Expert Opinion

1. Introduction
2. Mammalian Hedgehog signalling
3. Inhibitors of Hh signalling
4. Hedgehog in cancer
5. Hedgehog in breast cancer
6. Conclusions
7. Expert opinion

informa
healthcare

The Hedgehog signalling pathway as a therapeutic target in early breast cancer development

Sandra A O'Toole, Alexander Swarbrick & Robert L Sutherland†
†*Garvan Institute of Medical Research, Cancer Research Program, Darlinghurst 2010, NSW, Australia*

The Hedgehog (Hh) signalling pathway is a highly conserved developmental pathway, which plays critical roles in patterning of the embryo through epithelial to mesenchymal signalling and the maintenance of stem cells in the adult organism. There is increasing evidence that this pathway is dysregulated in many malignancies, including breast cancer. While there has been a significant decrease in mortality from breast cancer, a number of treatment challenges remain, particularly in those tumours which develop resistance to endocrine-based therapy, or which lack expression of hormone or c-erbB2/HER2 receptors. Therapeutic manipulation of the Hh pathway as a potential cancer therapy is attracting great interest, with preclinical studies and clinical trials underway in a range of malignancies. This review highlights important recent developments that affect the potential of the Hh pathway as a novel therapeutic target in early breast cancer.

Keywords: breast cancer, developmental pathways, early breast cancer, Hedgehog signalling

Expert Opin. Ther. Targets (2009) 13(9):1095-1103

1. Introduction

Significant advances have been made in the early diagnosis and management of breast cancer through mammographic screening programmes and novel adjuvant treatments resulting in an increased proportion of 'early breast cancers' (i.e., cancers that have not spread beyond the breast and draining ipsilateral lymph nodes). The greater than 25% reduction in breast cancer mortality over the last decade has been particularly attributed to the use of targeted therapies such as tamoxifen and related drugs in oestrogen receptor (ER)-positive disease [1]. More recently, the use of targeted therapy such as trastuzumab in c-erbB2/HER2-overexpressing tumours is also making a significant impact on survival in this poor prognosis subgroup [2,3]. However, further improvements in survival will need to target those tumours that lack expression of hormone or HER2 receptors (such as the aggressive basal-like carcinomas), and in those tumours that show resistance to therapy despite expressing appropriate targets, such as ER and HER2.

The relatively recent demonstration that pathways which regulate development during embryonic life are reactivated in cancer and regulate stem cells in adult life – the Hedgehog, Notch and wingless-type mouse mammary tumor virus integration site family (Wnt) signalling pathways (reviewed in [4]) – has generated great interest in their potential as new therapeutic targets in primary and metastatic breast cancer. The Hedgehog (Hh) signalling pathway is an ancient and highly conserved developmental pathway which has critical functions in development of the embryo, particularly in relation to epithelial to mesenchymal signalling. In the adult, the Hh signalling pathway plays an important role in the maintenance and regulation of stem cells, in normal tissue and in some malignancies [5-9].

10.1517/14728220903130612 © 2009 Informa UK Ltd ISSN 1472-8222

Aberrant Hh signalling is known to play a role in the development of a wide range of malignancies from basal cell carcinoma through to gastrointestinal, lung and brain tumours (gliomas and medulloblastomas) [8,10-13]. There is emerging evidence that dysregulation of Hh signalling also contributes to the development and progression of breast cancer. It is thought to do this through a number of mechanisms; in the dysregulation of 'cancer stem cells', through roles in drug resistance and in aberrant epithelial to mesenchymal signalling. This review focuses in particular on the potential role of Hh signalling in breast cancer development and progression with emphasis on epithelial to mesenchymal signalling and its implications for possible therapeutic modulation of the Hh pathway in breast cancer. The role of Hh signalling in modulation of cancer stem cells has been recently highlighted in a number of reviews [4,5,14] and will not be specifically addressed here. We initially present an overview of Hh signalling in early breast cancer and identify current reports with implications for therapeutic inhibition. Endocrine resistance is a major challenge in the management of breast cancer, and we also describe recent data that illustrate the potential role that Hh signalling plays in this area.

2. Mammalian Hedgehog signalling

The Hedgehog gene was first identified in mutational studies in *Drosophila melanogaster* larvae. One of the mutations identified resulted in denticles, or spikes, covering the back of the larvae, with an appearance suggestive of a hedgehog [15].

Three mammalian homologs of this gene were subsequently identified [16]; Sonic hedgehog (Shh) named after the Sega computer game character, Indian hedgehog (Ihh) and Desert hedgehog (Dhh) both named after different species of hedgehogs. These three ligands initiate Hh signalling. Shh is the most widely expressed of the ligands during development, and regulates development of the notochord, floorplate, developing mid- and fore-brain, as well as the branchial arches, heart and axial skeleton. Ihh stimulates endothelial cell formation in the yolk sac, and is involved in haematopoiesis and endochondral bone formation while Dhh plays a key role in male germline development [17]. Hh signalling in adults is significantly reduced compared with that in the embryo and neonate [18,19].

