gli1*$^{-/-}$*Gli2*$^{-/-}$ spinal cords are more severe than in the *Gli2*$^{-/-}$ counterparts,[63,64] indicating that Gli1 can functionally substitute for Gli2 in the spinal cord. One dose of Gli3 is sufficient to induce target genes in *Gli2*$^{-/-}$; *Gli3*$^{+/-}$ presomitic mesoderm,[59,63] indicating Gli3 can substitute for Gli2 activator function in vivo. Furthermore, *Gli2*$^{Gli3/Gli3}$ mutant mice show some signs of rescue in the neural tube, suggesting that Gli3 can function in vivo as an activator, albeit less potently.[58] Due to their dual role as transcriptional activators and repressors, loss of Gli gene functions is not functionally equivalent to loss of Hh signaling. Defects observed in somites, neural tube, lung and trachea of *Gli2*$^{-/-}$; *Gli3*$^{-/-}$ mutants likely reflect the simultaneous loss of positive Hh responses and inhibitory repressor functions.[53,58,59,66,67]

Divergence within Shh/Gli Signaling Cascade

Whether members of the Gli/Ci transcription family are the sole mediators of Hh responses in target cells has been a controversial issue.[75-79] In the larval fly eye, Hh signaling through *Ptc* and *Smo* is required for development, although target gene transcription occurs independently of *Ci* function.[78] Similarly, the minimal Hh-dependent regulatory regions of the *wg* promoter in flies and the *COUPTFII* promoter in mice do not contain consensus Gli binding sites.[76,77] However, recent work in both flies and mammals support that most, if not all, of Hh responses are Gli/Ci-dependent.[12,53,58,60] In the ventral spinal cord, patterning of all neuronal subtypes by the ventral-to-dorsal gradient of Shh is dependent solely on Gli function.[53,58,60] These studies suggest that most cases of apparent Gli/Ci-independent Hh responses can be attributed to derepression of Hh target gene expression in the absence of both Gli/Ci activator and repressor functions. Another possible branch of the Hh/Gli signaling pathway comes from studies suggesting that there are some Gli functions independent of Hh signaling. *Gli2* and *Gli3* are expressed more broadly than sources of Hh signals during development.[80] Although Gli repressor functions may be required in these regions to inhibit inappropriate activation of Hh targets, it is also possible that the activities of Gli proteins are subject to other signaling inputs. During *Xenopus* development, FGF activates *Gli2* and *Gli3* expression, which are required to maintain and pattern mesoderm through induction of key regulatory genes, including *Brachyury* and *Xhox3*.[81] The antagonistic relationship between Hh and BMPs (Bone Morphogenetic Proteins) observed during development may involve direct interaction of truncated Gli3 repressor and Smads, the mediators of BMP signaling.[82]

While recent studies have revealed cooperative functions for the Gli transcription factors in several developmental contexts, their distinct and overlapping roles in mediating Hh responses in the skin was not known. Given the importance of regulated Hh signaling during skin development and tumorigenesis,[1,3,7] understanding the functional interplay of Gli transcription factors and the transcriptional cascades they control in the skin is necessary.

Molecular Mechanisms of Embryonic and Adult Hair Development

Epidermal-Dermal Signaling during Embryonic Hair Follicle Morphogenesis

Despite their differences, epidermal appendages, including mammalian hair follicles or avian feathers, share a similar developmental programme. A reciprocal exchange of inductive cues occurs between mid-gestation ectoderm and mesenchyme, to determine where these structures will form and trigger their initial development (Fig. 2).[83] Recombination experiments suggested the first signal arising from the mesenchyme instructs regions of the pluripotent ectoderm to elongate into regularly spaced placode structures.[84] Epidermal signals from the placode cue underlying mesenchymal fibroblasts to organize into dermal condensates, or

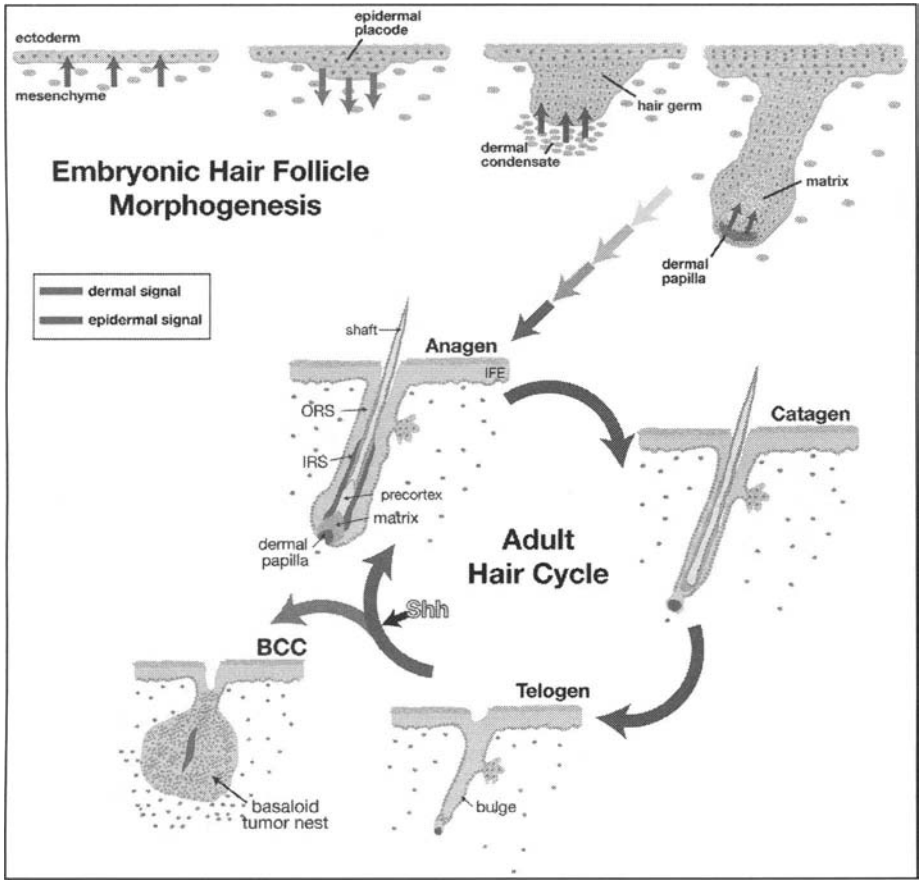

Figure 2. Initiation of embryonic and adult anagen hair follicle development proceeds through inductive epidermal-mesenchymal signals: Embryonic dermis instructs overlying ectoderm to initiate placode formation, a structure which signals to mesenchymal fibroblasts to form dermal condensate. Subsequently, the dermal condensate signals to follicular keratinocytes to stimulate proliferation and downgrowth into developing dermis. The dermal condensate will be enrobed by epithelial cells and mature to form dermal papilla. Proliferating hair progenitors (matrix cells) that are pushed away from contacting the dermal papilla will differentiate in response to lateral signaling between keratinocytes into the 6 epithelial cell types making up the hair shaft. *Shh* is expressed in early placode and remains expressed at the distal tip of developing follicles. Adult hair follicles undergo cyclic periods of growth (anagen), destruction (catagen) and rest (telogen). Matrix cells are thought to maintain their proliferative capacity through close association with dermal papilla during anagen. During catagen, withdrawal of growth signals results in massive apoptosis of the lower two thirds of the epidermal hair follicle. As a result, the dermal papilla is dragged through the dermis and comes to rest close to the permanent bulge region (telogen), during which the old hair shaft may be shed. At the onset of a new anagen phase, epidermal stem cells are stimulated to divide in response to signals from the dermal papilla. Epidermal progenitors proliferate to regenerate the matrix cells and their differentiated progeny necessary for a functional hair. Transient Shh signaling is required during the telogen-anagen transition and deregulated Hh responses can result in uncontrolled progenitor growth in BCC tumors. Key: *ORS*: outer root sheath; *IRS*: inner root sheath; IFE: interfollicular epidermis.

dermal prepapilla. A second "dermal message" from the condensate subsequently prompts epidermal cells in the placode to proliferate and grow down into the dermis, forming a hair peg, which will eventually engulf the dermal condensate to form a dermal papilla. This close epidermal-mesenchymal crosstalk is believed to stimulate further proliferation and differentiation of epidermal cells into many components of the mature hair follicle.[83] In the hair peg, epidermal cells that lose contact with the dermal papilla become outer root sheath (ORS) cells, a compartment that is contiguous with the basal layer of the interfollicular epidermis (IFE).[85] Matrix cells are highly proliferative epidermal cells that maintain contact with the dermal papilla and start to adopt distinct differentiation programs on withdrawal from the cell cycle. These differentiating cells move upwards in morphologically distinct concentric cylinders of cells that emerge at staggered intervals during postnatal development; the outer three cylinders of the inner root sheath (IRS) develop first, while the three central hair shaft layers arise later.[84] The final epidermal lineage to emerge is the oil-rich sebocytes, which populate the sebaceous glands, off the upper portion of hair follicles.

Adult Hair Cycle

Many aspects of embryonic epithelial-mesenchymal crosstalk are redeployed in adult skin where hair follicles cycle through periods of active growth (anagen), periods of regression (catagen) and rest (telogen) (Fig. 2). During anagen, matrix cells enrobing the dermal papilla continue to proliferate and differentiate, elongating the emerging hair shaft. Matrix cells have a finite proliferative capacity, influenced in part by the size and stimulatory output of the dermal papilla.[84,86] When the proliferative potential of matrix cells is exhausted, a destructive phase ensues resulting in apoptotic cell death of the lower two-thirds of the hair follicle.[87] During catagen, the dermal papilla also dramatically reduces in size and transforms into a quiescent cluster of cells.[88] However, the shrunken dermal papilla is pulled through the dermis trailing the lower portion of the follicle as it regresses to a resting position close to the permanent bulge region, the niche of epidermal stem cells.[86,89] The resting telogen hair follicle remains essentially inactive, with a small ORS-like hair germ and rudimentary dermal papilla, separated by a thick basement membrane.[90] Having lost its anchorage, the old hair shaft, or hair club, may be readily shed.[87] Although the exact nature of the trigger to enter the next anagen cycle remains unknown, it is hypothesized that in response to signals from dermal papilla, epidermal stem cells in the bulge region are transiently activated to proliferate and repopulate the lower portion of the hair follicle ("bulge activation hypothesis").[86,91] Subsequently, dermal progenitors are recruited to the dermal papilla from reservoirs in the adjacent dermal sheath[88] likely in response to epidermal signals from secondary hair germ. As anagen proceeds, a similar cascade of mesenchymal-epithelial cross-talk that leads to the production of matrix cells, inner root sheath and hair shaft lineages during embryonic hair follicle morphogenesis is redeployed.[90]

Shh Signaling in Hair Follicle Development

A putative candidate for the first epidermal signal required for induction of the dermal condensate is Shh, expressed early in the developing placodes.[92,93] Although overexpression of Shh can induce ectopic placode formation in chick skin[94,95] Shh overexpression in embryonic mouse skin results in disorganized hyperproliferative epithelial growths.[96] Furthermore, hair follicle induction is initiated in *Shh*$^{-/-}$ mutants, but subsequently arrests, resulting in small placodes with rudimentary mesenchymal condensates.[1,3,97] These findings suggest that Shh signaling is not required for initial specification events, but plays an important role in subsequent signaling required for dermal papilla development[97] and/or hair follicle downgrowth.[3] Consistent with this, expression of Hh target genes *Gli1* and *Ptc1* is detected in both epidermal and mesenchymal compartments of hair follicles throughout development.[3,7] Rudimentary *Shh*$^{-/-}$ dermal papilla contain fewer PDGFR-α-positive mesenchymal cells and fail to express differentiation markers, such as alkaline phosphatase and *Wnt5a*, suggesting that Shh signaling is required for proliferation and differentiation of papilla fibroblasts.[3,97,98] Epithelial-epithelial Shh signaling is required to promote proliferation of epidermal cells in developing hair

follicles.[3,99] Furthermore, terminal differentiation of interfollicular keratinocytes is unaffected in *Shh*$^{-/-}$ mutants, while disorganized hair follicle structures express low levels of hair keratins, indicating that Shh signaling is likely not required for epidermal lineage determination, but may regulate expansion of hair follicle progenitors.[1,3,99] During the adult hair cycle, *Shh* is induced in secondary hair germs at the telogen-anagen transition where transient activation of *Ptc1* is observed in the bulge region.[100] As anagen proceeds, asymmetric *Shh* expression in distal matrix cells is believed to promote proliferation of hair progenitor cells. Shh induces precocious anagen from telogen follicles[2] while blocking Shh signaling results in telogen arrest of postnatal hair follicles with reversible hair loss.[4] Interestingly, a novel role for Hh signaling in the maturation of postnatal sebocytes has recently been reported and is likely mediated through Ihh.[101,102] Shh signaling plays an important role in expanding epidermal progenitors and promoting dermal papilla maturation, likely to enable other signaling pathways to specify commitment to hair follicle lineages during embryonic and adult hair follicle development.

A Fine Balance: Gli Activator and Repressor Functions in Skin Development

During mouse embryonic hair follicle development, Gli2 is the major activator mediating Shh responses in the developing skin.[99] The *Gli2*$^{-/-}$ follicle defect phenocopies the arrested follicular development of *Shh*$^{-/-}$ mutant mice, with reduced Shh target responsive gene expression and decreased cell proliferation. Through tissue-specific transgenic rescue experiments in mutant background, we have shown that activation of Gli2 function in the epidermis by Shh signaling is essential for embryonic hair follicle development. Although the development of dermal papillae is severely affected in Gli2$^{-/-}$ skin and Gli2$^{-/-}$ dermal fibroblasts fail to respond to Shh in vitro, restoration of Gli2 activity in the epithelium is sufficient to fully rescue hair follicle development in *Gli2*$^{-/-}$ skin. Despite phenotypic similarities, epidermal overexpression of *Gli2* does not rescue *Shh*$^{-/-}$ follicles, suggesting that Gli2 activator function is Shh-dependent. ΔNGli2, a mutant form of Gli2 lacking the N-terminal repression domain, activates Shh target gene expression constitutively both in vitro and in vivo.[49] Importantly, ΔNGli2, but not Gli2, can induce *Gli1* and *Ptc1* expression in *Shh*$^{-/-}$ skin and induce ectopic expression of *Gli1* and *Ptc1* in regions normally lacking Shh response (Fig. 3). These results establish that ΔNGli2 functions as a constitutive activator of Hh target genes in vivo. Similarly, in *Drosophila*, the activator function of Ci has been shown to be Hh-dependent; an uncleavable form of Ci, which is incapable of forming the repressor form, cannot induce the expression of target genes in the absence of Hh signaling.[24]

While these results demonstrate the Shh-dependent activator function of Gli2, it remains unclear whether Gli2 also acts as a repressor in the absence of Shh signaling in the skin. Interestingly, Gli1 can compensate for the lack of Gli2 function in *Gli2*$^{Gli1/Gli1}$ mice during embryonic development, but remarkably, adult *Gli2*$^{Gli1/Gli1}$ mice develop skin defects, including alopecia and ulcers.[57] The molecular mechanisms behind this adult skin phenotype await further investigation, it is plausible that Gli2 may possess a repressor function, which is lacking in Gli1, and this Gli2 repressor function plays a key regulatory role in adult hair cycle.

Although ΔNGli2 can activate target gene expression and restore cell proliferation in *Shh*$^{-/-}$ epithelium, the rescue of hair follicle development is not complete in *K5Cre; ZIAPΔNGli2; Shh*$^{-/-}$ skin. Restored activator function of Gli2 in *K5Cre; ZIAPΔNGli2; Shh*$^{-/-}$ epithelium may compete with high levels of endogenous Gli3 repressor function for target gene control, hindering hair follicle development. *Gli2* and *Shh* mutants, which are both deficient for Gli activator function, display a similar but distinct arrest of hair follicle development; the more severe defects in *Shh* mutants could be compounded by the additional excess of Gli repressor activity. Consistent with the notion that Shh signaling acts to inhibit the repressor activity of Gli3,[27,49] there is a dramatic rescue of *Shh* mutant defects in the developing neural tube,[69,73,103] face and forebrain,[71] as well as limbs,[72,74] when Gli3 repressor function is removed in *Shh*; *Gli3* mutants.

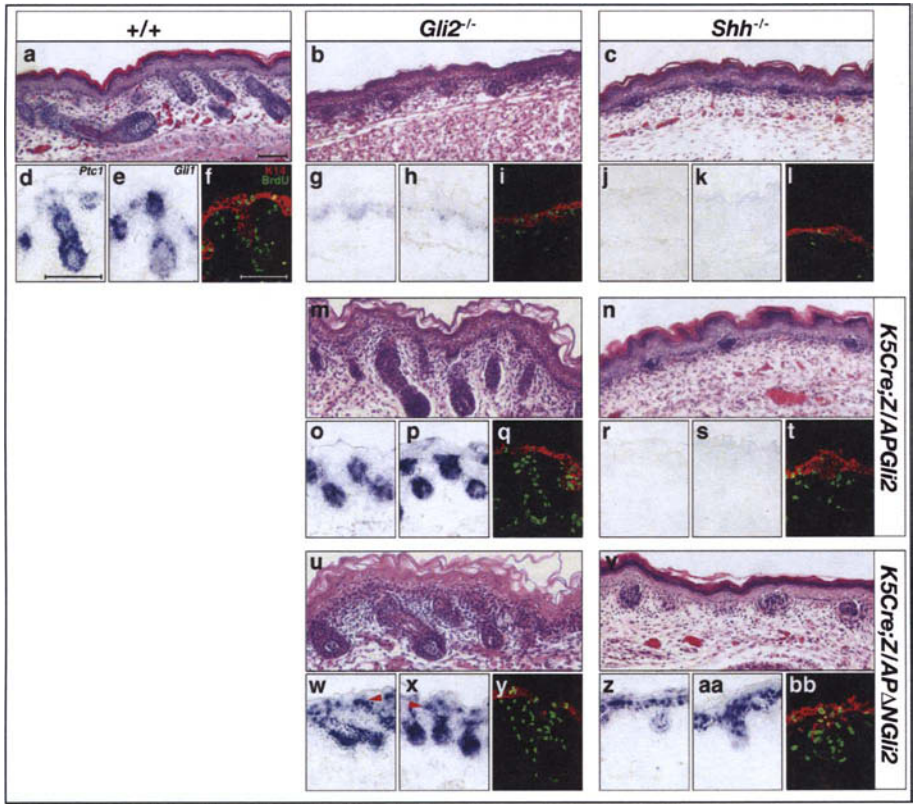

Figure 3. The activator function of Gli2 is required for hair follicle development and is Shh-dependent. Hematoxylin-eosin staining of dorsal skin sections from E18.5 wild type (a), *Gli2*$^{-/-}$ (b), *Shh*$^{-/-}$ (c), *K5Cre; ZlAPGli2; Gli2*$^{-/-}$ (m), *K5Cre; ZlAPGli2; Shh*$^{-/-}$ (n), *K5Cre; ZlAPΔNGli2; Gli2*$^{-/-}$ (u), and *K5Cre; ZlAPΔNGli2; Shh*$^{-/-}$ (v) mice. In situ hybridization analysis of Shh target gene expression, *Ptc1* (d, g, j, o, r, w, and z) and *Gli1* (e, h, k, p, s, x, and aa). Staining of BrdU (green) and K14 (red) in E18.5 wild type (f), *Gli2*$^{-/-}$ (i), Shh$^{-/-}$ (l), *K5Cre; ZlAPGli2; Gli2*$^{-/-}$ (q), *K5Cre; ZlAPGli2; Shh*$^{-/-}$ (t), *K5Cre; ZlAPΔNGli2; Gli2*$^{-/-}$ (y), and *K5Cre; ZlAPΔNGli2; Shh*$^{-/-}$ (bb) skin. Scale bars: 50μm. Reprinted with permission from: Mill P, Mo R, Fu H et al. Genes Dev 2003; 17(2):282-294. ©2003 CSHL Press Co.

To address the role of Gli repressor activity in the developing skin, we examined the development of vibrissae and pelage follicles in *Shh; Gli3* compound mutants. As seen in other systems, excess Gli3 repressor contributes to the *Shh* mutant skin phenotype, since removal of *Gli3* significantly alleviates the developmental arrest of *Shh* mutant follicles (Fig. 4, P.M. and C.c.H., manuscript in preparation). Transcription of early cell cycle regulators, including *N-myc* and *cyclin D2,* is derepressed in *Shh*$^{-/-}$; *Gli3*$^{-/-}$ mutants, contributing to the partial rescue of keratinocyte proliferation (P.M. and C.c.H., manuscript in preparation). However, transcription of late G_1 regulators is also Shh-dependent requiring additional Gli activator functions to allow for robust cell cycle progression and rescue of epithelial proliferative responses during hair follicle development. Our results suggest that Shh controls proliferation of the embryonic epidermis through a complex transcriptional cascade of multiple cell cycle regulators *via* both Gli-dependent gene activation and de-repression. There is growing support for a model where the balance of Gli activator and repressor functions determine Shh responses (i.e., the transcriptional read-out of Shh target genes). It has been shown that overexpression of a constitutive Gli3 repressor can override endogenous Shh signaling and associated activator

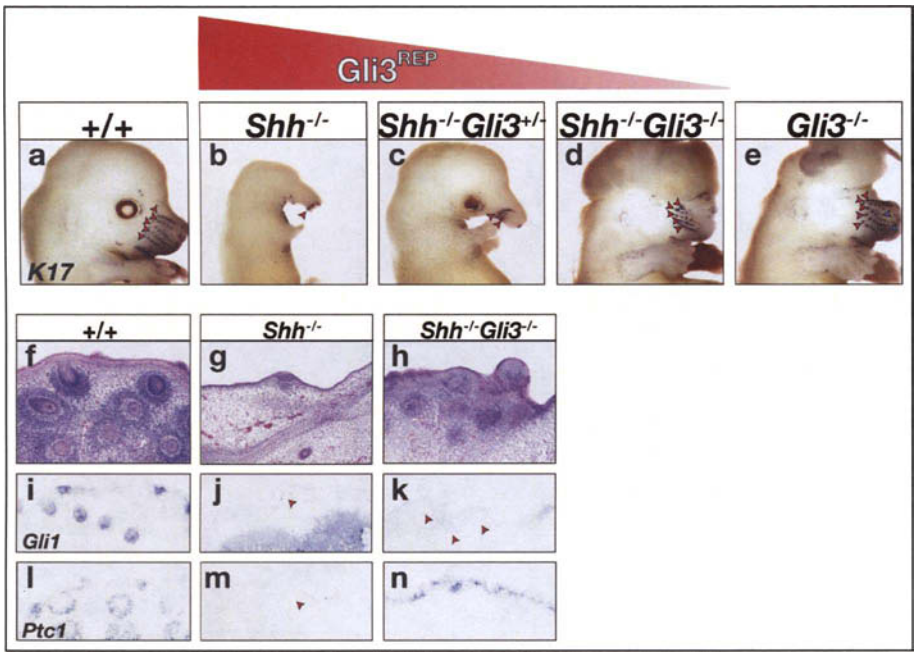

Figure 4. Arrest of vibrissae follicle development in $Shh^{-/-}$ mutants is due to excess Gli3 repressor activity. (a-e) *K17* whole-mount in situ hybridization of E14.5 embryonic vibrissae follicles (red arrowheads), in wild type (a), $Shh^{-/-}$ (b), $Shh^{-/-}$; $Gli3^{+/-}$ (c), $Shh^{-/-}$; $Gli3^{-/-}$ (d) and $Gli3^{-/-}$ (E), with ectopic tracts (blue arrowheads). Sections through E1.45 whisker pad of wild type (f,i,l), $Shh^{-/-}$ (g,j,m), and $Shh^{-/-}$; $Gli3^{-/-}$ (h,k,n) embryos. Expression of *Ptc1* (l-n) is derepressed in $Shh^{-/-}$; $Gli3^{-/-}$ vibrissae follicles, while expression of *Gli1* (I-k) is not affected.

function in dorsoventral patterning of the spinal cord.[73,104] Similarly, overexpression of a dominant repressor form of Gli2 (ΔCGli2) is sufficient to override endogenous Shh signaling and block Gli activator-directed hair follicle development.[101]

Dissecting Requirements of Signals and Deciphering Cross-Talk

The importance of interplay between the key developmental signaling pathways, such as *Wnt, Notch, BMP* and *Shh*, during skin development and homeostasis merits further investigation. Loss of *Notch1* in adult epidermis leads to upregulated *Gli2* expression and stabilization of nuclear β-catenin.[105] A yet unidentified Wnt signal is required to induce the placode expression of *Shh*,[106,107] which in turn induces *Wnt5a* expression in dermal papilla[98] during development and sustain high level *Wnt5a* expression in the stroma of BCCs.[108] Wnt signals can antagonize Hh responses through induction of *growth arrest specific gene 1* (*gas1*) expression, a glycosylphosphaditylinositol-linked membrane glycoprotein which binds Shh.[109] *Gas1* expression is induced surrounding developing epidermal appendages, such as teeth and vibrissae, in a domain distinct from the *Shh* source and partially overlapping with *Ptc1*.[109-111] Although the functional dependence on *Wnt* signaling in the developing mandible has yet to be demonstrated, *Gas1* expression may be key to sharpening Hh gradients allowing Hh responses to be periodically restricted developing teeth.[110] These signaling pathways individually play important roles in skin development and disease, but just how entwined the complex web of synergistic and antagonistic interactions between Shh and other key signaling cascades really has only begun to be understood.

Shh Signaling Plays a Conserved Role in the Development of Other Epidermal Appendages

The initial inductive steps in the morphogenesis of many epidermal appendages involves similar reciprocal epithelial-mesenchymal signals.[112] Vibrissae are large tactile follicles, which develop earlier than pelage (dorsal) follicles, in regularly patterned tracts in the mystacial pad. While the extent of vibrissae development in *Shh*$^{-/-}$ mutants is difficult to assess due to craniofacial defects,[1,3,99] cyclopamine treatment of wild type vibrissae cultures arrests their development[1] and ectopic expression of *Shh* can induce supranumary vibrissae follicles,[113] suggesting a role for Shh signaling in vibrissae follicle development. A proper balance of Gli activity is required to establish or maintain vibrissae tracts. *Gli2*$^{-/-}$ and *Gli3*$^{-/-}$ mutant vibrissae develop correctly (in contrast to *Gli2* mutant pellage follicle developmental arrest), albeit in reduced or expanded numbers, respectively.[99] Furthermore, a dosage dependent rescue of the vibrissae patterning defects in *Shh*$^{-/-}$ mutants is revealed by reducing Gli3 repressor function in *Shh*$^{-/-}$; *Gli3*$^{+/-}$ and *Shh*$^{-/-}$; *Gli3*$^{-/-}$ mutants, although the development and downgrowth of these structures is only partially restored (Fig. 4, P.M. and C.c.H., manuscript in preparation). Similar to the arrest of hair follicle development observed in *Shh*$^{-/-}$ mutants, both salivary gland and tooth development are also arrested at early rudiment stages.[114,115] Abnormalities in tooth development primarily arise from compromised planar (epithelial-epithelial) Shh responses, resulting in decreased epidermal proliferation and survival while differentiation proceeds unaffected.[114,116-119] Interestingly, while rudimentary teeth are observed in tissue-specific deletion mutants of *Shh* and *Smo*, a more severe phenotype is observed in *Gli2*$^{-/-}$; *Gli3*$^{-/-}$ which lack all signs of molar induction,[114,117,119] suggesting that both Gli activator and repressor functions may be involved in initial establishment and/or maintenance of these structures. In contrast, the development of some other epidermal appendages is less dependent on Shh signaling, as is the case with mammary glands and nails, likely due to redundant expression of *Shh* and *Ihh* such that target gene expression in these *Shh*$^{-/-}$ organs is unchanged.[120-123] However, a regulatory role for Hh signaling in postnatal mammary gland during pregnancy and lactation may still exist, as ductal dysplasias and hyperplasias spontaneously arise in *Gli2*$^{-/-}$ and *Ptc1*$^{+/-}$ mammary glands in response to different hormonal conditions.[124,125]

Shh: Stem Cells, Cell Cycle and Cancer

Regulation of Hh Responses Is Required for Maintenance of Adult Tissues

Deregulated Hh signaling has been reported in various human cancers of the brain (medulloblastomas, glioblastomas), muscle (rhabdomyosarcomas) and skin (BCCs), where Hh signaling may be normally required for proliferation and maintenance of specific adult stem cells.[126,127] Recently, elevated Hh responses have also been implicated in tumors arising from the upper gastrointestinal tract, including lung and pancreas, as well as from the prostate epithelium, where Hh signals likely play similar regenerative functions.[128-133] In response to physiological and stress stimuli, Shh signaling is likely required for progenitor cell expansion and tissue renewal, such as in the skin. However, tumors can be induced when these processes are not tightly regulated. Importantly, sustained Hh signaling is essential for tumor maintenance as specific inhibition of Hh responses with cyclopamine is sufficient to block tumor growth.[128-131,133-138]

Epidermal Stem Cells and Cancer

An important characteristic of postnatal skin and its appendages is that they are constantly turning over; keratinocytes are continually challenged by damaging environmental factors (UV irradiation, pathogens and chemical carcinogens) and physical stresses, which require ongoing repair. By virtue of its self-renewal capacity, epidermal stem cells can divide asymmetrically to sustain the pool of self-renewing cells and generate daughter cells that can terminally

differentiate to replenish lost keratinocytes.[86,139,140] Physically sheltered in the bulge region of the hair follicle, multipotent stem cells within this niche can repopulate all three epidermal progenitor lineages.[141,142] As long-term residents of the epidermis, epidermal stem cells are the most likely targets of skin carcinogenesis because they accumulate multiple genetic mutations that may be required for tumor formation.[140] However, in some cases, disturbing epidermal homeostasis by disrupting the stem cell compartment may also result in tumorigenesis. A tight regulation of when epidermal progenitors are competent to respond to periodic Shh signals ensures that proliferative responses are regionalized and restricted to hair follicle morphogenesis and anagen phase of the adult hair cycle (Fig. 6).[100,143,144] In contrast, deregulated activation of Hh responses in mouse skin is sufficient to induce hair follicle-derived tumors that resemble highly proliferative and undifferentiated masses of epidermal progenitor cells without secondary mutations.[96,145-148]

Basal cell carcinomas (BCCs) are the most common human malignancy and its incidence continues to increase.[149] Inactivating mutations in the human *patched* (*PTCH1*) gene were initially identified as the cause of nevoid basal cell carcinoma syndrome (NBCCS) which predispose affected individuals to the development of multiple BCCs.[8,9] Mutations in *PTCH1* have since been linked to sporadic human BCCs and other hair follicle derived neoplasias.[6,150] These studies provided the first molecular clues to the pathogenesis of these hair follicle derived tumors. Overexpression of Shh throughout the embryonic murine epidermis results in early BCC-like lesions and disturbances in hair follicle development,[96,143,151] emphasizing the importance of restricted Shh signal reception. A loss of temporal and spatial regulation of Shh signaling can lead to the formation of multiple tumors types, including BCCs.[5] In humans and mice, activated Hh responses in the skin as a result of loss-of-function *Ptc1* mutations or gain-of-function *Smo* mutations result in hair follicle-derived tumors.[146,148]

The downstream Gli transcriptional mediators of Hh signals likely play a key role in the development of skin tumors (Table 3). A hallmark of BCCs is high level expression of Hh target genes *Gli1* and *Ptc1*, in the absence of *Shh* signal (Fig. 5).[7,152] Epidermal overexpression of human *Gli1* in transgenic mice alone is sufficient to promote spontaneous tumor formation which resembles human BCC and other hair follicle neoplasias, including cylindromas and trichoblastomas.[147] Transgenic overexpression of *Gli2* in the epidermis results in spontaneous formation of BCCs,[145] while upregulation of endogenous *Gli2* in the skin, such as is observed in epidermal-specific *Notch1*$^{-/-}$ mice, also induces BCC formation.[105] Unlike Gli1, which lacks the N-terminal repression domain and functions as a constitutive transcriptional activator, Gli2 requires Shh-dependent activation to promote Hh target gene expression and responses.[49,99,144] Given the differences in tumor phenotypes between *K5Gli1* and *K5Gli2* mice, we hypothesized that the oncogenic potential of Gli2 requires a Shh-dependent activation trigger. As the expression of Shh in the adult skin is limited to hair follicles during the telogen-anagen transition,[100] conversion to Gli2^{ACT} in *K5Gli2* keratinocytes would be spatially and temporally restricted, such that hyperproliferation and transformation events yielding BCC progenitors would occur less frequently. In contrast, *K5Gli1*-overexpressing keratinocytes are ligand-independent, in that their activity is no longer limited to where and when Shh is expressed during the hair cycle, and could therefore target wider tumor progenitor populations.

To determine whether the difference in oncogenic potentials between Gli1 and Gli2 lay in their Shh-dependent activity, we have examined the effect of Gli2 and ΔNGli2 overexpression on epidermal homeostasis and tumorigenesis using 3 transgenic mouse models; *K5Gli2* which develop BCCs[145] and the previously uncharacterized adult phenotypes of *K5Cre; ZIAPGli2* and *K5Cre; ZIAPΔNGli2* mice.[99] The Keratin5 (*K5*) promoter drives transgene expression in the basal layer of the skin and hair follicles, targeting compartments believed to contain epidermal stem cells. Observations from these studies support three emerging themes of Gli activities in epidermal homeostasis.

Table 3. Mouse models of Hh-induced skin tumorigenesis. Additional table references: 207-229

Gene	Tissue	Transgene/ Mutant	Hh Status	Effect	Refs.
Shh	Basal epithelia	*K14-Shh*	Elevated Hh ligand, overwhelms negative regulation	Embryonic BCC-like tumors; perinatal lethality limits tumor studies	96
Ptc1	Global	*Ptc1$^{lac/+}$*	Decreased negative regulation of Hh responses	Low frequency spontaneous trichoblastomas and BCCs; Increased induction of BCCs and rare SCCs on irradiation treatment	5
Ptc2	Global	*Ptc2$^{TM1/TM1}$*	Decreased negative regulation of Hh responses	Male-specific alopecia and ulcered lesions develop with age	E.N. and C.c.H. unpubl.
Smo	Basal epithelia	*K5-M2SMO*	Ligand-independent activation of Hh responses	BCC-like proliferations in neonatal skin; perinatal lethality limits tumor studies	148
	Basal epithelia	*ΔK5-M2SMO*	Ligand-independent activation of Hh responses	Benign basaloid follicular hamartomas	146
Gli1	Basal epithelia	*K5-Gli1*	Unregulated GliACT	Spontaneous BCC-like tumors	147
	Basal epithelia	*K14-Gli1*	Unregulated GliACT, may be subject to endogenous Hh signal	HF tumors during anagen	100
Gli2	Basal epithelia	*K5-Gli2*	Increased Gli species, subject to endogenous Hh signals	Spontaneous BCC and basaloid follicular hamartomas; slower, milder tumor phenotype than Gli1 or DNGli2 overexpression	145,146
	Basal epithelia	*K5-Cre; Z/AP-Gli2*	Increased Gli species, subject to endogenous Hh signals	Spontaneous BCC and basaloid follicular hamartomas; slower, milder tumor phenotype than Gli1 or DNGli2 overexpression	P.M. and C.c.H. unpubl.
	Basal epithelia	*K5-ΔNGli2*	Unregulated GliACT	Spectrum of HF-derived tumors (BCCs, trichoblastomas, cylindroma, trichoepithelioma) ; rapid, aggressive tumor phenotype	162
	Basal epithelia	*K5-Cre; Z/AP-ΔNGli2*	Unregulated GliACT	Spectrum of HF-derived tumors (BCCs, trichoblastomas, cylindroma, trichoepithelioma) ; rapid, aggressive tumor phenotype	P.M. and C.c.H. unpubl.
	Basal epithelia	*K5-tTA; TRE-Gli2*	Inducible elevated Gli species, subject to endogenous Hh	Spontaneous BCCs requiring continuous Hh signaling for growth, transgene inactivation results in tumor regression	144

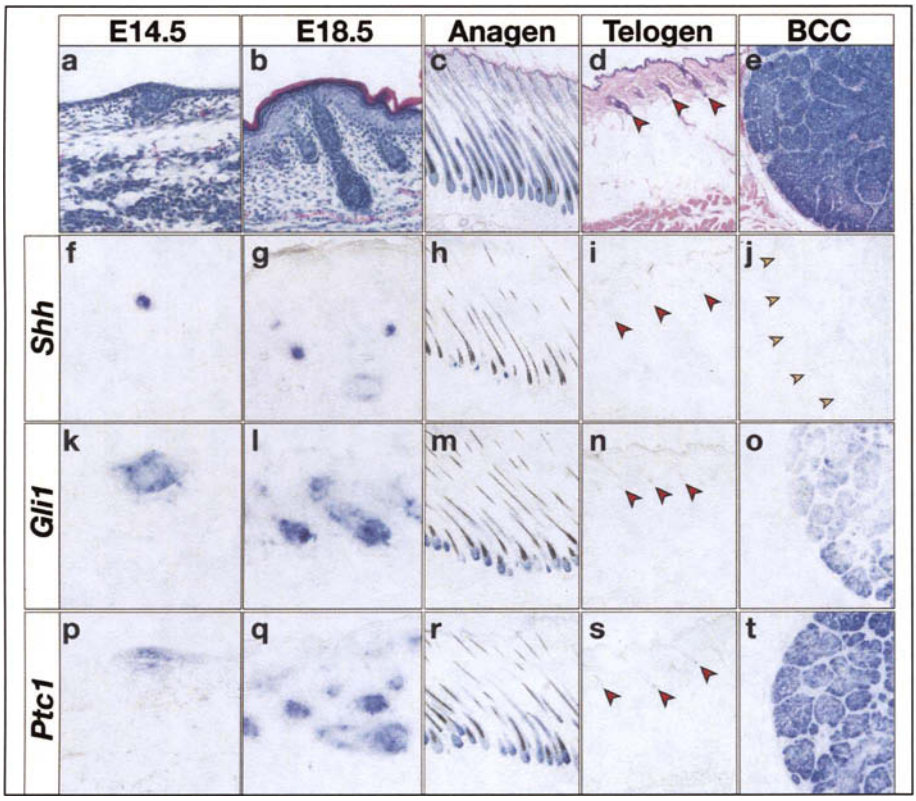

Figure 5. Ligand-dependent Hh target gene expression during embryonic and adult hair follicle development is restricted to where and when Shh is expressed, whereas ligand-independent target gene expression is observed in BCCs. (a-e) Hematoxylin and eosin. (f-j) *Shh* ligand expression. Target gene expression *Gli1* (k-o) and *Ptc1* (p-t). Throughout embryonic hair follicle morphogenesis (a,b,f,g,k,l,p,q) and adult anagen (c,h,m,r), *Shh* is expressed asymmetrically in the distal tip of the epidermal compartment, while expression of target genes is detected more broadly in both epidermis and mesenchyme. In telogen follicles (red arrowheads), no *Shh* expression is detected (i) and no expression of target genes (n,s) is observed at this resting phase. Similar to an embryonic hair germ, BCC tumor nests are composed of tightly packed, basaloid cells (e) expressing high levels of *Gli1* (o) and *Ptc1* (t) without expression of ligand in tumor islands (periphery marked by yellow arrowheads).

Ligand-Dependent versus Ligand-Independent Models of Gli2 Epidermal Tumorigenesis

Ligand-independent mutational activation of the Hh pathway, such as loss of function *Ptc1* or gain-of-function *Smo* mutations, is commonly linked to the formation of ectodermal tumors, including medulloblastoma and BCCs.[8,9,148,153-157] Alternately, ligand-dependent mechanisms lead to the formation of endoderm-derived tumors, such as small cell lung carcinoma, pancreatic and prostate cancer, where tumors express high levels of Hh ligand and target gene activation.[129-133,158] Results from our transgenic models suggest that differences in the oncogenic potential of Gli1 and Gli2 in vivo are due primarily to ligand-independent and ligand-dependent transcriptional activity, respectively. While *K5Gli2* and *K5Cre; ZlAPGli2* animals develop predominantly slow growing BCCs, *K5Cre; ZlAPΔNGli2* and *K5Gli1* animals develop heterogenous tumor phenotypes, with diverse tumors developing early and progressing aggressively. Deletion of the N-terminal repressor domain of Gli2, which is lacking in Gli1,

removes the Shh-dependent activation requirement for Gli2 transcriptional activator function, rendering it ligand-independent in vivo[99] and a more potent oncogene in these studies.

Gli2 overexpression results in predominantly BCCs and basaloid follicular hamartomas, while ΔNGli2 animals develop a wider range of malignant hair follicle derived tumors, trichoblastoma and cylindroma, in addition to BCC. Are these transgenes capable of transforming different target cells or do they induce different tumor cell fates in the same populations of progenitor cells? Despite differences in tumor phenotypes, the tumors that arise in both Gli2 and ΔNGli2 models are negative for markers of terminal differentiation. No tumors of differentiated hair follicle cell types, such as trichofolliculomas or pilomatricomas, which are derived from the hair shaft and inner root sheath lineages, respectively, were observed in ΔNGli2 animals. Moreover, marker analysis suggests that all tumors which develop in *K5Cre; ZlAPGli2* and *K5Cre; ZlAPΔNGli2* animals arise from the undifferentiated outer root sheath, indicating that both oncogenes likely target the same population of multipotent progenitor cells. Alternately, hyperactivation of Hh response genes by either Gli2 or ΔNGli2 could induce a (de)differentiation programme characteristic of outer root sheath progenitor cells in other cell lineages. Indeed, hair follicle derived basaloid tumor nests develop in the interfollicular epidermis of *K14GLI1*mice[100] and *ΔK5M2SMO* mice.[146] One of the earliest molecular alterations detected in both *K5Cre; ZlAPGli2* and *K5Cre; ZlAPΔNGli2* animals is the reexpression of *K17* in adult interfollicular epidermis, where it is normally extinguished in late gestation.[159] In contrast, K6, a marker for hyperproliferation was not observed. Thus, deregulated Hh activation throughout the epidermis appears to result in expansion of epidermal progenitor characteristics in *K5Cre; ZlAPΔNGli2* animals.

The Level of Gli Target Gene Activation Is Correlated to Tumor Phenotype

The level of Hh cascade activation is correlated to malignant potential of tumors in our transgenic models. The slower and less aggressive growth characteristics of *K5Gli2* and *K5Cre; ZlAPGli2* tumors correlates with low level activation of Hh target genes, including *Ptc1* and *CyclinD1* (Fig. 6). In *K5Cre; ZlAPΔNGli2* animals, more rapidly growing and locally destructive tumors express high levels of target gene activation. The magnitude of Hh responses likely plays a key role in determining the tumor phenotype: benign, indolent hamartomas which form in *ΔK5M2SMO* mice[146] and *K5Gli2* animals express very low levels of *Ptc1* and no *Gli1*, while benign trichoblastomas express high *Ptc1* and low *Gli1* and malignant BCCs express high *Ptc1* and high *Gli1*in *K5Cre; ZlAPΔNGli2* animals. The level of Hh signaling may similarly determine the phenotype of human lesions; human hamartomas express low levels *PTCH1* and undetectable *GLI1*,[146] while human BCCs express high levels of *PTCH1* and *GLI1*.[7]

Mouse models that disrupt Hh signaling in the skin are particularly interesting, given that most mouse strains are resistant to BCC development. Treatment of inbred strains with UV irradiation or topical chemical carcinogens readily induces cutaneous papillomas and SCCs, but rarely BCCs.[5,160,161] These findings suggest that there is something particular about hyperactivation of Hh responses in humans and mice that predisposes affected skin to spontaneous development of follicle-derived tumors.