Shh is the most widely studied and best characterized ligand and prior to secretion is dual cleaved and lipid modified; the full length 47 kDa protein undergoes autocatalytic cleavage by its C-terminal domain between conserved glycine and cysteine residues. There is a second cleavage resulting in an approximately 25 kDa C-terminal processing domain and an N-terminal signalling domain of approximately 19 kDa which has a cholesterol moiety at its C terminus. The N-terminal end of this 19 kDa signalling protein is then palmitoylated by an acyltransferase HHAT (reviewed in [20]). This dual lipid modification influences the range of diffusion of Hh ligand, although there are conflicting reports of the effects on short- and long-range signalling [21-26]. This modified 19 kDa protein referred to as ShhN is believed to be responsible for initiation of Hh signalling. The function of the 25 kDa C-terminal component, which is retained in the cytoplasm of the secreting cell, is not yet clear.

Shh can signal in an autocrine fashion, affecting the cells in which it is produced. However, paracrine Hh signalling requires secretion of Hh ligand via Dispatched (Disp) a 12-pass transmembrane protein, which like the receptor Patched (Ptch) belongs to the bacterial resistance-nodulation-division (RND) family of transport proteins. Abnormalities in Disp lead to loss of long-range signalling [27].

The receptor of the Hh pathway is Ptch, a twelve-pass transmembrane protein. There are two isoforms in vertebrates, which are 73% homologous. The function of Ptch2 is not yet clear and almost all actions of ShhN seem to be through binding to Ptch1 [28]. Ptch catalytically inhibits the seven-pass transmembrane protein Smoothened (Smo), rendering the pathway inactive in the absence of Hh ligand. Loss of Ptch (such as through inactivating mutations) results in constitutive activation of the Hh pathway. Recent evidence shows that much of the critical machinery of the Hh signalling pathway is associated with the primary cilium, an essential organelle for Hh signal transduction such that Smo when activated relocates to this structure from the cytoplasm (see also **Figure 1**) [29]. Interestingly, deficiencies of this organelle are phenocopies of loss of Shh [30].

Binding of Hh ligand inactivates Ptch, de-repressing Smo resulting in positive Hh pathway signalling. When Smo is inactive, a multiprotein complex constitutively processes the glioma-associated oncogene (Gli) proteins (Gli1 – 3) to transcriptionally repressive forms. Gli1 has a mainly activator role, while Gli3 is a repressor of the pathway. Gli2 can act either as an activator or repressor, with the mechanisms as yet poorly understood. Activation of Smo decouples this complex from microtubule domains and leads to stabilization of full length, transactivating Gli proteins that initiate transcription of Hh target genes, including Ptch and Gli [31]. A simplified schema of the mammalian Hh signalling pathway is shown in **Figure 1**.

Downstream targets of Hh signalling have not been well elucidated, and it is likely that these vary depending on tissue type and timing of development. However cyclin D1 and cyclin E are well documented Hh targets [32], as is c-Myc [31]. Hh signalling plays an important role in epithelial-to-mesenchymal signalling in development and reported stromal targets include IGF II and IGF binding protein 3 (IGFBP3) [33].

3. Inhibitors of Hh signalling

It has been estimated that aberrant Hh signalling contributes to the development of up to one third of all human malignancies [9] and thus there is considerable interest in

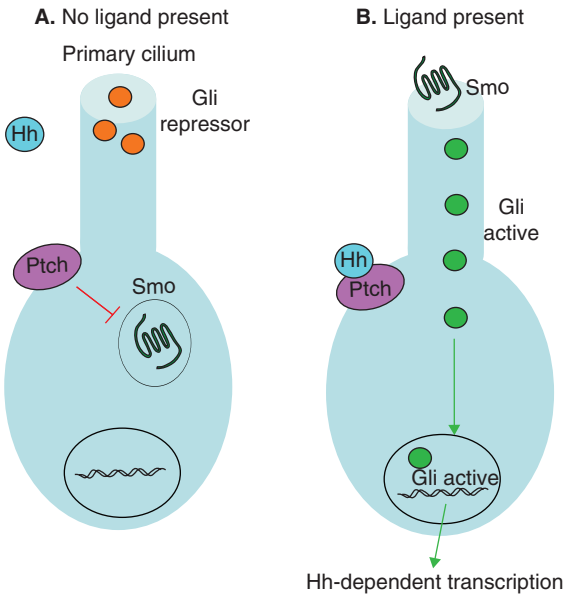

Figure 1. Simplified schema for mammalian Hedgehog (Hh) signalling. In the absence of Hh ligand **(A)** the Patched (Ptch) receptor represses Smoothened (Smo), which is located in the cytoplasm in its inactive form and glioma-associated oncogene (Gli) proteins are processed to transcriptionally repressive forms. With ligand binding **(B)**, Ptch releases its repression of Smo, which then relocates to the primary cilium and Gli is processed to transcriptionally active forms, which translocate to the nucleus resulting in activation of downstream target genes.