Epidermal Hyperproliferation May Require Additional Inputs for Tumorigenesis to Occur

Hyperproliferation without compensating apoptosis was observed in *K5Gli2, K5Cre; ZlAPGli2* and *K5Cre; ZlAPΔNGli2* animals (P.M. and C.c.H. unpublished results), perhaps due in part to upregulated expression of the anti-apoptotic gene, *bcl2* (data not shown).[145,162] *Bcl-2* may be a direct Hh target gene as several Gli-binding sites have been found in its promoter, and Gli-dependent induction of *bcl-2* occurs in cultured keratinocytes and mouse skin.[163,164] High proliferation rates without cell cycle specific checkpoints and correcting apoptotic events increase the likelihood of secondary mutation events. Deregulated Hh signaling in human patients appears to synergize with other genetic lesions to promote the development of BCCs.

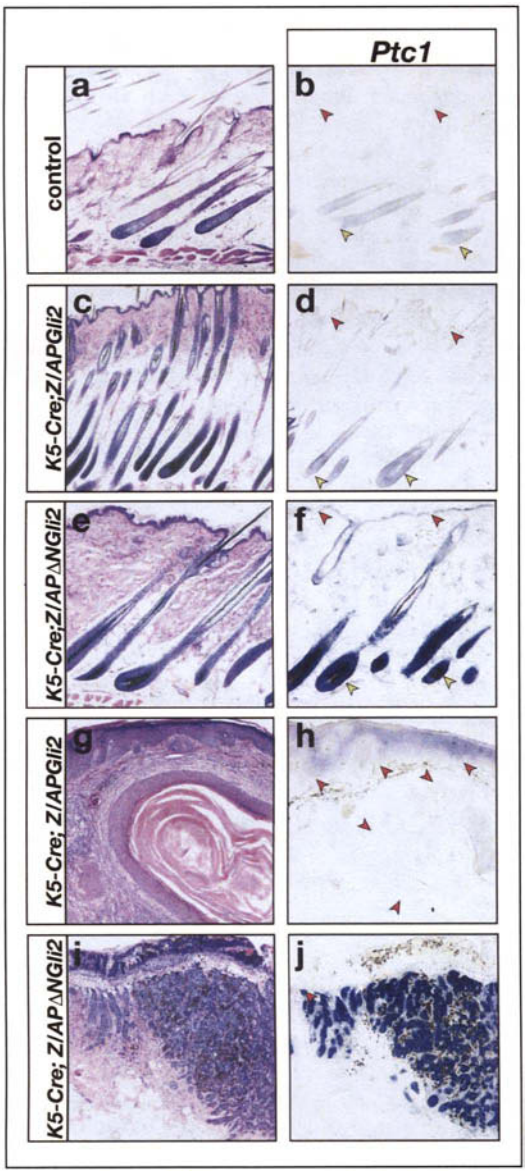

Figure 6. Higher levels of canonical Hh target induction genes in K5Cre; Z/APΔNGli2 skin and tumors are associated with more aggressive tumor phenotype. (a,b) In 4 mos *Z/APΔNGli2* control mouse skin, most hair follicles are in late anagen/early telogen so there is no expression of *Shh* (data not shown) or little/no expression of target *Gli1* (data not shown) and *Ptc1* (b). Endogenous *Gli2* levels are low (data not shown). (c,d) In 7 mos *K5Cre; Z/APGli2* dorsal skin, most hair follicles are not in anagen; there is no expression of *Shh* (data not shown) and no significant change in target gene expression (d), despite higher *Gli2* levels (data not shown). (e,f) In *K5Cre; Z/APΔNGli2* dorsal skin, hair follicles have deep broader bases characteristic of anagen follicles (e), despite no expression of *Shh* (data not shown). However, expression of constitutively active ΔNGli2 is sufficient to induce high *Ptc1* levels (f). (g,h) A slow-growing *K5Cre; Z/APGli2* ear tumor expresses low levels *Ptc1* (h), although no *Shh* is detected (data not shown). (u-y) Rapidly growing pigmented BCC in the *K5Cre; Z/APΔNGli2* animal (i,j), expresses similar levels of transgenic *Gli2* but much higher levels of target genes (j), independently of *Shh* expression (data not shown). Red arrowheads indicate interfollicular epidermis where Hh target genes are usually not expressed, while yellow arrowheads indicate hair follicle base, the source of Shh signal.

There is a high frequency of both *PTCH* and *TP53* mutations in both sporadic and inherited forms of BCCs.[165] However, the requirement for a "second genetic" hit in the mouse models of BCC are less clear. While similar *p53* mutations were identified in 40% of UV-treated $Ptc^{+/-}$ BCCs, these mutations did not appear to correlate with tumor aggression.[5] Mutational analysis of *p53* and *Ha-ras* genes did not reveal any mutations in known hotspots in *K5Gli1* tumors.[147] Additionally, the rapid rate of spontaneous tumor induction in *K5Cre; Z/APΔNGli2* mice and high tumor incidence in both *K5Cre; Z/APGli2* and *K5Cre; Z/APΔNGli2* animals argues against the requirement for additional mutation events in these tumor models.

Synergism with other growth factor signaling relays during developmental and disease processes is another recurrent theme emerging studies of Hh-related tumors. One such important

growth regulatory system is the PDGF/PDGFRα signaling relay. During embryonic hair follicle development, Shh signals are required to maintain high *PDGF-A* expression in the placode and drive proliferation of mesenchymal PGDFR-α-positive fibroblasts into developing dermal papillae.[97] In human and mouse BCC-like tumors, high levels of *PDGF-A* are observed in tumor islands, while *PDGFRα* is elevated in tumor stroma and surrounding basaloid nests (P.M. and C.c.H. unpublished data).[166] Furthermore, overexpression of Gli1 induces PDGFRα expression and stimulates its activation, driving strong proliferative responses in culture.[166] Blocking PDGFRα activity in a *Ptc*$^{-/-}$ BCC cell line is sufficient to significantly slow rates of proliferation. Maintenance of Hh-induced proliferation is likely an important function for PDGF receptor/ligand in the skin. Another good candidate is the insulin-like growth factor signaling relay (IGF/R), which is transcriptionally upregulated in various Hh-dependent tumors and required for induction of medulloblastomas in *Ptc*$^{+/-}$ mice.[8,167,168] While IGF2 appears to play a key role in proliferation of epidermal progenitors during hair follicle development and adult hair cycle,[169-172] and its expression can be induced by Gli1 overexpression in keratinocytes,[164] a synergistic relationship with Hh signaling in the skin has yet to be investigated. Together with high Shh signaling, activation of PI3-kinase activity through these growth factor receptors may inhibit GSK-3β targeting of labile Shh-induced cell cycle regulators for degradation and even affect Gli activity directly (P.M. and C.c.H., unpublished data).[18,173-176]

Hh Signaling and Cell Cycle Control: Tipping a Balance Towards Tumorigenesis?

In various developmental contexts, Hh signals have been linked to proliferative responses in target cells. Quiescent cells can be stimulated to enter the cell cycle in response to mitogenic signals, such as Shh, which induce the expression of D-type cyclins required to pass the G_1 restriction point. One potential mechanism for deregulated Hh signaling to induce tumorigenesis is through this influence on cell cycle control.[127] Hh signaling plays a conserved developmental role in controlling proliferation and patterning of the lung,[61,177,178] epidermal appendages,[3,94,99] muscle progenitors,[59,179,180] foregut[181,182] and brain.[134,183-185] In adult animals, Hh signals are additionally required for the proliferation of *Drosophila* ovary stem cells,[186] forebrain neural stem cells,[187-190] and mammalian hematopoeitic stem cells.[191] Despite its clear importance in controlling proliferation during development and disease, the molecular mechanisms by which Hh signals induce mitogenic responses in target cells are finally coming to light. In neural progenitors, Shh promotes proliferation through induction of D-type cyclins, although this effect is not sufficient to recruit cells into the cell cycle after arrest.[192] Similarly, Hh signaling in developing endochondral bone is required to promote proliferation of chondrocytes, in part through induction of *CyclinD1*.[193] Hh signals can also inhibit growth arrest via cell cycle inhibitor p21 and override terminal differentiation stimuli in cultured keratinocytes.[194] Interestingly, Ptc1 may directly participate in the G2-M checkpoint, as Ptc1 sequesters CyclinB1 at the membrane to inhibit cell proliferation in the absence of Hh ligand.[195] Binding of Shh to Ptc1 releases CyclinB1, allowing it to translocate into the nucleus to promote its M-phase-promoting-factor (MPF) activity.

How Gli functions mediate these Shh-mitogenic signals is less clear. In *Drosophila*, Hh signaling induces expression of *CyclinD* and *CyclinE*, where direct Ci binding to the *CyclinE* promoter mediates Hh's ability to induce DNA replication.[196] Aside from evidence for Gli-dependent regulation of *cyclinD1* and *cyclinD2*, there are few reports as to what additional Gli-dependent factors could be mediating cell cycle choices in response to Hh signaling in mammals (Fig. 7).[59,99] Our studies provide a molecular mechanism where Gli activator and repressor functions control multiple key regulatory decisions in the cell cycle. Gli3 repressor function plays an important role in ensuring early mitogenic targets, such as *N-myc* and *CyclinD2*, are not expressed in the absence of Shh-dependent growth signal. By removing Gli3 repressor activity in *Shh* mutants, it is sufficient to lift negative regulation, allowing for basal expression of some early cell cycle targets, including *cyclinD* and *N-myc*. Importantly, we have shown that

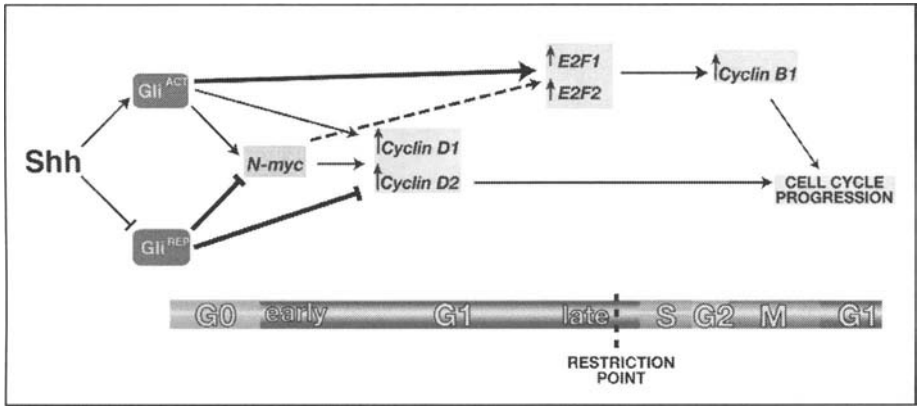

Figure 7. A model for Gli mediated Shh target gene expression controlling proliferation in developing hair follicle. Shh signaling controls cell cycle progression in the developing epidermis through transcriptional regulation of multiple cell cycle regulators. Although *Shh*$^{-/-}$; *Gli3*$^{-/-}$ and *Gli2*$^{-/-}$ follicles are partially rescued with respect to the expression of early cell cycle regulators, including *N-myc* and *CyclinD2*, they both lack Gli activator function that is essential for cell cycle progression through transcriptional control and post-transcriptional stabilization of later cell cycle regulators.

N-myc protein is not stabilized in *Shh*$^{-/-}$; *Gli3*$^{-/-}$ skin, suggesting that there are additional levels of control that require Shh-dependent Gli activator function. This result is consistent with a recent report that proliferation in cerebellar neuronal precursor cells requires both Shh-mediated transcriptional activation and phosphatidylinositol-3-kinase (PI-3K) mediated inhibition of N-myc degradation.[174] Furthermore, we found robust proliferation requires progenitors to move concertedly through the cell cycle, which involves the switch from repressor to activator E2Fs to activate G_1/S specific genes at the restriction point. This progression depends on sustained expression and/or their accumulation which additionally requires Shh-dependent Gli activator function (P.M. and C.c.H. manuscript in preparation).

During embryonic and adult hair follicle development, *Shh* induces proliferation of hair follicle progenitors through induction of a transcriptional hierarchy of cell cycle regulators, including *N-myc, CyclinD1, CyclinD2* and *E2Fs*. In contrast, hair follicles lacking *Shh* do not express these mitogenic markers, as is the case with arrested *Shh*$^{-/-}$ mutant hair follicles and adult telogen follicles, which show no significant proliferation (P.M. and C.c.H. unpublished results). Similar expression profiles of cell cycle target genes to those observed in anagen hair follicles are detected in *K5Cre; ZIAPGli2* and *K5Cre; ZIAPΔNGli2* tumors, where the level of expression of these genes correlates with the tumor proliferative index. It was recently reported that overexpression of Gli2 induces rapidly expression of similar mitogenic targets, including *CyclinD1* and *E2F1*, sufficiently to override growth arrest in contact inhibited keratinocyte cultures.[197] Hyperproliferative changes were observed early in *K5Cre; ZIAPGli2* and *K5Cre; ZIAPΔNGli2* skin prior to induction of tumors, including upregulated expression of *E2F1* (P.M. and C.c.H. unpublished results). All three epidermal lineages appeared to be sensitive to hyperactive Hh responses as shown by thickening of the interfollicular epidermis, sebaceous gland enlargement and alterations in hair follicle orientations. However, only hair follicle-derived tumors develop in *K5Cre; ZIAPGli2* and *K5Cre; ZIAPΔNGli2* skin. These results suggest that deregulated Hh signaling can act as a potent mitogen for multiple epidermal lineages, but its transforming ability is limited to progenitors destined for hair follicle fates.

Similar Shh-dependent Gli activator and repressor functions likely regulate proliferation at multiple cell cycle checkpoints in adult skin, where Shh proliferative responses are tightly controlled during hair cycle and deregulated during tumorigenesis. Furthermore, given that

Shh leads to induction and stabilization of *N-myc* associated with increased proliferation of granule cell precursors and medulloblastomas,[174,198] our results could provide a proximal "missing link" to these processes through opposing activities of the Gli transcription factors. The epidermis is an ideal developmental system to address these issues, as Shh plays primarily a proliferative role (P.M. and C.c.H. manuscript in preparation).[3,99,194,199] Understanding the transcription and stabilization profile of target cell cycle regulators controlled by Gli activator and repressor functions will provide important insights into many developmental and disease states, as well as potential therapeutic targets for treatment of pathological conditions where Hh signaling is deregulated.

Conclusions

Using a genetic approach complementing loss-of-function and gain-of-function mouse mutants, we have been able to dissect temporal and spatial requirements for Shh signaling in the skin. During hair follicle morphogenesis, Shh signaling is primarily required by the epidermis to promote proliferation of epidermal progenitors. Two important functions of Shh signaling are required for mitogenic responses to occur; (1) it promotes the generation of Gli activators, especially $Gli2^{ACT}$ and (2) it inhibits the generation of Gli repressors, primarily $Gli3^{REP}$. Interpreted by the cell as a "Gli code",[13] the summation of these Gli activities determines the transcriptional activity of target genes and ultimately, the decision to enter the cell cycle and proliferate. These studies have revealed novel Gli-dependent regulation of a transcriptional hierarchy of key cell cycle regulators, including the *D-type cyclins, N-myc* and *E2Fs*. Moreover, it has unveiled functional differences in how these target genes are regulated by Gli proteins. Expression of early cell cycle markers was derepressed in *Shh; Gli3* double mutants, but induction of later regulators, namely activator *E2Fs*, could not be induced without Gli^{ACT}. Consequently, robust proliferative responses were not observed. These results suggest that Shh signaling controls the cell cycle at multiple levels, to ensure that proliferative responses are restricted in time and space to regions of high Hh signal. The Hh-dependent balance of Gli activator and repressor functions also plays a key role in limiting proliferative responses to adult hair follicles at the telogen-anagen transition. Disturbing this balance, either by increasing the magnitude of Hh responses transiently at anagen in *K5Cre; Z/APGli2* mice or by extending Hh responses independently of the hair cycle in *K5Cre; Z/APΔNGli2* mice, is sufficient to overwhelm endogenous regulation. These changes disrupt epidermal homeostasis, triggering hyperproliferative responses and tumors in transgenic skin.

There appear to be distinct classes of target genes that are differentially regulated by Gli activator and repressor functions (Fig. 1). Mutant analysis has revealed that certain genes are induced by Gli2, while expression of other targets is inhibited by Gli3 in the embryonic skin. As seen in the developing neural tube,[69] expression of *Ptc1*, but not *Gli1*, is derepressed in *Shh; Gli3* vibrissae and pelage follicles. The expression of *N-myc* and *cyclinD2* is also derepressed in the developing hair follicles by removing Gli3 repressor activity in *Shh* mutants. Since expression of *Ptc1*, *N-myc*, and *cyclinD2* can also be induced by Gli activator function (P.M. an C.c.H. manuscript in preparation),[99] these genes represent a class of Shh targets that are regulated by both Gli activator and repressor. In contrast, the transcription of other Shh target genes, such as *Gli1* and *Wnt5a*, seem to be regulated solely by Gli activator function. Furthermore, it is possible that there is a third class of Shh target genes, which are regulated solely by Gli repressor. Shh can induce target gene transcription through promoting the Gli activator function and/by inhibiting the Gli repressor function. Our genetic studies in skin have revealed a Gli-dependent transcriptional cascade of cell cycle regulators in vivo, the next step will be to determine how these targets are directly or indirectly regulated by Gli activities.

An interesting aspect of the Hh intracellular response is the parallel induction of a positive transcriptional feedback loop through *Gli1,* which propagates and amplifies Hh transcriptional responses, and a negative signaling feedback loop through *Ptc1* and *Hip1* (Hedgehog binding protein) which serve to dampen Hh signaling at the cell surface (Fig. 8).[175,200]

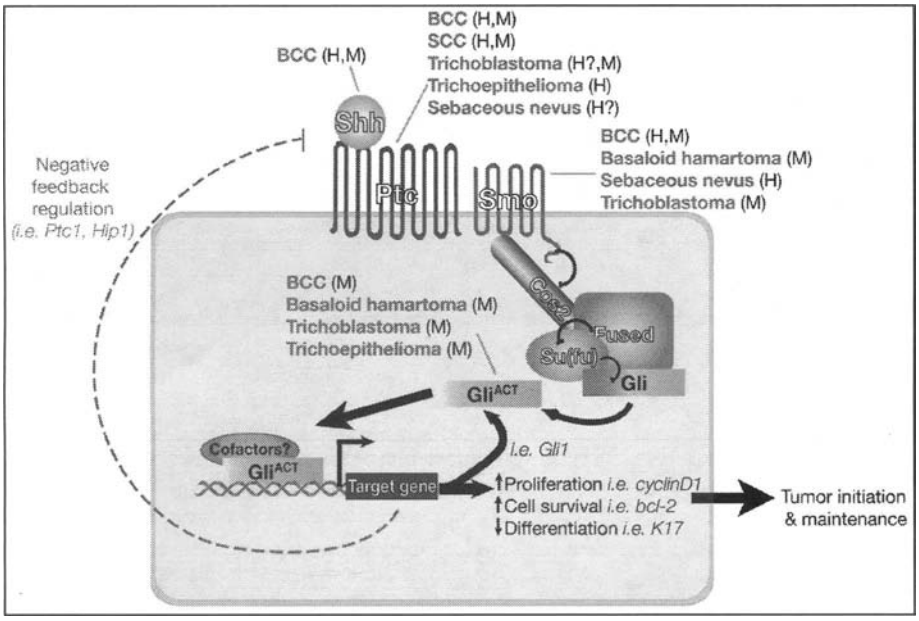

Figure 8. Hedgehog signaling and skin cancer. Alterations in various components of the Hh signaling cascade can result in both human (H) and mouse (M) skin tumors, by either inactivating negative regulators (i.e., *Ptc*) or deregulating positive factors (i.e., *Smo*). Such conditions result in the accumulation of Gli activator species, which drive expression of cell cycle regulators (i.e., *D-type cyclins*), survival factors (i.e., *bcl-2*) and stem-cell like characteristics (i.e., *K17*) required to support tumor initiation and maintenance. Hh signals induce expression of another set of target genes, including *Ptc1* and *Hip1*, which compose a negative feedback loop at the distal end of the pathway. However, once a tumor is established, it has become ligand-independent through the autoregulation of *Gli1* and leads to uncontrolled proliferation.

The interplay between these feedback loops create a "genetic switch", whereby a specific developmental decision is made, such as choice of cell fate, at a distinct threshold of Shh signal.[200] In disease settings where the negative regulatory loop is compromised, such as reported truncated or dominant-negative *Ptc* mutations,[201-204] the system may be incapable of turning itself "OFF" resulting in unregulated Hh responses and unchecked proliferation. Accordingly, high levels of negative feedback components *Hip1* and *Ptc1* expression are detected in mouse and human BCCs.[108] Alternately, increasing Gli activator species either artificially in transgenic mouse models (P.M. and C.c.H., manuscript in preparation)[145,162] or by unclear mechanisms in human skin[205] may overwhelm the endogenous negative feedback loop in a ligand-independent hyperactivation of Hh responses and proliferation. Consideration of these regulatory loops needs be incorporated into future therapeutic strategies for the prevention and treatment of Hh-related cancers. Initial reports of upstream antagonists, such as those opposing Smoothened functions including cyclopamine[136] and small molecule inhibitor CUR161414,[138] show effective BCC lesion regression with minimal side effects in animal and clinical trials. However, it is possible that some Hh-dependent tumors, such as those resulting from overexpression of Gli activator species in mice or loss of proximal negative regulator Su(Fu) in some human medulloblastoma patients,[206] will be refractory to these distal treatments that have little impact on limiting the hyperactive positive Gli feedback loop.[131] An effective alternate strategy could be to target this feedback loop directly, such as with *Gli1* RNA interference.[130] If the balance of Gli activity is ultimately the deciding factor in developmental and disease choices within a cell, better understanding of the molecular mechanisms which determine this set point of Gli activator and repressor levels needs to be the focus of future studies.

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CHAPTER 9

Shh Expression in Pulmonary Injury and Disease

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Abstract

The Hedgehog signalling pathway is crucial for normal vertebrate growth and development. Recent studies would suggest that signalling capability is retained in the post-embryonic organism. Shh signalling has been identified in the adult immune system, participating in $CD4^+$ T lymphocyte activation. Studies on fibrotic pulmonary disorders have demonstrated Shh in both human and mouse lung restricted to areas of active disease. Acute lung injury has also shown upregulated expression and together this data is highly suggestive for a functional role for Shh signalling in adult lung injury and disease. We propose that hedgehog signalling may contribute to epithelial injury and repair and act as an intermediary in cross-talk between damaged epithelium and the immune/inflammatory system.

Introduction

Work within our group has identified the up-regulated expression of the Hedgehog (Hh) signalling pathway in idiopathic pulmonary fibrosis (IPF) and murine models of fibrotic lung disease.[1] That Hh signalling may also play a role in lung injury is also suggested by the work of Watkins et al[2,3] in their murine model of acute lung injury, and further confirms a role for hedgehog signalling in post embryonic pulmonary tissues.

Another area of interest to our group is the role of Hh signalling in the immune system. Active hedgehog signalling has been identified in cells of the peripheral immune system.[1,4-6] Since these immune cells are integral to inflammation and repair in the damaged lung, these observations have led to the emergence of the hedgehog signalling pathway as a possible target for therapeutic intervention in conditions of pulmonary damage and disease. The aim of this chapter is to review divergent avenues of research into the hedgehog signalling pathway and relate them to observations made with this pathway at the pulmonary interface.

The Hedgehog Pathway

The hedgehog signalling pathway has been reviewed extensively elsewhere in this volume and in recent publications,[7] thus will be covered only briefly in this review. There are three vertebrate hedgehog signalling molecules, Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh). Of these, most studies have concentrated on the Shh signalling pathway. *Shh* mRNA generates an inactive 45kD precursor protein which auto-catalytically cleaves,

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and is post-translationally modified to produce a highly hydrophobic cholesterol/palmitate modified Shh signalling molecule. This signals either as a cell surface molecule or is secreted following association with the Dispatched molecule. Binding of its receptor Patched (Ptc), releases Ptc mediated inhibition of Smoothened (Smo), a G protein coupled receptor-like molecule, allowing Smo to influence gene expression through the GLI zinc finger transcription factors. Signalling upregulates Ptc in an auto-regulatory loop, similar to many developmental pathways.

Studies have shown Shh to be a crucial morphogen in a number of developmental systems, including the limbs, lung, gut, nervous and immune systems (for a review, see ref. 8). It is only relatively recently that a role for the hedgehog signalling pathway has been elucidated in the post embryonic organism.

Hedgehog Signalling in Normal Pulmonary Tissues

Adult lung tissue contains several cell types, including Type I and II epithelial cells, Clara cells, mesenchymal cells (fibroblasts and endothelium), and resident immune system cells including alveolar and interstitial macrophages. Immunolocalisation studies from our group have demonstrated the expression of Shh protein in the lung is restricted to focal patches of epithelium in areas of injury and repair. Ptc is detectable on bronchial and alveolar epithelium and in alveolar macrophages.[1] Watkins and colleagues have also identified normal expression of Ptc in the basal layer of bronchial epithelium.[3]

Continued expression of Ptc in the absence of ligand, might indicate regulation and/or signalling via another pathway.[9] However, data emerging from studies in the gastrointestinal tract would suggest that Hh signalling may continue in tissue, in the apparent absence of immunodetectable ligand.

Gastrointestinal tract Shh protein expression is localised to areas of regeneration, such as the fundic glands of the stomach.[10-13] Initially protein expression appeared to be absent from many areas of the adult mammalian tract such as the oesophagus, small intestine and colon, although some cells in these regions appeared to retain the ability to generate mRNA.[10,11,14] Further to this, many of these areas lacked apparent Ptc expression.[12] However, treatment of mice with cyclopamine, a Smo inhibitor, resulted in marked decreases in small intestine epithelial cell proliferation, as evidenced by BrdU incorporation and PCNA studies, suggesting active signalling.[11]

Of interest is the finding that in the mature colon, cyclopamine inhibition of Hh signalling results in a marked *increase* in colonic epithelial proliferation and reduced differentiation of the colonocytes. This alternate response to that of the small intestine may represent a Hh specific effect, with variation in Ihh/Shh expression, or may simply reflect the presence or absence of another signalling factor or downstream effector in the tissues of the colon.[14]

Explanations for signalling in the absence of ligand include the existence of another pathway upstream of Smo (indeed, Ptc-independent pathways have been postulated previously,[15]) or that Hh signalling continued, but at a concentration below immunohistochemical detection. Recent research would support the latter explanation, for Hh expression has now been immunolocalised to areas of the colon, previously described as negative.[10,12] This was achieved through variations in antibody concentration and technique. Thus, future improvement in antibodies, detection methods and specific inhibitors may further clarify this issue for both the gastrointestinal and pulmonary systems.

Hedgehog Signalling in Injury and Disease

Whilst the studies of Watkins et al demonstrated bronchiolar epithelial expression of the Shh signalling components,[3] we have demonstrated Shh restricted to type II like cells in the alveoli of patients with IPF.[1] Expression was localised to areas of remodelling epithelia, particularly those areas with underlying fibrosis. Interestingly, whilst an irritant induced murine model of fibrosis illustrated similar localisation, a murine model of allergen induced inflammation

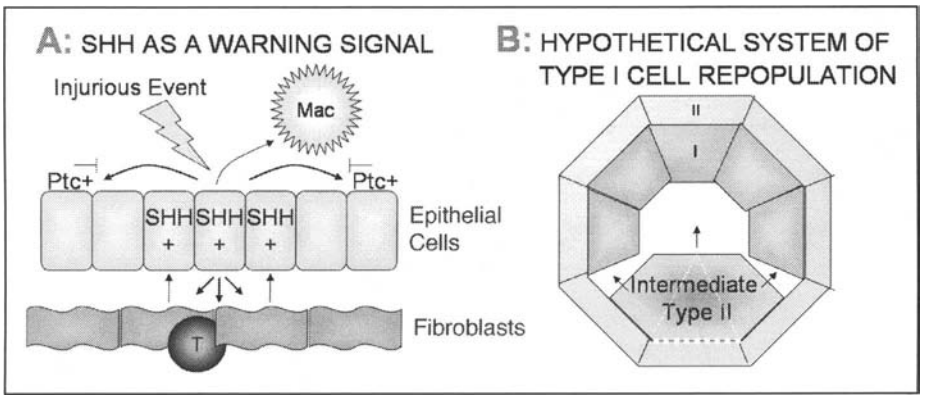

Figure 1. A) Epithelial cell Shh has the potential to signal to neighboring cells to warn of cellular injury, and generate matrix deposition and proliferation in underlying fibroblasts, preventing transudation in the event of epithelial shedding. Communication with Macrophage and T lymphocytes is also possible, as is fibroblast-epithelial signaling. B) Type II cells are restricted to junctional positions at the entrance to alveoli, as shown this aerial diagram. In the event of type I cell loss, type II cells form intermediate cells which differentiate and spread into type I cells. Shh upregulation may represent a differentiation inhibitor, to which cells adjacent to denuded areas are nonresponsive.

showed no such upregulation. Whilst both models illustrated evidence of inflammation, the allergen model lacked progressive fibrosis.[1,16]

The upregulation of Shh at sites of epithelial injury comes amongst a plethora of other modulating signals and could therefore represent a downstream marker of a much larger process initiated by the underlying mesenchymal/fibroblastic cells, which play a crucial role in epithelial maintenance. Mesenchymal cells produce specific epithelial growth factors such as keratinocyte growth factor (KGF) and fibroblast growth factor 10 (FGF-10). These are upregulated in response to injurious stimuli, resulting in increased epithelial proliferation and rapid reestablishment of an intact epithelial layer (for a review, see ref. 17). Studies using FGF-10, FGFR2b and Shh knockout mice have recently confirmed Shh to be downstream of FGF-10 signalling.[18] Shh expression on epithelium at sites of fibroblastic proliferation, such as that observed in IPF, may be a consequence of this repair process rather than an instigator of repair. Thus, Shh could be considered a regulatory factor in this instance, given its ability to downregulate FGF-10[19] (see Fig. 1A).

Alternatively expression at the alveolar surfaces of IPF patients might relate to a specific element of the disease process, which maybe mirrored in the FITC mouse model, where we observe a similar pattern of expression.[1]

IPF is a chronic fibrotic condition of the lung, associated with inflammation, where median survival after diagnosis is limited to five years. Although without known cause, progression is believed to have an immunological basis, linked in some way with aberrant epithelial mesenchymal interaction.[20] Previous work in this laboratory identified circulating antibodies to a 70-90KD protein in IPF patient serum, which were not observed in normal controls.[21] This protein was later localised to type II cells (Fig. 2), suggesting an autoantigen.[22] Experiments in vivo with a polyclonal antibody raised against the antigen and a human type II epithelial cell line showed upregulated tenascin and TGF-β production.[23] Tenascin is an extracellular matrix protein associated with active scar formation, whilst TGF-β can be pro-fibrotic. Both have been well characterised as being present in the lungs of patients with IPF,[24,25] and tenascin has been shown to be localised to areas of active fibroplasias or 'fibroblastic foci'.[23] It will be interesting to discover whether Shh is also upregulated by this interaction, as it would explain its localised expression, at areas of putative antibody interaction.

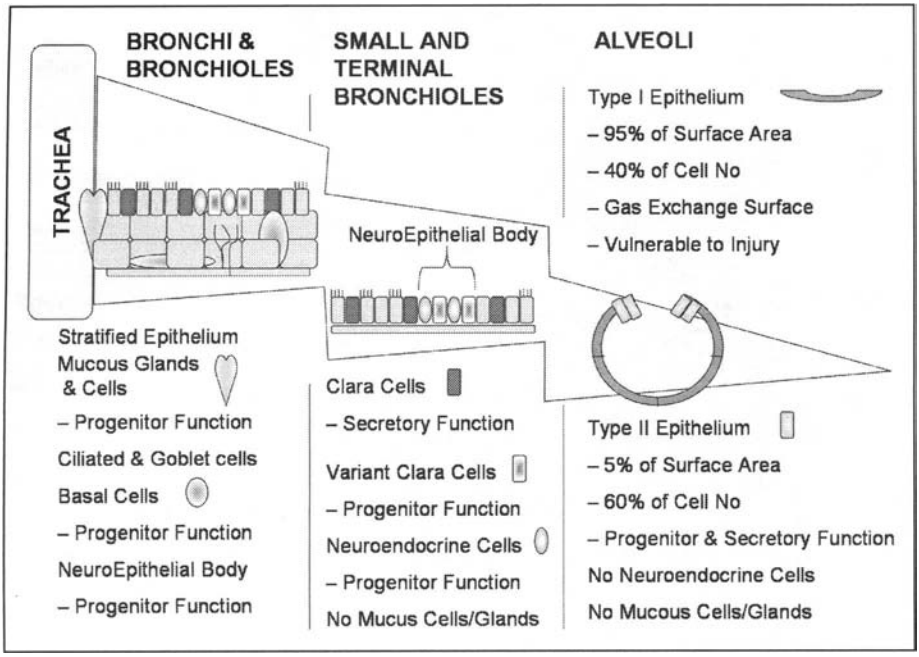

Figure 2. Cell types at the pulmonary surface.

Shh also has the potential to be profibrotic, Shh and FGF signalling are well linked in neural systems both pre and postnatally[19,26-28] and hedgehogs have been linked with collagen fibre induction in the neural system.[29] Shh also has substantial cell cycle modulating capabilities[30,31] and participates in well characterised proliferative mesenchymal interactions in the developing lung. Thus the localisation of Shh at fibrotic foci in IPF may also present a causal relationship with fibrosis in the IPF patient.

Similar hypotheses can be drawn from the observations made in the fibrotic FITC mouse model of IPF pathology.[16] Fibrosis and inflammation are believed to be induced in this model through the persistence of the intratracheally instilled irritant FITC, which lodges in the interstitium and binds resident proteins. Antibodies are raised to FITC, and mice develop a pathology similar to that of IPF patients, where Shh expression is localised to sites of damage.[1]

Interestingly, recent work has illustrated a requirement for cell confluence for Shh responsiveness, in a Ptc expressing Shh bioassay.[32,33] Thus, Shh and Ptc expression at a site of proliferative repair, may not automatically indicate that active signalling is occurring. Indeed, the broad expression area of Ptc in the lung might represent a "Shh sponge", to endocytose, in the absence of signalling, free Shh, as described by Torroja and colleagues.[34] However, whilst this may, in part, be true for some expression studies in the lung, work performed in our laboratory and that of others has identified alterations in target gene expression such as Ptc and Gli1, coincident with Shh expression, suggesting that at least some active signalling results from Shh expression.[3]

Shh would appear to share many of its effects with KGF and FGF-10,[30] most notably, stimulation of increased cellular resistance to injury and death.[35,36] Shh exhibits this effect in a number of cell types,[30,37,38] but has yet to be established at the pulmonary interface. Perhaps the best example of this activity is in Parkinson's disease, where exogenous administration of Shh in both a marmoset and mouse model of the disease results in improved locomotion and function.[39,40] Tsuboi and colleagues,[40] citing work by Miao et al,[38] have suggested that this is

likely due to Shh acting as a neuroprotective agent against dopaminogenic neuron cell death, which is central to disease progression.

In support of this Thilbert et al have shown that Shh can induce the anti-apoptotic protein Bcl-2 in keratinocytes, and that its presence prevents the apoptosis of neuroepithelial cells.[37] Therefore in a pulmonary situation of injury, such as exposure to environment oxidants, Shh release, along with KGF and FGF-10, may serve to temporarily increase surrounding epithelial cell resilience, and prevent complete denudation of an epithelial surface and the risk of secondary infection (Fig. 1A).

Sustaining viability of injured cells carries with it the increased probability of populating the epithelium with cells which have incurred genetic damage. Indeed, this may help to explain the higher incidence of small and nonsmall cell lung cancers following epithelial trauma, and the preponderance for the involvement of Shh signalling in these carcinomas.[41]

Upregulation of Hh signalling following injury, can be found in a number of model systems Pola et al[42,43] have demonstrated the upregulation of Shh and Ptc1 expression in a rodent hind limb ischemia model. Furthermore, the same group demonstrated that the administration of Shh induced robust neovascularisation, enhanced blood flow, and the upregulation of the angiogenic factors, vascular endothelial growth factor (VEGF) and the angiopoietins, Ang1 and Ang2,[43] although any direct association is a matter of conjecture.[44,45] The findings from other groups have been broadly in keeping with those of Pola et al[43] demonstrating that Shh is capable of inducing VEGF expression[44] and angiogenesis.[45]

Such angiogenic modulation could have important implications at the respiratory surfaces of IPF patients, where we have observed Shh expression in association with fibrotic foci.[1] Shh expression here may represent an attempt by the lung to revascularise and remodel the fibrotic architecture. Alternatively, it may represent a step in the progression of the fibrosis, where VEGF itself is found upregulated with epithelial injury.[46] Perhaps the greatest implication is in the neoangiogenic advancement of pulmonary malignancies, which are associated with the expression of Hh pathway components.[3]

Shh in the Immune System

A functional role for hedgehog signalling in the adult immune system is perhaps not surprising given the well documented role of Shh in lymphoid development and differentiation.[4,47,48] Work in our laboratory has found evidence for expression of Shh and Ptc in both resting and activated murine and human $CD4^+$ and $CD8^+$ T lymphocytes, human macrophages and secondary lymphoid tissue,[5,6] both at the mRNA and protein level. This lends support to a possible role for immune-epithelial cell interaction at sites of injury and repair.

It has been postulated that the expression of Hh in a large range of diversified tissues and cell types, might represent a common method of sensing the cellular environment and neighbouring cell health.[49] Given the expression of the Ptc molecule on human macrophages, it may be postulated that Shh expression acts as an advertisement of epithelial damage both to the macrophage, to facilitate attention, and to the adjacent cells as a warning signal for defence and repair initiation (Fig. 1A).

Further to this, Lowrey and coworkers[5,6] have demonstrated that exogenous Shh has the capacity to push sub-optimally activated peripheral $CD4^+$ T lymphocytes through further cell cycles, whilst addition of a blocking antibody to endogenous Shh results in decreased proliferation. Together these results would suggest that exogenous Shh administration enhances an endogenous stimulatory effect of Shh. Indeed, further work from our group has demonstrated that Shh can act as a costimulatory molecule to T-cells activated via the T cell receptor using anti-CD3 antibodies (Lowrey et al, unpublished observations). This explains the upregulation of the activation antigens CD25 and CD69, by exogenous Shh, along with the production of Interleukin 2 (IL-2), a T lymphocyte proliferative factor, interleukin 10 (IL-10) and Interferon gamma (IFN-γ). Thus Shh expression at sites of disease and damage has the potential to sustain

T lymphocyte mediated immune responses and even to influence the effector function of immune responses, through an IFN-γ bias.

Shh has also been shown to upregulate *Bcl-2* in T lymphocytes, a step linked with memory T lymphocyte generation,[4] and thus, Shh at sites of injury might also serve to ensure adaptive immunity to damaging antigens, or an exacerbation of autoimmunity. Given that many of the diseases associated with Shh expression contain elements of autoimmunity or immune dysregulation, this crosstalk could have important implications both for the initiation and maintenance of a disease process.

Hedgehogs and Pulmonary Progenitor Cells

Watkins and colleagues[3] depleted Clara cells from mouse airways via their sensitivity to naphthalene injury. This resulted in a transient peak in Shh and Gli expression 3 days post depletion that coincided with the reestablishment of a complete epithelial barrier. It was suggested that Shh signalling might be involved in this repopulation step, since this peak preceded an increase in the number of pulmonary neuroepithelial cells—a cell type identified as a potential epithelial progenitor.[41,50] Subsequently, Watkins and colleagues identified clusters of epithelial cells expressing Ptc and the neuroendocrine marker, Calcitonin Gene Related Peptide (CGRP), associated with Shh expressing cells in the developing lung.[3] It was suggested that this clustered interaction might continue into the adult pulmonary system, where Shh expression might influence neuroepithelial differentiation.

Presently, progenitor cell phenotypes are characterised via their longevity via nucleotide analogue incorporation, and are defined as label retaining cells. In the mouse these cells are localised to glandular submucosal glands in the distal trachea and bronchi.[51] In the absence of these glands, such as in the bronchioles and alveoli, LRCs occur in localised foci,[52] referred to as neuroepithelial bodies[41] (Fig. 2). These foci are populated by Clara Cell secretory protein (CCSP)-expressing epithelial cells, known as variant Clara cells (vCC), which are naphthalene resistant. Associated with these are CGRP-expressing epithelial cells which show intermediate morphology between a pulmonary Neuroendocrine Cells (PNEC) and a Clara cell[41,50] (Fig. 2). Infiltration of blood borne progenitors in this regenerative process, add a further level of complexity outwith the scope of this chapter, but covered elsewhere.[53]

Label retaining cells undergo hyperplasia in the event of Clara cell death, such as that induced by naphthalene, resulting in the rapid reestablishment of a functional epithelial cell layer. Selective depletion of vCC using a Herpes simplex virus thymidine kinase linkage system, prior to naphthalene injury, results in a failure to repopulate a differentiated ciliated epithelium, despite PNEC hyperplasia.[50] Whether this highlights the CCSP expressing cells as progenitor cells, producers of a signal necessary for PNEC progenitor cell function or as coprogenitors with the PNEC is still an area of conjecture.

As to a potential role for Shh in this process, epithelial coverage is completed at the peak of Shh expression, thus a causative role in nonspecific epithelial proliferation is unlikely, and this is confirmed in our studies where we observe bronchiolar Shh expression restricted to areas of complete epithelial coverage. It is interesting that coverage normally occurs in the absence of differentiation, which typically occurs following reestablishment of epithelial integrity. Given the necessity for confluence for Shh signalling in some cell lines, as mentioned previously, one might speculate that it is in this differentiation step that Shh might play a role.

Reasoning for such speculation arises through the well characterised progenitor modulating role of Hh in a range of post embryonic systems including T lymphocyte development[47] and haematopoiesis.[54] The work by Bhardwaj in haematopoiesis was notable for it clearly defined the downstream effector of Shh function as BMP-4, where Shh addition to an enriched population of human CD34+Lin-CD38- progenitor cells resulted in increased self renewal, albeit in combination with many other growth factors.

An example of Hh mediated progenitor modulation in injury comes indirectly from several sources.[55,56] Pepinsky et al demonstrated that Shh administration can accelerate nerve recovery

following sciatic nerve crush injury.[55] A Possible explanation for this response comes from studies by Bambakidos et al, using a rat demyelination model.[56] These authors observed increased proliferation of stem cell like progenitors in areas of chemical demyelination following direct Shh administration. This suggests that exogenous Shh at a site of injury induces proliferation in stem cell populations, although the specificity of this progenitor proliferation was not addressed in this study.

The manner in which hedgehog signalling might facilitate this stem cell influence has yet to be characterised, but is likely to lie in its ability to regulate differentiation. Evidence in support of such a role comes from studies of fracture repair, in which Ihh may play a role. Following a fracture, pluripotent mesenchymal cells invade and differentiate into osteoblasts, to initiate the production of hard callus comprising bone matrix, and chondrocytes for the formation of the soft callus. A balanced secondary chondrocyte differentiation step facilitates the remodelling of bone until the bone shape is restored.[57,58] Ihh expression is induced by 3 days post fracture persisting past 2 weeks in a murine model, with the greatest level of expression found in chondrocytes undergoing their secondary differentiation step.[59,60] Ihh expression here induces further chrondrocyte proliferation, but prevents further chondrocyte differentiation via PTHrP signalling.[61-63] Given the osteoblast upregulation of PTHrP in the presence of rShh shown by Jemtland and colleagues[64] and the osteoblastic lineage bias induced by Ihh,[65] it is likely that in this system, Ihh is central to restricting differential fates of invading pluiripotent mesenchymal cells and facilitates an accurate bone remodelling response.

Central to the stem cell maintenance function of Shh observed in haematopoiesis, is its ability to upregulate BMP-4. These molecules are crucial in early lung morphogenesis and thus have substantial signalling potential in the post embryonic lung. Indeed, studies in a mouse model of allergic inflammation have demonstrated an upregulation of BMP's at sites of inflammation, including BMP-4.[66] In vitro studies would suggest that BMP-4 has anti-proliferative effects in cancer derived cell lines,[67] however these effects can often be contradictory and dependant on the cosignals and cell types present in culture.

In vivo studies into pulmonary BMP functions are limited by the lethality of BMP-4 knockouts. However, innovative in vivo studies in this field from the lab of Brigid Hogan and colleagues have demonstrated a potential progenitor modulating function for BMP's in epithelium. Cells exposed to high levels of BMP-4 retained undifferentiated characteristics.[68,69] In light of these studies, were Shh to modulate progenitor function through the BMP's, it would likely be in conjunction with a number of other signals, such as FGF-10. Perhaps in a recapitulation of the process of cell fate designation in the embryonic lung.[68]

Shh and Type II Epithelial Cells

Studies thus far have focused on roles for Shh expression at the bronchi and bronchiolar levels, however immunohistochemical data suggest that the hedgehog signal might persist in the larger and smaller airway systems. Watkins and colleagues observed a persistence of Ptc signalling in the progenitor cells of the trachea and bronchi, whilst our lab has identified expression of Shh and Ptc in type II like cells of the terminal bronchioles and alveoli (Fig. 2).

Pulmonary epithelial regeneration occurs rapidly at the alveolar level. Here, in response to the removal of contact inhibition and KGF,[36] type II cells downregulate specific functional machinery, proliferate and spread out to become thinly spread type I cells, specialised for gaseous exchange. This process occurs almost continually as type I cells are highly susceptible to injury by exogenous factors, i.e., pneumotoxic environmental pollutants and by factors involved in both innate and adaptive pathogen clearance in the lung.

Whilst lack of contact inhibition and KGF are triggers for type II differentiation, no maintenance signal for type II cell number has yet been identified. Perhaps Shh has a modulating function here too. Certainly immunolocalisation in IPF patient biopsies has identified Shh upregulation in individual type II like cells amongst adjacent negative cells, with expression isolated to areas of injury.[1]

This makes for an attractive hypothesis. Damage upregulates type II cell expression of Shh, this induces type II proliferation and inhibits differentiation. However, type II cells adjacent to areas of denuded basement membrane have incomplete confluence and thus lose responsiveness to the Shh, allowing them to differentiate and cover the exposed area (Fig. 1B). However, were Shh a maintenance factor in this continual type II–I transition, it might be expected that normal lung might exhibit some limited immunohistochemical reactivity, and this is not observed. Whether this represents true absence of Shh protein, or the limits of immunohistochemical detection, remains to be determined.

Concluding Comments

The potential for post embryonic recapitulation of developmental signals in injury and disease presents many exciting targets for therapeutic modulation. This is particularly true for the pulmonary system, where the air interface facilitates the direct and rapid delivery of short half life Hh modulating agents to target cells. This will avoid the systemic complications of treatment that might be expected in the treatment of conditions such as multiple sclerosis and Parkinson's disease. Our knowledge of the Hh signalling pathway and its role in development has made great advancements over recent years and has led to the development of many new and exciting ideas which may identify functions for the Hh pathway in post-embryonic systems.

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CHAPTER 10

Human Correlates of GLI3 Function

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Abstract

The human phenotypes caused by mutations in GLI3 are a prototype for the utility of clinical analysis to unravel gene function. When the human studies were coupled with basic research, the pathway to understanding gene function was easier to discern and more rapidly accomplished. Outlining the series of discoveries that led to our current understanding of GLI3 and genotype-phenotype correlation is useful to demonstrate how these discoveries can be facilitated in the future.

The Delineation of the Greig Cephalopolysyndactyly Syndrome (GCPS)

The story of GLI3 and human pathology began with a 1926 publication[1] by the Edinburgh surgeon David Middleton Greig.* He described a series of patients with a clinical finding of "oxycephaly", defining this term as "general craniofacial synostosis". He recognized that these craniofacial findings often occurred with limb anomalies. However, he lumped a number of patients together who probably had what we now recognize to be Carpenter, Crouzon and Saethre-Chotzen syndromes into the category of "oxycephaly". One case (case VI) in his report also had cranial and limb anomalies but was described as an exception to the general concept of "oxycephaly". This girl had a "broad and voluminous forehead", her hands had short, broad thumbs with terminal phalangeal lacunae and numerous scars from previous attempts to correct cutaneous syndactyly, and her feet demonstrated broad great toes with scars from removal of a partially duplicated distal phalanges, with complete cutaneous syndactyly of toes 1 to 3 and partial syndactyly of toes 3 and 4. She was "considered to be rather above the average at school". Her mother was said to have "symmetrical webbing of the fingers of both hands" but no craniofacial findings. Greig mistakenly cleaved the craniofacial findings from the limb findings in this case: "The webbing of the digits was hereditary and the head affection a dysplasia of the basis cranii." He did not apply the concept of pleiotropy nor that of variable expressivity to his analysis. The application of current concepts of syndrome delineation lead us to conclude that this patient and her mother were affected by the disorder we now consider to be GCPS. In spite of the limitations of Greig's observations and analysis, the designation of "Greig cephalopolysyndactyly syndrome" was coined to honor this description. Ironically, Greig rejected the use of the compound term "acrobrachycephalosyndactylism", a term that is similar to that which we now combine with his name.

* The surname is pronounced "greg", with a trilled r. The surname of the composer Edward Grieg is pronounced "greeg".

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The heritable nature of the condition was affirmed by a number of authors, including a family of ten patients in four generations.[2] Primarily through the approach of clinical analysis of affected patients, it was confirmed that the typical manifestations included hypertelorism,* broad or prominent forehead, macrocephaly, with polysyndactyly. The polydactyly was most commonly preaxial polydactyly of the feet and postaxial polydactyly of the hands,** and varying degrees of cutaneous syndactyly, most commonly of digits 3 and 4 in the hands and when present, often involves all five digits of the feet.[4,5] Other less common manifestations include craniosynostosis (once thought to be a common manifestation), mild mental retardation, umbilical and inguinal hernias, hydrocephalus, and agenesis or hypoplasia of the corpus callosum.

The Molecular Basis of GCPS

The first insight into the molecular basis of GCPS was the observation that patients with interstitial deletions of the short arm of chromosome 7 manifested the syndrome.[6-8] These cytogenetic localizations were refined by molecular techniques and led to the determination that GCPS was caused by interruption of the GLI3 zinc finger transcription factor gene,[9] presumably through the mechanism of haploinsufficiency. The study of humans with *GLI3* mutations lay relatively dormant from that time up to the discovery of the etiology of Pallister-Hall syndrome.

The Clinical Delineation of Pallister-Hall Syndrome (PHS)

We set out to rigorously delineate the clinical features of PHS in preparation to undertake a positional cloning effort. The PHS phenotype was originally described in a cohort of six individuals who had polydactyly (typically central polydactyly# and sometimes postaxial polydactyly) and hypothalamic hamartoma with varying degrees of pituitary dysfunction, a shortened nose, imperforate anus, pulmonary segmentation anomalies, and laryngotracheal anomalies ranging from bifid epiglottis to laryngotracheal clefts.[10] In the early 1990s several case reports demonstrated that PHS was also inherited in an autosomal dominant pattern.[11-16] Subsequently, clinical diagnostic criteria were developed that specified that a proband had to have central polydactyly and a hypothalamic hamartoma.[17] Using this strict clinical definition, a positional cloning effort was undertaken on several large families with PHS, which led to its mapping to 7p14,[18] in a small candidate region that included *GLI3*. After all other genes were excluded, *GLI3* was sequenced, and frameshift mutations were found in two families.[19]

At this point, the question arose as to whether PHS and GCPS were distinct clinical entities. To approach this question, it is necessary to review some basic principles of clinical dysmorphology. First, pleiotropic developmental anomaly syndromes can be identified that are distinct clinical entities. Second, all syndromes have anomalies that are shared with some other syndromes, that is, they have overlapping manifestations. To define a distinct pleiotropic anomaly syndrome, one has to identify a pattern of anomalies that is typical for the disorder and demonstrate that it is distinct from existing syndromes. The anomalies described above seemed to cleave PHS and GCPS into two distinct syndromes (Table 1).

From these data, it appeared that GCPS and PHS had little overlap, although preaxial polydactyly was found in occasional patients with PHS, and postaxial polydactyly was

* Hypertelorism is an abnormally large interpupillary distance, commonly confused with telecanthus, which is an abnormally large distance between the inner canthi. Curiously, Greig wrote a paper on hypertelorism in 1924,[3] but did not mention this finding in the "oxycephaly" case description in 1926.

** Duplication of the thumb or great toe in humans is called preaxial polydactyly, while in developmental biology it is called anterior polydactyly.

Central polydactyly is synonymous with insertional or mesoaxial polydactyly.

‡ A hamartoma is a focal malformation that is composed of an abnormal mixture of tissue elements. It is not a neoplasm.

Table 1. Distinct clinical manifestations in GCPS and PHS

	GCPS	PHS
Hypertelorisim	+++	-
Preaxial polydactyly	+++	+
Postaxial polydactyly	++	++
Hypothalamic hamartoma	-	+++
Central polydactyly	-	+++
Imperforate anus	-	+

+++ common manifestation; ++ seen in some cases; + seen uncommonly; - not reported

common in both disorders. Another key criterion for a distinct disorder is that it breeds true. If the syndrome diagnosis changed among members of the same family (where the mutation is obviously constant), it should raise the possibility that the allele at the primary locus does not specify a distinct syndrome and that there are major modifiers that determine a range of varying manifestations that make the syndrome difficult to describe as a distinct entity. In the case of PHS and GCPS, both disorders bred true, that is, no person with a diagnosis of PHS had been reported from a family with GCPS, and vice versa. At the time that the *GLI3* mutations in PHS were discovered, all of the clinical data suggested that PHS and GCPS were distinct clinical entities. Importantly, the distinction of PHS and GCPS was qualitative, not quantitative. That is, no plausible spectrum could be hypothesized that put GCPS and PHS on a single scale of severity. Specifically, both GCPS and PHS had a wide spectrum of severity. Pallister-Hall syndrome varied from severe, neonatal lethal cases to mildly affected patients with insertional polydactyly, asymptomatic hypothalamic hamartomas, and asymptomatic bifid epiglottis. Greig cephalopolysyndactyly syndrome was recognized early on to comprise a wide spectrum of severity, including patients with very mild manifestations.[20] On the other end of the GCPS spectrum were patients with severe limb anomalies, craniofacial anomalies, CNS anomalies (agenesis of the corpus callosum), and mental retardation with or without seizures. Therefore, both disorders varied from mild to severe, but that variation did not encompass changes in the malformations that distinguish GCPS and PHS. On that basis, it was necessary to propose a biologic model that accounted for the generation of two distinct phenotypes.

Animal Models of GLI3 Phenotypes

Because the human clinical data were limited,* insight into the mechanism of GLI3 activity was needed. One of the earliest insights was that the mouse mutant extra toes (*Xtj*) was very similar to human GCPS,[21] which was later confirmed by the determination that *Gli3* was deleted in the *Xtj* mouse mutant.[22] A mouse model of PHS was also developed and it also demonstrated a qualitatively distinct set of malformations than those seen in the Xt mouse.[23] A key insight was also gained from work on *Drosophila melanogaster* when it was demonstrated that CI, the fly homologue of the human *GLI* genes, was subject to post-translational modification that converted ci from a transcriptional activator to a repressor.[24,25] The pathway controlling ci processing is regulated by hedgehog (HH), the fly homologue of the human hedgehog family that includes sonic hedgehog. Based on these data, a model was proposed whereby GLI3 was also post-translationally processed and had both activator and repressor functions.[26] Furthermore, the mutations in patients with GCPS and PHS had to affect these two functions in distinct ways, in order to explain why both disorders had qualitatively distinct phenotypes.

* Both GCPS and PHS are rare, probably affecting fewer than 1/100,000 persons.

Table 2. Distinct classes of mutations in PHS and GCPS

	GCPS	PHS
Translocations	4	0
Large deletions	19	0
Missense	5	0
Splicing	6	0
5′ frameshift	13	0
3′ frameshift	12	13

Currently published data show that the two disorders are associated with different classes of mutations (Table 2).[7,19,27-50] Note that only one class of mutations is associated with PHS, truncating mutations 3' of the zinc finger binding domains. In contrast, mutations in patients with GCPS include translocations, large deletions,[#] missense, and frameshift mutations predominantly 5' of the zinc finger domains, but also in other areas of the gene.* Therefore, the range of mutations in GCPS is broader than in PHS and this observation is consistent with the hypothesis that GLI3 haploinsufficiency causes GCPS. However, the hypothesis that *GLI3* haploinsufficiency causes PHS is untenable, as no PHS patients have been described with mutations that cause haploinsufficiency. We proposed that mutations in GLI3 that cause PHS do so by generating a constitutive repressor that is independent of regulation by SHH. Numerous studies have shown that GLI3 (and GLI2, but interestingly not GLI1) are subjected to posttranslational processing and GLI3 has both activator and repressor activities.[51-56]

While these data substantially validate the bifunctional hypothesis, many questions remain. First are the so-called nonsyndromic polydactylies. As noted above, clinical observations in the 1980s suggested that some individuals with GCPS have sufficiently mild craniofacial manifestations that many observers would find it difficult to recognize without detailed objective comparisons to unaffected relatives. Indeed, these features may be missed for two reasons. First, mild hypertelorism is considered a desirable trait (note that many models and actors have mild hypertelorism) and second, benign familial macrocephaly is one of the most common familial traits.[54] Therefore, if a child has mild GCPS caused by a de novo mutation in *GLI3*, general physicians and surgeons (the practitioners who would normally care for such a child) commonly miss the diagnosis of GCPS and instead diagnose the child with nonsyndromic polydactyly. In such a case, the clinical geneticist may not get involved with the family and make a diagnosis of GCPS until that child has children of their own.

For PHS the situation is less clear. As noted above, PHS does have a wide range of severity, including patients with polydactyly, asymptomatic hypothalamic hamartomas (detectable only by cranial MRI), and asymptomatic bifid epiglottis (detectable by direct or indirect laryngoscopy**). We have evaluated a number of patients with apparently nonsyndromic central polydactyly who were diagnosed with mild PHS only after cranial MRI and laryngoscopy was performed and showed asymptomatic hypothalamic hamartoma or bifid epiglottis (unpublished data). In addition, some patients are diagnosed with PHS because they have postaxial polydactyly (without central polydactyly), because they are related to a person with PHS in a

[#] Here we define "large" as being larger than the gene, which is ~300 kb.

* Unpublished data include more than 40 additional mutations that are consistent with these preliminary data.

** Remarkably, this lesion is often missed by anesthesiologists when these patients are intubated at the time of repair of their polydactyly.

pattern consistent with autosomal dominant inheritance.[17] The obvious implication is that there are likely to be individuals who have postaxial polydactyly and asymptomatic hypothalamic hamartomas who are not related to someone with central polydactyly. Such patients have syndromic polydactyly, but unless they are carefully evaluated, would carry a diagnosis of nonsyndromic polydactyly. This brings to mind the report of families with so-called PAP-A (post axial polydactyly type A, the "type A" designation indicating that the extra digit is comparable in size to the normal fifth digit)[58] who have mutations in *GLI3* that are similar to those in patients with PHS. Importantly, these individuals were not examined by cranial MRI or endoscopy so it is not known if they have asymptomatic hamartomas nor bifid epiglotti. Therefore, two hypotheses could explain the so-called nonsyndromic PAP-A patients with *GLI3* mutations. First, such patients may have undiagnosed mild PHS. Second, it is possible that they are correctly described as nonsyndromic and that PHS comprises a spectrum from severe, sporadic, neonatal presentations, through more typical patients, to mildly affected patients with small and asymptomatic hamartomas and finally, patients with nonsyndromic PAP-A. If the latter explanation is correct, other mechanisms must be invoked to explain the mildness of symptoms in the latter patients. One possibility is modifier genes. It is possible that variation in the expression of the normal and abnormal *GLI3* alleles would affect the expression of the phenotype. The latter would be expected to be in *cis* with the mutations and thus be linked in the families, whereas the former would be expected to vary with the variant in the wild type allele. The are a number of coding polymorphisms in *GLI3*, whose functional consequences are unknown, and the regulatory and splicing polymorphisms have not yet been studied. Thus, this area is ripe for further study.

Another area of study is other classes of mutations. For example, as it is now known that GLI3 processing occurs, it is reasonable to expect that there could be amino acid substitution mutations in the gene that would render GLI3 resistant to processing. Such mutations should cause a phenotype distinct from PHS or GCPS, although the nature of this phenotype is difficult to predict. Another interesting class of mutations are those that cause amino acid substitutions that interfere with binding of GLI3 to the cytoplasmic complex. Such mutations might be nulls and thus phenotypically resemble GCPS, but it is also possible that they will cause constitutive GLI3 activator function, the phenotypic consequences of which are again difficult to predict. In this light, the recent report of acrocallosal syndrome caused by a point mutation in GLI3 raises another set of questions.[59] The overlap of GCPS and acrocallosal syndrome was recognized by clinicians before the *GLI3* mutations were identified.[20] Part of this overlap is undoubtedly due to misclassification, primarily of sporadic cases. Acrocallosal syndrome is inherited in an autosomal recessive pattern and shares essentially all of the manifestations of GCPS except that agenesis of the corpus callosum, mental retardation, and seizures are common, if not universal. Prior to molecular diagnostics, if a sporadic patient presented with hypertelorism and preaxial polysyndactyly they were diagnosed with GCPS if they did not have mental retardation and seizures and acrocallosal syndrome if they did. In addition, the GCPS contiguous gene syndrome overlaps substantially with acrocallosal syndrome but is caused by large deletions in 7p that remove sufficient additional genes to cause CNS dysmyelination, mental retardation, and seizures (all of which are relatively nonspecific symptoms).[33,45] Nevertheless, the data are convincing that acrocallosal syndrome is a distinct clinical entity that has a phenocopy caused by mutations in *GLI3*. In addition to the GCPS contiguous syndrome patients, one patient with acrocallosal syndrome and a point mutation in GLI3 has been described.[59] No functional data on this mutant are available, but it may prove to be a dominant negative mutation of some kind. A final class of interesting patients is that with unclassified types of the oral-facial-digital syndrome (OFD). The OFD category describes a family of disorders with cleft palate, oral hamartomas, and frenulae, limb and other anomalies and currently comprises ten apparently distinct phenotypes.[60] There is an informal moratorium on designating additional subtypes of OFD because the boundaries of the entities are not clear and it is felt that molecular delineation will lead to a more rational distinction of the

various subtypes. Among patients with unclassified OFD, two patients have been described with frameshift mutations in *GLI3* (unpublished data).

In summary, the clinical and molecular analyses of patients with mutations in *GLI3* have provided a wealth of insight into the biology of GLI3. In addition, these findings have result in direct benefits of improved diagnosis, treatment, and recurrence risk assessments for families affected by these disorders. For the future, it is clear that there is much yet to be studied and learned about the manifestations of GLI3 dysfunction in humans.

Note Added-in-Proof

A manuscript describing the genotype-phenotype correlation was published while this chapter was in production. Johnston JJ, Olivos-Glander I, Killoran C et al. Molecular and clinical analyses of Greig cephalopolysyndactyly and Pallister Hall syndromes: Robust phenotype prediction from the type and position of GLI3 mutations. Am J Hum Genet 2005 76:609-22.

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CHAPTER 11

From Oligodactyly to Polydactyly:
Role of Shh and Gli3 in Limb Morphogenesis

Chin Chiang*

Abstract

Secreted molecules encoded by the *Hedgehog* (*Hh*) gene family have emerged as key signals in regulating the growth and patterning of invertebrate and vertebrate embryos. One of the most prominent features among Hh members is thought to reside in their ability to impose distinct cell fates in a concentration-dependent manner. This ability is highlighted by the critical and indispensable role of Sonic hedgehog (Shh) signaling in specifying the anterior-posterior polarity of the embryonic limb. Alteration of Shh expression and signaling activity can lead to profound developmental abnormalities in digit numbers and identity in mice and humans. In this chapter, we discuss the Shh regulatory mechanism that establishes the anterior-posterior polarity of the limb and how misregulation of this mechanism can lead to severe limb malformations in humans.

Introduction

All tetrapod limbs consist of three skeletal segments (Fig. 1), with the proximal stylopod (humerus in forelimb or femur in hindlimb) articulating a pair of zeugopods (radius/ulna or tibia/fibula), followed by the autopod (wrist/hand or ankle/foot). These complex structures are products of the outgrowth and patterning of paired forelimb and hindlimb buds which originate from the lateral plate mesoderm by inductive signals.[1,2] The anterior-posterior (A/P) asymmetry of the normal five-digit pentadactyl limb, as reflected by the thumb and small finger, is controlled by a group of specialized mesodermal cells located at the posterior margin of the limb bud referred to as the zone of polarizing activity (ZPA) (Fig. 1). Grafting of the entire ZPA to the anterior mesoderm results in a complete mirror-image duplication of digits[3] while partial ZPA grafts result in duplication of a subset of digits. The ability of cells in the ZPA to progressively specify an increase in the number of digits with more posterior identities suggested the existence of a diffusible molecule (morphogen) in the establishment of A/P polarity.[4]

A major advance in understanding the molecular control of A/P limb patterning came from the discovery of Shh, a secreted signaling molecule expressed in the ZPA. Shh is capable of inducing supernumerary digits and modifying digit identity when misexpressed anteriorly[5-7] (Fig. 1). Analysis of mouse embryos with a targeted deletion of *Shh* indicated an absolute requirement of Shh for ZPA function.[8] Additionally, the *Shh* mutant exhibited truncations of distal skeletal elements in the forelimbs while its hindlimbs displayed a single un-ossified but identifiable digit 1[8-10] (Fig. 3). These studies provide an important foundation for elucidating the molecular mechanism of human congenital limb malformations which encompass a broad

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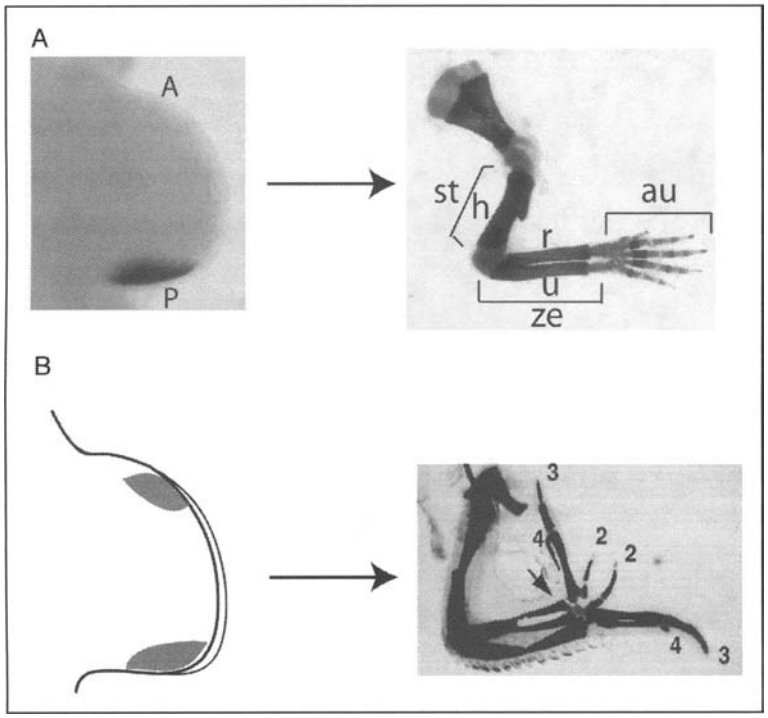

Figure 1. Shh mediates the zone of polarizing activity (ZPA) in the limb. A) the limb consists of three segments: the stylopodium (st), zeugopodium (ze) and autopodium (au), in a proximal to distal order as shown for the mouse forelimb. *Shh* expression in the ZPA at the posterior (P) margin of the limb bud is necessary for anterior-posterior (A/P) patterning of distal skeletal elements. B) Grafting of the ZPA or Shh-soaked beads to the anterior margin of the limb mesoderm (red) induces supernumerary digits (4-3-2-2-3-4) in chick.[8] Note that arrow in b highlights the duplicated radius (r). Abbreviations: h, humerus; u, ulna and distal is to the right. A color version of this figure is available online at http://www.Eurekah.com.

range of limb phenotypes from oligodactyly to polydactyly, with fewer or more than the normal number of digits, respectively.

Shh Expression and the Specification of Digit Identity

Restricted expression of *Shh* in the ZPA permits the generation of a Shh concentration gradient along the A/P axis of the developing limb bud. Analysis of several preaxial polydactylous (PPD) mutants provided evidence for the existence of a negative regulatory program that restricts *Shh* expression to the posterior margin of the limb bud. A common feature shared by these PPD mouse mutants is ectopic *Shh* expression in the anterior margin of the limb mesoderm, consistent with the generation of additional digits in the anterior side of the limb. Several of these mutants are now molecularly characterized including *Strong's luxoid (lst), Sasquatch (Ssq), Hemimelic extratoes (Hx), and M10081.*[11-13]

The *lst* gene, which encodes a paired-type homeodomain protein, was previously described as the *Aristaless-like4* (*Alx4*) gene.[14] In *Alx4* mutants, the critical arginine residue within the first helix of the homeodomain is substituted by glutamic acid. This mutation is predicted to result in *Alx4* loss-of-function due to disruption of DNA binding at target sites. In addition to developmental defects in the limb, *Alx4* mutants also display craniofacial and hair defects and failure of testicular descent into the scrotum.[15] *Alx4* is expressed in the anterior mesoderm opposite the ZPA, consistent with its role in repressing *Shh* expression in the anterior

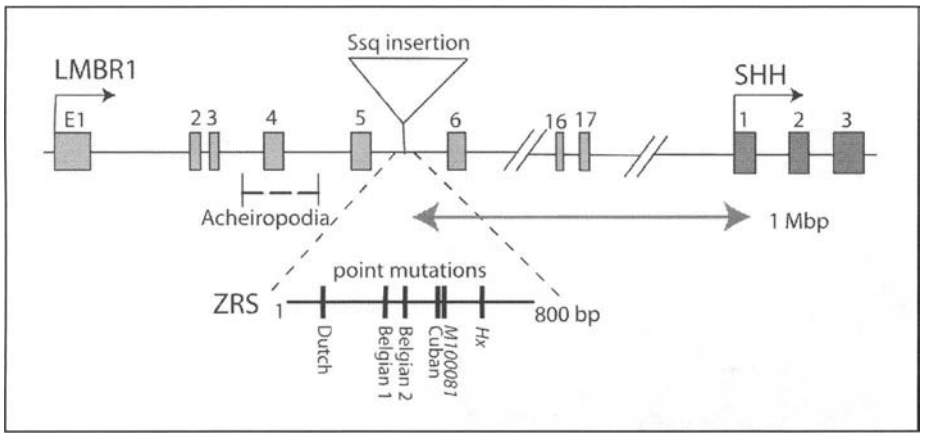

Figure 2. Genomic structure of the *SHH* and *LMBR1* loci. Numbers above the boxes denote exons. The *LMBR1* transcription unit consists of seventeen exons whereas *SHH* has three exons. PPD mutations in humans and mice are clustered in the 800 bp ZRS enhancer within intron 5 of the *LMBR1* gene. The locations of point mutations in four familial cases[12] and two mouse mutants[13] with PPD are indicated. The ZRS enhancer is located about 1 Mbp from the *SHH* gene. The critical region that is deleted in five familial acheiropodia patients is shown by the dotted line spanning exon 4 of *LMBR1*.[26]

mesoderm. Genetic epistasis experiments involving *Shh* and *Alx4* mutants demonstrated that the PPD phenotype indeed results from ectopic expression of *Shh*.[16] These observations indicated that Alx4 is a key transcription factor in the repression of *Shh* expression, possibly through interaction with negative regulatory elements in the *Shh* promoter.

In contrast to *Alx4*, the *Ssq* phenotype is restricted to the limb. The *Ssq* mutation is caused by a transgenic insertion in intron 5 of *Lmbr1* which is situated about one mega basepairs (Mbp) upstream of the *Shh* transcription unit on chromosome 5[17] (Fig. 2). *Lmbr1* encodes a putative transmembrane receptor-like protein that is expressed at low levels throughout the mouse embryo.[18,19] It was thought that the insertion at intron 5 would disrupt *Lmrb1* function which normally represses *Shh* expression in the anterior limb bud mesoderm. However, both *Lmbr1* gain- and loss-of-function studies fail to elicit phenotypes that are consistent with its proposed function.[18,20] Thus, observation from these functional studies suggests that the transgenic insertion likely disrupted a long-range negative regulatory element in the *Shh* promoter. This notion is supported by the fact that the dominant PPD phenotype of *Ssq* reverted to normal when the *Ssq* mutation was placed in cis to a *Shh* null allele in a recombinant chromosome.[17] Comparative analysis of the intron 5 sequence among different vertebrate species revealed a highly conserved region of approximately 800 bp that can direct reporter gene expression selectively in the ZPA of transgenic mouse embryos. Positional cloning of two additional PPD mutants, Hx and M100081, has identified single basepair mutations within the 800 bp conserved ZPA regulatory sequence (ZRS)[13] (Fig. 2). It is apparent from these studies that the ZRS contains both positive and negative cis-regulatory elements that restrict *Shh* expression to the ZPA.

Congenital Limb Malformations Associated with Shh Misregulation

In humans, PPD is the most common form of congenital limb malformations.[21] The majority of cases show an autosomal dominant mode of inheritance, but isolated sporadic occurrences have also been reported. The limb phenotype ranges from extreme preaxial digit duplications of the index finger to mild addition of phalanx to form the triphalangeal thumb.[22] A major locus for human PPD was mapped to chromosome 7q36 region, syntenic to mouse

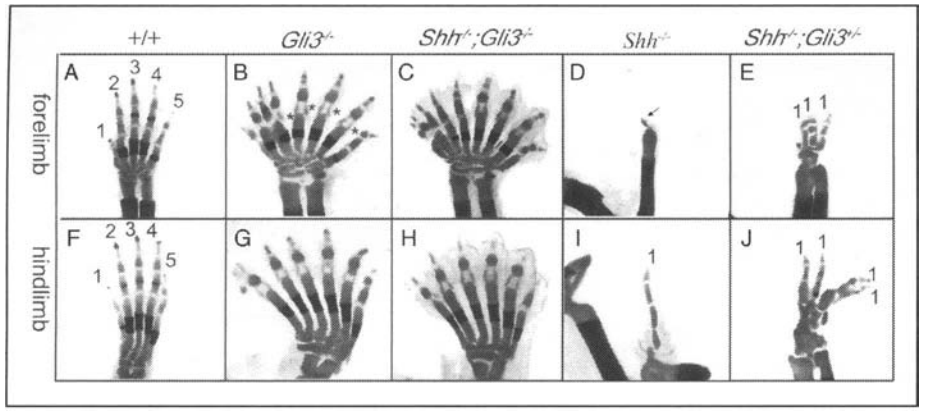

Figure 3. *Gli3* regulates digit number and identity. Skeletal analysis of forelimbs (A-E) and hindlimbs (F-J) of E16.5 wildtype (A,F), *Gli3*$^{-/-}$ (B,G), *Shh*$^{-/-}$;*Gli3*$^{-/-}$ (C,H), *Shh*$^{-/-}$ (D,I), and *Shh*$^{-/-}$;*Gli3*$^{+/-}$ (E,J) embryos. Wildtype autopod digits are numbered (A,F). B,G) *Gli3*$^{-/-}$ autopods are polydactylous. C,H) *Shh*$^{-/-}$;*Gli3*$^{-/-}$ limbs are polydactylous and digits lack normal identities. The syndactylous digits are short, thick, and appear more serially homologous than in wildtype; phalanges exhibit alternating gaps of unstained cartilage and bulbous stained material (black asterisks). *Shh*$^{-/-}$;*Gli3*$^{-/-}$ autopodia exhibits reduced, but characteristic A/P polarity; note invariant bunching of anterior digits and kinking of the posteriormost forelimb digit. Note that *Shh*$^{-/-}$;*Gli3*$^{-/-}$ and *Gli3*$^{-/-}$ limbs are virtually indistinguishable (compare B,G with C,H). D,I) *Shh*$^{-/-}$ autopods have a single unidentifiable element in the forelimb (arrow) and digit 1-like element in the hindlimb (I). E,J) *Shh*$^{-/-}$;*Gli3*$^{+/-}$ autopods develop three or four digits; all unfused digits are identifiable as digit 1, regardless of A/P position (modified from Litingtung et al, 2002). Anterior is to the left.

chromosome 5.[23-25] The discovery of a long-range limb enhancer led to the identification of point mutations in the ZRS in four unrelated families with PPD.[12] Intriguingly, these mutations are not clustered in a specific region. They are distributed throughout the 800 bp sequence of the ZRS[12] but do not overlap with the mutations found in the mouse PPD mutants described above[13] (Fig. 2). Additionally, none of the six point mutations in the ZRS appears to disrupt any known transcription factor binding sites. Therefore, it remains enigmatic as to how point mutations, insertion or deletion in the ZRS lead to ectopic *Shh* expression in the limb bud. It is possible that there are multiple silencer elements within the ZRS, and a mutation in any one of these elements activates *Shh* expression in the anterior limb bud. Alternatively, minor sequence perturbations in the ZRS could elicit structural changes of the chromatin, thus interfering with the normal function of transcription factors that are important for the asymmetric expression of *Shh* in the limb bud. Further studies are necessary to evaluate these possibilities.

In addition to PPD, the locus for the less common form of congenital limb malformation known as acheiropodia was also mapped to the *LMBR1* critical region on chromosome 7q36.[26] Acheiropodia is a limb-specific autosomal recessive disorder characterized by truncation of distal extremities resulting in the absence of bones in hands and feet. Detailed analysis indicated that affected individuals have a 4-6 kilo basepairs deletion that removes exon 4 of *LMBR1* as well as part of the surrounding introns[26](Fig. 2). It was suggested that disruption of *LMBR1* is likely responsible for the acheiropodia phenotype. However, abrogating or diminishing *Lmbr1* function by targeted mutation or by transgenic insertion as in *Sq*, failed to produce a phenotype that is consistent with acheiropodia.[17,27] While acheiropodia mutations are associated with a deletion spanning exon 4, the presence of a small deletion or point mutations in intron 5 was not formally examined. Therefore, the possibility remains that acheiropodia mutations are caused by the disruption of a limb-specific long-range enhancer element possibly in the ZRS. This view is also consistent with the observation that *Shh*$^{-/-}$ mice and acheiropodia patients have similar limb phenotypes.

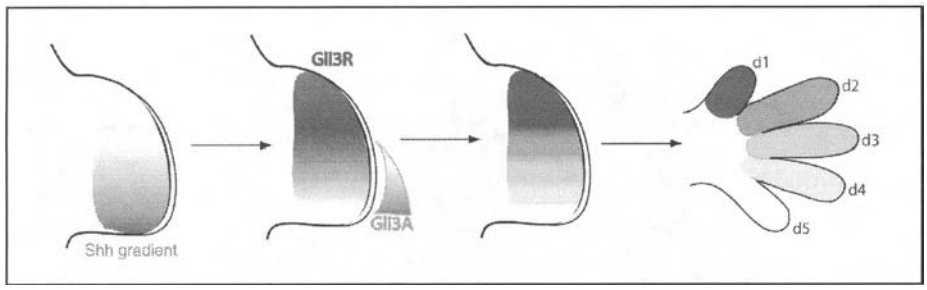

Figure 4. Model for Shh and Gli3 function in A/P patterning of the limb. Expression of *Shh* in the ZPA (posterior) generates a Shh protein concentration gradient (red) that in turn establishes an opposite gradient of Gli3R(blue) and Gli3A(purple) in the limb bud anteroposteriorly. The most anterior region of the limb bud (depicted at the top) expresses a high level of Gli3R, specifying digit 1 (d1), whereas the most posterior region does not express Gli3R (white), generating digit 5. The opposite Gli3A and Gli3R gradients generate three distinct threshold levels of target gene expression profiles to specify digit identities 2-5. A color version of this figure is available online at http://www.Eurekah.com.

Shh Activity in Limb Patterning Is Mediated by Gli3

Genetic and biochemical studies indicated that Patched (Ptch), a twelve-pass transmembrane receptor for Shh, functions as a negative regulator of Shh signaling by suppressing the activity of another seven-pass transmembrane protein, Smoothened (Smo). Shh binding to Ptch relieves Ptch-mediated inhibition of Smo which in turn triggers Shh signaling resulting in the activation of downstream target gene expressions mediated by the Gli family (Gli1, 2, 3) of transcription factors.[28] Functional analysis of single and double mutant combinations of *Gli1-Gli3* demonstrates that *Gli1* and *Gli2* perform no significant A/P patterning function in the limb.[29,30] In contrast, the homozygous dominant mouse mutant *extra toe* (Xt^J) that harbors null alleles of Gli3, dies at birth and exhibits limb polydactyly with syndactyly (fusion of soft tissues)[31,32] (Fig. 3). These abnormal limb phenotypes were causally attributed to ectopic *Shh* expression in the anterior limb mesoderm.[33,34] However, detailed skeletal analysis of $Gli3^{-/-}$ limbs indicated that their digits did not exhibit identities consistent with ectopic Shh signaling, instead the digits are serially homologous and lack wild type identities[35] (Fig. 3). Furthermore, mice lacking both *Shh* and *Gli3* develop polydactylous limbs that are virtually indistinguishable (compare Fig. 3B,G and C,H) from $Gli3^{-/-}$ limbs.[35] Therefore, polydactyly in $Gli3^{-/-}$ is a direct effect of lack of Gli3 function, and not due to ectopic *Shh* expression. These studies clearly indicate that Gli3 is the primary mediator of Shh signaling, and is required in limb skeletal patterning to control the two aspects of autopod morphology: digit number and identity.

How is the Shh signaling gradient interpreted by Gli3 in the specification of digit number and identity? Gli3 is a bi-functional transcription factor capable of activating or repressing Shh target genes in a Shh-dependent manner.[36-39] Shh inhibits Gli3 processing into its repressor form (Gli3R), hence promoting the accumulation of full-length Gli3 protein (Gli3A) which presumably functions as an activator.[40] Analysis of Gli3 protein profiles in dissected wild-type limb bud fragments revealed a polarized distribution of Gli3R and Gli3A across the A/P axis of the limb which bears correlation with the Shh signaling gradient.[35,40] In contrast, $Shh^{-/-}$ mutants express Gli3R uniformly in the limb,[35] consistent with the existence of a Gli3R gradient. Because all digits generated from mice that lack Shh function such as $Shh^{-/-}$ or $Shh^{-/-};Gli3^{+/-}$ develop as digit1,[35] this observation suggests a requirement of Gli3R in digit 1 formation.

Based on the polarized distribution of Gli3A and Gli3R in the limb bud, it was proposed that transcription of Shh target genes is mediated by the relative balance of Gli3A and Gli3R activities[35] (Fig. 4). According to this model, different threshold levels in the ratio of Gli3A to Gli3R (Gli3A:Gli3R), generated in response to graded Shh signal, direct distinct target gene

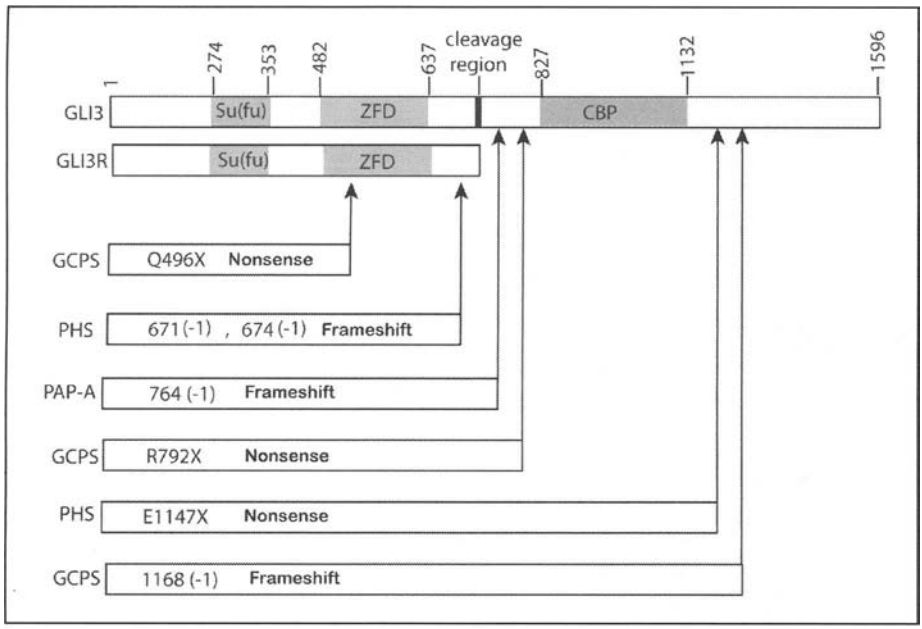

Figure 5. Examples of GLI3 mutations in GCPS, PHS and PAP. Schematic diagram of GLI3 and GLI3R proteins with several known functional domains such as the suppressor of fused [Su(fu)] binding domain, zinc finger domain (ZFD) and CBP binding domain. The types of mutations are indicated as nonsense, missense or frameshift. The number designation for each GLI3 mutation specifies the altered amino acid residue. Data are from references 42, 43, 46 and 47.

expression profiles that are associated with particular digit identities. Thus, a higher Gli3A:Gli3R threshold level will generate digits with posterior identities while a lower threshold level will generate digits of anterior identities. This model is consistent with the finding that homozygous *Gli3*$^{-/-}$ or *Shh*$^{-/-}$;*Gli3*$^{-/-}$ limbs exhibit a complete loss of wild-type digit identities. Further support for this model comes from analysis of *talpid2* (*ta*^{2}) chick mutant limbs which show uniform expression of Shh target genes and high Gli3A:Gli3R levels across the A/P axis.[40] Accordingly, *ta2* limbs exhibit polydactyly and uniform posterior digit identities.[41]

Congenital Limb Malformations Are Associated with Gli3 Mutations

Mutations in human GLI3 have been identified in Greig Cephalopolysyndactyly syndrome (GCPS),[42] Pallister-Hall syndrome (PHS)[43] and postaxial polydactyly type A (PAP-A).[44] GCPS and PHS are autosomal dominant conditions displaying defects in growth and development of the head, limb and visceral organs, while PAP-A affects only the development of the limb. The hallmarks of GCPS are preaxial polydactyly with facial anomalies such as hypertelorism and frontal bossing. These characteristics are distinct from PHS which displays central or postaxial type polydactyly, hypothalamic hamartoblastoma and, occasionally, neural midline defects such as abnormal pituitary and callosal agenesis. The clinical manifestations of these three syndromes are quite distinct, but all exhibit polydactyly and syndactyly.

It has been proposed that defined truncations of the GLI3 protein are associated with specific phenotypes[45] (Fig. 5). The dominant preaxial polydactyly phenotype in GCPS patients and in mouse *Xtj*$^{+/-}$ is caused by genetic mutations that disrupt the function of one Gli3 allele. In contrast, PHS and PAP-A syndromes are associated with mutations predicted to generate altered Gli3 proteins with truncations carboxy-terminal to the zinc finger domain (ZFD) (see Fig. 1). These truncated proteins will not be regulated by Shh at the level of processing. Thus,

the distinct characteristics of PHS and PAP-A are possibly due to enhanced production of GLI3R proteins, whereas GCPS phenotypes are caused by GLI3 haploinsufficiency (Fig. 1A, structurephenotype model). Since individuals with PAP-A have a milder phenotype than those with PHS, this observation suggests that the sites mutated in PAP-A may attenuate repression by truncated GLI3 proteins.

However, recent analysis of additional families displaying these syndromes suggests that the molecular mechanism leading to a clinical manifestation may be more complex than predicted based on the structurephenotype model.[46-48] For example, familial patients carrying a nonsense mutation in codon 792 (R792), predicted to harbor a GLI3 truncation right after the zinc-finger domain, were diagnosed with GCPS instead of PHS (Fig. 1B). These observations suggest that the expression of distinct phenotypic traits in these syndromes likely reflects how a particular mutation affects GLI3 transcriptional properties.