therapeutic inhibition of the pathway, with a number of inhibitors in clinical trials [34]. The first inhibitor of the Hedgehog pathway identified was cyclopamine [35], derived from the corn lily (*Veratum californicum*). Pregnant ewes which ate the corn lily had a higher incidence in offspring with severe forebrain and facial development defects including a single eye 'Cyclops' (holoprosencephaly) [36]. Cyclopamine is a small molecule inhibitor of Smoothened [37], and a number of compounds have been identified or synthesized which have similar mechanisms of action [38]. Subsequently, several approaches to inhibition of the pathway have been developed:

1. Prevention of Hh ligand binding to Ptch receptor, for example using the monoclonal antibody 5E1 [39].
2. Inhibition of Smoothened via cyclopamine and related compounds such as jervine and 3-Keto-*N*-(aminoethyl-aminocaproyl-dihydrocinnamoyl)cyclopamine (KAAD-cyclopamine) [38] as well as a number of synthetic analogues [34] some of which are in clinical trials. Much of the preclinical and clinical trial work on Hh inhibitors undertaken to date focuses on inhibitors of Smo.
3. Inhibitors of Gli transcription - small molecule inhibitors of Gli-transcription have been described (GANT61 and GANT58) as well as a number of plant derived compounds (zerumbone, physalin F and B and a range of pentacyclic triterpenes).

4. Hedgehog in cancer

There are two main mechanisms by which Hh signalling is aberrantly regulated in malignancy (illustrated in **Figure 2**); ligand-independent, resulting from mutation in Ptch or downstream signalling pathway components (**Figure 2A**), and ligand-dependent, due to upregulation of Hh ligand. Ligand-dependent pathway signalling is thought to include both autocrine (**Figure 2B, i**) and paracrine (**Figure 2B, ii**) mechanisms of activation.

The first mechanism of aberrant Hh signalling discovered was through identification of mutations in Ptch, an inhibitor of Hh pathway activation, in the genetic disorder Gorlin's syndrome. Gorlin's syndrome is a rare condition associated with skeletal, skin and neural abnormalities as well as a propensity to develop multiple skin tumours – basal cell carcinomas (BCCs). Ptch mutations were then identified as the underlying cause of the majority of spontaneous BCC [40,41], with activating mutations of Smo accounting for approximately 10% of sporadic BCC [42]. Malignancies caused by mutation in Hh pathway genes are referred to as being 'ligand-independent'. Mutations of the Hh pathway associated with malignancy have also been reported in a subset of paediatric brain tumours, medulloblastomas and rhabdomyosarcoma (reviewed in [4]).

A second mechanism of abnormal Hh signalling, referred to as 'non-Gorlin's' or 'ligand-dependent' is seen in a rapidly expanding range of malignancies. It was first identified in studies of small cell lung cancer (SCLC) where Watkins and colleagues [43] first demonstrated co-expression of Shh ligand and Gli1 in human tumours and cell lines, producing evidence of persistent activation of Hh signalling in lung cancer with no evidence of Ptch1 mutation. Cyclopamine was only able to inhibit growth of those SCLC cell lines showing coexpression of both Shh and Gli1 *in vitro* as well as in immunodeficient mouse xenografts, suggesting an active and intact Hh signalling pathway. This group also demonstrated autocrine and juxtacrine mechanisms of Hedgehog pathway activation in SCLC [43].

Thayer and colleagues [10] further delineated a role for ligand-dependent Hedgehog signalling in pancreatic cancer. They found Pdx-Shh mice, with expression of Shh directed to pancreatic ducts, develop lesions equivalent to human pancreatic cancer precursor lesions, while precursor and invasive lesions from human cancers expressed Shh, Ptch and Smo. This group identified Ptch in both the stromal and epithelial components, consistent with a possible role of the Hedgehog signalling pathway in paracrine and autocrine tumour stimulation.

Hh signalling plays a particularly important role in epithelial-to-mesenchymal signalling during development of organs such as the lung [44] and prostate [45] and there is considerable interest as to the cellular compartment in which the Hh signal is initiated and in which it is received in malignancy. Some recent reports regarding Hh signalling

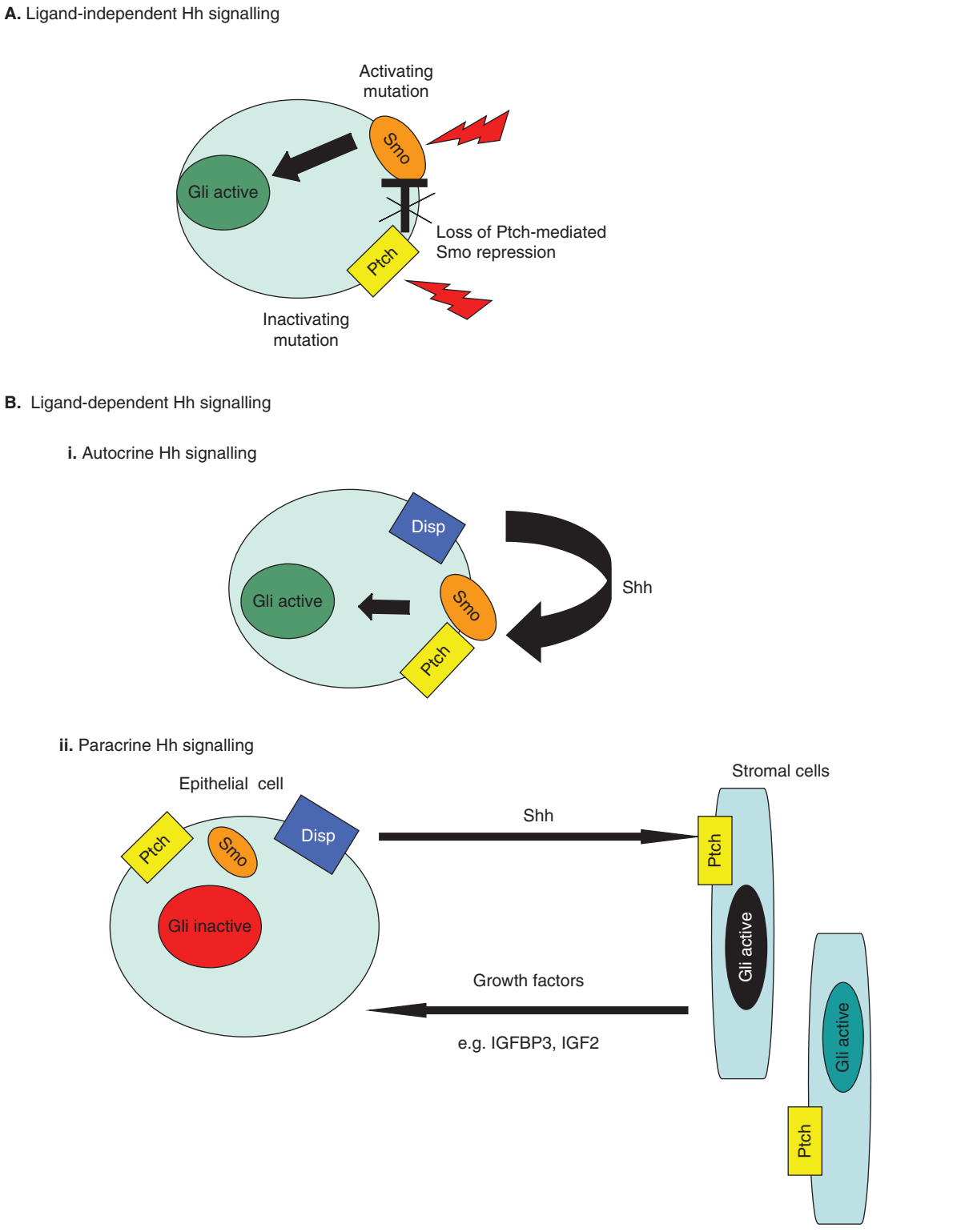

Figure 2. Proposed mechanisms of Hedgehog (Hh) signalling in malignancy. (A) In ligand-independent Hh signalling, inactivating mutations of Patched (Ptch) or activating mutations of Smoothened (Smo) result in constitutive pathway activation. **(B)** In ligand-dependent signalling, increased secretion of ligand via Dispatched (Disp) is associated with pathway activation within the malignant epithelial cell – autocrine signalling **(i)** or in the adjacent stromal cells which secrete growth factors affecting the epithelial cell compartment – paracrine signalling **(ii)**. Gli: glioma-associated oncogene, IGFBP3; IGF binding protein 3.

in cancer have provided evidence suggesting tumour stroma is the main target of Hh ligand secretion.

In view of the known pattern of Hh signalling during development, with Hh ligand secreted by epithelium to the mesenchyme/stroma, Yauch and colleagues [46] investigated whether a similar mechanism of paracrine signalling was present in a number of epithelial malignancies. They first examined the response of two pancreatic cell lines to a range of Hh pathway antagonists; 'HhAntag' a small molecule inhibitor of Smo from Curis, cyclopamine and 5E1. The first cell line expressed Smo, and had been previously shown to be cyclopamine-sensitive while the second cell line was Smo negative, and relatively cyclopamine-insensitive. Yauch and colleagues [46] found non-specific inhibition of cell growth with HhAntag and cyclopamine, unrelated to Gli mRNA levels (as a readout of an active Hh pathway) in both cell lines and no effect of 5E1 on cell growth, suggesting that the actions of the Smo antagonists were non-specific and off-target.

Microarray expression analysis identified high levels of Hh ligands in some ovarian, endometrial, colorectal and pancreatic carcinoma specimens, validated by RT-PCR, and also seen in cell lines representative of these malignancies [46]. Co-culture of these cell lines in a fibroblast GLI reporter assay showed paracrine Hh signalling from epithelial cells to the stromal cells. This was supported by xenograft studies of pancreatic cell lines, with differential expression of Hh ligands, into Ptch1–lacZ/Rag2$^{-/-}$ reporter mice which showed evidence of stromal Ptch expression immediately adjacent to the epithelial tumour cells only in the ligand-expressing cell lines [46].