Conclusion

Polydactyly is one of the most common types of limb malformations in humans. Analyses of mouse mutants have provided insights into the molecular mechanism leading to polydactyly as observed in humans. The emergence of two conceptual classes of polydactyly provides a link between developmental mechanisms and phenotypic traits.[35] Group 1 mutations result from ectopic *Shh* expression in the anterior limb bud mesoderm but do not disrupt Gli3 function. Group 1 mutation is characterized by preaxial polydactyly with mirror image digit duplications, which is exemplified by mouse mutants such as *lst*, *Ssq*, *Hx*, M100081 and the human PPD locus. Additional mouse mutants such as *Recombination induced mutant 4 (Rim4), X-linked polydactyly (Xpl)* and *luxate (lx)*[33,49] also belong to Group 1, and elucidating the molecular nature of these mutants will likely provide mechanistic insights into the negative regulatory program that controls *Shh* expression in the limb.

In contrast, Group 2 mutations disrupt Gli3 function by a Shh-independent mechanism thereby generating a spectrum of supernumerary digits. Group 2 polydactylous limbs lack mirror image duplications and their supernumerary digits are always syndactylous. The phenotypic variations in GCPS, PHS and PAP-A syndromes likely reflect how a particular Gli3 mutation affects its transcriptional activities and target gene expression. Functional characterization of these mutant proteins in mice will likely provide novel insights into the developmental mechanisms of polydactyly in humans.

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CHAPTER 12

The Genetics of Indian Hedgehog

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The hedgehog proteins are highly conserved between species and act as key morphogens during development, along with other molecules such as fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs) and Hox proteins. In mammals there are three known family members, sonic hedgehog (Shh), desert hedgehog (Dhh) and indian hedgehog (Ihh). While these proteins are highly homologous and can substitute for one another in a variety of functional assays,[1] they elicit distinct biological responses in vivo and in vitro.[2] Studies have shown that Ihh is critical for proper development of several tissues including the pancreas,[3] and the endochondral skeleton.[1,4,5] There is also evidence that it is involved in hematopoiesis, vasculogenesis[6] and colonocyte differentiation.[7] Thus far, mutations of the *IHH* gene have been identified in two hereditary conditions, brachydactyly type A1 (BDA1)[8-11] and acrocapitofemoral dysplasia (ACFD).[12] Surprisingly, despite the range of tissues that IHH modulates developmental and differentiation processes of, the only system that appears to be affected in these conditions, is the skeleton.[8,12]

Normal bone development can occur by one of two processes, intramembranous, or endochondral ossification. The first process involves direct ossification of mesenchymal tissue at the site of the future bone and occurs primarily in the flat bones of the skull. Endochondral ossification, by contrast, requires a cartilage intermediate and is the primary process by which bone development occurs in the axial and appendicular skeletons. As the limb grows along the proximodistal axis, mesenchymal cells begin to condense to form the basic shapes of the future bone. At the core of these condensations, cells differentiate into chondrocytes and start to secrete a cartilaginous matrix (reviewed in ref. 13). Periphery cells also differentiate and secrete a perichondrial sheath that surrounds the cartilage anlagen. Since bone does not have the capacity to grow, formation of a cartilaginous structure is essential for skeletal growth prior to bone deposition. Defects in proper cartilage analgen formation, or in mesenchymal condensation, can result in shortened, abnormally shaped or absent bones.[14] Shortly after formation of the cartilage analgen, cells in the center of the structure exit the cell cycle, and mature into hypertrophic chondrocytes. The terminally differentiated chondrocytes finally die through apoptosis and the calcified matrix is replaced with trabecular bone. As the process of hypertrophy extends outwards from the center of the bone, the zone of proliferating chondrocytes is restricted to the apical ends. Secondary ossification centers may form at the distal ends of the bone, leaving a small region between the primary and secondary ossification center that continues to proliferate. This region is called the epiphyseal growth plate and is the only portion of the skeletal element that retains growth potential. The cells within this region arrange themselves in columns including a zone of resting chondrocytes followed by zones of proliferating, prehypertrophic, hypertrophic and terminally differentiated chondrocytes. The conversion of calcified cartilage to trabecular bone matrix continues until adolescence when the epiphyses close and the growth plate is finally replaced by bone.

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Within the growth plate, the transition of chondrocytes from the proliferative to terminally differentiated states is a highly regulated process. Maintenance of appropriate numbers of cells within each fraction is necessary to ensure normal growth and development. Ihh is a key modulator of proliferation and differentiation within the growth plate. Mice homozygous for an *Ihh* null allele (*Ihh*$^{-/-}$ mice) show a reduced capacity for chondrocyte proliferation and a shift in the fraction of hypertrophic chondrocytes towards the axial ends.[4] The absence of a bone collar in these mice further demonstrates a role for Ihh during osteogenesis, although it does not appear necessary for initiation of osteoblast differentiation.[4,5] Finally, it has been shown that Ihh can directly promote chondrocyte proliferation and may be necessary for driving growth along the longitudal axis of the bone.[15]

Brachydactyly Type A1

The brachydactylies are a group of congenital malformations that affect normal bone development resulting in shortened digits. They are categorized into a number of different types based on the bones and digits involved.[16,17] Brachydactyly type A1 (BDA1) is characterized by bilateral shortness of the middle phalanges in digits two through five that is at least 2 standard deviations below normal. The observed phenotype is variable and can range in severity from a mild variant, in which the shortness of the middle phalanges is more apparent than in other digital bones, to a severe variant, in which the middle phalanges are absent or fused to the terminal phalanges. In both mild and severe forms of BDA1, the shortening is most prominent in digits two and five, followed by digits four, then three. Similarly, if terminal symphalangism is present, it is more common in digits two and five than four and three.[17] Although shortening of the middle phalanges is the hallmark feature of BDA1 a number of other skeletal features have been reported including short stature,[18-21] radial/ulnar clinodactyly,[22-27] shortening of the first, third,[10,25] or fifth metacarpal,[24] and premature epiphyseal closure.[17,19,24,28] In a comprehensive analysis of affected individuals from an American family with BDA1, Haws and McKusick further described sloping of the distal end of the radius, an absence of the styloid process of the ulna, abnormally shallow acetabulum and glenoid fossa, and an elliptical, rather than round, humerous head.[28] Incidents of scoliosis and BDA1 have also been reported in at least three families.[20,27,29]

BDA1 has the distinction of being the first human trait described in terms of autosomal dominant Mendelian inheritance.[30] Despite this, little was known about the genes involved until recently. In 2000, Yang et al described linkage of BDA1 to chromosome 2q35-36 in two Chinese families with the trait.[25] This led to the subsequent identification of *IHH* mutations at nucleotide positions 283 and 391 of the coding sequence in families I and II, respectively.[8] An additional mutation at nucleotide position 300 was identified in a third family, further supporting a role for IHH in the pathophysiology of BDA1.[8] The three mutations were predicted to cause amino acid substitutions at Glu95 (Glu→Lys), Asp100 (Asp→Glu) and Glu131 (Glu→Lys). Since the initial report of *IHH* mutations, two additional allelic variants have been identified in individuals with BDA1.[9,11] The first, a G→A transition at nucleotide position 298, was identified in descendents of some of the earliest reports of BDA1 inheritance,[9,18,22] and is predicted to cause an aspartic acid to asparagine amino acid substitution at codon 100.[9] It has since been identified in four additional kindreds and likely represents an ancestral mutation (unpublished observation and ref. 10). The second variant, an A→G transition was identified at nucleotide position 284 of the *IHH* coding sequence, in an American family of Scandinavian descent.[11] It is predicted to cause a glutamate to glycine amino acid substitution at codon 95.[11] The Asp100Asn and Glu95Gly variants both cause substitutions of identical amino acids to those described in the first report of BDA1 mutations, although they result in different substitutions.[8,9,11] This suggests that these two amino acids may be hot spots for BDA1 mutations and that they are likely important for normal IHH function during endochondral ossification.[9]

Although the tertiary structure of the human IHH protein has not yet been characterized, the crystal structure of Shh predicts that Glu95, Asp100, and Glu131 are closely situated to

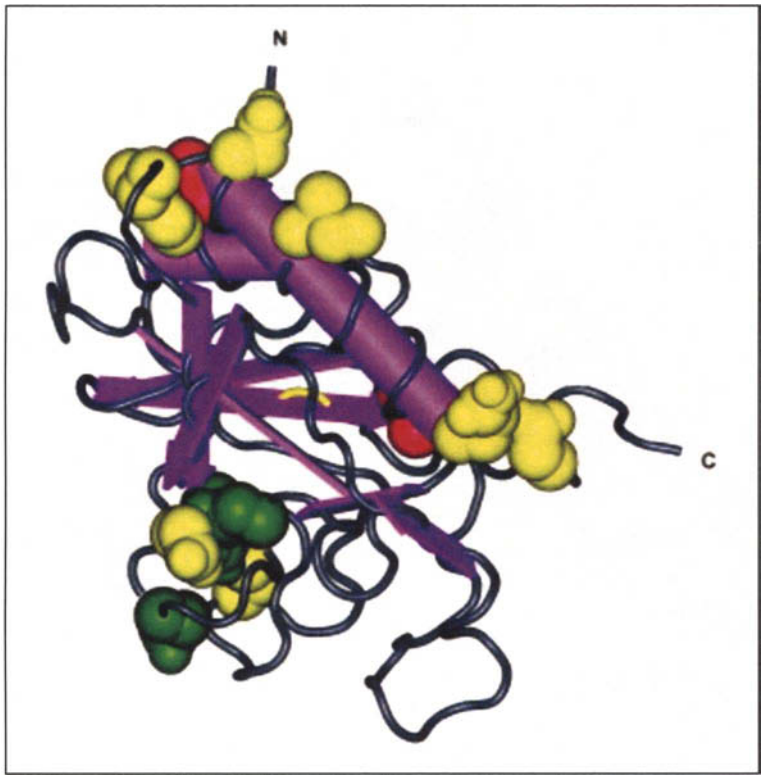

Figure 1. Tertiary structure of murine Shh N-terminal signalling domain indicating location of amino acids mutated in human disease. Based on high degrees of similarity between the murine Shh and human hedgehog N-terminal domains, the tertiary structure of murine Shh has been used to model the locations of disease-causing mutations reported in three human diseases, BDA1, ACFD, and holoprosencephaly. The latter disease is caused by mutations of the *SHH* gene. Green, red and yellow molecules indicate the position of amino acids that are mutated in BDA1, ACFD and holoprosencephaly, respectively. (Reproduced with permission from Hellemans J, Coucke PJ, Geidion A. Am J Hum Genet 2003; 72:1040-1046, University of Chicago Press. ©2003 by The American Society of Human Genetics.)

one another in an external groove of the protein[31] in an area known to facilitate binding of the hedgehog proteins to the Patched (PTCH) receptor (see Fig. 1).[8,32,33] Gao et al predicted that mutations at these sites may alter IHH function by either disrupting interactions with it natural receptor(s), or by enhancing its ability to interact with alternate receptors.[8] Attenuation of IHH signaling may further decrease the growth potential of developing bones by reducing the number of proliferating chondrocytes, and/or increasing the number of cells differentiating from the proliferative to hypertrophic stages. As the middle phalanges are the last phalanges to ossify, they may be more susceptible to reduced chondrocyte numbers than other phalanges.[17]

Mutations of the *IHH* gene account for only a subset of the BDA1 incidents described to date, indicating that the trait is genetically heterogenous.[11,34] This is further supported by the identification of a second locus on chromosome 5p13.3-13.2.[34] A third locus may also exist, based on the identification of an American family with BDA1 that lacked detectable *IHH* mutations in the coding region, and whose trait was not linked to the chromosome 5p locus.[11] The identification of BDA1 mutations at loci other than *IHH* is important as this information may contribute significantly to our understanding of endochondral ossification in humans.

Characterization of alternate BDA1 loci may also provide information about the IHH signaling pathway, or parallel biological pathways.

Acrocapitofemoral Dysplasia (ACFD)

ACFD is an autosomal recessive skeletal dysplasia characterized by short stature, brachydactyly (short middle and distal phalanges), a narrow thorax, and a relatively large head.[35] Cone-shaped/ abnormal epiphyses and premature epiphyseal closure occur in the hands, the proximal portion of the femur and to a variable degree in the shoulders, knees and ankles of affected individuals.[35] The most consistent finding among individuals diagnosed with ACFD, is a round, egg-shaped femoral head with a shallow, almost absent femoral neck.[35]

ACFD was first described in March 2003, in two consanguineous families with five affected individuals.[12,35] Homozygosity mapping of individuals from these families was used to define a 5 cM critical region on chromosome 2q35-q36. Using a positional candidate gene approach, the same group subsequently identified a C→T transition at nucleotide 137 of the *IHH* gene (P46L) in family one, and a T→C transition at nucleotide 569 of the same gene (V190A) in family two. Both mutations caused amino acid substitutions in the amino terminal portion of the protein responsible for local and long range signaling.[12,36,37] Hellemans et al hypothesized that the mutations cause ACFD by diminishing IHH signaling and thus increasing the rate of chondrocyte differentiation.[12]

Despite being heterozygous for the above mutations, the parents of each affected individual did not show signs of ACFD. The authors did note, however, that the parents from family one had short middle phalanges (up to -2.5 SD in digits 2-4 and up to -5 SD in digit 5), which were neither rudimentary nor fused to the terminal phalanges.[12] The parents from family two were asymptomatic except for mild shortening of the metacarpals and proximal phalanges (> 2 SD) in two individuals.[12]

Comparison of Human IHH Disorders

As BDA1 and ACFD are allelic, we may expect to observe some phenotypic similarities between the two conditions. The most consistent similarity between individuals affected with each condition, is brachydactyly involving the middle phalanges. Short stature and scoliosis have also been reported in association with both conditions,[12,18,21,22,24,35] but do not occur consistently in all cases. Skeletal surveys are seldom reported in case studies of BDA1, making it difficult to compare other phenotypes between the two traits. However, in 1963, Haws and McKusick[28] published an extensive description of three individuals with BDA1, who were descendants from the first kindred used to demonstrate Mendelian dominant inheritance in humans. In addition to analyses of the hands and feet, Haws and McKusick provided detailed descriptions of malformations to the distal ends of the radius, the ulna and the tibia, to the femoral and humeral heads and to the glenoid fossa, and acetabulum.[28] Of particular interest, was the description of a mushroom appearance to the femoral head and an unusually short femoral neck,[28] which is similar to the abnormalities described in ACFD affected individuals.[12,35] Since the report by Haws and McKusick, an IHH mutation has been identified in affected individuals from this family (Asp100Asn)(unpublished), suggesting that these malformations occurred due to defective IHH, and not another BDA1 gene. Similar abnormalities of the radius, tibia, ulna, glenoid fossa, acetabulum and humerus were noted in a young girl with a complex condition including BDA1, dwarfism, ptosis, mixed partial hearing loss, microcephaly, and mental retardation.[21] However, due to the lack of radiographic evidence, it is not possible to assess whether femoral abnormalities occur in other incidents of BDA1, or whether it is isolated to individuals from these families. Similar malformation of the middle phalanges, the vertebral column and the femoral head in individuals with BDA1 and ACFD suggest that IHH is critical for normal patterning of these skeletal elements.

The observation of dominant and recessive allelic variants within a single gene, which cause distinct malformations or syndromes, is not uncommon. Dominant mutations of the gene

GDF5/ CDMP1 have been identified in both brachydactyly type C and DuPan Syndrome, while recessive mutations of the same gene are known to cause Grebe type chondrodysplasia, and acromesomelic dysplasia, Hunter-Thompson type (reviewed in ref. 13). In many of these cases, the dominant phenotype is milder than the recessive one, and may reflect the presence of wild type protein in the dominant conditions. A similar relationship between allelic dominant and recessive conditions exists between BDA1 and ACFD. While the BDA1 phenotype is largely restricted to the middle phalanges, with some exceptions, the ACFD phenotype affects a broader range of skeletal elements. In addition to short middle phalanges, individuals diagnosed with ACFD exhibit short distal phalanges, microcephaly, and a narrow thorax.[35] Short stature is also more pronounced in individuals with ACFD, than those with BDA1.[35] Although these differences in phenotype may be partially due to inheritance of dominant versus recessive mutations, Hellemans et al predicted that they may also occur because of differences in location of the respective mutations.[12] They showed that while the mutations causing BDA1 occur clustered together in a single pocket along the IHH protein surface, the mutations identified in individuals with ACFD are predicted to affect amino acids on the opposite face of the protein (refer to Fig. 1).[12] Due to the lack of in vitro or in vivo data, it is difficult to determine what effect these mutations have on normal IHH function. The mild phenotype observed in some carriers of the ACFD mutations, and lack of phenotype observed in others, suggests that either the mutant protein retains some activity, or that haploinsufficiency of the IHH protein is not sufficient to greatly affect bone developmental processes. In contrast, the *IHH* mutations identified in individuals with BDA1 are inherited in an autosomal dominant pattern, indicating that the wild type IHH gene cannot complement for these mutations. Gao et al predicted that BDA1 mutations may disrupt normal endochondral ossification in the digits through haploinsufficiency of the wild-type IHH protein.[8] However, if the recessive mutations identified in ACFD carriers also cause haploinsufficiency, but do not cause an obvious phenotype, than it is more likely that the dominant BDA1 mutations disrupt IHH signalling through a dominant-negative effect.

IHH Animal Model

Although an animal model with specific BDA1 or ACFD mutations has not been described, analysis of the *Ihh*$^{-/-}$ mouse can provide important insights into how the mutant protein may contribute to the physiology of these two malformations. An *Ihh*$^{-/-}$ mouse was generated by St-Jacques, Hammerschmidt and McMahon by replacing the first exon of the gene with a neomycin resistance cassette.[4] The mutation was lethal and half of the *Ihh*$^{-/-}$ embryos died between 10.5 to 12.5 days post-coitum (dpc), likely due to circulatory abnormalities. Most of the remaining *Ihh*$^{-/-}$ embryos survived to term but died shortly after birth because of respiratory failure that may have resulted from shortening of the ribs.[4] Those embryos that survived to birth were substantially smaller than their wildtype littermates and showed pronounced shortness of their snout, tail, and limbs. Rounded skulls, misshapen endochondral bones and improper joint formation were also observed.[4] Unfortunately, because these mice lacked proper segmentation of the digital bones, it is not possible to compare the brachydactylous phenotype of individuals with either BDA1 or ACFD to the *Ihh*$^{-/-}$ mice. Several similarities between the *Ihh*$^{-/-}$ mice and ACFD-affected individuals were apparent, however, and include dwarfism of the long bones, a narrow thorax, and misshapen bones. St-Jacques, Hammerschmidt and McMahon showed that *Ihh*$^{-/-}$ mice had reduced amounts of chondrocyte proliferation compared to wildtype littermates.[4] They also showed delayed chondrocyte differentiation, disorganization of cells in the growth plate, and a shift of hypertrophic cells to the apical ends.[4] Similar growth plate abnormalities in BDA1- and ACFD-affected individuals may in part account for the short bones observed in each condition. Reduced chondrocyte proliferation and displacement of the hypertrophic zone could stunt growth of the skeletal element by diminishing cell numbers within the growth plate. Disorganization of cells throughout the growth plate could also contribute to the development of short bones by altering the

ability of developing skeletal elements to expand lengthwise through cell expansion during hypertrophy. The observation of irregular, "cone-shaped" epiphyses and premature epiphyseal closure in individuals with both BDA1 and ACFD[17,19,24,28,35] supports the hypothesis that IHH mutations in both conditions cause abnormalities of growth plate organization and proliferation. Fitch further hypothesized that, in individuals with BDA1, shortness may be more pronounced in the middle phalanges than in other bones since these bones are the last to ossify and may be more susceptible to the affects of shortened cartilage analgens.[17]

One of the primary differences observed between *Ihh*$^{-/-}$ mice and individuals with *IHH* BDA1 or ACFD mutations relates to the synthesis of bone matrix. While null mice lacked osteoblasts and subsequently did not produce a bone collar,[4,5] similar observations in individuals with either ACFD or BDA1 have not been reported. Moreover, BDA1 and ACFD mutations do not appear to affect pancreatic development or circulation in humans despite reports of an annulus encircling the duodenum caused by an extension of the ventral pancreas in 42% of *Ihh*$^{-/-}$ embryos,[3] and circulatory abnormalities in *Ihh*$^{-/-}$ embryos that die between 10.5 and 12.5 dpc.[4] The lack of phenotype in BDA1 and ACFD beyond those observed in the skeleton suggests that either the IHH mutants identified thus far retain some function that allows normal activity in other tissues or there are redundant pathways in humans that do not occur in mice. Further experimentation is required to address this issue.

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CHAPTER 13

Sonic Hedgehog Signaling in Craniofacial Development

Dwight Cordero, Minal Tapadia and Jill A. Helms*

Introduction

Facial malformations comprise a significant proportion of the anomalies observed in children born with birth defects. Although congenital facial disfigurements are relatively common and have been exhaustively categorized, science is just now beginning to unravel the genetic and environmental etiologies as well as the molecular mechanisms underlying the clinical manifestations. The Sonic Hedgehog (SHH) pathway is one important signaling pathway involved in craniofacial development. The aim of this chapter is to provide a concise review of craniofacial development within the context of recent advances in our knowledge regarding SHH signaling in facial morphogenesis. We will discuss what is known about the functions of SHH in normal and abnormal craniofacial development in humans and how nonhuman model systems are being used to build a foundation in which to understand the mechanisms underlying craniofacial malformations in humans.

The Face in Myth and Reality

In Homer's Odyssey, the Cyclops Polythemus is depicted as a terrifying, mysterious, one eyed man-eating monster (Fig. 1).[1] The tale was intended, like many mythologies, to explain phenomena which were not clearly understood at the time but which certainly existed in human and animal populations (Fig. 2). Centuries later, we are beginning to elucidate the scientific mysteries that underlie the physical malformations that gave rise to the myth of the Cyclops and that are still observed today. We now know that cyclopia and other less severe craniofacial disorders can result from disruptions in the SHH pathway caused by genetic or environmental etiologies, or a combination of these factors.

Cyclopia is the most extreme manifestation of the congenital disorder holoprosencephaly (HPE), which occurs when the forebrain fails to separate into two distinct cerebral hemispheres. One clinical aspect of this disorder is the wide spectrum of phenotypes involving midline patterning defects observed in fetuses and newborns (see Fig. 3). The spectrum of facial phenotypes ranges from cyclopia with a proboscis, to lesser degrees of facial dysmorphologies consisting of microopthalmia (small eyes), coloboma (congenital abnormality of the eye caused by a lack of complete optic cup closure), hypotelorism (eyes positioned close together in comparison to standard measurements), midface hypoplasia, cleft lip and palate (Fig. 3A,B), and a single incisor.[2,3] At the other end of the spectrum, individuals with HPE can have a normal or nearly normal appearing face (Fig. 3C,D). In this chapter we use HPE as a prototype in which to think about the molecular mechanisms governing normal and abnormal craniofacial development.

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Figure 1. The Cyclops Polythemus. The Cyclops Polythemus was depicted in the ancient texts as being a monster with horrific facial features. Several centuries later, modern science is unraveling the molecular and cellular basis for a number of human craniofacial disorders, such as Holoprosencephaly, which may have given rise to the myth of the Cyclops. Reproduced, with permission, from reference 3a.

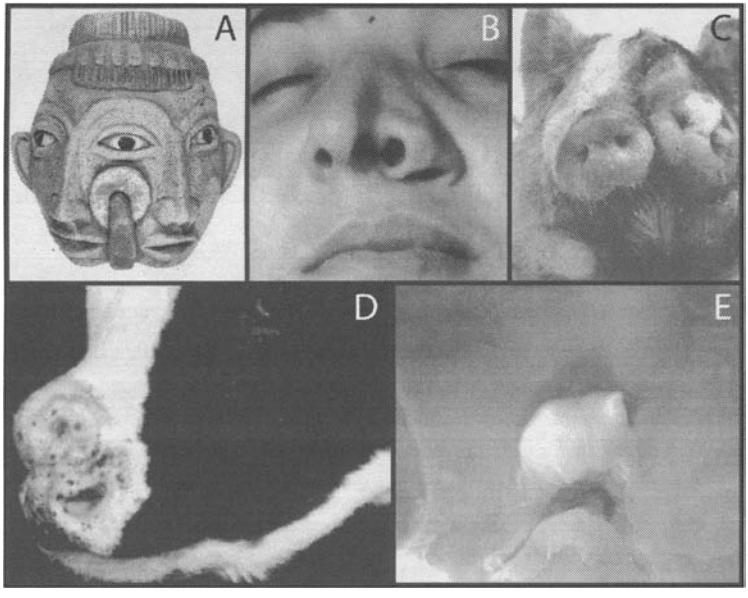

Figure 2. Facial duplications. Facial duplication has also held a special fascination and involves humans and other animals. Duplications shown in: A,B) human; C) pig; D) cow; E) chick.

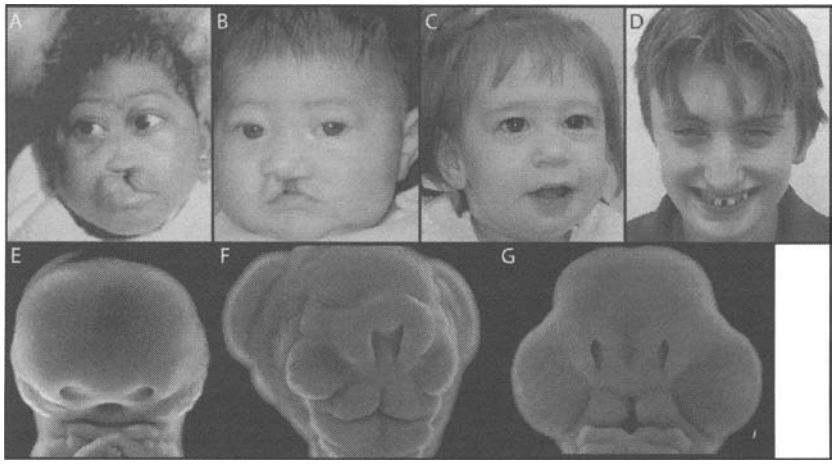

Figure 3. Spectrum of facial phenotypes associated with holoprosencephaly (HPE). The craniofacial phenotypes observed in patients with Holoprosencephaly (HPE) (A-D) do not always correlate with the underlying forebrain abnormality and can be recapitulated using the chick model system (E-G). A) Children with alobar HPE (the most severe forebrain abnormality in HPE) can manifest with facial malformations including microcephaly, midface hypoplasia, hypotelorism and midline cleft lip and palate as seen in this child. Patients with alobar HPE may exhibit more severe facial malformations such as cyclopia (not shown). B) This child has semilobar HPE (the intermediate form of forebrain malformation in HPE) yet exhibits facial malformations similar to the child in (A). C) A child with lobar HPE (the least severe forebrain malformation in HPE) presents with relatively normal facial features. D) The variation in the clinical phenotype among patients with the same anatomic classification of HPE is seen in this child who also has lobar HPE, but compared to (C) has mild hypotelorism and midface hypoplasia and abnormal dentition. E,F) Stage 25 chick embryos treated with cyclopamine at stage 15 exhibit features reminiscent of lobar HPE, including a single telencephalic vesicle, midface hypoplasia, and hypotelorism. G) Stage 25 chick embryos treated with cyclopamine at stage 17 exhibit features reminiscent of semilobar HPE, including microcephaly, hypotelorism and mild midface hypoplasia. A) courtesy Dr. Alan Shanske; B-D) courtesy Dr. Jin Hahn; E-G) reproduced, with permission, from reference 73.

HPE can be classified based on anatomic or genetic criteria. Either classification scheme is valid within the given context of the patient but the classification criteria should be noted when discussing HPE and the various aspects of the disorder.[4] For example, in the classic or anatomic classification of HPE, the degree to which the telencephalon (forebrain) fails to separate into individual hemispheres has been used as the criteria to define alobar, semilobar, or lobar HPE. For many years, the severity of the facial malformations in HPE was thought to directly correlate with the severity of these brain malformations. This viewpoint led to the postulate, "the face predicts the brain".[5]

With advanced imaging technologies we now realize that the face does not always predict the anatomic status of the brain, nor does the brain always predict the severity of facial manifestations in HPE. In the genetic classification, the type of HPE is defined based on cytogenetic deletions or mutations in genes known to cause HPE such as *SHH*, *ZIC2*, *PTCH*, *TGIF*, or *SIX3*. For example, mutations in *SHH* are designated as HPE3[6] and may manifest with severe anatomic anomalies of the forebrain and face while in other cases, the same mutation can result in lesser degrees of brain malformations and a spectrum of facial dysmorphologies which do not necessarily meet the anatomic criteria for HPE.[4] One is left with an incomplete understanding, therefore, of how mutations in *SHH* can generate such a remarkable diversity of craniofacial phenotypes. In the next section we will discuss how the ability to detect genetic alterations in the SHH pathway raises several new questions about the roles of SHH signaling in brain and facial development.

The Craniofacial Consequences of Disrupting SHH Signaling: The Human Experience

Genetic Disruptions

A number of mutations in the SHH pathway that lead to HPE[2,7-10] and associated facial phenotypes,[10-13] have been identified. It has been proposed that the severity of HPE phenotypes is dependent on the extent to which the function of the proteins in the SHH pathway are altered. Traiffort and her group recently addressed the functional aspects of HPE-causing *SHH* mutations in an effort to understand their functional consequences.[13] She found that *SHH* mutations in patient's with HPE fell into one of three classes: those mutations that influenced zinc binding of the protein, those that affected auto-processing of SHH, and those that adversely altered SHH stability.[13] Her study confirmed what scientists and clinicians had come to accept:[7] there is no clear genotype-phenotype correlation in HPE. This finding strongly suggests that additional genetic and/or environmental factors are important determinants of a given HPE phenotype.

In addition to SHH, mutations in other genetic components of the SHH pathway can result in HPE. For example, perturbations in *Patched* (*PTCH1*), which encodes the receptor for SHH, and the zinc finger transcription factors *GLI2 and GLI3* are known to cause HPE and other craniofacial dysmorphologies. Mutations that render the PTCH1 receptor unable to bind SHH[14] or adversely alter intracellular interactions of PTCH1 may lead to the repression of SMOOTHENED (SMO) and inhibition of SHH signaling, resulting in HPE.[11] To date, no mutations in *GLI1* have been identified as causative for HPE, however, patients with mutations in *GLI2* exhibit features of HPE and pituitary anomalies.[15]

HPE is not the only disorder that results from aberrations in the SHH pathway. Mutations in *Ptch* which lead to truncations of the C-terminus render the protein unable to repress Smoothened activity and the net result is constitutive activation of the SHH pathway.[16] Basal cell nevus syndrome (also known as Gorlins' syndrome) is an autosomal dominant disorder caused by such a gain-of function mutation in *PTCH1*.[17,18] The most serious complication of this disorder is the predisposition to basal cell carcinomas and medulloblastomas. These patients may also exhibit dysmorphic facial features consisting of strabismus, cleft palate, and keratocysts of the jaw.[19,20]

Mutations in *GLI3* also cause a number of craniofacial syndromes. Two disorders are Greig cephalopolysyndactyly syndrome (GCPS) and Pallister-Hall syndrome (PHS), both of which have distinctive craniofacial dysmorphologies.[21-23] GCPS syndrome is an autosomal dominant disorder characterized by macrocephaly, a broad nasal root, hypertelorism, a prominent forehead, pre-and postaxial polydactyly, and syndactyly of the hands and feet.[24,25] The syndrome shares some clinical features with Pallister-Hall syndrome (PHS) such as craniofacial abnormalities, postaxial polysyndactyly and inheritance in an autosomal manner.[22,26] However, PHS is clinically distinguishable from GCPS in that PHS patients may have a hypothalamic hamartoblastoma (tumor), do not exhibit hypertelorism or a broad nasal root,[22] and occasionally have midline cleft lip and palate holoprosencephaly. Studies suggest that GCPS syndrome results from functional haploinsufficiency of *GLI3*[21,27,28] while PHS results from the production of a truncated GLI3 protein.[22] Truncation mutations can, however, cause GCPS. Based on *GLI3* truncation mutations and the clinical phenotypes of these disorders, a model which showed that mutations in *GLI3* can mimic the bi-functional nature of *Drosophila* Cubitus interuptus (Ci) was generated.[23] It was found that full length GLI3 localizes to the cytoplasm and activates *PTCH1* expression, whereas the mutant GCPS-GLI3 protein had no effect on *PTCH1* expression, consistent with haploinsufficiency in this disorder. However, the mutant PHS-GLI3 protein repressed GLI3 activated *PTCH1* expression.[23]

Smith-Lemli-Opitz Syndrome (SLO) is an autosomal recessive disorder, which may be associated with perturbations in SHH signaling. HPE occurs in approximately 5% of SLO

patients.[29-31] SLO is due to a defect in 3 beta-hydroxy-steroid–Δ7-reductase (7-DHC reductase) that leads to impaired cholesterol synthesis.[32,33] Cholesterol plays many roles during embryonic development, and most importantly for this discussion, in SHH signaling. Cholesterol appears to play a role in the cellular transport of Shh and in the generation of or in allowing clustering of the morphogen to create areas of higher and lower concentration in a cell.[34] Although the relationships between cholesterol and SHH are not completely characterized, perturbations in cholesterol metabolism may alter SHH function.[35,36]

Teratogens and Their Effect on SHH Signaling

A number of teratogens have been identified which appear to exert their adverse effects on craniofacial development via the SHH signaling pathway. The untoward consequences of maternal teratogen exposure depend upon three factors: first, the genetic susceptibility of a given embryo to a teratogen; second, the developmental stage of the embryo at the time of exposure; and third, the mechanism of action of the teratogen. While birth defects are caused by inadvertent exposure to teratogens, these substances are being increasingly exploited in the laboratory setting to gain insights into both normal and abnormal craniofacial developmental processes.

Alcohol is a known teratogen, yet prenatal exposure still occurs at a daunting rate, making it one of the most common causes of preventable birth defects and mental retardation.[37] Fetal Alcohol Syndrome (FAS) occurs between 0.2-1.5 per 1000 live births and is characterized by growth restriction, abnormalities of the central nervous system, and a stereotypical facial dysmorphology. The craniofacial features observed are microcephaly, microopthalmos, hypoplastic philtrum and maxillary hypoplasia.[38-40] The underlying molecular mechanisms resulting in the clinical manifestations of FAS remain enigmatic, although recent studies are providing more clues. Exposure to ethanol alters the embryonic expression patterns of *Shh* in the craniofacial complex.[41] The net effect of altering *Shh* expression appears to be the premature death of cranial neural crest cells.[41] Although these studies were conducted in nonhuman models they may provide a molecular or cellular explanation for how alcohol adversely affects human brain and facial development and will be discussed in detail later in this chapter.

Malformations of the craniofacial region are also seen in cases of excess and deficient vitamin A, and the phenotypes of the effected individuals appear to be remarkably similar to individuals with FAS.[40,42] Public attention to the teratogenic potential of vitamin A and its derivatives came in the early 1980s when women became pregnant while taking the drug Accutane (Isotretionoin) for treatment of acne.[43-45] In addition to increased spontaneous abortions, children born to mothers who took Accutane had birth defects involving the brain, heart and face. Exogenous retinoic acid, the active metabolite of vitamin A, has been shown to disrupt the expression of *Shh* in chicken embryos[46] and may play a role in the clinical manifestations seen in either excessive or deficiencies in vitamin A or its derivatives.

A group of drugs used to treat hypercholesterolemia, the Statins, pharmacologically lower cholesterol biosynthesis by inhibiting HMG-CoA reductase, and may be associated with limb and central nervous system (CNS) malformations such as HPE when taken during the first trimester of pregnancy.[36] Presumably the teratogenesis is due to decreased cholesterol bioavailability needed for a number of processes during embryogenesis. Theoretically this may affect autoprocessing or transport of Hedgehog (Hh) proteins thus adversely influencing Hh activity. The teratogenic potential of these drugs in humans requires further clinical and basic scientific investigation.

Taken together, these clinical findings indicate a strong correlation between disruptions in SHH and craniofacial malformations. These studies also implicate a wide variety of drugs that can have a teratogenic effect, and do so presumably by altering SHH signaling. In order to rigorously test our understanding of how SHH functions in craniofacial development, however, model systems have had to be developed which allow us to alter spatially and temporally SHH signaling. Some of these models are presented in the following section.

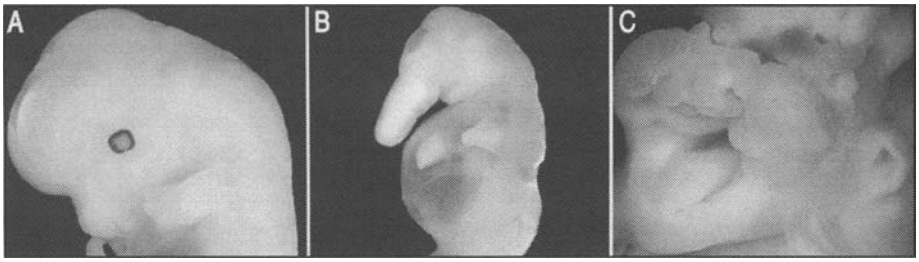

Figure 4. Targeted disruption in genes in the Sonic Hedgehog (Shh) pathway results in craniofacial dysmorphologies in the mouse. A) Wild type mouse embryo at stage 15.5 reveals normal craniofacial anatomy. B) Stage 15.5 *Shh* null embryo with grossly apparent abnormality of the forebrain, anopthalmia, proboscis, and hypoplasia of the maxillary and mandibular prominences. The fetus is severely growth restricted and has distal limb anomalies. C) Stage 14.5 embryo with *Ptch1* null background partially rescued by a transgene expressing *Ptch1*. The resultant alteration in Shh activity leads to abnormal frontonasal process derivatives such as the nose, hyperplastic maxillary processes and bilateral clefting of the lip and palate. The mandible does not appear to be affected by the underlying genetic disruptions.

Models to Study Craniofacial Development

As in other biological disciplines, deducing the constituents of a biological pathway and understanding their functions is the first step in understanding normal as well as abnormal processes during embryonic development. Because it is not always possible to use human subjects, the use of model systems such as chickens (Figs. 3E-G, 5A-F, 6), mice (Fig. 4), fruit flies, worms, and fish are invaluable.

Although the models in these systems exhibit widely disparate facial appearances, accumulating evidence suggests that most genes involved in craniofacial development are evolutionary conserved across species. Therefore, despite differences between mouse and man, or birds and fish, this remarkable amount of conservation allows us to unravel the mechanisms underlying normal and abnormal craniofacial development using a number of species. Consequently, a considerable amount of the knowledge about human craniofacial development is attributable to the extensive study of developmental processes in such model systems. We will discuss recent experimental data from these model systems, which has lead to our understanding of both normal and abnormal craniofacial morphogenesis.

Craniofacial Consequences of Shh Perturbation: The Model Experience

Genetic Disruptions

A number of mice have been generated in which mutations in the Shh pathway result in HPE[47] and other craniofacial malformations (see Fig. 4). Targeted disruption of the *Shh* gene in mice (Fig. 4B) has revealed that Shh plays a number of critical roles in brain, axial skeleton, limb and craniofacial development. Null mutations in Shh lead to malformations of the brain, vertebral column, limbs as well as cyclopia and malformations involving the first pharyngeal arch derivatives[48] (Fig. 4B).

As in humans, mutations in molecules downstream of the Shh ligand also result in malformations (see Fig. 4C). For example, mutations in the murine *Ptch1* gene result in open and overgrown neural tubes.[49] Mice homozygous for the *Ptch1* mutation exhibit ventralization of the neural tube and the ventral forebrain, with dorsal expansion of the ventral forebrain marker *Nkx2.1*.[49] A number of mice heterozygous for the *Ptch1* mutation develop tumors of the cerebellum known as medulloblastomas.[49] These studies reveal the importance of the interplay between Shh and Ptch in regulating growth and patterning during neural plate and neural tube

development.[49] However, the ability to study the roles of these molecules during later craniofacial development is limited because of the disruption of early neural patterning in *Shh* mutant mice[48] and loss of viability past E9.5 in mice homozygous for *Ptch1*.[49]

To address the role of Hedgehog (Hh) signaling on the cranial neural crest cells (CNCCs) during craniofacial development without disrupting early neural patterning, the ability of the CNCCs to respond to Hh signaling was removed by generating Wnt-1-Cre; conditionally null Smoothened mice (Wnt-1-Cre; $Smo^{n/c}$).[50] Embryos carrying the conditional knockout of smoothened exhibited extensive loss of craniofacial structures. This experimental strategy demonstrated the requirement of Hh signaling in postmigratory CNCCs and the roles of Hh signaling on craniofacial development during later stages of embryogenesis.[50] These authors also show that Fox genes play a role in mediating Hh activity during craniofacial development.

To circumvent embryonic lethality in *Ptch1* null mice, Scott et al partially rescued the phenotype by overexpressing *Ptch1* using a metallothionein promoter to drive *Ptch1* expression in a Ptch-null background.[51] They utilized two lines of mice that differed in their copy number (i.e., high and low) of the *Ptch1* transgene, and were able to obtain viability up to stage E14-14.5.[51] Use of the line with a lower copy number, and therefore less *Ptch1* expression/rescue, lead to an increase in viability to E12.5 but the embryos still had neural tube defects (NTDs). Embryos with the higher copy number survived to E14.5 without NTDs.[51]

Further examination of these high copy number transgenic mice,[52] found evidence of maxillary hyperplasia reminiscent of the large maxillary processes in $Gli3^{-/-}$ mutant mice (Fig. 4C). This observation suggests that growth of the maxillary processes, from which the sides of the face and the secondary palate are derived, are finely regulated by the activity of the Shh pathway. We also noted that the mandibular process, which gives rise to the lower jaw, was unaffected by *Ptch1* disruption (Fig. 4C).[52] These findings suggest that the factors controlling growth in the different facial processes may be unique and separable. Conversely, overexpression of *Ptch1* in a *Ptch1* wild type background causes inhibition of the induction of Shh target genes, which manifests as a dorsalization of the neural tube along with a failure of tube closure around the telencepahlon.[53]

Shh signaling has also been altered in mouse models by mutations in *Gli3*.[54] Mice carrying a *Gli3* null mutation are reminiscent of human GCPS.[54] As such, insight into the molecular etiologies underlying the GCPS phenotype has been aided by studying these "Extra-toes" mice.[54] In these *Gli3* null embryos *Fgf8* domains are upregulated in the anterior neural ridge and facial primordia, and these regions display reduced apoptosis.[55] Conversely, *Fgf8* expression is decreased in $Shh^{-/-}$ mice,[55] which suggests that in addition to its ability to limit the activity of Shh, Gli3 may also restrict the expression of *Fgf8*. In *Gli3* null mice, *Pax9* expression is expanded in the medial nasal process, consistent with the hypertelorism phenotypes of GCPS syndrome patients.[55]

Mutations in *Gli2* result in failure of the floor plate to develop.[56] *Gli2* mutant mice also show a decrease in Shh signaling, as manifested by a ventral shift in expression of *Nkx2.2* and *Pax6* in the neural tube.[56] This suggests that Gli2 protein functions as a transcription activator to regulate the expression of Shh-responsive genes.[56] There are no data currently available on the function of Gli2 in the craniofacial complex, separate from its earlier role in neural tube patterning.