One criticism of this approach is its heavy reliance on cells in long-term culture. It is well known that there are significant changes in gene expression with even short-term *in-vitro* culture [47,48] and thus the data observed by Yauch and colleagues [46] may be confounded by this. However, they also performed primary xenograft experiments in which tumour samples from a range of human malignancies that had never been cultured and using species-specific probes to detect human tumour compared with mouse stroma and found that there was significantly greater expression of SHH and IHH in the epithelium compared with stroma, while mouse stroma showed elevated levels of Ptch1 and Gli1 compared with epithelium. There was no correlation with ligand expression and PTCH1 mRNA in the human epithelial cells, suggesting that ligand is produced by the epithelial tumour compartment but received in the adjacent mouse stroma, where markers of Hh pathway activation were seen.

Therapeutic trials of oral HhAntag in primary xenografts of human pancreatic and colonic adenocarcinoma demonstrated impressive reductions in tumour growth with a concomitant downregulation of canonical Hh target genes in the stroma, but not the epithelium. A similar result was observed with colon cancer cell line xenografts treated with 5E1. These data strongly support a paracrine role for Hh signalling in at least some malignancies and show that blockade of paracrine signalling is a promising therapeutic approach.

There are a number of criticisms of this paper [49] and there is extensive conflicting evidence in the literature that suggests that paracrine Hh signalling may not be the only pattern of aberrant signalling in epithelial malignancies. Cell-autonomous and juxtracrine (epithelial-to-epithelial) Hh signalling has been demonstrated in lung cancer [43], a range of gastrointestinal carcinomas [12], including pancreatic carcinoma [10], prostate carcinoma [11] and even in non-epithelial malignancies such as melanoma [8] and glioma.

5. Hedgehog in breast cancer

There are a number of recent reviews documenting the accumulating evidence for aberrant Hh signalling in breast cancer [4,7,50]. This review gives a brief overview of selected earlier reports documenting evidence for aberrant Hh signalling in early breast carcinogenesis, but particularly highlights more recent data.

The earliest reports that Hh signalling may contribute to breast carcinogenesis came through the studies of Lewis and colleagues in mice with heterozygous disruption of Ptch1 which demonstrated marked abnormalities in mammary ductal structures including hyperplasias and dysplasias similar to human breast lesions [51]. More recently members of the same group [52] studying mice with constitutive activation of human Smo under control of the mouse mammary tumor virus (MMTV) promoter, found that mammary ductal cells showed increased proliferation, altered differentiation and developed ductal dysplasias. Earlier, this group had also demonstrated that the mammary ducts of mice with loss of Gli2 showed a range of histological alterations similar to micropapillary ductal hyperplasia in the human breast [53] and it seemed that the role of Gli2 was particular to the stroma, as duct changes were not seen when epithelium with Gli2 deleted was transplanted into a wild-type mouse stroma.

Interestingly, although hyperplasia and dysplasias all resulted from aberrant expression of a number of Hh signalling pathway components, each of these resultant lesions seems to show a distinct and different morphology. In our own mammary graft reconstitution studies, transplantation of retrovirally directed Shh expression in mammary epithelium resulted in hyperplasia [54], with a similar appearance to that noted in mice with Ptch1 deficiency (MT Lewis pers. commun.). Whole mammary gland transplantation from Ptch1-deficient mice into athymic mice with intact Ptch1 maintained their dysplastic phenotype, but transplantation of just the epithelium from Ptch1-deficient mice into wild-type cleared fat pad resulted in loss of the dysplasia, suggesting a stromal requirement for Ptch1 [51]. It is interesting that the development of ductal hyperplasias with a similar appearance may result from Shh overexpression in the epithelium [54] or from loss of the pathway inhibitory receptor Ptch1 in the stroma [51]. This may be consistent with a paracrine pattern

of Hh pathway activation, although this is speculative. However, the pattern of Hh pathway activation in breast cancer is far from established. There are conflicting reports regarding whether cell-autonomous (autocrine) or paracrine activation, or a combination of both mechanisms is seen in breast cancer.

Initiating mechanisms of Hh pathway activation in breast cancer have also not been clearly established. Early studies show that mutational activation seems to be a rare event. Wicking and colleagues [55] found no evidence for the H133Y missense mutation in Shh in 44 breast carcinomas and 8 breast cancer cells lines and Vorechovsky *et al.* [56] found no evidence of mutations in Shh, or Smo. While there are data suggesting that ligand-dependent activation is a major mechanism of pathway activation [57], deletional analysis has shown loss of the PTCH1 locus in almost one third of breast cancers in a recent study in Indian women [58], while amplification of the 12q13-q15 locus, which includes Gli1, has been reported as a relatively frequent event, and may be seen in up to 20% of tumours, in a study using microarray-based comparative genomic hybridization in a small number of advanced breast cancers [59].

Epigenetic events may also play a role in activation of the Hh pathway in breast cancer. Wolf and colleagues [60] demonstrated that treatment of two breast cancer cell lines with demethylating and histone-deacetylating agents resulted in upregulation of the tumour suppressor PTCH and observed that Ptch protein expression was lost in 58% of invasive carcinomas and was associated with methylation of the promoter region.

The first association between abnormal expression of Hh signalling pathway components in human breast cancer was reported by Kubo and colleagues [57] when they examined expression of a relatively small number of invasive carcinomas. They found expression of Shh, Ptch and Gli in the epithelial cells of virtually all carcinomas, in comparison with normal breast epithelium, which was negative for these markers. This is consistent with our own observation, that by immunohistochemistry, almost all invasive ductal carcinomas express Shh, although we found quantitative differences in the intensity of expression [54]. Kubo and colleagues [57] did not comment on the presence or absence of stromal staining. This group also investigated expression of Shh as well as Ptch1 and Gli by immunocytochemistry and immunoblotting and identified that some cell lines expressed both ligand and downstream pathway components, suggestive of cell autonomous activation. Furthermore, the growth of a number of breast cancer cell lines could be inhibited by cyclopamine (at a concentration of 10 μM), suggesting that Hh pathway is a promising therapeutic target in breast cancer.

However, these data should be interpreted with caution in light of the studies of Zhang *et al.* [61] from the Lewis group who evaluated the expression of Hh pathway components using quantitative reverse transcription-PCR (RT-PCR) and the growth responses of a collection of Smo-positive and Smo-negative breast cancer cell lines. Quantitiative RT-PCR showed a mixed pattern of Hh pathway components in the breast cell lines with little correlation between ligand expression and downstream pathway components such as Smo and Gli. Zhang *et al.* [61] also tested the ability of ShhN ligand to induce Hh pathway activation in these cell lines, and only one, T-47D, showed induction of Hh network gene expression.

Based on these findings, the authors suggested that only T-47D with evidence of intact Hh pathway signalling would be growth-inhibited by cyclopamine treatment. However cyclopamine inhibited the growth of many of the breast cancer cell lines (ER-positive and -negative), and this growth inhibition was unrelated to the presence or absence of Smo and was seen only at relatively high concentrations (> 10 μM). Counter-intuitively, the single cell line with evidence of an intact Hh pathway was relatively insensitive to cyclopamine, showing growth inhibition only at the very highest concentrations (20 μM). Unexpectedly, this cell line, T-47D showed increases in Hh network genes after 4 days of treatment with cyclopamine. The lack of correlation between cyclopamine growth inhibition and Hh pathway activity was also confirmed by an inability of ShhN ligand to rescue growth inhibition in almost all the cell lines. Dual treatment of T-47D cells with cyclopamine and ShhN ligand showed even more significant inhibition of Hh network genes than treatment with cyclopamine alone.

These data are the opposite of the expected effects of a Hh pathway inhibitor and strongly suggest that, at least at higher doses, cyclopamine is acting on as yet undetermined molecular targets. They also support the hypothesis, that at least in the artificial setting of long-term cultured breast cancer cell lines, autocrine Hh pathway activation is not seen. These findings are supportive of the data of Yauch and colleagues [46]. However, the Zhang [61] study also revealed the intriguing finding that tumourigenic breast cancer cell lines showed much greater growth inhibition with cyclopamine treatment than non-tumourigenic breast cell lines, which were relatively insensitive to treatment. Although these data cast doubt on the specificity of these 'Smo inhibitors' at least in cell-culture assays, they suggest that cyclopamine has a selective effect on malignant cell growth, compared with non-transformed, immortalized 'benign' cells.

Although these data further 'muddy the waters' of the therapeutic potential of Smo inhibitors, it is important to highlight that cell culture assays are unlikely to accurately reflect effects of these compounds in a dynamic, *in vivo* physiological system, such as mouse models, with intact epithelial-to-mesenchymal signalling. The testing of Hh inhibitors in *in vivo* systems, in view of the increasing appreciated importance of epithelial-to-mesenchymal signalling, may more accurately reflect probable effects in clinical settings.

Hormonal treatment is a mainstay of breast cancer therapy, with a major effect on improved survival over the past decades. Recent evidence suggests that Hh signalling plays an important role in the hormonal regulation of breast cancer

and possibly a role in the development of resistance to endocrine therapy. Zhao and colleagues [62] reported that Gli1 may be involved in the transition of breast cancer cells to oestrogen-independent growth. A striking negative correlation was seen between expression of ERα and Gli1, such that ER-positive cell lines had the lowest levels of Gli1 and ER-negative cell lines showed the highest levels of Gli1 at the mRNA level. Furthermore, Gli1 protein was undetectable on Western blot in estrogen-dependent cell lines, and seen only in estrogen-independent cell lines. *In vitro* studies also demonstrated that stable transfection of Gli1 into ERα, estrogen-dependent cell lines increased cell growth in estrogen-deficient medium, through induction of cell cycle progression. Gli1 overexpression was also associated with an attenuated response to antiestrogen-induced growth inhibition, with a 70% decrease in inhibition after treatment with Tamoxifen in the highest expressing Gli1 clone.