Abnormal Shh signaling is also observed in talpid3 chicken mutants.[57,58] These mice exhibit polydactyly and abnormal facial morphology consisting of fused nasal placodes and hypotelorism as well as hypoplastic maxillary primordia which are fused in the midline.[58]

Teratogenic Disruptions

For several years, teratogens have been used in a number of model systems in order to study developmental mechanisms and to understand how teratogens exert their adverse effects. In this section we present three known environmental teratogens and one compound which is not currently recognized as such, and describe how they alter Shh function. A short discussion of

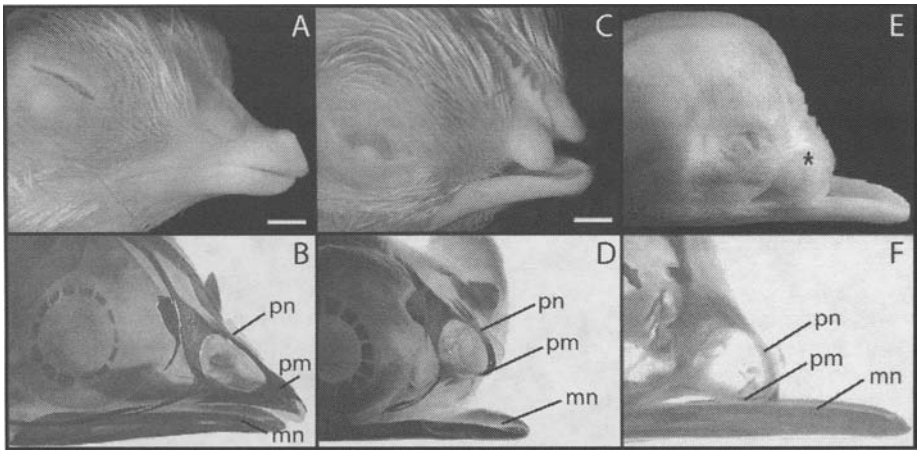

Figure 5. Sonic Hedgehog (Shh) signaling in the craniofacial complex is required for normal patterning of the upper beak. A,C,E) are oblique views of stage 41 chick embryos and, B,D,F) are corresponding skeletal structures of these embryos visualized with alcian blue and alizarin staining. A,B) Control embryos reveal the normal gross morphology and skeletal structures. C,D) The morphologic consequences of exposing stage 17 embryos to cyclopamine consist of mild microcephaly and hypotelorism as well as malpositioning and truncation of the distal aspect of the upper beak. Proximal elements such as the dorsal component of the premaxillary bone (e.g., the nasal process of the premaxilla (pn)) is intact, while the body of the premaxillary bone (pm) is shifted ventrally. The mandible (mn) is unaltered in cyclopamine-treated embryos. E,F) Following placement of 5E1 cells, which produce antibodies to Shh at stage 9.5, microcephaly, abnormal eye development and abnormalities in the distal upper beak are seen (E). The gross phenotype is consistent with the morphological changes seen following inhibition of Shh signal transduction with cyclopamine (compare to (C,D)). A-D) Reproduced, with permission, from reference 73; E-F) Reproduced, with permission, from reference 106.

their use and implications is presented here; in the case of cyclopamine, more detail will be provided in a separate section.

The first teratogen we will discuss is perhaps the best known: alcohol. Prenatal alcohol consumption in humans leads to FAS and lifelong physical and mental impairment. Although education about drinking in pregnancy is ongoing and abstinence is preventive, pregnant women continue to drink and their children continue to bear the consequences. Model systems to study FAS are uncovering the mechanisms underlying these impairments and may involve alterations in Shh signaling. Administration of ethanol to chicken embryos resulted in down-regulation of *Shh, Ptch, Gli1, Gli2,* and *Gli3* expression in the developing head, neural crest cell death and craniofacial defects.[41] *Shh* overexpression or administration of recombinant Shh can rescue neural crest cell death and in general, lessen the craniofacial manifestions of the alcohol exposure.[41]

Alcohol may also exert effects by interfering with retinoic acid (RA) metabolism, in effect usurping the function of retinol dehydrogenase and causing decreased RA in the embryo.[59] The consequences of retinoid disruption have been studied in some detail in the face. For example, in the chicken teratogenic doses of RA inhibit *Shh* expression and abolish polarizing activity in the frontonasal and maxillary processes.[46] Inhibiting endogenous retinoic acid receptor activity in the heads of chicken embryos leads to loss of *Shh* expression in the facial ectoderm.[60] The morphological consequences of this biochemical disruption are decreased outgrowth of the mid-and-upper facial structures.[60] In zebrafish, exogenous retinoic acid administration results in an initial decrease in *Shh,* but later an ectopic expression of the morphogen.[61] Taken together, these data suggest that RA may exert its teratogenic effects through

the Shh signaling pathway and that the response of Shh to RA may be biphasic in some model systems.

Another potent teratogen is cyclopamine (11-deoxojervine), a steroidal alkaloid extracted from the plant *Veratrum californicum*.[62-64] As its name suggests, cyclopamine is capable of inducing cyclopia when embryos are exposed during early development (see Figs. 3E-G, 5C,D). Cyclopamine first came to attention in the 1950s when it was observed that pregnant ewes grazing on *Veratrum californicum* on day 14 of their gestation gave birth to sheep with cyclopia.[65] This led to the eventual purification of cyclopamine and other steroidal alkaloids responsible for this defect. Since that time, cyclopamine has been a powerful tool to study the effects of altering Shh signaling in the brain and face in a number of model systems (avian, murine, amphibian, and fish) (see Figs. 3E-G, 5C,D).[64,66-73] Cyclopamine inhibits Shh signal transduction by binding directly to Smo and possibly altering its protein confirmation.[74] The efficacy of steroidal alkaloids to inhibit Shh transduction and phenocopy mutations in *Shh* was shown by exposing gastrulation-stage chick embryos to another steroidal alkaloid, jervine, that recapitulated the anomalies seen in the $Shh^{-/-}$ mouse.[75] In the chicken model, temporal interruption of Shh with cyclopamine reproduced the spectrum of HPE-associated phenotypes seen in humans (Fig. 3E-G).[73] The ability of these compounds to block Shh may also have clinical implications in human diseases because some cancers are associated with uncontrolled Shh signaling.

Drugs that interfere with cholesterol metabolism are the third class of potential teratogens. Cholesterol is important in many aspects of embryonic development,[35] and is involved in autoprocessing and transport of Shh.[76] The compounds AY9944 and BM15.766 block the function of Δ7-reductase, which itself is required for cholesterol synthesis. Rats exposed to AY9944[77] or BM15.766[78] produce the same biochemical disruptions as seen in SLO and may lead to HPE.[79]

The low-density lipoprotein receptor family member Megalin (gp330) is important in embryonic development.[80,81] Megalin is expressed in the visceral endoderm, neural ectoderm, neural plate and neural tube.[82] It mediates endocytosis of ligands, targeting them for lysosomal degradation or transcytosis.[81,83] Megalin has been shown to bind to Shh with high affinity and mediate endocytosis.[83] It also binds to complexes of retinal-binding protein.[84,85] The receptor-associated protein (RAP) is a chaperone, which regulates the expression and processing of LDL receptor-related protein and can bind to Megalin.[86] Administration of the receptor-associated protein (RAP) to gastrulation stage chick embryos produces cyclopia (Cordero and Helms, unpublished observations). The teratogenic effects elicited by exogenous RAP appears to result from inhibition of the receptor-ligand interaction between Shh and megalin[83] and or with other complexes and therefore most likely accounts for the resultant phenotype. Megalin may therefore provide a link between Shh signaling and RA. The significance of megalin-Shh interactions is discussed in the final chapter of this book.

Alternative Methods of Disrupting Shh

A number of different approaches to disrupting Shh signaling are also being applied experimentally and the reader is referred to the articles below for a detailed description of these techniques. One method that has been used successfully to block Shh activity is the use of anti-Shh antibodies (Fig. 5E,F).[87-89] RNA interference (RNAi) or gene knock-down is another method to silence a gene of interest.[90-93]

Embryologic Aspects of Normal Craniofacial Development: An Introduction

During development of the craniofacial complex, the transformation of single pluripotent neural crest cells into a complex three-dimensional structure such as the face requires an elaborate interchange between tissues that form the face.[73,94,95] Shh plays a number of important roles in pattern formation and growth of the face, by mediating interactions within and between these tissues.

In general, the fate of neural crest cells in the face is controlled by communication with nearby cells/tissue. The fates of these craniofacial neural crest cells are highly dependent upon the developmental time point of which this fate information is received, and one mechanism by which signaling molecules such as Shh control patterning and growth of the face is through organizing centers and epithelial-mesenchyme interactions.

Organizing Centers

During embryonic development, groups of cells have the capacity to induce biochemical and morphological changes in neighboring cells. Based on this function, these groups of cells have been referred to as "organizers" or "organizing centers". Organizers are critical determinants of cell fate and provide positional information that is required for the establishment of body axes. The organizers' ability to alter the fate of other cells was initially shown in 1924 by Spemann and Mangold, who demonstrated that transplanted dorsal blastopore lip of the amphibian induced a secondary developmental axis.[96] Since that time, a number of embryonic organizing centers have been identified which exert their developmental influences through Shh. These centers include: the notochord, the zone of polarizing activity in the limb bud, Hensen's node, the tail bud, and the frontonasal ectodermal zone. Shh secreted by these organizing centers may establish concentration- or activity-dependent gradients that determine different cell types and positional information. Shh can act directly on target cells to produce distinct cellular responses in a concentration-dependent fashion,[97] but the concentration of Shh may not correlate directly with the gradient of activity sensed by the target cells. It is possible that the concentration gradient can be established or influenced by the levels of Ptch1[97,98] and growth-arrest specific protein 1 (Gas1), both of which bind to Shh and influence Shh activity.[99]

The organizing properties of Shh have been demonstrated in the neural tube.[100] During neural tube development Shh is initially secreted by the notochord, which subsequently induces the floor plate cells of the neural tube to secrete Shh.[101] A concentration gradient of Shh is established within the neural tube, which specifies progenitor cell identity and neuronal fate through a set of homeodomain transcription factors.[102] Shh establishes ventral polarity in the neural tube and in conjunction with bone morphogenic proteins (Bmps) secreted from the roof plate[103] delineates dorsal-ventral polarity of this structure. Development of the forebrain may involve similar signaling mechanisms.[104]

Organizer centers which are located outside or within the craniofacial complex provide information for patterning and morphogenesis of the face, most likely through the establishment of morphogen gradients similar to those of the notochord and floor. Because the structural anatomy is quite distinct between the neural tube and face, additional mechanisms may influence just how this Shh-mediated organizing activity is established and interpreted. These anatomical distinctions are discussed in more detail in the following paragraphs.

The prechordal plate mesendoderm underlying the forebrain is a source of Shh which patterns the forebrain.[30,105] Loss of Shh in the prechordal plate in *Shh*$^{-/-}$ mice results in severe neural plate alterations which causes brain malformations and cyclopia.[48] In turn, Shh in the ventral forebrain has a significant influence on morphogenesis of the frontonasal primordium, from which the middle and upper face develop.[73,106] The endoderm also has head organizing activity[107] and presumably is also a source of Shh signaling crucial to head development.

Discrete domains of the craniofacial epithelia exhibit Shh-mediated organizing activity.[46] This was demonstrated by grafting Shh-expressing facial ectoderm into the anterior region of a limb bud, where the tissue exhibited organizing activity as assayed by its ability to induce digit duplications.[46] Precisely how this organizing activity is established is still not clear. We subsequently determined that this facial organizing center is delineated by a boundary between *Shh* and *Fibroblast growth factor 8* (*Fgf8*) expression,[108] and referred to this region as the frontonasal ectodermal zone, or FEZ (Fig. 6A).

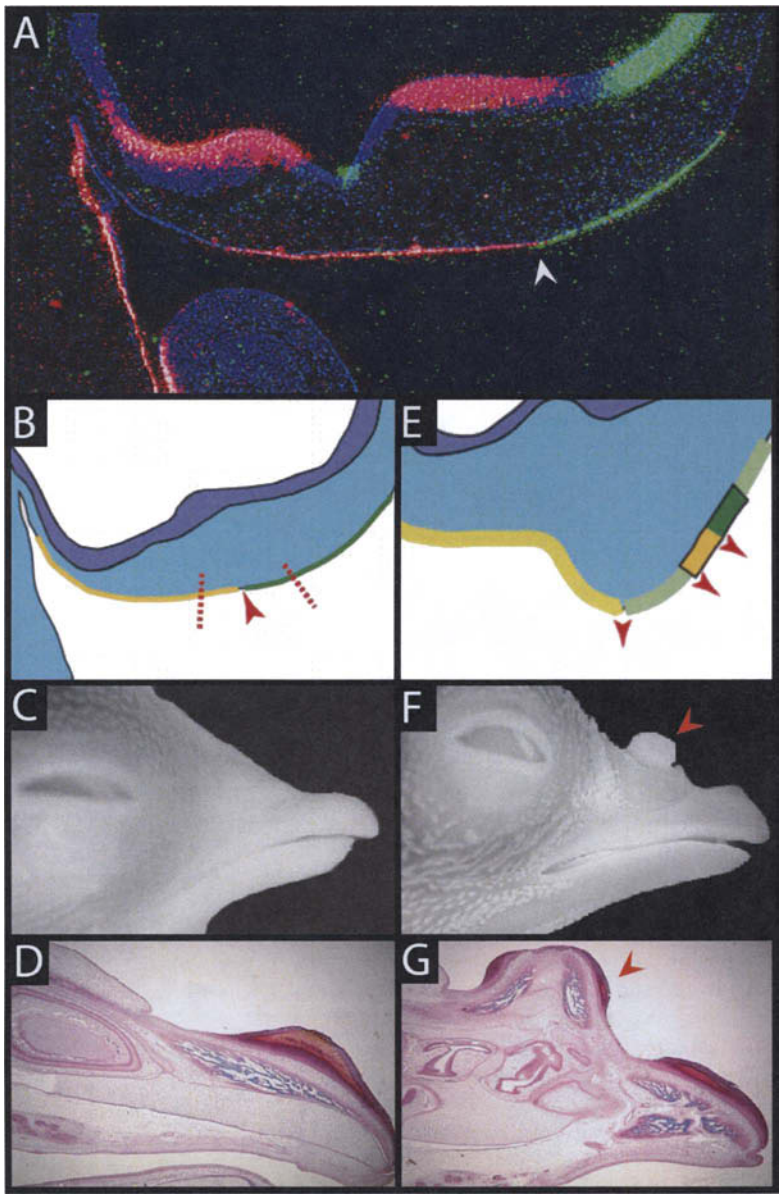

Figure 6. Transplantation of the facial ectodermal zone (FEZ) demonstrates its importance in patterning of the frontonasal primordia. A) In situ hybridization on a sagittal section of a stage 20 chick embryo; red corresponds to a *Shh*-expressing domain and green corresponds to a *Fgf8*-expressing domain. B,E) are representations of similar lateral sections of stage 20 and stage 25 chick embryos, respectively. The FEZ is indicated by dotted lines, where yellow corresponds to the *Shh*-expressing domain and green corresponds to the *Fgf8*-expressing domain; the junction between *Shh* and *Fgf8* is indicated with an arrowhead. C,F) Photographs of stage 36 control and transplanted embryos, respectively. Arrowhead indicates formation of an ectopic beak from transplanted FEZ. D,G) Trichrome stained sections of stage 36 control and transplanted embryos, respectively. Again, arrowhead indicates formation of an ectopic beak from transplanted FEZ. A-G) Reproduced, with permission, from reference 108.

The FEZ plays a critical role in establishing dorsoventral patterning of the frontonasal primordium.[108] Heterotopic transplantation of the FEZ from stage 20 quail embryos into stage 25 chick embryos provoked a repatterning of first pharyngeal arch mesenchyme which resulted in the generation of an ectopic beak whose dorsal-ventral polarity directly correlated with the orientation of the *Shh* and *Fgf8* expression domains (Fig. 6).[108]

Epithelial-Mesenchymal Interactions

The importance of epithelial-mesenchymal interactions in craniofacial development was shown by classic recombination studies between the oral epithelium and underlying mesenchyme.[109,110] As with all epithelial-mesenchymal interactions, they provide the correct spatial and temporal cues necessary to generate normal anatomic structures and their relationships. Shh in conjunction with a number of growth factors including Bmps, Wnts, and Fgfs mediates these epithelial-mesenchymal interactions.[111] The importance of Shh signaling during development of the forebrain facial prominences,[46,60,73,106] palate,[112] teeth,[88,113] taste papillae[114] and submandibular salivary glands[115-117] are becoming more evident and important in understanding clinical manifestations in several craniofacial disorders.

Forebrain Development and Shh

The commencement of the craniofacial region is considered to coincide with gastrulation. At this stage, the embryonic ectoderm is divided into neural and non-neural ectoderm. The neural ectoderm will give rise to the forebrain, whereas the non-neural ectoderm will give rise to the epidermis of the face and the rest of the body.[117] The close spatial proximity of these two ectodermal regions gives rise to an intimate molecular and structural relationship between the developing forebrain and the presumptive facial regions.

During development of the central nervous system, the anterior prosencephalon becomes the telencephalon (cerebral hemispheres and basal ganglia) and the diencephalon (thalamus, hypothalamus and pituitary gland).[118] The prechordal plate mesendoderm is located in the ventral midline of the forebrain beneath the neural plate, and is a source of Shh. Shh from the prechordal plate is critical for establishing ventral polarity during dorsal-ventral patterning of the forebrain and proper development of the dorsal telencephalon. During normal development of the dorsal telencephalon, the roof plate undergoes invagination (commonly referred to as cleavage), which in conjunction with growth of the telencephalon, leads to two separate cerebral hemispheres.[119,120] Interruption of Shh can result in the failure of invagination of the dorsal telencephalon and a single hemisphere.[10] The mechanism by which Shh influences the dorsal telencephalon is unclear since *Shh* is expressed ventrally. A number of other roles for Shh during neurogenesis exist but are outside the scope of this chapter.[121-123]

In avians, *Shh* expression is initially restricted to the ventral diencephalon at stage 15 (Fig. 7C,D).[73] At stage 17, a separate domain of *Shh* is induced in the ventral telencephalon, which is separated from the diencephalic domain by a *Shh*-negative optic recess (Fig. 7E,F).[73] At stage 20, Shh expression is first detected in the facial ectoderm (Fig. 7G,H).[73] This progressive format to *Shh* expression in the craniofacial tissues (Fig. 7) underscores how many tissues are responsive to this morphogen.

Craniofacial Development and Shh

In mammals, structures of the face develop from the facial prominences (Fig. 8). They include the paired maxillary, the paired lateral nasal, the paired mandibular and a single frontonasal. These primordia consist of undifferentiated cranial neural crest cells or mesenchyme enclosed by epithelium. The mesenchyme is derived from undifferentiated cranial neural crest cells that originate from the dorsal neural tube.[124] These cells migrate into specific regions of the facial primordia and differentiate into the cartilaginous and osseous structures of the face.[94,95,124-127] During their migration cranial neural crest cells are interposed between the neuroectoderm and facial ectoderm until reaching their final destination, either

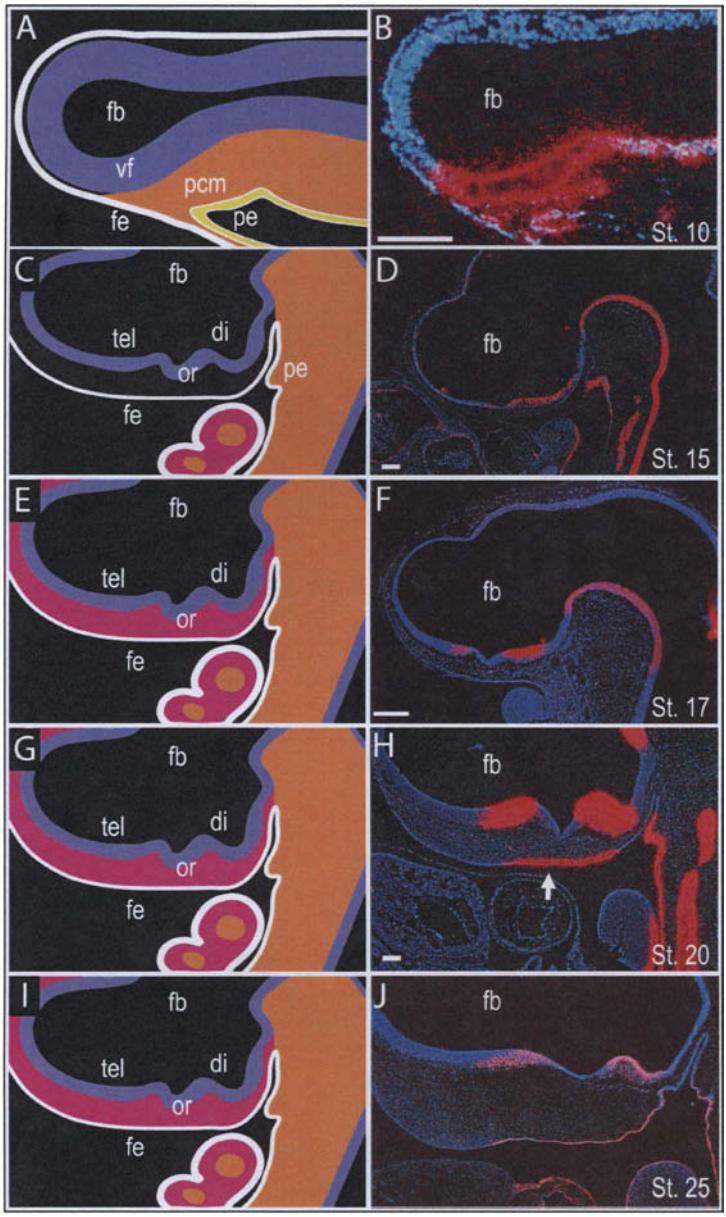

Figure 7. Ontogeny of *Sonic Hedgehog* (*Shh*) expression in the chick craniofacial complex. A,C,E,G) are representations of the (B,D,F,H) actual midline sagittal embryonic sections. In figures (B,D,F,H) red represents *Shh* expression as obtained by in-situ hybridization with S^{35}. A,B) At stage 10 *Shh* is expressed in the ventral prosencephalon (vf), oral ectoderm and pharyngeal endoderm and the prechordal mesoderm (pcm). C,D) By stage 15 the prosencephalon is comprised of the telencephalon (te) and the diencephalon (di). At this stage *Shh* transcripts are localized to the (di). E,F) At stage 17, *Shh* is now expressed in the (te). G,H) Around stage 20, *Shh* is expressed in not just in the diencephalic and telencephalic neural ectoderm, but also in the facial ectoderm (arrow). I,J) *Shh* expression remains in the neural ectoderm and facial ectoderm at stage 22. Panels B,D) reproduced, with permission, from reference 73.

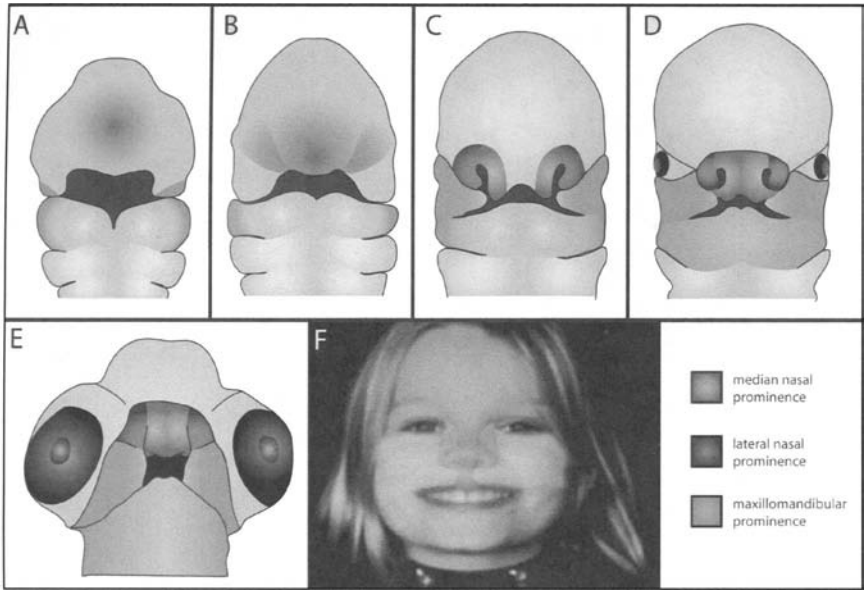

Figure 8. Development of the craniofacial primordia. A-D) Representations of frontal views of mouse embryos showing the prominences that give rise to the main structures of the face. The frontonasal (or median nasal) prominence (pink) gives rise to the forehead (A), the middle of the nose (B), the philtrum of the upper lip (C) and the primary palate (D), while the lateral nasal prominence (blue) forms the sides of the nose (B,D). The maxillomandibular prominences (green) give rise to the lower jaw (specifically from the mandibular prominences), to the sides of the middle and lower face, to the lateral borders of the lips, and to the secondary palate (from the maxillary prominences). E) Frontal view of a chick embryo, also showing which prominences give rise to different facial structures. F) Frontal view of a human child, with different facial structures colored to indicate the prominences from which each structure developed. A-F) Reproduced, with permission, from reference 147.

the pharyngeal arches or the frontonasal primordium. Developmental instructions are received from the surrounding epithelium during their passage and following arrival have a profound influence on their developmental fates.[128] In the avian model system cranial neural crest cells are thought to provide patterning information that directs morphology of regions of the craniofacial complex.[129,130]

As a consequence of both passive and active cell movements, the forebrain neuroectoderm and the facial ectoderm remain in close proximity, being displaced "in register" during head flexure. Thus the brain, rather than being a passive scaffold, actually influences facial patterning via a complex dialogue with facial tissues. This new view is laying the foundation for investigations into how genetics and epigenetics influence the dynamic interactions between the brain and face.

When *Shh* expressing ectoderm in the frontonasal primordium is removed, the result is inadequate outgrowth of the middle and upper face (Fig. 9D-I).[131] Conversely, supplying exogenous Shh causes an expansion in the mediolateral width of the face (Fig. 9J-L).[131] When over-expression of *Shh* is localized to the facial ectoderm, by delivering Shh via a retrovirus, the same morphogenic consequence of a widened frontonasal primordium is observed (Fig. 9J-L).[132] Similar effects can also be produced by manipulating genes that regulate Shh activity, as seen in *Gli3*$^{-/-}$ mice.[54,133] But how Shh is established in the face, and how this facial domain coordinates growth and development along with the forebrain domain of Shh has, until recently, remained unclear.

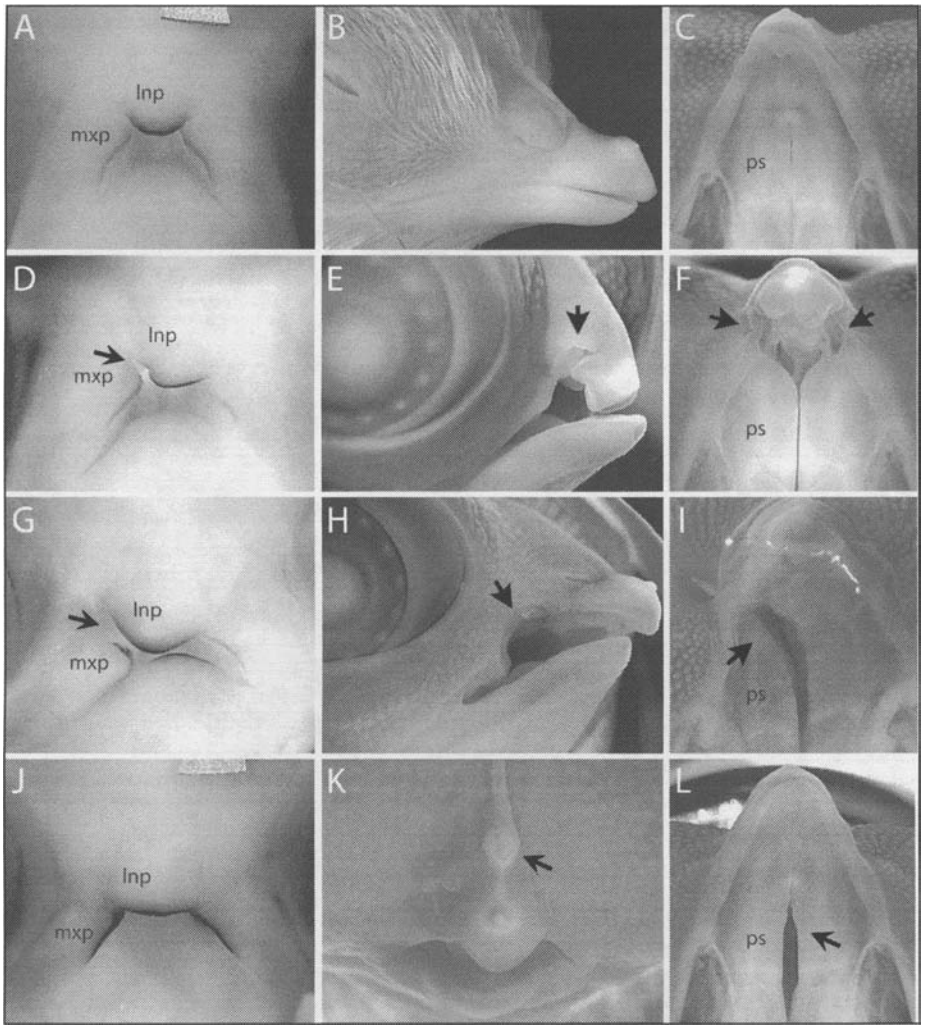

Figure 9. Consequences of removing and overexpressing Shh in stage 30-35 chick embryos. A-C) Frontal (A), oblique (B), and ventral (C) views of normal stage 25 chick embryos. D-F) Consequences of removing *Shh*-expressing frontonasal (fnp) ectoderm at stage 20. Removal of fnp ectoderm causes a loss of *Ptc* expression (arrow, D) by stage 30. Excision also causes a visible cleft (arrow, E) between the maxillary process (mxp) and lateral nasal process (lnp) at stage 35, as well as bilateral cleft palate (arrow, F). G-I) Consequences of removal of *Shh*-expressing mxp ectoderm. Removal of mxp ectoderm causes a loss of *Ptc* expression (arrow, G) by stage 30. Excision also causes a similar cleft (arrow, H) between the mxp and lnp by stage 35, as well as unilateral cleft palate (arrow, I). J-L) Consequences of injecting RCAS-*Shh* fibroblasts into fnp ectoderm. Ectopic administration of *Shh* results in considerable widening of the upper beak (J) along with growth of an ectopic beak (arrow, K). Hypertelorism (not shown) also results from expansion of the fnp, and the palatal bones are positioned more laterally (arrow, L). A-L) Reproduced, with permission, from reference 131.

Shh in mice results in early neural plate patterning defects that result in a single telencephalic vesicle and severe craniofacial malformations (Fig. 4B).[48] The early loss of Shh at the neural plate stage in this model precludes delineation of the role(s) of Shh in later facial development.

In order to circumvent this limitation imposed by the interruption of the neural plate, other model systems and techniques have been used to interrupt Shh at later stages of embryonic development.

The chick was recently used to study the effects of temporal inhibition of Shh transduction on facial morphogenesis, by administering cyclopamine at specific developmental stages. The stages chosen for cyclopamine treatment were based upon the elucidation of the dynamic expression pattern of *Shh* in the brain and face.[73] If Shh was blocked prior to initiation of expression in the telencephalon, brain anomalies as well as facial defects such a severe hypotelorism, microopthalmia, and microcephaly were noted (Figs. 3E,F).[73] However, when Shh was blocked after its induction in the ventral telencephalon but prior to its expression in the facial ectoderm, no gross phenotypic brain anomalies were observed and the facial malformations were less severe than those treated at earlier stages (Fig. 3G, 5C,D).[73] When Shh was blocked after its induction in facial ectoderm then the embryos exhibited only subtle facial anomalies (data not shown).[73] Taken together, these data suggest that the brain and face are differentially susceptible to perturbations in the Shh signaling pathway. This is clinically relevant since some patients with mutations in SHH sometimes have abnormal brains and facial features while others have normal-appearing brains and abnormal facial phenotypes.

In another set of experiments conducted to look at the role of Shh specifically emanating from the forebrain, hybridoma cells producing antibody (5E1)[134] to Shh were injected into the brain of stage 9.0-9.5 embryos and evaluated at state 41 (Fig. 5E,F).[106] This treatment blocked Shh emanating from the ventral forebrain.[106] We found that the resultant facial dysmorphologies (Fig. 5E,F) following cyclopamine or 5E1 administration are not secondary to increased neural crest cell apoptosis in the frontonasal primordium, but to a molecular alteration in the FEZ.[106] For example, inhibition of Shh in the ventral forebrain neuroectoderm blocked *Shh* expression in the facial ectoderm.[106] This molecular alteration was accompanied by a proximal expansion of the *Fgf8* domain, which interrupted the molecular boundaries of the FEZ.[106] The morphological consequences of interrupting this organizing center are truncation of the upper beak, ventralization of the premaxillary bone, and decreased expansion of the medial-lateral axes (Fig. 5E,F).[106]

The intervening mesenchyme is critical for either the induction or the maintenance of *Shh* expression in the face, as shown by blocking the ability of neural crest cells to respond to a Hedgehog signal, as shown by Jeong[50] and as discussed previously.

Palatogenesis and Shh

The definitive palate in mammals consist of the primary and secondary palate.[135] The primary palate forms from the fusion of the medial nasal prominences.[135] The secondary palate gives rise to the majority of the hard and soft palate are formed from the palatal shelves which extend from the maxillary processes.[135] These shelves structurally consist of an epithelial surface surrounding neural crest cells or mesenchyme. As a result of mesenchymal cell proliferation the palatal shelves initially grow vertically downward on either side of the tongue. Subsequently, the vertical palatal shelves elevate to a horizontal position above the tongue. In the final stage of palate development, the now horizontally-oriented, elevated palatal shelves fuse to form the palate.

Shh plays a role in palatal development and appears to be required for signaling interactions within this structure. Fibroblast growth factor (Fgf) signaling, specifically Fgf10 and the Fgf receptor 2b (Fgfr2b) together with Shh, are implicated in the outgrowth of the palatal shelves.[112] *Fgfr2b* is expressed in the epithelium of the palatal shelf between E12-14, and at lower levels in the mesenchyme of the nasal side of the palate. *Fgf10*, which functions as a ligand for Fgfr2, is expressed in the adjacent underlying mesenchyme.[112] Null mutations in *Fgfr2*[136] and *Fgf10*[137] result in decreased cell proliferation in the palatal mesenchyme, down regulation of *Shh* in the palatal epithelium, and clefting secondary to decreased outgrowth of the palatal shelves. In vitro experiments using recombinant FGF10 show that Fgf proteins can induce *Shh* expression

in the palatal epithelium and can cause increased expression of *Ptch1* and cell proliferation in adjacent mesenchyme.[112] An increase in cellular proliferation is also elicited with application of exogenous Shh to wild type mesenchymal palatal explants.[112] Thus in the palate, Shh is a downstream target of Fgfr2/Fgf10. The clinical implications of these findings may be that alterations in Shh and/or Fgfr2/Fgf10 signaling are involved in select cases of cleft palate, which are presently described as idiopathic. Surprisingly however, studies of newborns with isolated oral clefts suggest that mutations in *SHH* are not a common cause of nonsyndromic oral clefts;[138] however, further investigation is warranted.

Odontogenesis and Shh

Shh plays a number of roles during tooth development such as the location and generation of the teeth. Though the roles of Shh are slightly different in teeth than in palate, Shh is involved in reciprocal signaling interactions between the epithelia and the mesenchyme. During odontogenesis in the mouse, *Shh* expression is limited to localized thickenings of oral epithelium that will give rise to teeth. However, *Shh* activity is absent from the diastema mesenchyme, an edentulous region between developing teeth. The spatial relationship of Shh to the presence or absence of teeth highlights the importance of its spatial activity during odontogenesis. Reciprocal interactions between the oral epithelium and mesenchyme occur during tooth development.[113,139-141] Mandibular mesenchyme appears to have the ability to decrease and/or limit Shh signaling via interactions with the epithelium.[113] When mandibular processes were cultured without the overlying epithelium, *Ptch1* and *Gli1* were up-regulated, and the expression of *Gas1* was down-regulated in the underlying diastema mesenchyme.[113] Taken together, these data suggest that *Gas1* in the mesenchyme has an antagonistic effects on Shh signaling in the epithelium, a relationship that has been observed in other tissues.[99]

Shh appears to initiate tooth bud formation[88] and to influence the morphology of teeth. For example, when Shh signaling is blocked in the epithelium the number of teeth that develop is substantially reduced.[88] Blocking Shh activity at slightly later stages allows some teeth to form, but the superficial epithelium of the developing tooth buds often exhibits apoptosis.[88] The role of Shh during tooth morphogenesis is extensively covered in a later chapter.

Papillogenesis and Shh

The tongue develops from contributions of the first four pharyngeal arches and involves complex epithelial-mesenchymal interactions. The surface of the tongue has multiple types of papillae, which provide mechanical functions (filiform) and others that contain tastebuds and are known as gustatory papillae (fungiform and circumvallate). Papillary development begins when localized regions of epithelial cells begin to proliferate, giving rise to epithelial thickenings called placodes. The placodes subsequently evaginate into the mesenchyme thereby forming the raised papillae which consist of an epithelial surface and a mesenchymal core.[89,142]

Shh plays very similar roles in tooth and papillae with respect to both early patterning and later epithelial-mesenchymal interactions. Murine in situ studies have shown *Shh*, along with *Ptch1* and *Gli1*, are expressed diffusely in the developing tongue during early stages of embryogenesis (Fig. 10).[114,143] Subsequently, *Shh*, *Bmp2* and *Bmp4* are localized to discrete regions of the anterior tongue epithelium which correspond to where fungiform papillae will ultimately form (Fig. 10).[114,143] *Ptch1* is expressed in the mesenchyme underlying *Shh*-expressing epithelium and in areas surrounding the fungiform papillae.[114] These data suggest that Shh specifies the locations of the fungiform papillae and is involved in epithelial-mesenchymal interactions and may play a role in papillary spacing. Shh may also play a role in directing the morphogenesis of the papillae by limiting the extent of papillary growth, both in the placodal epithelium and in the mesenchyme.[89] Inhibiting Shh signal transduction with cyclopamine or 5E1 results in significantly enlarged papillae and the development of papillae in ectopic regions, the interplacodal areas.[89,144] This suggests that Shh normally signals to Ptch1 in the mesenchyme of the periplacodal regions that result in repression of papillary development in these areas.[89]

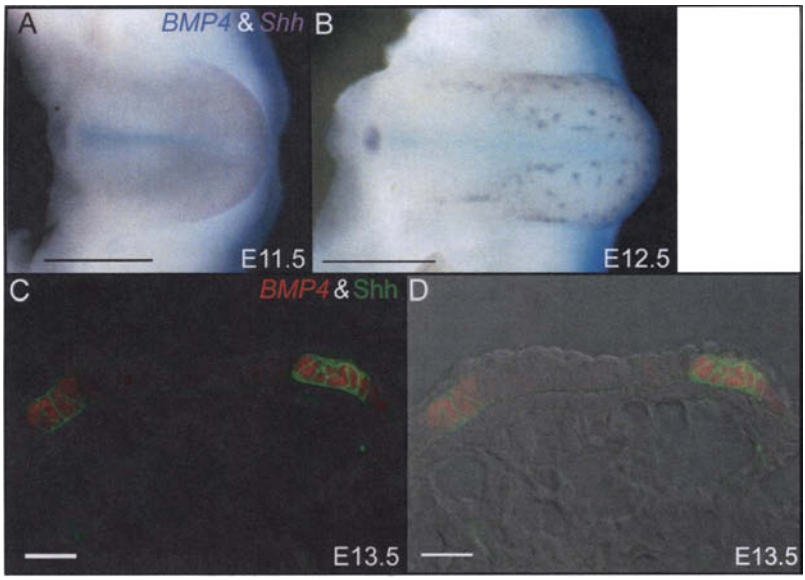

Figure 10. Gene expression in the developing tongue. *BMP4* and *Shh* are both expressed in early developing tongues. A,B) Double X-gal/*in situ* hybridization staining for *BMP4* and *Shh* expression, respectively, in $BMP4^{LacZ}$ tongues. $BMP4^{LacZ}$ staining is blue and *Shh* is purple. A) At E11.5, *Shh* is expressed broadly in the tongue in a different pattern than $BMP4^{LacZ}$, which is restricted to the midline mesenchyme. B) At E12.5, no difference in $BMP4^{LacZ}$ and *Shh* expression is detected. C) Single image plane confocal micrograph of double-label immunohistochemistry for β-galactosidase (red) and Shh (green) in E13.5 $BMP4^{LacZ}$ fungiform placodes, with a corresponding Nomarski image overlaid in (D). $BMP4^{LacZ}$ and Shh are present in all of the same cells in the developing papillary placodes. $BMP4^{LacZ}$ is present in the cell nuclei because it carries a nuclear localization signal. Shh is present along the cell surface. Scale bars: A,B 500 μm; C,D 20 μm. Pictures courtesy Dr. Thomas Finger and Dr. Joshua Hall, and reproduced, with permission, from reference 89.

Submandibular Gland Development and Shh

The submandibular glands (SMG) are located on each side of the lower jaw and produce saliva consisting of mucin, salts and amylase. Embryonic development of these glands depends on epithelial-mesenchymal interactions and branching. The stages of SMG development are classified as prebud, pseudoglandular, canalicular and terminal bud; each stage is delineated by particular morphological characteristics.[145] Initially, there is downward growth of the oral epithelium into the mesenchyme forming a bulb like structure.[115] Branching morphogenesis subsequently occurs as a result of cellular interactions.[116,146] The Shh pathway is important in SMG development.

The expression of *Shh, Ptch1, Smo* and *Gli3* are localized to the epithelia at all stages of SMG development.[117] The requirement for Shh in branching of SMG has been shown by knockout of the *Shh* gene and biochemical inhibition of Shh transduction.[117] Evaluation of the SMG in E18.5 $Shh^{-/-}$ mice reveals tiny dysplastic structures with decreased branching.[117] Decreased branching is also produced when cyclopamine is administered to cell culture explants of SMG primordia.[117] When Fgf8 is added to the media of these explants the branching morphology is rescued suggesting that Fgf8 in the SMG is downstream of Shh.[117]

It is also of interest that the expression of *Ptch1, Smo* and *Gli1* in SMG are different from other organs in which branching occurs. Although *Shh* is expressed in the epithelium of SMG and other branched organs, *Ptch1, Smo* and *Gli* are expressed in the epithelium of SMG but expressed in the underlying mesenchyme of other branching organs.[117] It remains unclear why the SMG is different in this respect.

Future Directions

One third of birth defects involve the craniofacial region. Fortunately, the past decade has witnessed exponential growth in our knowledge of craniofacial development due to the integration of data from a number of disciplines. A range of experimental strategies and abundant clinical data demonstrate the requirement for Shh signaling in normal craniofacial morphogenesis. We are just at the beginning of our journey into uncovering the many developmental complexities of the craniofacial region. Delineating the molecules and their actions involved in craniofacial development will continue to provide insight into the etiologies of congenital disorders that are now regarded as idiopathic. This knowledge will also provide novel treatment methodologies and more accurate diagnostic methods in both prenatal and postnatal periods.

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CHAPTER 14

Important Role of Shh Controlling Gli3 Functions during the Dorsal-Ventral Patterning of the Telencephalon

Jun Motoyama* and Kazushi Aoto

Abstract

The dorsal-ventral patterning of the telencephalon is crucial for normal brain function because it determines the proportion of two different basic types of neurons: glutamatergic excitatory neurons and GABAergic inhibitory neurons. The secreted protein sonic hedgehog (Shh) is required for ventral cell specification, whereas the zinc finger transcription factor Gli3 seems to be important for dorsal cell type specification. Recent studies suggest how both Gli3 and Shh control the normal proportion of dorsal and ventral cell types to generate appropriate tissue size and shape. These observations may offer new insights into our understanding of the graded function of Shh during brain development.

Introduction

Embryogenesis involves a complex coordination of cell fate specification, proliferation and differentiation that is determined by the actions of a legion of genes. The sonic hedgehog (Shh) signaling cascade is important in many developmental processes of the central nervous system, and dysfunctions in this pathway can lead to birth defects and brain tumors.[1-7] Although the roles of the individual genes of Shh signaling cascade in each of these developmental processes have started to be defined, how these gene functions combine to translate the dynamic assembly of cells into tissues remains unresolved. Recent genetic studies using combined mutant mice have provided new insights into how several genes control the normal proportion of different cell types to generate appropriate tissue size and shape. Here, we review the role of Shh and Gli3 during dorsal-ventral patterning of the mouse telencephalon revealed by the analyses using not only *Gli3* and *Shh* single mutants but also *Gli3/Shh* double mutant mice.

Shh Signaling and Dorsal-Ventral Patterning of the Telencephalon

Sonic hedgehog (Shh) is a secreted protein that controls the patterning and proliferation of many tissues in developing human and mouse embryos. Many of the components that mediate the Shh signaling in these embryos were first discovered in their homologues affecting early embryonic development in *Drosophila*. In *Drosophila*, hedgehog (Hh) is a secreted protein that is required for pattern formation during many processes of fly development. Two transmembrane proteins, patched (Ptc) and smoothened (Smo), are involved in the reception of the Hh signals[8,9] and a transcription factor, cubitus interrupts (Ci), is the ultimate transducer of Hh

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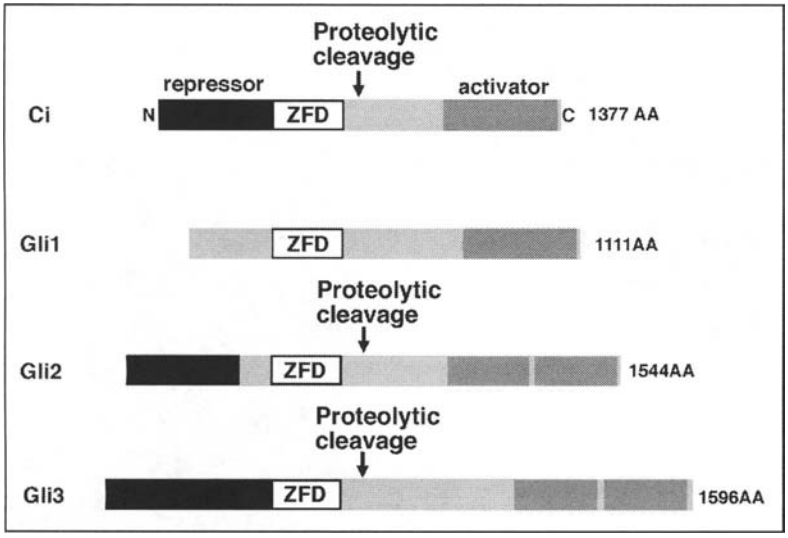

Figure 1. Schematic diagram of Ci/Gli family protein structure. All members have the zinc finger domain (ZFD) and the activator domain in the C-termini (gray). Ci/Gli proteins, except for Gli1, have the repressor domain in the N-termini (black) and act as repressors after processing in the proteolytic cleavage site (arrow).

signaling inside target cells.[10] In the absence of Hh, Ptc inhibits Smo function, allowing proteolytic cleavage of Ci which then suppresses the transcription of target genes.[11] However, when Hh binds to Ptc, the repressive action of Ptc on Smo is blocked, Ci proteolysis is inhibited, and Ci is converted into a transcriptional activator. Several features of Hh signal transduction are evolutionarily conserved in vertebrates, and when this pathway is impaired birth defects and tumorigenesis result. Shh is encoded by one of three mammalian orthologous genes to *Hh*, *desert hedgehog*, *Indian hedgehog* and *sonic hedgehog*. Mice and humans have at least three *Ci* homologues (*Gli1, Gli2, and Gli3*) which encode transcription factors mediating Shh signaling.[12-17] Of these, Gli2 and Gli3 can be proteolytically processed and seem to have transcriptional activator and suppressor functions[18,19] (Fig. 1). Mouse *Gli* genes were introduced in the fly wing disc to examine whether the processing of mouse Glis can be regulated by *Drosophila* Hh signaling.[18] Gli1 and Gli2 can function as activators of Hh signaling in the fly disc. Gli2 and Gli3 are processed in the fly wing disc, but the processing of Gli2 is observed even within the posterior compartment expressing Hh signaling, suggesting that it is not regulated by Hh signaling. On the other hand, the proteolytic cleavage of Gli3 is regulated by Hh signaling in a similar manner to how Ci is regulated by Hh in *Drosophila*.[18] Gli1 seems to have only an activator function[18] (Fig. 1). Also in the developing limb buds in mouse and chick embryos, proteolytic cleavage of Gli3 is observed and its distribution appears to be regulated by Shh from the posterior region of the limb bud named the ZPA (zone of polarizing activity).[19] Thus, not only in Drosophila but also in mouse and chick, proteolytically processed Gli3 appears to be a specific antagonist of Shh signaling.

The dorsal-ventral patterning of the telencephalon determines the proportion of two different types of basic neurons, glutamatergic excitatory neurons and GABAergic inhibitory neurons, which are necessary for normal telencephalon function. Fibroblast growth factor 8 (Fgf8) is an essential regulator for the dorsal-ventral patterning of the telencephalon. At the head fold stage, around 8.0 dpc, the presumptive forebrain is determined and the cells in the anterior-most aspect start expressing *Fgf8*. Expressing cells in the anterior neural ridge are important for outgrowth of the telencephalon territory in the forebrain region (Fig. 2A,B). Depending on the

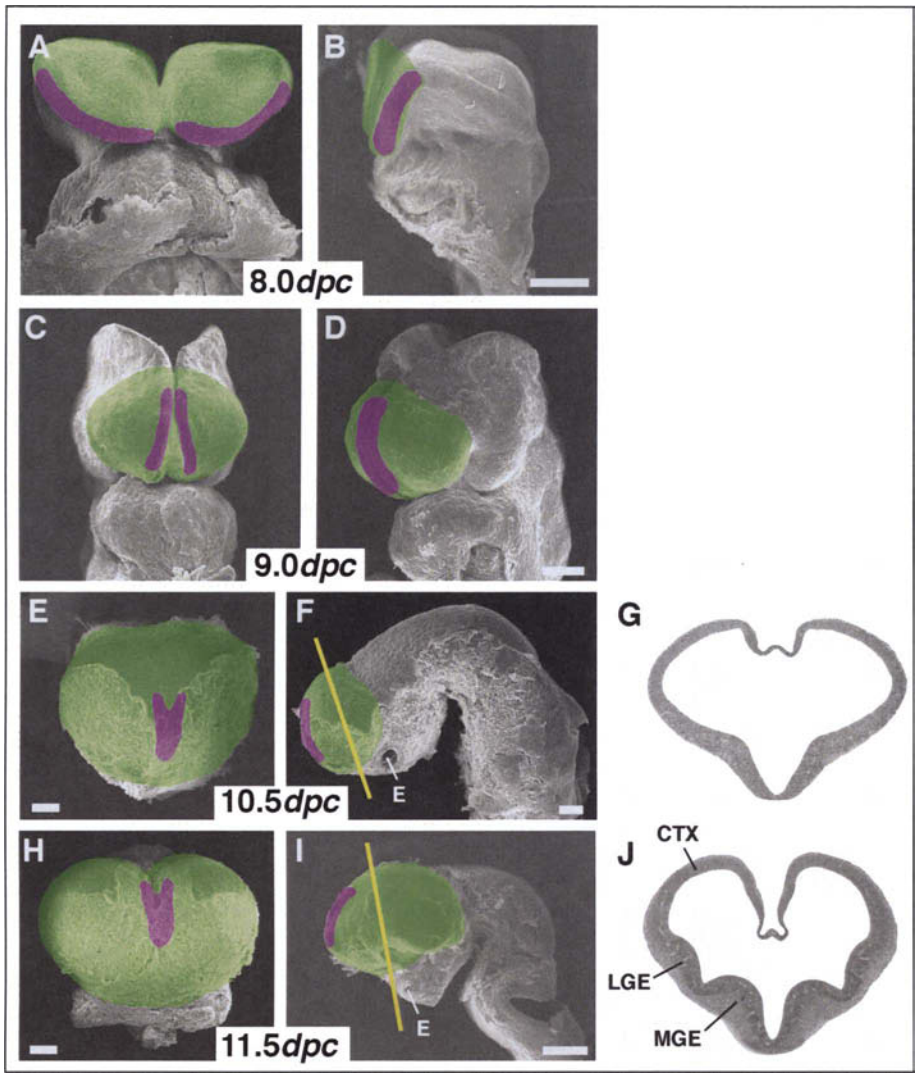

Figure 2. Distribution of *Fgf8* expression during telencephalon development. Scanning electron micrographs of embryonic telencephalon isolated from different stages during embryogenesis. Green area indicates the presumptive telencephalon in frontal (A, C, E, H) and lateral views (B, D, F, I). Purple area indicates the domain of *Fgf8* expression. G, J) Transverse section at yellow line in (F) and (I) are shown. Mesenchymal cells and surface ectoderm are removed in (E, F, H, I). E: eye; MGE: medial ganglionic eminence; LGE: lateral ganglionic eminence; CTX: cortex. Scale bars: 0.1 mm in (A, B, C, D); 0.2 mm in (E, F, H); 0.5 mm in (I).

effect of the Fgf8, the surrounding neural plate grows rapidly and forms a pair of brain vesicles that becomes the presumptive telencephalon[20,21] (Fig. 2C-G). The dorsal part of the telencephalon develops into the neocortex and the hippocampus, mainly generating excitatory glutamatergic projection neurons. The ventral telencephalon develops into lateral and medial ganglionic eminences (Fig. 2H-J). The majority of the neurons generated in the ganglionic

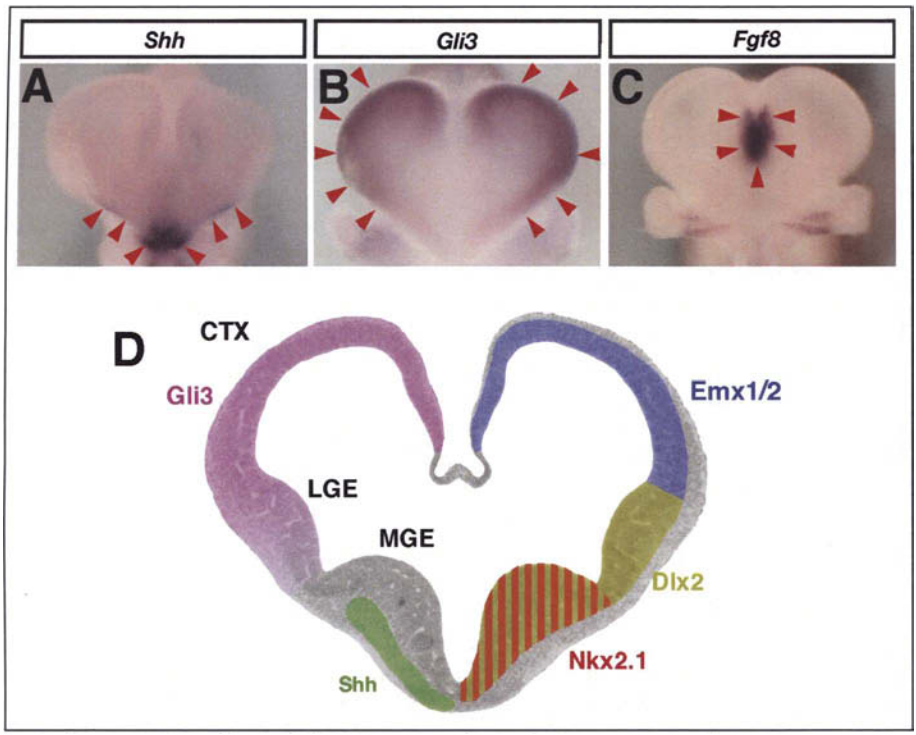

Figure 3. Distribution of *Shh*, *Gli3* and *Fgf8* mRNA in the telencephalon of wild type mice at 11.5 dpc (A, B, C) and schematic diagram of genes expression in the telencephalon (D). Frontal views of the telencephalon after whole mount in situ hybridization are shown in (A-C). *Shh* is expressed in the ventral midline, while *Gli3* expression is in the presumptive cortex (arrow heads in A, B). Strong *Fgf8* expression is detected in the intermediate zone of the telencephalon (arrow heads in C). In the schematic diagram each color code indicates the expression of regional brain marker; *Shh* (green), *Gli3* (pink), *Nkx2.1* (red), *Dlx2* (yellow) and *Emx1/2* (blue). MGE: medial ganglionic eminence; LGE: lateral ganglionic eminence; CTX: cortex.

eminences are inhibitory GABAergic interneurons, which tangentially migrate to the neocortex and make a circuit with excitatory projection neurons. Thus, this dorsal-ventral patterning is important to maintain the proper balance between two different types of basic excitatory and inhibitory neurons.

Shh and Gli3 are required for this dorsal-ventral patterning of the telencephalon. Shh is expressed in the ventral midline and Gli3 is in the dorsal telencephalon[22] (Fig. 3A,B,D). Shh is thought to be essential for directing the formation of the ventral telencephalon, because the medial and lateral ganglionic eminences are severely affected and a single forebrain vesicle develops with missing midline structures in *Shh-/-* mouse embryos[23] (Fig. 4B). However, the severe brain malformations observed in *Shh-/-* mutants are restored in *Gli3/Shh* double mutant embryos[22,24,25] (Fig. 4C). This finding suggests that the phenotype in *Shh-/-* single mutant mice includes secondary defects. Moreover, the restored phenotype also suggests that Gli3 function is involved in the secondary defects observed in *Shh-/-* mutant mice (Fig. 4B). The important question is how Gli3 functions with Shh to regulate the normal proportion of different cell types in order to generate appropriate tissue size and shape during telencephalon development.

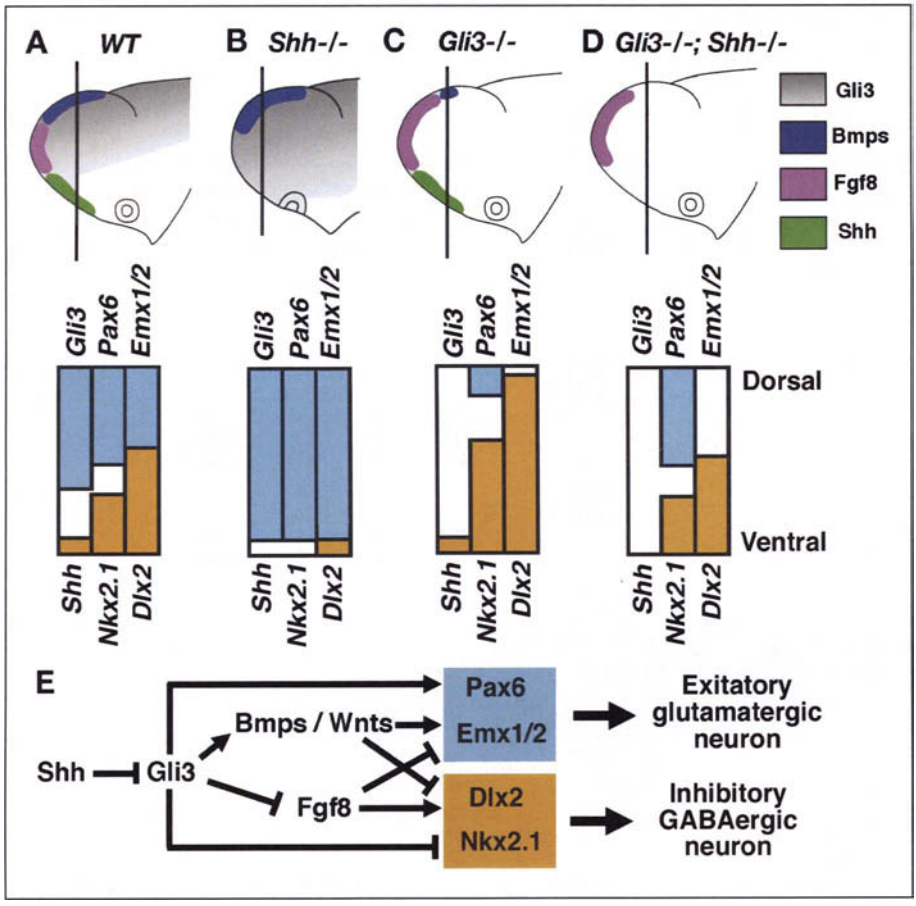

Figure 4. Dorsal-ventral patterning defects in Shh and Gli3 single mutants can be explained by the disrupted balance between Shh and Gli3 functions. A-D) Effects of Shh and Gli3 loss-of-function mutations on the dorsal-ventral patterning of the telencephalon. Summary of progenitor expression domains in the telencephalon at 11.5 dpc based on lateral views (top) or frontal sections at the line (bottom). B) In the *Shh-/-* telencephalon, *Bmps, Gli3, Pax6 and Emx1/2* expression is expanded toward the ventral telencephalon, while *Fgf8* and *Nkx2.1* expression is almost absent. *Dlx2* expression is also obviously reduced. C) By contrast, in *Gli3-/-* embryos, *Bmps, Pax6 and Emx1/2* expression in the dorsal telencephalon is obviously reduced. The expression domains of *Fgf8, Dlx2* and *Nkx2.1* are expanded in *Gli3-/-* mutant embryos (C). D) In comparison with *Shh-/-* mutant embryos, embryos missing both Shh and Gli3 functions substantially restore dorsal-ventral patterning of the telencephalon. The expression of *Nkx2.1, Dlx2* and *Fgf8* is restored in the double mutant embryos (D). E) Model for Shh and Gli3 function in mouse telencephlon development. Gli3 directly or indirectly induces cortex genes (*Pax6* and *Emx1/2*) and inhibits *Nkx2.1* and *Dlx2* in the ganglionic eminence of the ventral telencephalon. There appear to be two functions of Gli3 as an activator of dorsal cell types and as a suppressor of ventral specification, that seem to be related to the promotion of *Bmps/Wnts* expression and suppression of *Fgf8* expression (E). The important balance between Shh and Gli3 is maintained by the role of Shh to suppress Gli3 function. The genes expressed in the cortex and the ganglionic eminence are required for the development of the excitatory glutamatergic neurons and the inhibitory GABAergic neuron, respectively.

Gli3 as an Activator of Dorsal Cell Types

What is the role of Gli3 protein in the developing brain? Gli3 seems to be required for the development of dorsal cell types and is essential for the suppression of ventral specification in the telencephalon based on its expression pattern and the phenotype observed in *Gli3-/-* single mutant mice[22,26,27] (Figs. 3, 4D). During telencephalon development, *Gli3* is expressed in the dorsal edge of the neural plate in 8.0 dpc embryos and restricted to the dorsal region (Fig. 3C,D). The mouse *Gli3 Xt^J/Xt^J (-/-)* mutant is a model for human Greig cephalopolysyndactyly syndrome because it shows several features of the disease described by Johnson.[28] In *Gli3-/-* mutant embryos, the number of cells in the dorsal telencephalon, marked by *Emx1/2* expression (Fig. 3D), is obviously reduced, resulting in a severe reduction of the cortex, olfactory bulb and hippocampus[26,27] (Fig. 4D). On the other hand, ventral cell types marked by *Nkx2.1* and *Dlx2* expression are expanded[22,25,29] (Figs. 3D, 4D). Based on these observations, it appears that Gli3 is required for dorsal cell type specification. While the dorsal telencephalon is greatly reduced, the medial and lateral ganglionic eminences in the ventral telencephalon are clearly expanded in *Gli3-/-* mutant embryos[22,29] (Fig. 4D). Thus, Gli3 may have at least two major functions in the developing telencephalon: promotion of dorsal cell types and suppression of ventral cell types (Fig. 4E).

This malformation observed in *Gli3* mutant brain may be explained by the up-regulation of *Fgf8* during telencephalon development, because Fgf8 can promote the development of ventral cell fates and suppresses dorsal cell specification[22,26,27,30] (Figs. 3C, 4D). The up-regulation of *Fgf8* is observed in the neural tube of *Gli3-/-* embryos from 8.5 dpc, and it becomes more obvious at later stages[22] (Fig. 4D). The timing of brain malformation correlates with *Fgf8* up-regulation, suggesting that the up-regulation induces the growth retardation of dorsal telencephalon and also promotes the development of the ventral telencephalon (Fig. 4E). Thus, Gli3 may promote the proliferation and differentiation of dorsal cells by suppression of *Fgf8* expression because Fgf8 participates in the inhibition of dorsal cell development.

On the other hand, it is also highly possible that reduced *bone morphogenetic protein (Bmp)* and *Wnt* gene expression observed in *Gli3-/-* mutant mice is involved in abnormal dorsal telencephalon development. Bmp and Wnt proteins are secreted proteins normally expressed in most dorsal cells of the telencephalon and diencephalon, and they are known to be necessary for dorsal cell type specification in the brain[31-33] (Fig. 3D). Because *Bmp* and *Wnt* genes expression is down-regulated in *Gli3-/-* mutant mice, it appears that Gli3 is required for the activation of the expression of these in the dorsal telencephalon and diencephalon[26,27,32] (Fig. 4D). Gli3 may be required for the promotion of dorsal telencephalon development via the activation of *Bmp* and *Wnt* genes (Fig. 4E).

Gli3 as a Suppressor of Ventral Cell Type

Gli3 may function as a suppressor of ventral specification, because the number of ventral cells expressing *Nkx2.1* and *Dlx2* is increased in *Gli3-/-* mutant embryos[22,25,27,29] (Fig. 4D). The suppressor function of Gli3 in the spinal cord was clearly shown by Persson et al (2002)[34] and Meyers and Roelink (2003).[35] In *Gli3-/-* mutant embryos, the number of V0 and V1 interneuron progenitors expressing Nkx6.2 is increased in the intermediate zone of the spinal cord. On the other hand, dorsal cell types, motor neurons, V3 interneuron progenitors and the floor plate are not affected, suggesting that Gli3 suppresses the generation of V0 and V1 interneuron progenitors during normal spinal cord development. The next question is whether the specification of these interneuron progenitors is suppressed by an intact or a proteolytically processed Gli3, because Shh may block the proteolytic cleavage of Gli3 in the intermediate zone of the spinal cord. To identify which form of Gli3 is responsible for the suppression of V0 and V1 interneuron progenitors, mutant mice with targeted mutation of Gli3 have been generated.[34,36] In these mutant mice only the processed form of Gli3, which is missing the C-terminal activator domain by premature termination of translation of Gli3 C-terminal of the zinc finger region, is expressed.[36] Interestingly, their spinal cord displays normal dorsal-ventral patterning,

clearly indicating that the processed form of Gli3 is sufficient to suppress and maintain the proper amount of V0 and V1 interneuron progenitors during the normal spinal cord development.[34]

The artificially processed Gli3 was also ectopically introduced into the ventral side of the chick spinal cord to examine the effect on other ventral cell types.[34,35] Consistent with the finding in the targeted mutation of Gli3, the ectopic expression of the processed form of Gli3 can block the generation of V0 and V1 progenitors.[34,35] Moreover, the processed form of Gli3 can also block the development of motor neurons, V3 interneurons and oligodendrocyte progenitors in a cell-autonomous manner. However, *Shh* expression in the floor plate is not affected. Based on these observations, the proteolytic cleavage appears to turn Gli3 into a suppressor against not only V0 and V1 interneuron progenitors also against other ventral cell types. The suppressor function may not be required for the regulation of V2, V3 interneuron and motor neuron progenitors during normal development, because their development is not affected in *Gli3-/-* mutant spinal cord.[34] However, in the telencephalon of *Gli3-/-* mutant mice, the expression of *Fgf8, Dlx2* and *Nkx2.1* is increased in the intermediate zone and ventral region, strongly suggesting that the processed form of Gli3 is required for the maintenance of the proper amount of these ventral cell types during normal telencephalon development.[22,25-27,29]

Importance of the Balance between Gli3 and Shh

Suppressor function of Gli3 against the ventral progenitors may be responsible for the absence of these progenitors in *Shh-/-* single mutant mice, which has been strongly suggested by the restoration of these cells in *Gli3-/-;Shh-/-* double mutant mice.[24] Litingtung and Chiang (2000) showed that V2 inter neurons and motor neurons were restored in the spinal cord of *Gli3-/-;Shh-/-* double mutants.[24] This finding strongly suggests that the absence of motor neuron and V2 interneuron progenitors in *Shh-/-* single mutant mice is caused by the suppression of their specification by Gli3. Similar suppressor effect of Gli3 has also been revealed in the brain, as there is a severe lack of midline structure and expansion of dorsal cells in the brain of *Shh-/-* single mutants that is dramatically restored in *Gli3-/-;Shh-/-* double mutants[22,25] (Fig. 4B, C). The ventral-most aspect marker *Nkx2.1* in the medial ganglionic eminence is restored in the double mutant telencephalon.[25]These findings suggest that Shh signaling is not directly required for the specification of some ventral cell types, such as the cells in medial and lateral ganglionic eminences. Shh may be indirectly required for the ventral cell types to block the Gli3 suppressor function in the ventral telencephalon.

The activity gradient of Shh may regulate the amount of proteolytically processed Gli3 protein in the ventral region. The studies using an artificial processed form of Gli3 showed that ectopic expression of proteolytically processed form of Gli3 can block the generation of interneuron and motor neuron progenitors in the spinal cord.[34,35] It has been known that Hh regulates the processing of Gli3 in a similar manner to how Ci is regulated by Hh in *Drosophila.*[18] Based on these observations, the amount of proteolytically processed Gli3 proteins may be less in the ventral region of the spinal cord and brain by the action of the activity gradient of Shh. Intriguingly, not only blocking the development of ventral cell types, the processed form of Gli3 can also induce a ventral-to-dorsal shift in progenitor cell identity in a cell autonomous manner, suggesting that the amount of proteolytically processed form of Gli3 is involved in directing of dorsal-ventral patterning.[34,35]

Future Perspectives

These studies suggest that a combined action of Shh and Gli3 function is fundamental in telencephalon development (Fig. 4E). There appear to be two functions of Gli3, as an activator of dorsal cell types and as a suppressor of ventral specification, that seem to be related to the promotion of *Bmp/Wnt* and suppression of *Fgf8* expression (Fig. 4E). Gli3 may be involved in specific aspects of each of these processes. Although the studies with mutant phenotypes suggest a dual function, it has yet to be confirmed directly. It is possible that the promotion of

differentiation into dorsal cell types itself inhibits the differentiation into ventral cell types, and vice versa.

Gli3 function seems to be essential for dorsal cell specification, because the reduction of dorsal cells observed in the *Gli3-/-* telencephalon is not restored in the *Gli3-/-;Shh-/-* double mutants[22] (Fig. 4C,D). The regulation of *Bmp/Wnt* expression by Gli3 appears to be essential in determining dorsal cell types. Importantly, in embryonic neuronal progenitors isolated from the dorsal telencephalon, the inhibition of Bmp signaling was reported to be sufficient to determine ventral cell types shown by using cell culture system.[37] Blocking Bmp signaling in the neuronal progenitors isolated from embryonic dorsal telencephalon with a dominant-negative Bmp receptor Ib can induce differentiation into inhibitory interneurons, even if Shh signaling is also blocked by cyclopamine, an inhibitor of Smoothened function.[37] Thus, for dorsal progenitor cells, inhibition of Bmp signaling is necessary and sufficient for the induction of a dorsal-to-ventral shift in progenitor cell identity. Based on the mutant phenotype analyses and ectopic expression experiments, we already know that Gli3 is required for the activation of *Bmp* genes expression[27] and that the processed form of Gli3 can induce a ventral-to-dorsal shift in progenitor cell identity.[34,35] These findings suggest that Shh is required for inhibition of dorsal cell specification by inhibiting Bmp signaling via the conversion of Gli3 repressors into activators only in the ventral region. The spatial and temporal distribution of Gli3 repressor form regulated by the activity gradient of Shh may therefore control the normal proportion between dorsal and ventral territories.

Because most of the results summarized here are based on analyses of tissues from mutant animals, many unanswered questions about the functions of Gli3 and Shh proteins remain. Confirmation of the cellular functions of Gli3 is essential to extend these results to the molecular level.

The results presented here indicate that the proper balance between Shh and Gli3 functions is important for normal dorsal-ventral patterning of the neural tube and brain. As Shh and Gli3 inhibit each other, when one is missing severe malformation results as the remaining one expands to the other's territory. Recent findings using double mutant studies revealed that both Shh and Gli3 regulate only a set of specific cell types during neural tube and brain development. Moreover, when both are absent many cell types located in the intermediate zone of the brain and neural tube develop normally, indicating that Shh and Gli3 can only modulate the dorsal-ventral patterning established by other signaling pathways. Bmp/Wnt signaling and their antagonists are essential for neural plate development and its specification before the expression of Shh in the neural plate cells has been established.[38,39] It is likely that other signaling pathways participate in dorsal-ventral patterning in the telencephalon, but their precise roles and whether they act in concert or in parallel with Shh signaling requires further investigation.

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CHAPTER 15

Regulation of Early Events in Cell Cycle Progression by Hedgehog Signaling in CNS Development and Tumorigenesis

Anna Marie Kenney and David H. Rowitch*

Abstract

Hedgehog signaling is essential for proliferation of neural precursor populations in the developing central nervous system (CNS) and is etiologic in cerebellar brain tumors. Here we will contrast general strategies of cell cycle regulation by growth factors in the developing CNS with the emerging concept of a noncanonical Hedgehog "proliferative pathway" as suggested by published studies. Mechanisms utilized by Sonic hedgehog signaling to promote cell cycle progression in CNS progenitors during development and in adult stem cell populations also contribute to CNS tumorigenesis.

Mitogenic Signaling in the Developing CNS

A fundamental problem in development is how to precisely regulate the size of neural precursor pools so as to generate sufficient mature progeny to sculpt a central nervous system (CNS) complex enough to handle the organism's needs for motion, sensation, reaction, and perhaps learning and thought. Some primitive organisms, such as *C. elegans*, restrict the actual number of cells generated, with each cell having a defined role in the developing and mature organism.[1] Vertebrates have evolved a different mechanism, in which neural precursors are produced in vast excess with the unnecessary cells undergoing subsequent apoptosis.[1] Rather than being restricted in overall number, generation of neurons is tightly temporally restricted. Thus, large populations of neuronal precursors are supplied at specific places and times, such that they have the opportunity to mature and form synapses with the appropriate targets. Superfluous neurons—those failing to make successful synaptic connections—die. During later refinement of CNS circuitry, these synapses may be eliminated or remodeled, but the initial selection of surviving cells is based on strength of synapse formation.

How pools of CNS progenitors respond to temporal and spatial cues to undergo high levels of proliferation is only now beginning to be understood. As detailed below, localized CNS precursor pool expansion is likely to occur as a result of coinciding (1) microenvironmental mitogenic stimuli, (2) expression of appropriate receptors and signal transduction machinery, and (3) intrinsic cell cycle regulators, or "clocks". These final two criteria may be defined as "cellular competency" to proliferate. In the following section, we discuss the identity and function of molecular mediators of neuronal progenitor proliferation competency.

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Precursors of the Embryonic and Postnatal Brain

The development of the vertebrate CNS occurs in four major phases. The first specifies the site of the nervous system and biases the ectoderm toward a neural fate known as neural induction. In the second phase, neurulation occurs followed by an expansion of the stem cells to generate a pool of neural progenitors. At this stage, commitment to a particular cell fate (e.g., neuronal subtype or glial cell) does not preclude further proliferation.[2,3] Next, the period of symmetric division progressively switches to *asymmetric division*, whereby one daughter cell permanently exits the cell cycle and differentiates into its mature phenotype, while the other daughter cell retains its proliferative capacity. Last, at birth, except for a few specialized regions, including the rostral migratory stream, cerebellar cortex, hippocampal dentate gyrus, and cortically located glial precursor cells,[4,5] the brain enters a state of replicative quiescence.

Unlike some somatic cell types (e.g., hepatocytes and myocytes), neurons are unable to re-enter the cell cycle after completing terminal differentiation. Indeed, aberrant re-entry into the cell cycle is associated with apoptotic cell death, a process that has been implicated in the pathology of certain neurodegenerative diseases.[6,7] Although this lack of cell cycle flexibility was initially interpreted to mean a lack of turnover of CNS cell structures, it is now understood that pools of undifferentiated "neural stem cells (NSCs)" persist throughout the lifetime of vertebrates.[5] Collectively, NSCs provide the basis for neuronal precursor expansion during CNS development, and remain a persistent population in adults to potentially replace lost neurons or generate new cells if required, e.g., during learning processes and following injury.[4]

As noted above, neuronal precursors undergo both symmetric and asymmetric types of cell division. The determinants driving symmetric or asymmetric division in neural stem cells are not well understood, particularly in higher-order species such as mammals. Unequal inheritance of cell-cycle regulatory components and signal transduction machinery is likely to play as a role. For example, the heritable distribution of Notch protein[8] and the Notch modulator, numb,[9] have been implicated in determining whether daughter cells of asymmetric division retain their proliferative capacity or take on a differentiated neuronal cell fate. Since commitment of neural precursors to a specific fate does not preclude continued proliferation, basic mechanisms of cell cycle progression are likely to be similar between neural progenitors with a defined fate and uncommitted precursors. Therefore, general lessons learned from studying a committed population, such as cerebellar granule neuron precursors, may be extended to other types of neural stem cells. Here, we will address the broad question of how undifferentiated neuronal progenitors initially respond to mitotic signals and shift from a quiescent phase into the first and second phases of active cycling.

Regulation of the Cell Cycle by Mitogens in CNS Precursors

In neural precursor cells, extrinsic and intrinsic signals regulate the balance of proliferation or growth arrest through effects on the cell cycle machinery. The mitotic cell cycle is composed of four phases: one dedicated to synthesis of the genomic DNA (S), one to mitosis (M), and two gap (G1and G2) phases (Fig. 1). The first gap phase, G1, occurs between the end of mitosis and S phase. During early G1, critical decisions are made to commit to another round of cell division, exit the cell cycle permanently, or transiently exit to a G0 phase. Once committed to a new cell cycle, a cell requires sustained stimuli to reach a restriction point,[10] after which the cell no longer requires mitogenic stimulation to copy its DNA and continue through the cell cycle. The second gap phase, G2, between the end of S phase and the beginning of mitosis, allows cells to repair replication errors and strand breaks made during DNA synthesis and prepare for mitosis. M phase is typically the shortest phase of the cell cycle, and is followed by reentry into G1, or exit into quiescence (G0).

"Immediate Early Genes" Carry Out Activation of the Cell Cycle Apparatus by Mitogenic Signaling

In the developing CNS, cell cycle entry is likely to occur as a result of signaling pathway activation by extracellular factors. Bone morphogenic proteins (BMPs),[11-13] Notch pathway

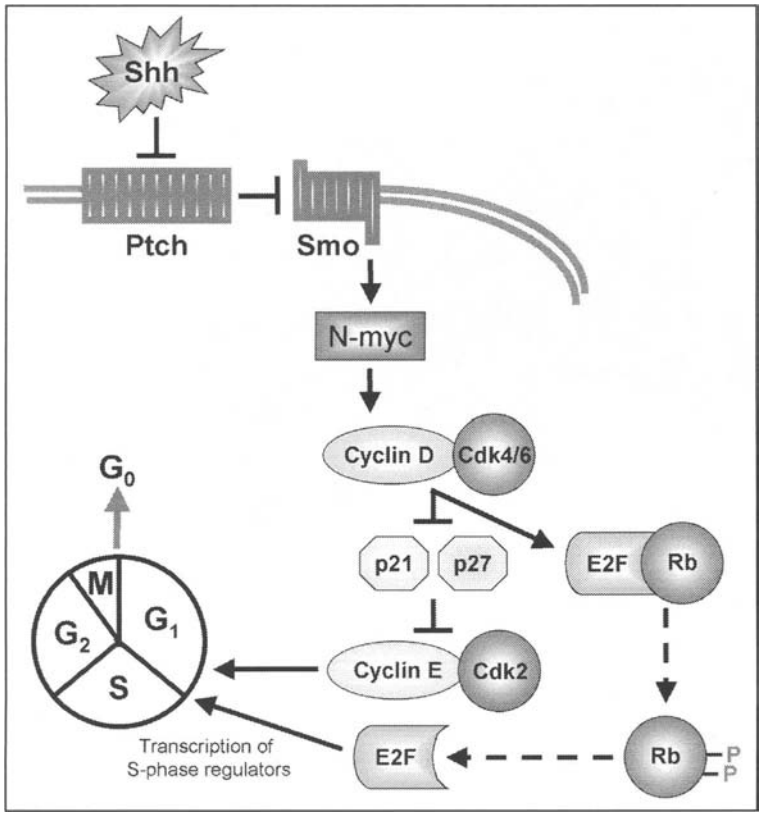

Figure 1. Scheme for cell cycle regulation in CGNP and effects of Shh on G1 phase progression through effects of N-myc. The cell cycle is divided into 4 phases: G1, S, G2, and M. Entry into the cell cycle and progress through the earliest phases is mediated by G1 cyclins and cdks acting downstream of immediate early genes. Schematic depicting the cell cycle phases and associated proteins regulating passage through each phase. Mitogens induce cell cycle progression by inducing expression of immediate early genes (iegs), such as N-myc in the case of Shh signaling. Iegs up-regulate D-type cyclins which form complexes with cdks 4 and 6. Cyclin D:cdk4/6 promotes progression through the G1 phase of the cell cycle by inactivating the ckis p21 and p27, which causes activation of cyclin E:cdk2 complexes. Phosphorylation of cyclin E:cdk2 substrates is necessary for entry into and progression through S-phase. CyclinD:cdk4/6 also phosphorylates Rb, thus releasing E2F transcription factors from Rb-mediated inhibition. E2F proteins regulate expression of genes required for S-phase, including some involved in DNA replication.Rb phosphorylation releases E2F transcription factors from inhibition. Activity of cyclin A:cdk2 regulates progression through S-phase into G2, when cyclin A may also associate with cdk1. G2 is marked by an increase in levels and activity of mitotic cyclins, including cyclin B, in complex with cdk1.

ligands,[14] neurotrophins,[15] epidermal growth factor (EGF) and fibroblast growth factor (FGF) family members,[16] Wnts,[17] and Sonic hedgehog[18] are among the many factors shown to be important for regulating neuronal precursor proliferation. The capacity of these factors to promote proliferation is often cell-type specific. For example, neurotrophins promote proliferation of cortical progenitors,[15] whereas activation of the Sonic hedgehog pathway is powerfully mitogenic for precursor cells in the cerebellum,[19-21] retina,[22] and forebrain.[23,24] Recently, adult hippocampal neural stem cells[25] and post-natal telencephalic progenitors[26] have also been shown to divide in response to Sonic hedgehog signaling, demonstrating a mitogenic role for hedgehog signaling throughout life.

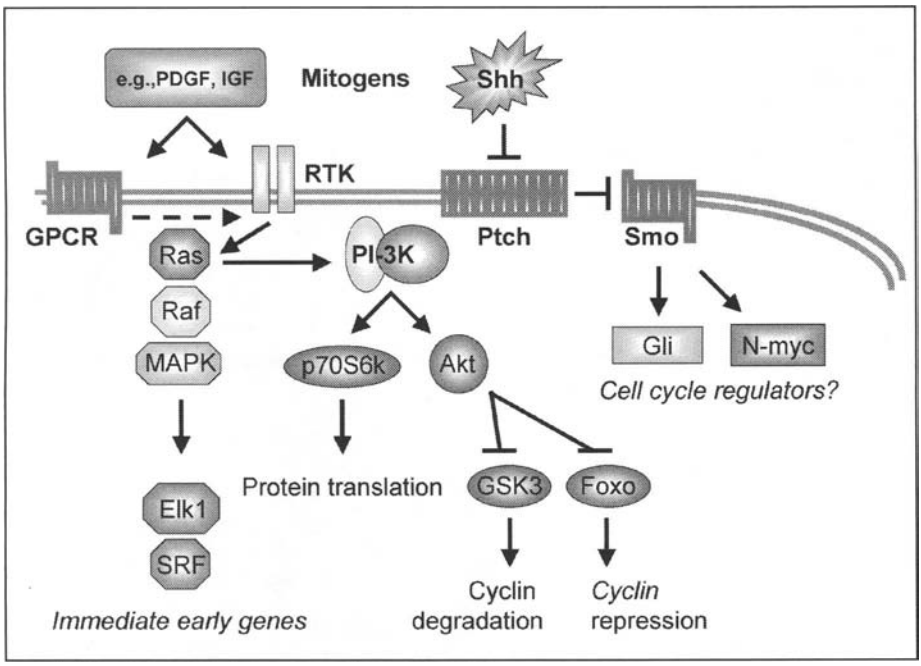

Figure 2. Many well-characterized mitogens induce cell cycle progression by activating receptor tyrosine kinases (RTKs). Schematic showing selected examples of how activation of RTKs promotes proliferation through signaling cascades regulating protein synthesis, proteolysis, and gene transcription. Many growth factors utilizing nonRTK receptors, such as G-protein coupled receptors (GPCRs), also promote activity of pathways downstream of Ras. Indeed, some GPCRs directly activate the PDGF or EGF RTKs through intracellular scaffolding mechanisms. The hedgehog pathway's proliferative effects are less clear. Shh signaling leads to nuclear localization of Gli transcription factors, which might activate or repress gene expression for cell cycle regulatory components. In contrast, activation of N-myc expression by Shh (Fig. 1 and text) provides a direct connection to the cell cycle apparatus via a noncanonical pathway.

Many well-understood mitogens access the cell cycle through classically defined receptor tyrosine kinase-activated (RTK) pathways.[27,28] When ligand binding activates such RTKs, a cascade of intracellular phosphorylation events results in activation of pathways regulating protein synthesis, and inhibition of pathways promoting cell cycle exit. The protein-synthesis dependent induction of so-called "immediate early genes (iegs)" also occurs in response to mitogens (Fig. 2). The name "immediate early genes" reflects the fact that mRNAs for such factors were initially characterized by rapid transcription within minutes of serum or specific mitogen exposure in synchronized cells, without requiring new protein synthesis.[29,30] Transcription of these genes occurs directly as a result of activation and nuclear localization of cytoplasmic proteins responsive to specific mitogens, including those activating pathways through mechanisms other than RTK binding. Intracellular cytoplasmic sensors, for example Smad proteins downstream of TGF-β,[31] or β-catenin downstream of wnt,[32] are latent transcriptional regulators, whose ability to activate immediate early gene transcription depends on interaction with nuclear-localized entities, such as chromatin remodeling complexes. The mechanisms through which RTK-activating mitogens such as platelet-derived growth factor (PDGF) promote proliferation have been well studied. By contrast, how the Shh pathway regulates the cell cycle is less clear (Fig. 2).

Many of the iegs induced by mitogens, such as members of the fos, jun, and myc families, are themselves transcription factors, whose function is to activate genes controlling entry into G1 of the cell cycle. Other known ieg targets include metabolic regulators, which will increase levels of protein synthesis required for proliferation.[33] As described below, N-myc is an ieg with a critical role in Shh-mediated cerebellar precursor proliferation.

G1 Phase Progression Is Mediated by D-Type Cyclins

Among the earliest responders to ieg activity are D-type cyclins. Indeed, cyclins D1 and D2 are known to be transcriptional targets of myc.[34,35] Because their induction is dependent upon activity of upstream regulators, D-type cyclin upregulation is referred to as a "delayed early response."[36] In quiescent G0-phase cultured cells, D-type cyclin expression is rapidly elevated in response to mitogenic stimulation, and is typically maintained through the length of the cell cycle,[37] or until growth factors are removed. In primary cerebellar granule cell cultures, protein synthesis-dependent up-regulation of cyclins D1 and D2 can be observed within one to three hours of Shh stimulation,[38,39] with levels dropping when Shh is removed. The timing of cyclin D1 up-regulation in primary cerebellar cultures is consistent with the kinetics of the delayed early response in other cell types to mitogenic stimuli. For example, increased levels of cyclin D1 can be detected in serum-stimulated keratinocytes after 1 hour.[36] These observations indicate that hedgehog-stimulated proliferation of neuronal precursors utilizes mechanisms conserved with other mitogenic pathways.