These data are consistent with our finding in subgroup analysis that ER-positive patients treated with tamoxifen whose tumours showed the highest levels of Shh expression showed significantly poorer survival in Kaplan-Meier analysis [54] and suggest that activation of the Hh signalling pathway plays a role in the development of endocrine resistance in breast cancer. However, we also observed that high Shh tumour expression was associated with a poor survival in chemotherapy-treated patients, and thus the potential of Shh as a specific predictive marker of endocrine resistance in human breast cancer warrants further investigation. We also observed that Shh expression was highest in the 'triple negative' basal-like tumours which lack expression of ER, progesterone receptor and HER2 receptors. However, there is limited data in this area and there is a pressing need for more studies to further elucidate the role of Hh signalling in therapeutic resistance.

6. Conclusions

The field of Hh signalling is currently fraught with contradictory data and a lack of consensus of the mechanisms of signalling in a range of malignancies, including breast cancer. However, despite the confusion, some very promising data suggests that aberrant Hh signalling plays an important role in the development and progression of breast cancer. There is also early *in vitro* data to suggest that dysregulation of Hh signalling plays a role in the acquisition of resistance to endocrine therapy in ER-positive tumours. If this is proven to be correct, there may be potential for manipulation of the Hh pathway in mitigation of this endocrine resistance in breast cancer.

Although significant progress has been made in improved survival from breast cancer, further gains are likely to be made only if additional therapeutic targets are identified and exploited to tackle the troubling areas of 'triple negative' breast cancer, resistance to endocrine therapy and the treatment of metastatic breast cancer.

There is an accumulating mass of data to support a role for the Hh signalling pathway in these areas. However, more studies are needed, particularly in more physiological, *in vivo* systems with intact Hh signalling mechanisms; the need for which are reinforced by the often surprising and contradictory findings on the effects of Hh pathway inhibitors in *in vitro* assays.

7. Expert opinion

There is mounting evidence that aberrant Hedgehog signalling plays an important role in the development and progression of breast cancer. However, the field is still in its infancy, with often conflicting reports on the types of abnormal Hh signalling and their potential mechanisms. A greater understanding of these processes is required in order to design appropriate systems for rigorous preclinical testing of current and future Hh inhibitors as potential anticancer agents in clinical settings. Recent data have illustrated the flaws of using traditional cell culture assays of drug response, where interactions with the tumour stroma cannot be evaluated, in investigating the therapeutic potential of Hh pathway modulation. Studies carried out in systems with intact epithelial/stromal signalling, such as transplantation and transgenic animal models, are likely to yield more meaningful results in relation to possible clinical settings while data obtained from simple cell monolayer culture should be interpreted with caution. Nontheless, *in vitro* studies identifying that modulation of Hedgehog signalling induces estrogen-independent proliferation and an attenuated response to antiestrogens implies a potential association with endocrine resistance.

Tamoxifen and related therapies have had a major effect on breast cancer mortality, and the mechanisms of resistance and new therapeutic strategies to treat endocrine resistance are a major focus of breast cancer research. More work is needed to expand on these early reports of a relationship between Hh pathway activation and the acquisition of hormonal resistance with the promise that Hh antagonists have a role in this area. Again appropriate animal models of Hh signalling in the mammary gland and breast cancer may help in the elucidation of mechanisms and the translation of these findings into clinical application.

Declaration of interest

The authors would like to thank the Cancer Institute NSW, the National Health and Medical Research Council, the RT Hall Trust and the Petre Foundation for their financial support of this work.