D-cyclins are nuclear proteins,[40] which function in several ways to promote progression from G0 into and through early phases of G1. In complex with cyclin dependent kinases (cdks) 4/6, D-cyclins sequester the cdk inhibitors (ckis) p21 and p27, which can block S-phase entry though their inhibition of cyclin E/cdk2 activity.[41,42] Thus, cyclin D:cdk4/6 activity is necessary for cyclin E activation.[43,44] A critical role for D-type cyclin/cdk complexes is to initiate hyperphosphorylation and inactivation of the retinoblastoma protein (pRb), a tumor-suppressor[45] (Fig. 1). The D-type cyclin family comprises three proteins, whose expression and activity during development and in response to mitogens is highly cell-type specific.

Studies of mice lacking members of this family have shown that D-type cyclins play an important role in development of many tissues, including the CNS. Specifically, cyclin D1 is required for normal retina and mammary gland development;[46,47] cyclin D2 is important for development of the ovaries and testes;[48] and D3 plays an important role in the lymphoid compartment.[49] Consistent with its important role in mammary development, de-regulated activity of cyclin D1 is observed in breast cancers.[50] Cyclin D1-mutant animals display neurological abnormalities, although their cerebella appear phenotypically normal. Loss of cyclin D2 results in impaired survival of several cerebellar cell types.[51] Interestingly, combined loss of cyclins D1 and D2 causes markedly perturbed cerebellar development, most likely attributed to defective granule cell precursor proliferation.[52] Cyclin D3 ablation has no effect on brain development.[52]

Cyclin E Plays a Role in Late G1 and the Transition to S-Phase

Increased expression of cyclin E is also observed during G1. Like cyclin D, cyclin E is a nuclear protein.[53] However, unlike D-cyclins, cyclin E is rapidly degraded upon entry into S-phase.[43,54] Activity of cyclin E and its partner, cdk2, occurs later in G1, and functions to facilitate entry and passage through S-phase. Cyclin E activation does not occur as a direct response to mitogens, but is instead regulated by cyclin D-mediated inactivation of the cdk 2 inhibitors p21 and p27 (Fig. 1). Interestingly, mice lacking p27 show marked cerebellar hyperplasia and increased proliferative response to Shh, demonstrating an important role for cyclin E activity in the hedgehog proliferative pathway.[55] In vitro, granule cell precursors treated with Shh show increased levels of cyclin E.[38] Like D-cyclins, cyclin E/cdk 2 can phosphorylate pRb. Replacing cyclin D1 with cyclin E results in rescue of cyclin D knockout phenotype, indicating

that a major function of cyclin D is its activation of cyclin E.[44] In addition to pRb, cyclin E substrates include numerous proteins involved with S-phase progression,[56,57] including E2F transcription factors,[58] which regulate expression of molecules initiating DNA replication. Over-expressing cyclin E in fibroblasts accelerates progression through G1, likely due to early activation of S-phase regulators.[53]

There are two known cyclin E family members, cyclins E1 and E2. In mice, ablation of either family member alone is not lethal—although cyclin E2 knockout males exhibit reduced fertility.[59] Mutation of both cyclin E1 and E2 causes placental defects, and live pups are not born. When experiments were performed such that double mutant animals could develop in utero with wild-type placental tissue, they died on the first day of life. Analyses of embryonic development revealed cardiovascular abnormalities.[59] Since the animals died before the onset of CGNP expansion, development of the cerebellum could not be analyzed. Analysis of fibroblasts derived from cyclin E mutant embryos revealed an inability to reenter the cell cycle upon mitogenic stimulation. Given that cerebellar granule neurons cannot be induced to divide in response to Shh after leaving the cell cycle, it is tempting to speculate that this may be due to an irreversible "shut-down" of cyclin E. Indeed, such a "shut down" could be accomplished by strongly increasing levels of ckis, such as p21 or p27. High expression of p27 is observed in post-mitotic granule cells.[55]

The Transition from G1 to S-Phase Requires Rb Inactivation

Both cyclin D and cyclin E in complex with their cdk partners can phosphorylate the tumor suppressor retinoblastoma gene product, pRb. Rb is phosphorylated at several sites,[60] and this phosphorylation is sustained throughout the cell cycle until the cell exits mitosis.[61] In G1, phosphorylation of Rb by cyclin D is likely to be more relevant than phosphorylation by cyclinE:cdk2, as cyclin D is dispensable for proliferation of Rb-null cells, whereas cyclin E is not, indicating that cyclin E's other functions are critical for cell cycle progression. In its hypophosphorylated form, pRb prevents transition from G1 to S-phase, by interacting with and blocking function of specific proteins. Most well known of pRb substrates are the E2F transcription factors, many of which promote S-phase progression through activation of their target genes (Fig. 1). However, Rb is also involved in down-regulating activity of the Ras pathway,[62] which is critical for survival and proliferation in the CNS. This function of Rb is separable from its E2F-binding role. Moreover, Rb can also function as a corepressor with CDP/cut/cux proteins, blocking activation of cell cycle regulated histone promoters.[63] CDP/cut/cux family members are expressed in the CNS and are implicated in cell fate specification and differentiation,[64,65] consistent with a role for their cooperation with pRb in this process. Recently, work in osteosarcoma cell lines has shown that phosphorylation of Rb is also important for entering G1 from G0.[66] Cyclin C/cdk3 mediates this phosphorylation. It is not known whether a similar process occurs in proliferating neural precursors. However, it is interesting to speculate that suppression of cyclin C/cdk3 activity may be part of the mechanism preventing differentiated neurons from reentering the cell cycle.

Rb is a member of the so-called "pocket protein" family, which also includes p107 and p130. The "pocket" refers to a highly conserved protein-protein interaction domain, through which binding to E2F factors is mediated. These proteins show specific developmental expression patterns and tissue distributions.[67] P107 and p130 are more highly related to each other than to pRb, and ablation of either of these proteins in the mouse causes no apparent phenotype during development or adulthood. However, combined mutation of p107 and p130 results in defective bone formation and perinatal death due to respiratory defects. The bone formation defect was attributed to excessive proliferation of chondrocytes, indicating that like pRb, p107 and p130 have anti-proliferative functions. Neither p107 nor p130 have been implicated in cancer.[68]

In contrast, mutation of Rb in humans results in retinoblastoma, as well as several other types of tumors,[69] including brain tumors.[70] Several groups have generated mice with homozygous inactivation of Rb.[71-73] These animals die by day 15 of gestation, with defects in many

tissues, including the hematopoetic compartment. The nervous system is also highly affected: widespread neuronal cell death was observed. A close analyses of Rb-deficient mouse nervous system development showed that many of the dead cells in the nervous system had recently attempted to enter the cell cycle,[74] and that neuronal cell death occurred during a time at which, during normal development, Rb is hypophosphorylated in the nervous system. These findings indicate that Rb's cell cycle inhibitory function promotes survival of post-mitotic neurons. Cell death in the absence of Rb occurs through pathways, which involve p53, and others which do not.[75] Interestingly, combining loss of p53 with loss of Rb in the developing cerebellum results in medulloblastomas of granule neuron precursor origin, suggesting that the p53 and pRb tumor suppressor proteins may cooperate in promoting normal cell cycle exit in the developing cerebellum.

SHH Signaling Is Required for Growth of Cerebellar Granule Neuron Precursors and Is Etiologic in Medulloblastoma

The cerebellum has pivotal roles in coordination of posture and locomotion, head and eye movements and a wide range of routine and skilled motor activities. Several lines of evidence further indicate cerebellar functions in learning, cognition and language. The stereotypical laminar organization of the cerebellar cortex—with its limited number of input and output lines, and only a half dozen major classes of neurons—has aided efforts to trace successive stages of cerebellar ontogeny and molecular mechanisms involved in its development (Fig. 3).

Purkinje cells and the cells of the cerebellar nuclei originate from precursors of the rhombic lip and caudal midbrain.[76] The precursors for granule cells are generated in rhombomere 1 of the embryonic hindbrain and migrate dorsally to form the outer layer of the cerebellum, or external granule layer (EGL). Certain transcription factors including *Math-1* have been demonstrated to be essential for early granule cell development.[77] Proliferation of granule cells is largely postnatal.[78] Proliferating granule cell precursors are readily detected in the cerebellum by virtue of their position in the EGLa compartment. Post-mitotic cells initially become *NeuroD1*-positive and reside in the EGLb compartment. They then migrate to the IGL, comprised of differentiated granule cells that label with the mature neuronal marker, NeuN, and the granule cell marker, Zic. In humans, granule cell differentiation is completed by approximately 18 months of age. The equivalent process in the rodent cerebellum takes place within 1 month of birth.[78]

SHH Signaling Is Essential for CGNP Proliferation

During mammalian central nervous system (CNS) development, multipotent precursor cells undergo division, cell fate specification, and maturation in response to extrinsic cues. The secreted signaling molecule Sonic hedgehog (Shh) is essential for development of organizing structures at the ventral midline (e.g., floorplate) and the specification of neurons and glia.[79] In addition, recent evidence has indicated that Shh regulates the proliferation of granule neuron precursors in the cerebellum.[19-21] Proliferative effects associated with Hedgehog pathway activation have also been described in the developing neural tube[79-81] and retina.[22]

The active Shh signal is produced by autoprocessing and cholesterol modification,[82] and binds to a receptor complex composed of at least two transmembrane proteins, Patched and Smoothened.[77,83] Shh binding to Patched is thought to relieve Patched-mediated inhibition of Smoothened activity, resulting in the activation of transcriptional targets by members of the *Gli* family.[84] Smoothened belongs to the family of serpentine G-protein coupled receptors (GPCRs). Shh signaling can be inhibited experimentally by increasing cyclic AMP levels or protein kinase A (PKA) activity.[85-87] Developmental effects of Shh can be mimicked in vivo by expression of pertussis toxin[88] or dominant negative PKA,[89] suggesting that an inhibitory G protein ($G_{\alpha i}$) may be the target of Smoothened. However, a specific heterotrimeric G-protein downstream of Smoothened has yet to be identified,[90] and endogenous cyclic AMP levels do not respond to Hedgehog pathway activation.[91] Conserved components of the Hedgehog

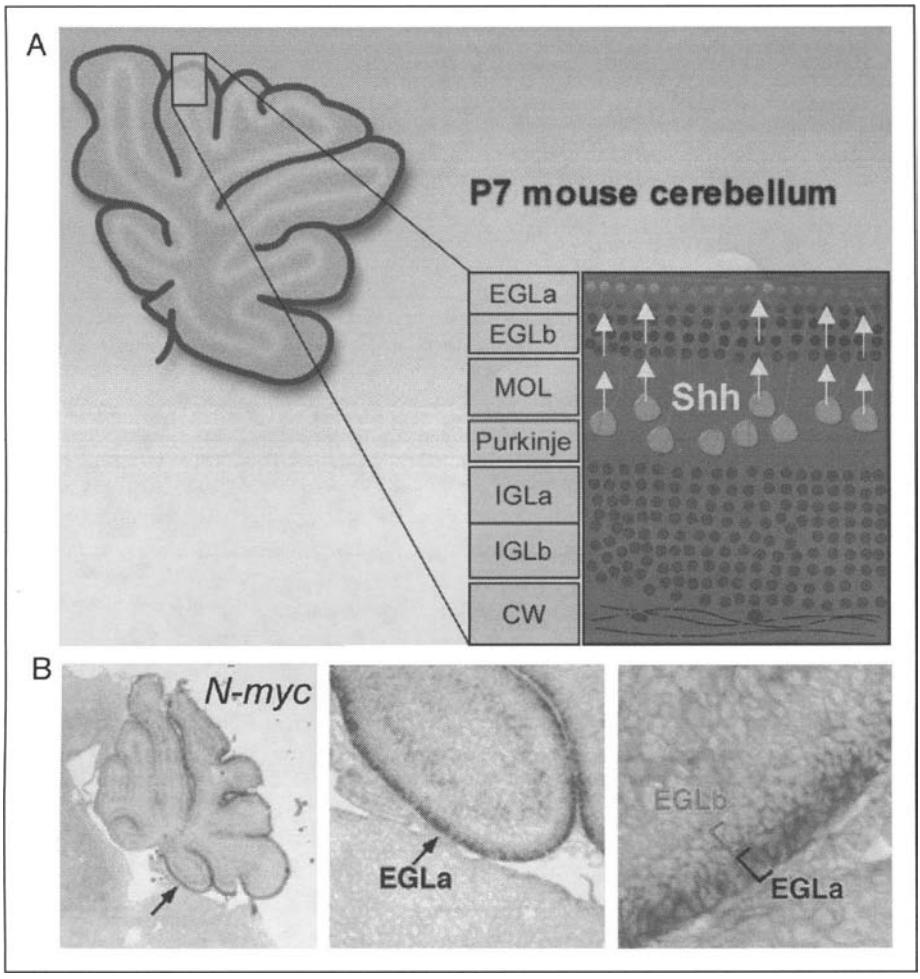

Figure 3. Structure of P7 mouse cerebellum and expression of mRNA transcripts for *N-myc* the EGL layer in situ. The layered architecture of the cerebellum is apparent in the cartoon of a parasagittal section at low power. A) High power view showing layers of the developing cerebellum. The EGLa comprises proliferating Math-1-positive granule cells. Commensurate with maturation to NeuroD1-positive nonproliferating cells, they migrate to the EGLb layer. Subsequent stages of maturation are recognized by the markers Zic and NeuN, and further migration to the IGL. The Purkinje neurons are the source of Shh proteins, which are exported to the EGLa compartment through a poorly understood mechanism. The cerebellar white matter (CW) layer is indicated. B) N-*myc* is expressed in proliferating precursors of the developing cerebellum. N-*myc* expression is observed during the proliferative phase of cerebellar development at PN7, but is undetectable in the PN15 or adult (not shown) mouse cerebellum. Low power views show the distribution of N-*myc* mRNA transcripts primarily in the EGLa region.

signaling pathway include Fused and Suppressor of Fused.[92,93] These proteins are thought to retain the Shh-activated transcription factors Gli2 and Gli3 (orthologues of *Drosophila ci*) in the cytoplasm via Costal2-mediated interactions with microtubules.[94,95]

Until recently, the Shh signal transduction pathway was not known to share common targets with any known mitogenic intracellular signaling pathways. Indeed, it has been proposed that proliferative effects of Shh on retinal precursors are indirect, perhaps involving synthesis of

a secondary mitogen.[22] However, a secondary mitogen need not be invoked, as transactivation of receptor tyrosine kinase (RTK) pathways by GPCRs is well described.[27,96-98] Upon phosphorylation by GPCR kinases (GRKs), GPCRs can utilize the intracellular domains of RTKs as scaffolds for stimulating activation of the mitogen-activated protein kinase (MAPK)/extracellular-signal regulated kinase (ERK) pathway, a target of many extracellular mitogenic stimuli.[27] Independent of RTK scaffolding, some GPCRs can activate the MAPK pathway through PI-3 kinase activation.[99] This mechanism involves the β/γ subunits of heterotrimeric G-proteins. Given the resemblance of Smo to a GPCR, and recent evidence that Smo activates and endogenous Gα, it is possible that Shh proliferative signaling through Smoothened may also regulate MAPK in neuronal precursors. However, it is unlikely that this mechanism plays a role in Shh-mediated cell division, as inhibiting MAPK activity does not affect Shh-mediated proliferative effects on cerebellar granule neuron precursors.[38]

SHH is required for granule cell precursor proliferation during cerebellar development.[19-21] Purkinje cells arborize towards the EGL and are considered the relevant source of SHH signal for granule cells in the EGL (Fig. 1). The Hedgehog pathway is highly conserved (Fig. 2). Genetic and biochemical characterization in *Drosophila* has yielded important insights into its organization in higher vertebrates.[100,101] This has led to the proposal that SHH relieves Patched-mediated inhibition of Smoothened activity, resulting in the activation of transcriptional targets by members of the *Gli* family.[84]

Inappropriate Activation of HH Signaling Results in Brain and Other Tumors

Previous work in the fields of developmental biology and human genetics has revealed that certain mechanisms essential during formation of the embryonic CNS can also become inappropriately activated at later stages resulting in tumors of the cerebellum. Activating mutations of the Wnt and Hedgehog signal transduction pathways, in particular, have been associated with medulloblastoma, a cerebellar tumor most commonly affecting children. Loss-of-function mutations in human *PATCHED (PTC)* are associated with activation of the Hedgehog signal transduction pathway and promotion of a neoplastic state.[18] Three to five percent of children with Gorlin's syndrome, caused by inherited mutations of *PTC,* develop medulloblastoma.[102] Inactivating mutations of *PTC* have also been found in sporadically occurring medulloblastoma;[103] mice heterozygous for targeted mutations of *Patched,* in which SHH targets are potentially upregulated, develop cerebellar tumors at a low incidence (10-15%).[100] Interestingly, combination of the *PTC+/-* allele with loss of the tumor suppressor *p53* results in a dramatic increase, with tumor incidence approaching 100%.[104] Although the combination of *PTC+/-* mutation and *p53-/-* is not found together in human tumors, these data suggest that other "second hit" mutations might facilitate tumor progression in combination with deregulated SHH signaling (Fig. 2). Indeed, experimental mutations in Rb and p53 also promote medulloblastoma formation in mice.[105] Further work is needed to identify the authentic collaborative mutations that may occur within human medulloblastoma.

Pomeroy et al have recently shown by oligonucleotide microarrays that the desmoplastic sub-type of medulloblastoma is specifically associated with activation of Hedgehog gene targets.[106] This sub-type comprises ~20% of all medulloblastoma cases in the US, and is thought to result from loss-of-function mutations of the Hedgehog pathway suppressors, *PTC* and *SUFU.*[107] The mechanisms connecting Hedgehog signal transduction to molecular regulators of the cell cycle during development and tumorigenesis are beginning to be understood through biochemical analyses of Shh proliferative effects on responsive neuronal precursor populations, as well as phenotypic analysis of mouse genetic models.

Understanding Sonic Hedgehog Pathway Interactions with the Cell Cycle Apparatus

The links between the Shh pathway and the cell cycle apparatus have been less clear than with many other mitogens so far studied. Downstream effectors of cell cycle progression have been highly conserved through evolution, making it likely that the Shh pathway intersects with

similar molecular sensors to manifest its mitogenic role in competent neuronal progenitors. However, the studies of canonical Shh pathway, as defined by genetic analyses, have failed to clearly indicate this mechanism, necessitating the use of biochemical approaches. Here we will examine the candidate mediators of Shh effects on regulation of G1 progression, initially identified in primary mouse CGNP cultures and subsequently validated in vivo.

During the phase of granule cell expansion in the mouse, it is relatively straightforward to prepare primary cultures consisting of approximately 90% pure CGNPs. With additional purification, cultures up to 98% pure can be obtained.[108] CGNPs can be maintained in a proliferative state in vitro in the presence of Shh for a week. It is important to note that the presence of Shh does not constitute an absolute block to proliferation: even with Shh treatment, many CGNPs exit the cell cycle and go on to differentiate,[38] most likely due to intrinsic cell cycle clock mechanisms.[109] However, sufficient numbers of CGNPs proliferate in vitro in response to Shh to make these cultures ideal for performing biochemical analyses of cell cycle regulatory events, modeling effects of signal transduction pathway activation on proliferation, and ectopic expression studies using retroviral infection. By taking advantage of these easily accessible, nearly homogeneous, and highly manipulatable cells, we and others have been able to make great strides in defining mediators of Shh proliferative effects on neuronal precursor cells.

Is There a Role for GLI Activity in Vertebrate HH Proliferative Signaling?

Signaling through the Hedgehog pathway results in activation of Gli zinc-finger transcription factors,[110] which are cytoplasmically localized until activated. The topic of Gli signaling is covered in several other chapters and will only be touched on briefly here. Gli1 behaves as a transcriptional activator, whereas Gli3 generally functions as a repressor of hedgehog signaling.[111,112] Although Gli2 has been shown to have activator and repressor properties distinct from *Gli1*,[111] *Gli1* can functionally compensate for *Gli2* during CNS development.[113] It has been suggested that all three are retained in the cytoplasm, and processed or relocalized in response to hedgehog signaling.[110-112,114]

Gli proteins are implicated in regulation of neural proliferation, as *GLI1* was originally identified as a locus amplified in a low percentage of human gliomas.[115] Over-expression of *Gli* is sufficient to cause skin tumors and neural hyperplasia,[116] as well as granule cell precursor proliferation,[39] and transcriptional effects of GLI have been observed on D-type cyclins by gene expression profiling of a transformed rat kidney cell line.[117] Indeed, these experiments suggest that Gli1 can directly upregulate *cyclin D2*. Moreover, Oliver et al have shown that retroviral expression of Gli1 is sufficient to maintain high levels of CGNP proliferation in vitro, in the absence of Sonic hedgehog, suggesting that Gli1 can regulate components of the cell cycle regulatory apparatus. However, *Gli1* function is not required during cerebellar growth[118] or tumorigenesis[119] demonstrating that regulation of D-type cyclins and cerebellar growth is not *Gli1*-dependent in vivo.

These experiments do not rule out an important role for *Gli2*, which is expressed in a similar pattern to *Gli1* and may compensate for loss of *Gli1* function. It is interesting to note, however, that proliferation and cyclinD1 levels are normal to increased in spinal cord progenitors of compound mutant animals lacking all Gli function.[120] In vitro, Gli2 is less potent than Gli1 in its ability to promote CGNP proliferation.[39] Thus, the role of the canonical Shh signaling pathway in mediating Shh mitogenic effects remains controversial, and is likely to be a complicated question to unravel given the combination of activator and repressor functions carried out by Gli proteins. It is also possible that Shh signaling through Ptc and Smo regulates cell cycle progression through novel mechanisms. Indeed, as described below, recent work raises the possibility that Shh access to the cell cycle via the proto-oncogene N-myc may not involve Gli proteins.

N-MYC as a Key Component of HH Signaling

Identification of Shh signaling intermediates that function in cell cycle regulation is key to understanding the role of this pathway in CNS proliferation during development and tumorigenesis. We and others have shown that treatment of CGNP cultures with Shh results in rapid upregulation of D-type cyclin genes, and that regulatory effects on G_1 cyclins require protein synthesis.[38] Like classical mitogens, which promote proliferation through upregulation of immediate early genes, Shh directly induces the proto-oncogene N-myc in primary cultures of CGNPs. N-*myc* is a member of the myc family, which also includes c-*myc* and L-*myc*. Activity of myc proteins is known to promote proliferation and/or transformation in many cell types.[121] Complete loss of myc activity results in severely compromised cell cycle regulation.[122]

When expression of myc family members was examined by in situ hybridization, only N-*myc* was expressed in the proliferative zone of the cerebellum, suggesting a potential role for N-myc in CGNP proliferation in vivo (Fig. 3). N-myc mRNA was detected in the same region of the developing cerebellum as cyclin D1, and Gli1, demonstrating activation of the Shh pathway in proliferating CGNPs in vivo (Fig. 3). Moreover, N-myc expression was only detectable during the time of cerebellar expansion, being completely downregulated by P15 (Fig. 3). We observed N-*myc* upregulation in CGNP cultures and the dorsal embryonic spinal cord exposed to Shh, suggesting that signaling via N-*myc* may be a general feature of Shh-induced proliferation in the CNS. However, in contrast to known general transcriptional targets of Shh (e.g., *Gli* genes), we note that Shh signaling effects on N-*myc* regulation are unique to immature, proliferating precursor cells. Thus, cellular competence is evidently a critical determinant of the transcriptional response of N-*myc* to Shh.

Increased levels of *N-MYC* mRNA have been reported in certain cases of human medulloblastoma.[123] More recently, *N-MYC* upregulation has been observed in the desmoplastic form of medulloblastoma, the specific subtype of medulloblastoma associated with pathological activation of the Hedgehog pathway.[106] In contrast, *N-MYC* expression was not elevated in the majority of other (nondesmoplastic) medulloblastoma types analyzed. Consistent with findings in humans, we observed dramatic N-*myc* upregulation in medulloblastomas from *Ptc* heterozygotes.[100] Together, these findings are strong evidence that similar mechanisms operate downstream of Hedgehog signaling during central nervous system development and tumorigenesis. Our results further suggest that *N-MYC* expression in desmoplastic medulloblastoma is a direct consequence of Hedgehog pathway activation.

Evidence That N-myc Activity Is Required for the Full Shh Proliferative Response in CGNPs

To establish a role for N-myc in the Shh proliferative pathway in developing cerebellar precursors, it will be necessary to examine cell cycle progression in the absence of N-*myc* function. However, mice homozygous for N-*myc* null or certain hypomorphic alleles die in utero or at birth,[124-127] preventing assessment of N-*myc* function during the proliferative post-natal phase of CGNP development. Recently, Knoepfler et al have used lox-cre technology to conditionally target murine N-*myc* in the developing CNS, resulting in severe hypoplasia of cerebellum and defects in granule cell precursor proliferation.[128] These findings indicate that N-*myc* function is necessary for cerebellar development. Yet, they leave open the question of whether N-myc activity is required downstream of Shh signaling during the post-natal phase of granule cell expansion. In vitro studies suggest this is likely to be the case, as dominant negative inhibition of N-myc results in drastically reduced cerebellar precursor proliferation,[39,129] and cre retrovirus infection of CGNPs cultured from N-myc$^{fl/fl}$.mice causes near complete cessation of Shh-inducible proliferation (A.M. Kenney, D. Rowitch, P. Knoeptler and R. Eisenman, unpublished). Cre-mediated ablation of N-myc in early post-natal granule cell precursors is likely to shed light on this issue in vivo.

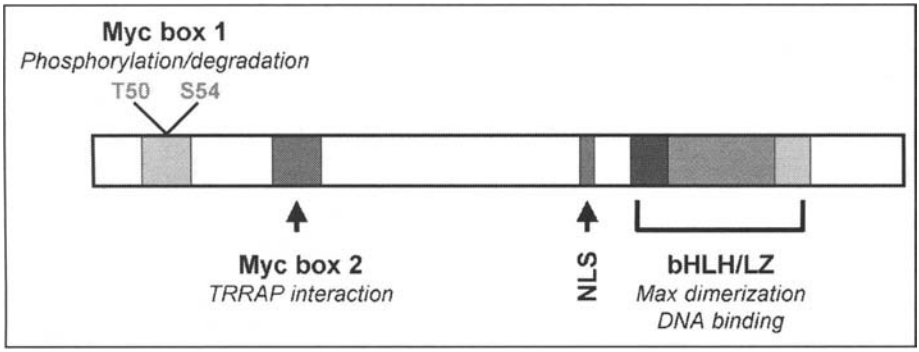

Figure 4. N-myc protein structure contains regions highly conserved among myc family members. A schematic illustrating functional domains of N-myc, which migrates at approximately 50 KD on SDS-PAGE. The amino terminal comprises the transactivation domain. This portion of the protein includes the myc box one, which contains phosphorylation sites regulating myc stability. C-myc box one has been proposed to interact with Bin1, a protein involved in differentiation.[177] Bin1 interaction with N-myc has not been investigated. Myc box 2 mediates interactions with TRRAP, a protein involved with chromatin remodeling and whose interaction with myc is required for activation of certain target genes.[178] TRRAP interaction promotes histone acetylation. This region of N-myc is required for promoting CGNP proliferation. The carboxyl terminus of myc proteins contains basic helix-loop-helix and leucine zipper domains responsible for dimerization to Max, and DNA binding.

In studies of myc biology, over-expression of Mad family members is an established method for inhibiting myc functions in proliferation, transformation and apoptosis.[130-133] These studies indicate that the repressive activity of Mad family members is specific, and Mad proteins are unlikely to behave as general transcriptional repressors; indeed, Mxi over-expression cannot inhibit transformation by E1a, another potent oncoprotein.[134] *Mxi* over-expression was shown to abrogate the mitogenic effects of Shh in vitro,[135] raising the possibility that Mad family members may regulate CGNP cell cycle exit during cerebellar development in vivo. In keeping with this, expression profiling studies indicate up-regulation of several *Mad* family members at PN 7, coinciding with the major wave of CGNP differentiation (Q. Zhao, A. Kho, I. Kohane and D. Rowitch, unpublished observations).

Although the levels of CGNP proliferation in vitro are reduced in the presence of dominant negative N-myc,[39,135] absolute proliferation arrest is not observed. This is not surprising, since some residual N-myc activity is likely to remain. When myc activity is reduced, but not eliminated, myc-dependent processes can still occur.[136] Indeed, cultured fibroblasts lacking any myc family member remain capable of proliferation albeit with an extended cell cycle length.[122] Thus, abrogation of N-myc activity might not necessarily lead to cell cycle exit within the time frame of the in vitro studies performed. Taken together, the finding of decreased CGNP proliferation with specific N-myc antagonists in vitro, coupled with data that demonstrate essential functions for N-*myc* during cerebellar development in vivo,[128] strongly suggest that N-*myc* functions as an integral component of a Shh regulated pathway during proliferation of granule neuron precursors.

Mechanisms Underlying Cell Cycle Regulation by N-myc in Proliferating Neuronal Precursors

Myc proteins are characterized by specific functional domains (Fig. 4), including a carboxyl terminus DNA binding domain and a helix-loop-helix/leucine zipper domain, which mediates dimerization with myc's obligate partner, Max (Myn in the mouse)[137]).[121] Two highly conserved amino acid regions, termed Myc box 1 and Myc box 2, reside in the amino terminal transactivation domain. Myc box 1 contains two phosphorylation sites with important roles in

regulating myc stability. Myc box 2 associates with TRRAP protein,[138] which functions to recruit histone acetyltransferase to myc's transactivation domain.[139] This interaction is required for the transforming potential of c-myc and N-myc.[138] The Myc box 2 domain also appears to be required for N-myc's proliferative function in CGNPs, as over-expression of an N-*myc* mutant lacking Myc box 2 cannot rescue CGNP proliferation in the absence of Shh signal, and interferes with CGNP proliferation in the presence of Shh.

It is likely that N-myc activates yet to be identified genes involved in cell cycle regulation. Recently, *Id2* was shown to be an N-myc target in neuroblastoma.[140] Some c-myc targets include genes such as *cyclinD2*[35] and *cdc25*,[141] which control cell cycle progression. Metabolic regulators that enhance cell growth may also be targets of c-myc.[34] Finally, c-myc has been implicated in repression of genes that promote cell cycle exit and differentiation.[142] Given the high level of relatedness between N-myc and c-myc protein structure, and the observation that N-myc can rescue many of the defects in c-myc null mutant mice,[143] it is possible that they have similar transcriptional targets.

We observed increased levels of *cyclin D1* in N-*myc*-infected cells despite the presence of cyclopamine. N-*myc* is a direct transcriptional target of Shh signaling, whereas *cyclin D1* mRNA upregulation was inhibited by cycloheximide.[38] N-myc could be a candidate regulator of *cyclin D1* expression. Conversely, N-myc may repress or inhibit expression of proteins involved in cell cycle exit. Indeed, mice conditionally mutant for N-myc in the CNS show high levels of cki expression in the brain, consistent with premature cell cycle exit.

In summary, several studies indicate that N-*myc* is a direct downstream target of the canonical Shh signaling pathway in proliferating CGNPs. N-*myc* expression was also found in medulloblastomas of *Ptc* mutant mice, and in humans with Shh-pathway associated medulloblastomas. N-myc function is a key component of a Shh proliferative pathway essential for normal expansion of CGNP populations. These studies identify a direct connection between the Shh signaling pathway and a cell-intrinsic regulator of the cell cycle apparatus in primary neuronal cells and cancer cells. Proximal events in the progression from Smoothened activation to upregulation of N-*myc* expression remain to be determined. Identification of N-myc transcriptional targets will be critical for understanding of Shh-mediated cell cycle progression in CNS development. Moreover, because cerebellar granule cells are postulated to be the cell-of-origin for medulloblastoma, effective means to inhibit N-myc activity might provide new approaches to control the growth of cerebellar tumors. In the following section, we will cover work on the overall regulation of N-myc activity levels in CGNP, which involves combinatorial interactions between Shh and PI-3kinase signaling.

Regulating N-myc Protein: Post-Translation Modification and Turnover

Identification of the intracellular events that integrate effects of divergent signaling pathways is critical for a comprehensive understanding of growth control in both normal and neoplastic neuronal progenitors. To better understand interactions between the Shh and other signaling pathways during neuronal precursor proliferation, we have focused on determinants of N-myc protein turnover and cell cycle progression in primary CGNP cultures. C-myc, L-myc and N-myc all feature residues in the highly conserved amino-terminal myc box 1 (MB1) domain that could function as phospho-acceptor sites. Mutation of *C-MYC* amino-terminal phosphorylation sites in humans is associated with Burkitt's and other aggressive lymphomas,[144,145] suggesting that disruption of the regulatory role played by these sites may contribute to human disease. Previous analysis of c-myc has implicated phosphorylation of amino-terminal sites in c-myc turnover[146,147] using rat embryonic fibroblasts, cell lines or nonmammalian cell systems.[148] In contrast, N-myc phosphorylation had not previously been established.

We found that dual phosphorylation on threonine 58 and serine 54 promotes N-myc protein turnover and timely cell cycle exit in CGNP primary cultures. Signaling by Sonic hedgehog appeared to not be directly involved in N-myc phosphorylation, suggesting that N-myc protein turnover is regulated separately from its mRNA induction by Shh in CGNPs. N-myc

mutants unable to be phosphorylated at either or both sites are extremely stable, and promote prolonged proliferation in cultured CGNPs. These results suggest that kinases acting at these sites may promote cell cycle exit through their ability to increase N-myc degradation. N-myc threonine 50 is located within a consensus sequence for phosphorylation by glycogen synthase kinase 3β (GSK-3β). Phosphorylation at this site requires a priming phosphorylation event at S54, another characteristic of GSK-3β activity. Moreover, GSK-3β is known to antagonize proliferation in general, by promoting proteolysis of cyclin D1,[149] as well as downregulating hedgehog signaling by inducing proteolysis of Ci, the fly ortholog of Gli.[150] Indeed, we found that inhibiting GSK-3β activity prevented phosphorylation of T50.[129]

The identity of the kinase acting at S54 remains to be determined, although several candidates for the analogous site (S62) in c-myc have been proposed. These include MAPK, JNK, and members of the cdk2 family. In vitro kinase assays have shown that MAPK is capable of phosphorylating S62. However, modulating activity of the MAPK pathway in cultured cells has no effect on c-myc S62 phosphorylation.[151] As discussed above, the MAPK pathway appears to play no role in CGNP proliferation, suggesting it may not be involved in N-myc phosphorylation. Indeed, preliminary studies have found that activation or inhibition of this pathway does not affect N-myc phosphorylation or stabilization in CGNPs (DHR and AMK, unpublished obs).

The activity of GSK-3β is kept in check by the phosphoinositide 3 (PI-3) kinase pathway. IGF1-mediated activation of the PI-3 kinase pathway is critical for long-term survival of cultured CGNPs,[152] and under our culture conditions, insulin is present at levels sufficient to activate the IGF receptor. We found that short-term withdrawal of insulin substantially destabilized N-myc in cultured CGNPs, and this de-stabilization could be prevented by substitution of insulin with IGF. IGF signaling is important for CNS development, and increased IGF activity results in cerebellar hyperplasia.[153,154] In addition to enhancing growth by promoting survival, IGF-mediated activation of PI-3 kinase also has positive effects on the cell cycle regulatory apparatus. PI-3 kinase negatively regulates forkhead transcription factors, which can promote cell cycle exit by repressing cyclin D.[155]

The finding that IGF-stimulated PI-3 kinase activity can stabilize N-myc through inhibition of GSK-3β provides additional insight as to the molecular mechanisms underlying the pro-proliferative effects of PI-3 kinase signaling in neuronal precursors.

Several extracellular signals have been proposed to modulate proliferative effects of Shh in CGNP cultures, including those that activate RTK, Notch, and chemokine receptors.[14,21,156] The precise intracellular mechanisms underlying such interactions are unclear. However, many of the signaling pathways implicated in cerebellar growth control have direct or indirect effects on the PI-3K pathway. For example, even though our work has ruled out a direct role for the Shh pathway in phosphorylating N-myc, it is known that several transcriptional targets of Shh include proteins which regulate IGF signaling and PI-3K activity,[39] suggesting an indirect role for Shh signaling in regulating N-myc stability. Control of N-myc expression and turnover provides an example of how regulation of a single intracellular target can integrate activities of divergent signaling pathways in the developing brain (Fig. 5).

Conclusions and Future Prospects

Our review has focused on how the Shh signaling pathway promotes entry into and progression through the G1 phase of the cell cycle in proliferating neuronal progenitor cells. In cerebellar granule cells, Shh induces expression of the proto-oncogene N-myc, which in turn activates crucial mediators of G1 progression, including cyclins D1 and D2. In keeping with this mechanism, we have proposed that cerebellar granule cell cycle exit is regulated, at least in part, by amino-terminal phosphorylation of N-myc, which promotes its destabilization. N-myc phosphorylation requires activity of GSK-3β; a kinase with several previously defined anti-proliferative and hedgehog-antagonistic roles. GSK-3β activity is negatively regulated by the PI-3K pathway, which is known to be required for cerebellar granule proliferation and survival. Together, the available data indicate that Shh and PI-3 kinase act at distinct steps of

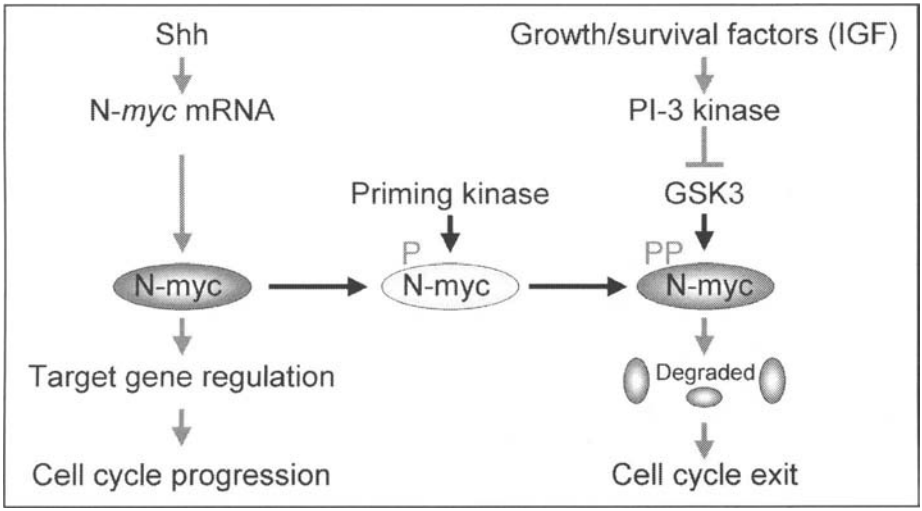

Figure 5. Integration of signaling pathways promotes proliferation during cerebellar development. Schematic illustrating how two pathways with known positive effects on neuronal precursor proliferation can cooperate by convergence on a common target. Shh signaling induces expression of the proto-oncogene transcription factor N-*myc*. N-myc is necessary and sufficient for maintaining CGNP proliferation. Maintenance of N-myc protein levels in vitro is regulated in a Shh-independent manner. We identified a role for the PI-3K pathway in stabilizing N-myc protein. In cultured CGNPs, IGFR signaling activates the PI3 kinase pathway, which inhibits GSK3-mediated phosphorylation of N-myc T50 and its subsequent degradation. The kinase priming N-myc for GSK3 phosphorylation, by acting at S54, remains to be identified. Other growth factors (e.g., SDF-1) or cell-cell interactions (e.g., via integrins) are candidates for PI-3 kinase regulation and inhibition of GSK3 activity in vivo. The combined effect of concerted Shh and PI-3 kinase activity is to promote G1 progression and precisely control the timing of cell cycle exit. A similar theme may be found in medulloblastoma tumorigenesis, as both PI-3K and hedgehog pathway activation are associated with this disease.

mRNA transcript upregulation and protein stabilization, respectively, to regulate overall activity and levels of a common target, N-myc.

Common Mechanisms in CNS Development and Tumorigenesis: Sonic Hedgehog Signaling as a Therapeutic Target in Medulloblastoma

In this review, we have emphasized many parallels between regulation of the cell cycle in developing CGNP and in human cases of medulloblastoma. We have recently taken a global approach to this question and compared many thousands of genes upregulated both in desmoplastic and classic variants of medulloblastoma to a spectrum of genes normally expressed during mouse cerebellar development, as assessed by oligonucleotide microarrays. Genomically, human medulloblastomas were closest to mouse PN 1-10 cerebella; normal human cerebella to mouse PN 30-60 cerebella. Furthermore, metastatic medulloblastomas were highly associated with mouse PN 5 cerebella, suggesting that a clinically distinct subset of tumors is identifiable by molecular similarity to a precise developmental stage. Together, our findings indicate a global recapitulation of tissue-specific developmental programs in medulloblastoma, and the utility of tumor characterization on the developmental time axis to identify novel aspects of clinical and biological behavior.[157] Although further work is required to expand on these observations, for example by testing roles for individual genes in mouse models of medulloblastoma, it is clear that important parallels between CNS development and tumorigenesis exist at the genetic and mechanistic level.

The established participation of Hedgehog signaling in medulloblastoma,[107,158] coupled with the availability of inhibitors specific for Hedgehog-Smoothened signaling[159,160] has raised the exciting possibility that such inhibitors may be useful clinically. The Hedgehog inhibitors jervine and cyclopamine were originally isolated from plants, and synthetic inhibitors of Hedgehog signaling have been developed commercially.[161] Providing preclinical data is forthcoming from mouse studies, it seems likely that such Hedgehog pathway inhibitors will soon be available for pediatric patients with medulloblastoma. Recently, the Shh pathway has been shown to be mitogenic for NSC populations and required for maintenance of the stem cell niche.[25] This data indicates that a possible adverse side effect of Hedgehog pathway blockade in children could be a depletion CNS neural stem cell pools, of particular concern for long-term effects on neurodevelopment and cognition. Such potential side effects must be weighed against the overall toxicity of currently used therapies such as chemo- and radiotherapy. In addition to general blockade of Shh signaling, it may be possible to target the downstream mediators of Hedgehog signaling with specific roles in cell cycle regulation. For example, our work suggests that inhibition of PI-3 kinase activity may be sufficient to destabilize N-myc. Further elucidation of Hedgehog mitogenic effects, including how the Shh pathway functions in context of other pathways, may provide further clues for therapeutic intervention in human tumors.[162] This issue takes on broader significance when one considers that Shh-Smo signaling is implicated in a wide variety of human cancers.[163,164]

N-myc as a Nodal Point for Interacting Pathways Regulating CGNP Proliferation

The events we have studied in CGNPs are likely to be recapitulated in human tumors of cerebellar origin. For example, N-myc is up regulated in Shh pathway-associated medulloblastomas in mice and humans. Moreover, evidence of increased activity of the PI-3K pathway is also observed in human and mouse medulloblastomas,[106,165] as well as other types of brain tumors, such as adult gliomas.[166] Such increased activity, with the consequence of inhibiting GSK-3β, may promote tumor cell growth by stabilizing N-myc and other cell cycle regulatory proteins, including D-type cyclins. These connections suggest several targets for designing specific therapies against medulloblastomas, including the Shh pathway itself, N-myc, and the PI-3K pathway.

Cell growth control is a tightly regulated process. Although much attention has been paid to the importance of the Shh pathway in regulating proliferation of neuronal precursors, it is necessary to keep in mind that Shh effects do not occur in a vacuum, and that proliferation, cell cycle exit, and cell fate outcomes result from finely tuned responses to Shh interactions with other signaling pathways operating in these cells. A major area of future exploration will be devoted to identifying nodes of cooperative or antagonistic interactions of the Shh signaling pathway with other major regulators of proliferation and brain development.

N-myc itself exemplifies such a node. We have already described how the PI-3 kinase pathway cooperates with Shh to promote N-myc-mediated proliferation, through stabilizing effects on N-myc's phosphorylation state. However, such stabilization may also be achieved through the action of phosphatases, removing the phosphate groups at T50 and S54, which direct N-myc's degradation. Although phosphatases targeting N-myc have not been identified, PP2A has recently been implicated in regulation of c-myc stability.[167] Microarray studies have identified SET1, an endogenous PP2A inhibitor, as a target of Shh in proliferating cerebellar granule cells.[168] By regulating the expression of phosphatases and their inhibitors, Shh could indirectly promote continued proliferation, or timely cell cycle exit, facilitated by N-myc stabilization or degradation.

Phosphatases such as PP1 and PP2A are highly expressed in the developing and mature brain, as are endogenous inhibitors of their activity.[169,170] Phosphatase access to their substrates may be affected by the substrate's intracellular localization, conformation, or interactions with other proteins. The prolyl isomerase Pin1 is an example of a protein that could prevent or promote phosphatase access to N-myc. Pin1 is widely expressed, and has been implicated in

positively regulating activity of proteins important in neuronal precursors,[171] such as tau and β-catenin. Pin1 binds to phosphorylated serine or threonine residues, which are followed by a proline residue. Pin1 affects isomerization around the proline, which has been shown to affect the localization, de-phosphorylation, and activity of several cell cycle regulatory proteins, including cyclin D1.[172] A recent study has also suggested a role for Pin1 in regulating myc stability, though these results have yet to be validated using in vivo models and endogenously expressed protein.[167] However, Pin1 over-expression is implicated in many human cancers, and it is generally understood to have a pro-proliferative role. Interestingly, Pin1 is inactivated by PKA,[173] a classic hedgehog pathway antagonist. In vivo, such inactivation and disruption of Pin1:N-myc interaction could promote cell cycle exit in Shh-stimulated cerebellar precursors.

As a Shh-induced target, N-myc may also play a role in the timing of CGNP differentiation. The ability of BMP signaling to promote neuronal differentiation has been well-established. Recently, Angley et al have demonstrated an important role for BMP4 in the post-natal differentiation of CGNPs.[11] The timing of BMP pathway effector (Smad proteins[174]) expression overlaps with the phase of Shh-induced CGNP proliferation in vivo. Shh and BMP are also known to oppose eachother in regulating cell cycle progression in forebrain embryonic neural progenitors.[13] Smads cooperate with the transcription factor Miz1, which induces expression of cdk inhibitors, thereby promoting cell cycle exit.[175] Interaction of Miz1 with myc results in repression of these genes. We have observed Miz1 expression in cultured CGNPs (AM Kenney and D Rowitch, unpublished observations. Whether Miz interacts with N-myc has yet to be determined, but given the highly conserved nature of myc proteins, it is possible that N-myc's anti-differentiation properties could arise in part from its ability to titrate levels of Miz1 and repress cki expression, until the strength of BMP-mediated Miz1 activation dominates, thereby permitting CGNP growth arrest. It is also worth noting that BMP signaling can promote stabilization of PTEN,[176] a negative regulator of PI-3 kinase activity, suggesting that BMP signaling could antagonize Shh-mediated proliferation by promoting N-myc destabilization through increased GSK-3β activity.

Whether activation of differentiation-promoting pathways such as BMP signaling can be used as an anti-tumor therapy remains to be seen, but it is intriguing to speculate that such strategies could be employed to prevent tumor expansion or promote apoptosis in brain tumors. What is clear from many studies in recent years is that developmental biology provides a vehicle to both ask and answer provocative questions about CNS tumorigenesis and anti-tumor therapy. An exciting possibility is that other CNS cancers, such as glial tumors, will show parallels to their counterpart glial precursors in the developing brain, analogous to the parallels we have discussed here between cerebellar precursors and medulloblastomas.

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CHAPTER 16

Modulating the Hedgehog Pathway in Diseases

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Introduction

The hedgehog (Hh) pathway is a signaling system that regulates a wide range of develop mental processes.[1] Hh proteins act as morphogens to induce cell differentiation in a dose dependent manner, control cell proliferation, or alter cell shape. Aberrant regulation of the Hh signal, caused by mutation in components of the pathway, has been shown to lead to various developmental disorders[2] or more recently to cancer.[3,4] Most components of the Hh pathway were first identified in flies and later shown to be conserved in higher organisms. Although still incomplete, a picture of how the hedgehog signal is transmitted at the cellular level has started to emerge, allowing for potential therapeutic approaches to the treatment of diseases involving this pathway.

Hedgehog proteins undergo autoproteolytic cleavage to generate an NH2-terminal fragment that is cholesterol modified at its COOH-terminus and palmitoylated at its NH2-terminus.[5] Secretion and diffusion of this dually lipid modified protein requires the activity of Dispatched,[6] a multi-transmembrane protein with homology to Patched (see below), and heparan sulfate proteoglycans,[7] a major component of extracellular matrix.

Reception of the signal transmitted by Hh or its three mammalian orthologs, sonic hedgehog (Shh), desert hedgehog (Dhh) and Indian hedgehog (Ihh), is mediated by two multitransmembrane cell surface proteins, Patched (Ptch) and Smoothened (Smo). Ptch is a twelve transmembrane domain protein with features similar to those of a transporter, which, in the absence of ligand, represses the activity of Smo, a seven-transmembrane protein. Upon binding of Hh to Ptch, the inhibition of Smo is relieved and the Hh signal is transmitted via a protein complex that includes a number of components thought to be specific to the pathway: Costal2 (cos2), an atypical kinesin-like protein, Fused, a serine-threonine protein kinase, Suppressor of Fused (SuFu) and the zinc-fingers transcription factor cubitus interruptus (Ci) or Gli1,2 and 3 in mammals. Additional proteins, including PKA, GSK3β and CK1α also play a critical role in regulating the activity and nuclear localization of Glis (for review see ref. 8).

Germline mutations in the *PTCH1* gene lead to a condition known as basal cell nevus syndrome (BCNS or Gorlin syndrome) where patients develop large numbers of basal cell carcinomas (BCC) on their skin and suffer higher incidence of other tumor types such as medulloblastoma.[9,10] Most sporadic BCC tumors have high Hh pathway activity,[11] often as a result of mutation in *PTCH* followed by loss of heterozygosity. Approximately 10% of sporadic BCCs do not have a *PTCH1* mutation, but harbor a specific heterozygous mutation in *SMO*,[12] which makes SMO less sensitive to PTCH1 mediated inhibition. Rare potential gain of

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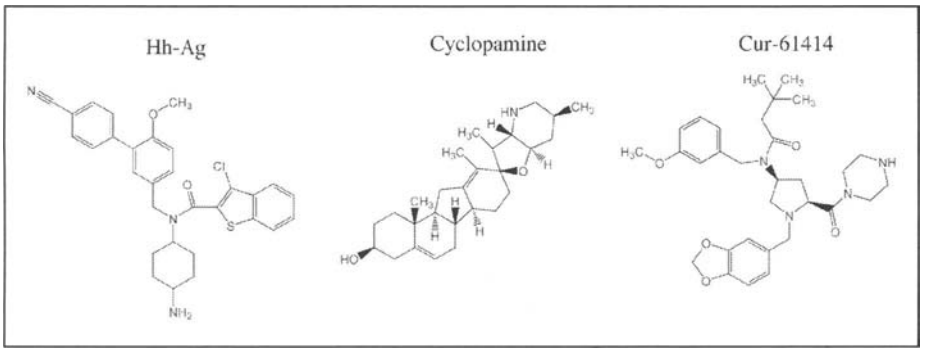

Figure 1. Small molecules modulator of the Hh pathway.

function mutations in *SHH* itself have been described but it has yet to be demonstrated how they could contribute to increased Hh activity and tumorigenesis.[13] Mutations in downstream signaling components of the pathway may also contribute to increased Hh signaling activity and tumor formation. However, to date only a few instances have been documented. *GLI1* was initially discovered as an amplified oncogene in human glioma[14] but this appears to be a rare event. More recently, mutations in *SUFU*, a negative regulator of the pathway involved in tethering Glis in the cytoplasm, were identified in some cases of familial medulloblastomas[15] suggesting that SUFU may function as a tumor suppressor.

In addition to some tumors bearing mutations in SHH or downstream Hh pathway components, other tumor cells may themselves synthesize high levels of Hh ligand and rely on its autocrine or paracrine activities for growth or survival. Small cell lung carcinomas, as well as tumors derived from the upper gastrointestinal tract, particularly pancreatic tumors were shown to require increased Hh signaling mostly through ligand stimulation.[16-18] Indeed, these tumors were shown in various in vitro and in vivo experiments to be sensitive to inhibition of Hh ligand by a neutralizing monoclonal antibody or by cyclopamine (Fig. 1), a steroidal alkaloid which binds to SMO and specifically inhibits the Hh pathway.[19] Together, these observations indicate that agents capable of inhibiting the Hh pathway at the level of Hh itself, or preferably downstream of PTCH, at the level of SMO could have an application in the treatment of Hh-dependent cancers.

Identification of Small Molecule Hedgehog (Hh) Antagonists

The observation that steroidal alkaloids such as cyclopamine and jervine inhibit Hh signaling suggested that it should be possible to identify additional small molecules that modulate the Hh pathway. To identify such small molecules, approximately 200,000 compounds were screened in a cell-based assay for activation (see below) or inhibition of Hh activity.[20] In this assay, a stable mouse 10T1/2 cell line expressing luciferase under the control of a Gli-1 DNA binding sequence was employed. Hh signaling was initiated by the addition of recombinant Hh protein, test compounds were added at the same time and luciferase activity was quantified after an overnight incubation. Numerous hits were identified, but since the goal of the screen was to identify potential therapeutics, and not simply useful reagents to probe the Hh pathway, they were prioritized by their drug-like nature. Even with this added filter, several quite potent chemical classes of antagonists were identified.

The first objective of the Hh antagonist program was to derive a topical treatment for BCC. One compound class, represented by the aminoproline Cur-61414 (Fig. 1), was highly soluble and seemed likely to have sufficient permeability to penetrate well into the skin lesions. This antagonist does not inhibit frizzled,[20] the Wnt receptor that shows the greatest sequence similarity to smoothened. In other studies, Cur-61414 was found not to compete in assays that

measure the binding of numerous other ligands to their cognate G-protein coupled receptor. Therefore, Cur-61414 appears to be a specific antagonist of the Hh pathway.

Cur-61414 has been tested in a variety of standard in vitro activities to confirm its ability to inhibit Hh signaling but, most cogently, has been tested in two cell culture models of BCC. In the first model, Williams et al recapitulated a previously described transgenic mouse model of BCC where Shh was overexpressed in basal keratinocytes[21] by exposing embryonic mouse skin punches to recombinant Shh. In this model, Cur-61414 was able to prevent the formation of BCC-like lesions produced after several days of constitutive activation of Hh signaling. Cur-61414 also cause the disappearance of preformed lesions via decreased proliferation and increased apoptosis.[22] Importantly, both effects were restricted to cells in which the Hh pathway was hyper-activated, leaving surrounding normal skin unaffected.

Similar effects were observed using another BCC model. Aszterbaum et al showed that long-term irradiation of *Ptch* heterozygous mice (a model of Gorlin syndrome) resulted in BCC-like skin lesions.[23] These mice were UV-irradiated for more than 6 months and lesion-bearing skin punches were placed in culture. Again treatment of skin explants from the mice with Cur-61414 produced a selective induction of apoptosis in the lesions without obvious effects on adjacent skin.

Using the screening procedure described above, an additional class of potent small molecule Hh antagonist intended to be used systemically, was identified.[20] In this case, it was important that these antagonists be sufficiently potent and preferably orally available. The compounds were also optimized for plasma half-life and other properties associated with effective tumor therapy and have now been shown to be effective in inhibiting growth and inducing apoptosis of medulloblastoma cells in vitro and in vivo.[24]

Identification of Hh Small Molecule Hh Agonists

The best-characterized function of the Hh pathway is to establish the number and type of cells in the brain and spinal cord during embryonic development.[25-27] For example, spinal cord motor neurons and midbrain dopaminergic neurons both owe their existence to the activity of Shh. A small number of experiments have confirmed that this pathway continues to have the potential to contribute to the regulation of cell number and cell-type in the adult nervous system. For example, Lai et al have shown that over-expression of Shh in the adult rat brain continues to stimulate stem cell proliferation and neural differentiation.[28] Other studies concluded that, when injected into the striatum, Shh protein could enhance the function of dopaminergic neurons in models of Parkinson's disease.[29]

These data raised the possibility that activators of the Hh pathway might be of therapeutic value in treating disorders of the nervous system by promoting cell survival, maintaining neural differentiation or by accelerating the appearance of new neurons after neuronal damage. Since viable central nervous system (CNS) therapeutics are most likely to be small molecules, a reporter gene screens similar to the one used to identify small molecule Hh antagonists was employed to identify potential agonists of the Hh pathway. One of the weak hits identified in the screen was subjected to extensive medicinal chemistry, culminating in the synthesis of sub-nM EC_{50} compounds with good oral availability and CNS penetration.[20] Interestingly, even weak agonists could stimulate proliferation of cerebellar granule neural progenitors and induce differentiation of ventral neural cell-types in spinal cord explants, both known Hh-mediated activities.[20] Additionally, it was demonstrated that retinoic acid-pretreated mouse ES cells could be stimulated to differentiate into motor neurons with either recombinant Shh or with the small molecule Hh agonists.[30]

Continued chemical modification of the Hh-Ag class led to the synthesis of compounds that were more active, on a molar basis, than the normal Hh protein ligands. Understanding the Hh-Ag mechanism of action therefore became quite important. Genetic epistasis studies suggested that Hh-Ag targeted a component of the Hh signaling pathway at or near the level of Smo. Direct cross-linking and, eventually, binding experiments using radiolabeled Hh-Ag

confirmed that it bound to Smo, confirming the hypothesis that Smo is a readily druggable component of the Hh signaling pathway.

Once orally available agonists able to readily cross the blood-brain barrier were identified, they were tested in various CNS disease models. Surprisingly, they have proven to be effective in decreasing the amount of neuronal cell death in different excitotoxicity models, including stroke. Given 6 hours after middle cerebral artery occlusion (a standard stroke model), the Hh agonists are able to markedly decrease the size of the infarct that result from the ischemia and subsequent reperfusion. The mechanism of action underlying this neuroprotective effect is not entirely clear at this point.[31]

When given orally, even in a single dose, the agonists stimulate proliferation of neural stem cells in the hippocampus and subventricular zone.[32] That is, the agonists offer the potential ability to stimulate the appearance of new neurons in the adult nervous system. In the context of a stroke-damaged brain, this could be of significant therapeutic consequence. Thus, small molecule Hh agonists offer the potential of being both neuroprotective and neuroregenerative.

Conclusion

Recent progress in the understanding of the Hedgehog pathway and the development of molecules that modulate its activity opens new avenues for the treatment of a number of devastating diseases. Inhibitors of the pathway, small molecules or antibodies may prove useful for highly aggressive cancers such as pancreatic tumors or medulloblastoma, the most common childhood brain tumor. On the other hand, small molecule agonists of the pathway provide new therapeutic options for the treatment of neurodegenerative disorders such as Parkinson's disease.

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CHAPTER 17

Hedgehog Signaling in Endodermally Derived Tumors

Marina Pasca di Magliano and Matthias Hebrok*

The Hedgehog Signaling Pathway

A relatively small number of intercellular signaling pathways, including the Hedgehog (Hh), transforming growth factor β (TGF-β), fibroblast growth factor (FGF), Wnt, and Notch pathways, interact to regulate embryonic development and organogenesis. In contrast to the other pathways, the mammalian Hedgehog signaling pathway is relatively compact, comprised of only three known ligands, Sonic (Shh), Indian (Ihh), and Desert Hedgehog (Dhh), and two receptors, patched 1 and 2 (Ptch1/2; (Fig. 1).[23] In the absence of the ligands, Patched (Ptch) inhibits the activity of a second transmembrane protein, Smoothened (Smo). As a consequence, transcription factors of the Gli family remain inactive in the cytoplasm through interaction with a protein complex that includes Suppressor of fused su(fu).[44] Upon ligand stimulation, Ptch mediated inhibition of Smo is alleviated and a cascade of events that is not fully understood results in the activation and translocation of Gli transcription factors into the nucleus where they activate the transcription of target genes. Some of these are components of the Hedgehog pathway itself, including Gli, Ptch and the Hedgehog interacting protein (Hhip). Both Ptch and Hhip are membrane proteins that attenuate the pathway by binding to and blocking diffusion of Hedgehog ligands.[8,10,15,16] Therefore, the Hedgehog signaling pathway regulates itself through a negative feedback mechanism that results in increased expression of Ptch and Hhip in Hedgehog responsive cells.

Hedgehog Signaling in Adult Endodermal Organs

The importance of a functional Hedgehog signaling pathway during embryogenesis becomes apparent in transgenic mice lacking *Smo*, a component of the pathway through which all signaling appears to be mediated. Only one *Smo* gene has been identified in vertebrates and homozygous mutant embryos die shortly after gastrulation.[55] Similarly, loss or reduction of Hedgehog activity has been implicated with developmental defects in a variety of different organs (see ref. 23). Here, we will focus on the role of the Hedgehog pathway during the formation of endodermal organs. While all three Hedgehog ligands are expressed in endodermal cells during embryogenesis,[5] no apparent defects in endodermal tissues have been noted in mice carrying homozygous mutations in *Dhh*.[6] By contrast, mice that lack either *Sonic* or *Indian Hedgehog* display numerous defects along the gastrointestinal tract[20,42] as well as loss of the branching morphogenesis in the lung.[29,39] Increasing evidence suggests that Hedgehog signaling functions in a concentration-dependent manner and different levels of the pathway

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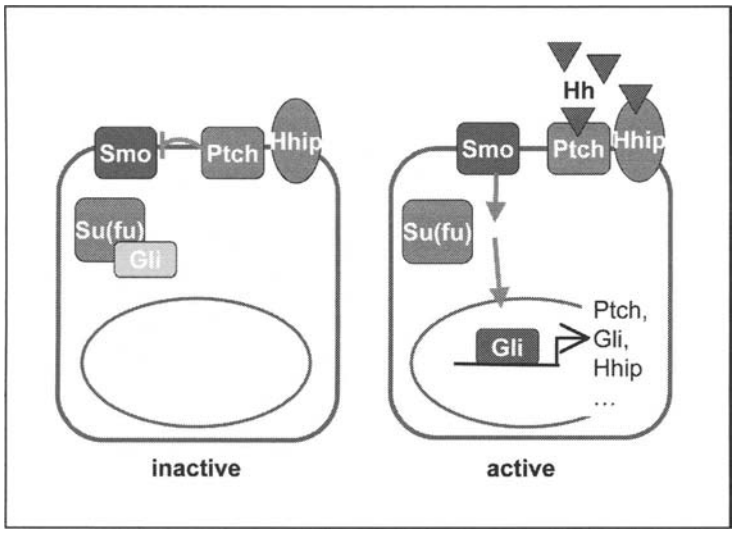

Figure 1. Hedgehog (Hh) signaling pathway. In the absence of ligands, Patched (Ptch) suppresses the activity of Smoothened (Smo), another transmembrane protein that is essential for Hedgehog signal transduction. As a consequence, Gli transcription factors, the downstream mediators of the Hedgehog pathway, are kept inactive within a cytoplamic complex that includes suppressor of fused (Su(fu)). Upon ligand binding, Ptch mediated inhibition of Smo is alleviated and Smo activation results in signals that lead to Gli translocation into the nucleus. Here, Gli factors activate target gene transcription, including *Ptch*, *Hhip* and *Gli* itself. Both Ptch and Hhip have been shown to attenuate Hedgehog signaling via sequestering of Hedgehog ligands (Hh), including Sonic, Indian, and Desert Hedgehog.

have been implicated in generating molecular boundaries required for proper organogenesis.[18] In the foremidgut area, both *Shh* and *Ihh* are expressed in forming stomach and duodenal tissues while the endodermal cells developing into pancreatic structures are devoid of both ligands at early stages.[2,5,19] Ectopic activation of Hedgehog signaling via tissue manipulation and forced expression of *Shh* under control of the *Pancreatic and duodenal homeobox gene 1* (*Pdx-1*) promoter results in loss of pancreatic marker genes and transformation of pancreatic mesenchyme into duodenal mesoderm.[2,19] Interestingly, stepwise increase in Hedgehog activity in transgenic mice carrying mutations in *Hhip* or *Hhip* and *Ptch* alleles, both inhibitors of Hedgehog signaling, result in increasingly severe phenotypes in lung and pancreas,[9,24] indicating that Hedgehog pathway activity has to be tightly controlled to allow proper organ formation.

While a lot of progress has been made in understanding which embryonic tissues respond to Hedgehog signaling, much less is known about the role of hedgehog signaling in adult tissues. A number of recent studies have shown that the pathway remains active in a subset of adult organs, mostly confined to a subset of cells within each organ. At least in some organs, the activity of the pathway in mature tissues appears to be lower than what has been shown in embryonic tissues (Fig. 2), thus posing a potential problem in identifying cells that receive and respond to Hedgehog ligands. However, previous reports had shown that Ptch, the receptor and antagonist of the pathway, is also a transcriptional target and thus a faithful and quantitative marker of Hedgehog-responsive cells. Most studies have used transgenic mice in which the bacterial *LacZ* gene has been used to replace the *Ptch* gene.[17] While the homozygous *Ptch* knock-out is early embryonic lethal, the heterozygous mice survive and staining for galactosidase activity allows identification of cells with active Hedgehog signaling. Interestingly, the Hedgehog pathway is down-regulated in endodermal organs and only few cells are marked by

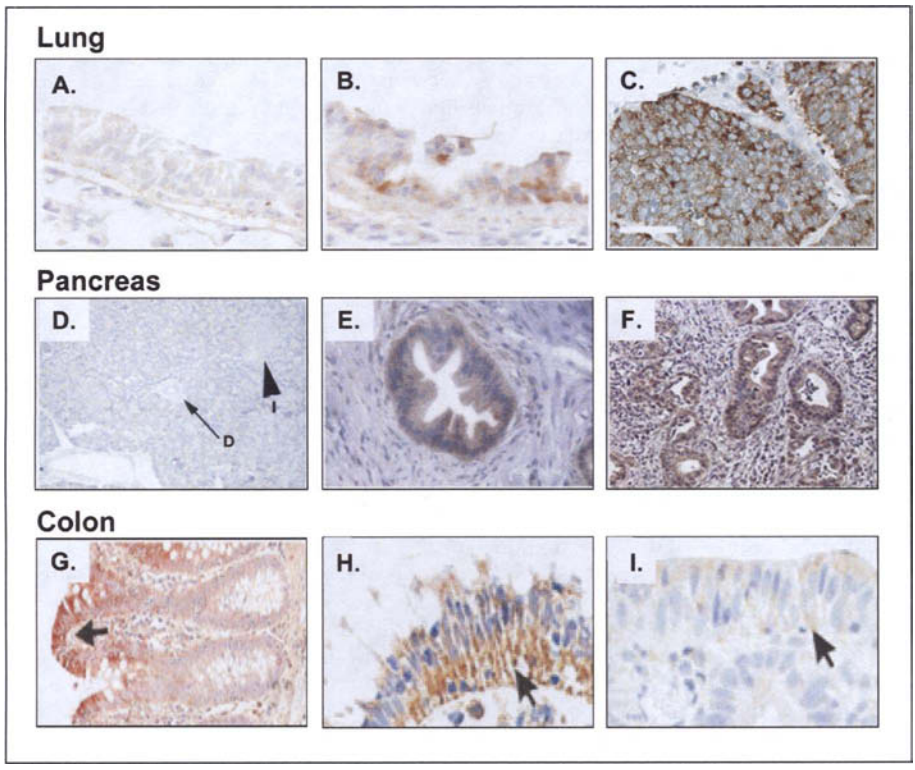

Figure 2. Hedgehog signaling in adult endodermal organs. A-C) Lung. A) Shh expression is undetectable in normal lung tissue. B) Upon tissue damage induced by naphtalene treatment a subset of cells becomes Shh positive. C) In contrast to normal tissue, small cell lung cancers (SCLC) are marked by significant expression of Shh. D-F) Pancreas. D) Similar to lung, expression of Shh is undetectable by immunohistochemistry in normal human pancreas. E) Pancreatic intraepithelial lesions are marked by a noticeable increase in Shh expression levels and the majority of pancreatic adenocarcinoma show high levels of Shh expression (F). G-I) Colon. G,H) By contrast to lung and pancreas, expression of Ihh is maintained in adult colon where it is confined to differentiated colonocytes. I) Colonic cancer have lost Ihh expression, suggesting that Hedgehog signaling in colon tissue might have anti-oncogenic functions. The arrow marks Ihh-positive, differentiated colonocytes in G,H, and colonocytes in colon adenoma that have down-regulated Ihh expression in I. Figures 2 a-c were originally published in Watkins et al, Nature 2003; 422:313-317; Figures 2 d-f were originally published in Thayer at al, Nature 2003; 425:851-856; Figures 2 g-i were originally published in van den Brink at al, Nature Genetics 2004; 36:277-282. All the figures were reproduced with permission from the Nature Publishing Group.

β-galactosidase activity. In the adult lung, *Ptch* is expressed in small clusters of cells, believed to constitute the neuroendocrine precursors within the airway epithelium, in the basal layer of the bronchial epithelium.[53] Neither *Shh* nor *Gli* are detectable in the adult lung. In the stomach and gall bladder some *Ptch* positive cells are found in the epithelium.[4] *Ptch* expression is observed at very low levels in the murine pancreatic ducts and the endocrine cells of the islet of Langerhans.[20,50] However, immunohistochemistry is not sensitive enough to detect Ptch, Shh, and Smo in human pancreatic tissues.[49]

An exciting possibility is that Hedgehog signaling marks a population of progenitor cells in several endodermal organs. While this hypothesis awaits confirmation from cell isolation and transplantation studies, publications analyzing other adult organs, mainly the brain, have noted

that Hedgehog signaling promotes cell proliferation of progenitor cells to maintain tissue homoeostasis.[28] Intriguingly, tissue damage seems to activate Hedgehog signaling in these cells, suggesting that the level of pathway activation directs the proliferative response in these cells. In the brain, Shh controls proliferation of cerebellum granule neuron precursors[11,52] as well as the proliferation of progenitors of the tectum and neocortex.[12] Moreover, Hh signaling in the postnatal telencephalon is required to promote proliferation and maintain the populations of progenitor cells.[30,36]

Hedgehog Signaling in Cancers of Endodermal Origin

Lung

The normal role of Hedgehog signaling in subsets of cells in mature tissues is a matter of intense research and could lead to important insights into how embryonic signaling pathways are reemployed to maintain tissue homeostasis. Growing evidence suggests that deregulated Hedgehog signaling within adult tissues can initiate or support tumor formation in mice and humans.[43] In general, increased pathway activity, either through sporadic mutation (Smo, Su(fu)) or in familial conditions (Gorlin's syndrome, mutation in *Ptch*), has been shown to lead to tumor formation in the skin, the cerebellum and skeletal muscle.[22,47,54] More recent publications have addressed the role of Hedgehog signaling in tumors of endodermally derived organs, such as lungs and organs in the gastrointestinal tract.

Lung cancer, the leading cause of cancer death world wide,[25] is classified as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) based on the histological appearance of the cancer cells. Small cell lung cancer is a highly lethal malignancy believed to derive from the Hedgehog-positive neuroendocrine cells, a specialized progenitor population located within the airway epithelium (Fig. 2).[53] In normal airway epithelium, small clusters of Ptch positive cells are observed that do not stain for Shh or Gli expression. Following lung damage, a process during which specialized cells of the lung epithelium (Clara cells) are depleted, the expression of both Shh and Gli is upregulated and putative airway progenitor cells start to proliferate.[53] In SCLC, Hedgehog signaling molecules can be detected, both in primary human tumors and in tumor-derived cell lines. Furthermore, SCLC cells secrete Shh ligand and inhibition of ligand-receptor binding with anti-Shh blocking antibodies inhibits pathway activation, suggesting that Hedgehog signaling regulates its activity via an autocrine loop in these cells.[53] In addition, SCLCs are susceptible to treatment with cyclopamine, an alkaloid derived from a plant of the lily family (Veratrum californicum) that physically binds to Smoothened and inhibits Hedgehog signal transduction at this level.[7,46] Cyclopamine-mediated inhibition results in reduction of cell survival and prevents tumor growth. Thus, the observation that cyclopamine treatment blocks the growth of the cancer cells opens up the possibility to develop new therapeutic approaches that rely on inhibition of the Hh pathway. However, it is important to note that Hedgehog signaling is usually not active in non-small cell lung cancer (NSCLC). Thus, it is likely that only distinct cell types within the adult lung, and most likely other organs, carry the ability to respond to upregulation of Hedghog ligand expression with increased proliferation while other cells types remain quiescent. This also suggests that the growing number of Hedgehog-antagonists will only block tumor progression in a subset of cancer types (Fig. 3).

Hedgehog Signaling in Gastrointestinal Tumors of the Pancreas and Colon

Recently, activation of the hedgehog pathway has been associated with several tumors of gastrointestinal origin.[4,49] Tumors originating from oesophagus, stomach, biliary tract, and pancreas show increased Hedgehog activity. Most tumor cell lines derived from tumors of these tissues express both Shh and Ihh Hedgehog ligands. Similar to the situation found in SCLCs, cell proliferation can be inhibited using either anti-Shh/anti-Ihh blocking antibodies or cyclopamine while purified ligands stimulate cell replication. It is noteworthy that in contrast to the other gastrointestinal tumors, colon tumors are not marked by increased Hedgehog

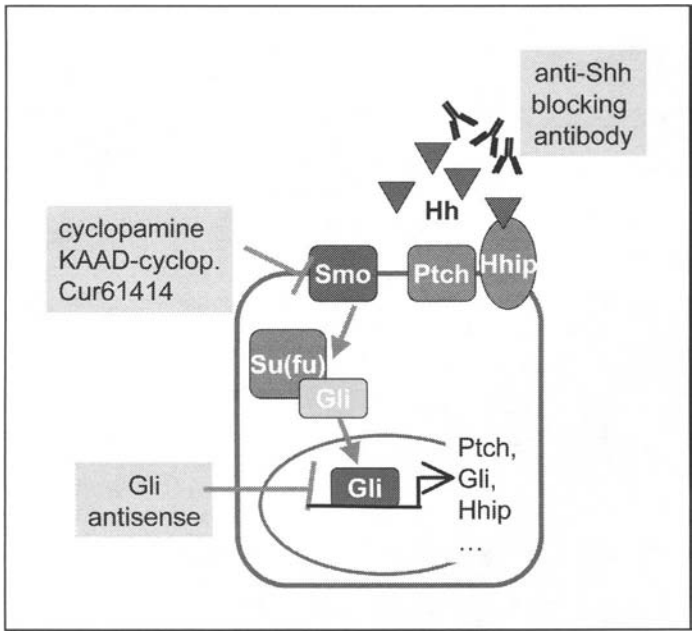

Figure 3. Hedgehog signaling inhibitors. An increasing number of Hedgehog inhibitors has been identified that block Hedgehog activation at different levels.[38] Inhibition of Hedgehog signal transduction could result in novel therapeutic treatments for endoderm-derived tumors.

signaling. Below, we will discuss the different mechanisms by which Hedgehog signaling might influence cancer formation and growth by comparing its role in pancreatic adenocarcinoma and colon tumors.

Pancreas

Pancreatic adenocarcinoma is a devastating disease for which no effective therapy is currently available.[3] The origin of the progenitor cell that forms this cancer is still controversial, however, the epithelial nature and expression of duct markers suggests that tumors might derive from pancreatic duct cells. Histological analysis of a large number of tumor patients has resulted in a progression model of precancerous lesions, known as pancreatic intraepithelial neoplasia (PanIN 1-3).[21] During progression from normal duct epithelium to carcinoma in situ, pancreatic duct cells first loose their normal cuboidal morphology and cell polarity, accumulate mucin, and finally evaginate from the epithelium to metastasize into the adjacent organs. The different PanIN lesions are also characterized by the accumulation of genetic mutations in tumor suppressor genes, including K-ras, DPC4, and p16.[3] Immunohistochemical analysis has shown that Hedgehog signaling components are overexpressed in a significant percentage of PanIN 1 lesions, suggesting that deregulation of the pathway is one of the early steps during pancreatic cancer formation.[49] Additional evidence for increased Hedgehog signaling as an initiating event comes from studies of transgenic mice that express *Shh* under control of the *Pdx-1* promoter. Pancreas formation in these mice is severely compromised and transgenic animals usually die within the first weeks after birth.[2] Nonetheless, forced Hedgehog activation results in morphological changes reminiscent of human PanIN lesions.[49] Analysis of a large series of pancreatic cancer cells lines derived either from primary or metastatic adenocarcinomas shows that all lines express components of the Hedgehog signaling pathway, indicating that the pathway is still active at later stages of tumor formation. Inhibition of the

pathway with cyclopamine blocks proliferation and induces apoptosis in a subset of the cancer cell lines, demonstrating an essential role for Hedgehog signaling during tumor growth and survival.

While cyclopamine is extremely effective in blocking proliferation of a subset of pancreatic cancer cell lines, about half of the cell lines do not respond to this treatment. It is currently unclear whether the cyclopamine resistant lines do not depend on Hedgehog activity for their growth or whether mutations within the pathway downstream of Smoothened render them insensitive to this drug. In different tumors, such as medulloblastoma and basal cell carcinoma, *Ptch* is often mutated or deleted, leading to constitutive activation of Smoothened.[22] Moreover, gain-of-function mutations of Smoothened have been identified.[54] In some cases, mutations in suppressor of fused, another negative regulator of the pathway, have been found in medulloblastoma.[47] So far, gastrointestinal cancer, including pancreatic adenocarcinoma, have not been analyzed for mutations in Hedgehog signaling components and it is thus unclear whether such mutations are implicated in familial cases of these tumors. It is interesting to note that all GI derived tumors and cell lines analyzed were found to express, and depend on the continuous presence of, Hedgehog ligands. The possibility that these tumors ensure their growth and survival via an autocrine signaling loop might provide clues for therapeutic interventions.

Colon

As a general rule, ectopic activation rather than silencing of Hedgehog signaling has been implicated in tumor formation.[38,43] One possible exception is colon cancer in which Hedgehog signaling might play a protective role by counteracting the activity of another embryonic signaling pathway, the Wnt pathway.[51]

In normal colon tissue, progenitor cells are located at the base of a crypt structure. These cells proliferate to self renew and to give rise to cells that undergo terminal differentiation, a process that depends on the presence of Indian Hedgehog. Thus, in contrast to other gastrointestinal tissues, Hedgehog signaling does not induce cell proliferation but cell differentiation. van den Brink and colleagues have recently shown that Ihh functions at least in part by repressing the activity of the Wnt pathway known to be deregulated in colon cancer cells.[51] Similarly, Wnt signaling represses Ihh expression, thereby establishing a delicate balance between progenitor cells higher in Wnt activity and differentiated cells higher in Hedgehog activity (Fig. 4). The importance of this delicate balance is confirmed by the opposite phenotypes of two knock-out mice: in $Tcf4^{-/-}$ mice, which lack active Wnt signaling in the colon, the progenitor cell compartment gets rapidly depleted after birth, as all the cells in the colon initiate terminal differentiation.[27] By contrast, *Ihh* knock-out embryos are characterized by actively proliferating cells that fail to differentiate into mature colonocytes.[51] While this scenario provides an elegant explanation of how mutual inhibiton of the Wnt and Hedgehog pathways allows simultaneous maintenance of undifferentiated, progenitor cells and differentiated colonocytes other studies have noted expression of both *Ihh* and *Shh* transcripts at the base of the developing crypts during embryogenesis.[42] Future studies will have to address whether adult crypt cells produce active Ihh and Shh protein and whether Hedgehog signaling provides different clues for developing versus adult colon tissue.

Emerging evidence suggests that the balance between Wnt and Hedgehog signaling is also implicated in formation of colon cancer. Colon cancer cell lines are negative for Hedgehog signaling activity and Ihh expression is lost in colon polyps and tumors.[4,51] Numerous studies have shown that increase in Wnt signaling, marked by nuclear β-catenin, is sufficient to cause colon cancer. As expected from the mutual inhibition of Hedgehog and Wnt signaling observed in normal colon tissue, the cells that have lost Ihh expression show staining of beta-catenin within the nucleus. Thus, it is possible that Ihh reduction, normally required to ensure proper differentiation of colonocytes through inhibition of overt Wnt signaling, would allow uncontrolled proliferation of crypt progenitor cells. According to this hypothesis, Hedgehog signaling in colon cancer would work as a tumor suppressor rather than an oncogenic pathway (Fig. 4). While intriguing, further research is needed to address some of the unresolved issues. For

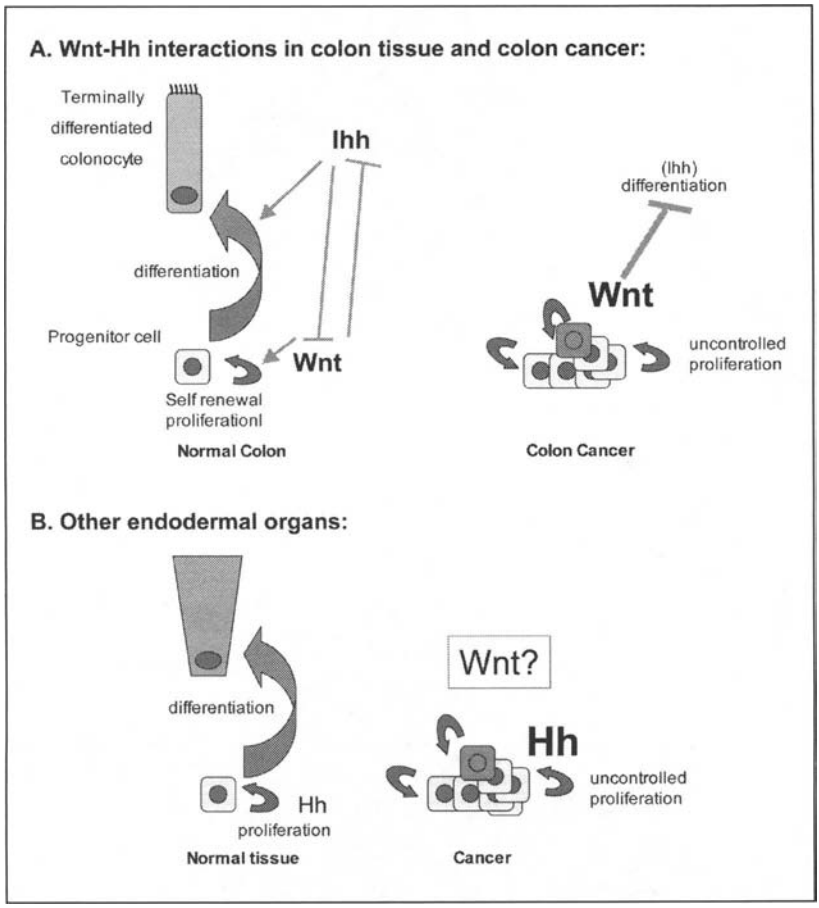

Figure 4. Hedgehog/Wnt interactions in gastrointestinal organs. A) Recent studies suggest that Wnt and Hedgehog signaling antagonize each other in colon to maintain homeostasis of undifferentiated progenitor cells (Wnt positive) and differentiated cell types (Hedgehog positive). A change in this balance, e.g., through activating mutations in the Wnt pathway, results in reduction of Ihh expression and subsequently an increase in progenitor cell proliferation that can lead to colon cancer. B) By contrast, increased Hedgehog signaling has been implicated in tumor formation in other endodermal organs, including esophagus, lung, stomach, and pancreas. Thus, the normal role of Hedgehog signaling in these organs might be to maintain proliferative capacities of a subset of cells. The role of Wnt signaling in these organs and the interaction between the Hedgehog and Wnt pathway have not been elucidated.

example, it would be interesting to determine whether colon cancer cells carry mutations in Hedgehog signaling components that reduce the activity of the pathway. Also, a subset of colon cancer cell lines do express *Ptch* and *Gli*, transcriptional targets of Hedgehog signaling[41] and it will be important to analyze whether Wnt and Hedgehog signaling behave in antagonistic fashion in all colon cancers. Furthermore, it might be important to understand whether cell proliferation and differentiation in other organs, and therefore ultimately tumor formation, is also regulated via this inhibitory feedback between the Wnt and Hedgehog pathways (Fig. 4).

Hedgehog Interactions with Other Pathways

During embryogenesis Hedgehog and Wnt signaling have been shown to activate each other's activity in various organs and recent evidence suggests that similar interactions also occur in adult tissues and tumors.[37,40,45] An example for positive interactions between these pathways comes from studies of cerebellum-derived medulloblastoma. Medulloblastoma are often caused by misregulation of the Hh pathway and are also marked by active Wnt signaling. Similarly, nuclear beta-catenin has been detected in several cases of basal cell carcinomas, another tumor known to be caused by ectopic activation of the Hedgehog pathway.[48] Additional evidence comes from the observation that several Wnt pathway components are upregulated when BCC-like skin tumors are induced in late xenopus tadpoles by expression of human Gli1.[33] Thus, the reinforcing and antagonizing regulation of the Wnt and Hedgehog in different tumors suggest that the nature of these interactions is tissue dependent.

Previous studies have noted the similarities between the Wnt and Hedgehog pathways.[35] In both pathways, the ligands are lipid modified and Smoothened and Frizzleds, the Wnt receptors, are related proteins. Furthermore, intracellular signaling components, e.g., glycogen synthase kinase 3 (GSK-3), appear to be shared by both pathways.[14] While the exact mechanism that regulates the interaction between the two pathways is as yet unknown, one intriguing possibility involves the function of one component of the Hedgehog pathway. Suppressor of fused (Su(fu)), an inhibitory component of the Hh signaling pathway, is known to bind Gli transcription factors and prevent their activation.[13,26,34] More recently, Su(fu) has also been shown to bind beta-catenin, and prevent it from entering the nucleus.[31] Translocation of beta-catenin to the nucleus is essential for Wnt activation, as it leads to the formation of a β-catenin-TCF transcription factor complex that activates transcription of the Wnt target genes. Thus, Su(fu) might act as a negative regulator of both the Wnt and the Hh pathway. Interestingly, a mutation of Su(fu),which is not able to bind beta-catenin, has been described in medulloblastoma,[47,48] thereby providing a possible link between the activation of both pathways in this type of cancer. Evidence for a positive interaction between both pathways in endodermal organ-derived tumors is currently missing and future studies might address this question.

Both the Notch and the Hh pathway play an important role in pancreatic cancer.[4,32,49] While direct interactions between these pathways in pancreatic tumors have not been reported, circumstantial evidence suggests a link between both pathways. Within pancreatic tissue, EGF receptor signaling has been shown to activate the Notch pathway; in a positive feedback loop, the Notch pathway is required to activate TGFα, a ligand for the EGF receptor.[32] Studies addressing the relation between Hh and EGF signaling in mammalian tumors are currently missing, however, recent evidence indicates that Shh and EGF cooperate to regulate the proliferation of neural stem cells.[36] In addition, Hh signaling is known to induce expression of EGF receptors and ligands in Drosophila.[1] Thus, it is tempting to speculate that Hedgehog signaling might regulate Notch activity in pancreatic and other gastrointestinal cancers via activation of the EGF signaling pathway. This would be particularly intriguing as Notch induction has been associated with the activation of β-catenin and Lef-1, markers of active Wnt signaling, in skin. Understanding the interactions between embryonic signaling pathways is important as more and more pathway inhibitors become available. In the best case scenario, distinct combinations of pathway inhibitors will provide novel therapeutic options for treatment of diverse cancers.

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