Bibliography

1. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005;365(9472):1687-717
2. Smith I, Procter M, Gelber RD, et al. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. Lancet 2007;369(9555):29-36
3. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 2005;353(16):1659-72
4. Zardawi SJ, O'Toole SA, Sutherland RL, Musgrove EA. Dysregulation of Hedgehog, Wnt and Notch signalling pathways in breast cancer. Histol Histopathol 2009;24(3):385-98
5. Liu S, Dontu G, Wicha MS. Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Res 2005;7(3):86-95
6. Liu S, Dontu G, Mantle ID, et al. Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res 2006;66(12):6063-71
7. Hatsell S, Frost AR. Hedgehog signaling in mammary gland development and breast cancer. J Mammary Gland Biol Neoplasia 2007;12(2-3):163-73
8. Clement V, Sanchez P, de Tribolet N, et al. HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. Curr Biol 2007;17(2):165-72
9. Beachy PA, Karhadkar SS, Berman DM. Tissue repair and stem cell renewal in carcinogenesis. Nature 2004;432(7015):324-31
10. Thayer SP, di Magliano MP, Heiser PW, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature 2003;425(6960):851-6
11. Karhadkar SS, Bova GS, Abdallah N, et al. Hedgehog signalling in prostate regeneration, neoplasia and metastasis. Nature 2004;431(7009):707-12
12. Berman DM, Karhadkar SS, Maitra A, et al. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature 2003;425(6960):846-51
13. Berman DM, Karhadkar SS, Hallahan AR, et al. Medulloblastoma growth inhibition by hedgehog pathway blockade. Science 2002;297(5586):1559-61
14. Wicha MS, Liu S, Dontu G. Cancer stem cells: an old idea–a paradigm shift. Cancer Res 2006;66(4):1883-90
15. Nusslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in Drosophila. Nature 1980;287(5785):795-801
16. McMahon AP, Ingham PW, Tabin CJ. Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol 2003;53:1-114
17. Cohen MM Jr. The hedgehog signaling network. Am J Med Genet A 2003;123A(1):5-28
18. Machold R, Hayashi S, Rutlin M, et al. Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron 2003;39(6):937-50
19. van den Brink GR, Bleuming SA, Hardwick JC, et al. Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. Nat Genet 2004;36(3):277-82
20. Varjosalo M, Taipale J. Hedgehog: functions and mechanisms. Genes Dev 2008;22(18):2454-72
21. Dillon R, Gadgil C, Othmer HG. Short- and long-range effects of Sonic hedgehog in limb development. Proc Natl Acad Sci USA 2003;100(18):10152-7
22. Gallet A, Ruel L, Staccini-Lavenant L, Therond PP. Cholesterol modification is necessary for controlled planar long-range activity of Hedgehog in Drosophila epithelia. Development 2006;133(3):407-18
23. Goetz JA, Suber LM, Zeng X, Robbins DJ. Sonic Hedgehog as a mediator of long-range signaling. Bioessays 2002;24(2):157-65
24. Lewis PM, Dunn MP, McMahon JA, et al. Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. Cell 2001;105(5):599-612
25. Li Y, Zhang H, Litingtung Y, Chiang C. Cholesterol modification restricts the spread of Shh gradient in the limb bud. Proc Natl Acad Sci USA 2006;103(17):6548-53
26. Zeng X, Goetz JA, Suber LM, et al. A freely diffusible form of Sonic hedgehog mediates long-range signalling. Nature 2001;411(6838):716-20
27. Kawakami T, Kawcak T, Li YJ, et al. Mouse dispatched mutants fail to distribute hedgehog proteins and are defective in hedgehog signaling. Development 2002;129(24):5753-65
28. Riobo NA, Manning DR. Pathways of signal transduction employed by vertebrate Hedgehogs. Biochem J 2007;403(3):369-79
29. Wang Y, Zhou Z, Walsh CT, McMahon AP. Selective translocation of intracellular Smoothened to the primary cilium in response to Hedgehog pathway modulation. Proc Natl Acad Sci USA 2009;106(8):2623-8
30. Huangfu D, Liu A, Rakeman AS, et al. Hedgehog signalling in the mouse requires intraflagellar transport proteins. Nature 2003;426(6962):83-7
31. Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes Dev 2001;15(23):3059-87
32. Duman-Scheel M, Weng L, Xin S, Du W. Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. Nature 2002;417(6886):299-304
33. Ingram WJ, Wicking CA, Grimmond SM, et al. Novel genes regulated by Sonic Hedgehog in pluripotent mesenchymal cells. Oncogene 2002;21(53):8196-205
34. Garber K. Hedgehog drugs begin to show results. J Natl Cancer Inst 2008;100(10):692-7
35. Cooper MK, Porter JA, Young KE, Beachy PA. Teratogen-mediated inhibition of target tissue response to Shh signaling. Science 1998;280(5369):1603-7
36. Keeler RF, Binns W. Teratogenic compounds of Veratrum californicum (Durand). V. Comparison of cyclopian effects of steroidal alkaloids from the plant and structurally related compounds from other sources. Teratology 1968;1(1):5-10
37. Chen JK, Taipale J, Young KE, et al. Small molecule modulation of Smoothened activity. Proc Natl Acad Sci USA 2002;99(22):14071-6
38. Mahindroo N, Punchihewa C, Fujii N. Hedgehog-Gli signaling pathway inhibitors

as anticancer agents. J Med Chem 2009;52(13):3829-45

39. Ericson J, Morton S, Kawakami A, et al. Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell 1996;87(4):661-73
40. Unden AB, Stahle-Backdahl M, Holmberg E, et al. Fine mapping of the locus for nevoid basal cell carcinoma syndrome on chromosome 9q. Acta Derm Venereol 1997;77(1):4-9
41. Unden AB, Zaphiropoulos PG, Bruce K, et al. Human patched (PTCH) mRNA is overexpressed consistently in tumor cells of both familial and sporadic basal cell carcinoma. Cancer Res 1997;57(12):2336-40
42. Xie J, Murone M, Luoh SM, et al. Activating Smoothened mutations in sporadic basal-cell carcinoma. Nature 1998;391(6662):90-2
43. Watkins DN, Berman DM, Burkholder SG, et al. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. Nature 2003;422(6929):313-7
44. Watkins DN, Berman DM, Baylin SB. Hedgehog signaling: progenitor phenotype in small-cell lung cancer. Cell Cycle 2003;2(3):196-8
45. Shaw A, Bushman W. Hedgehog signaling in the prostate. J Urol 2007;177(3):832-8
46. Yauch RL, Gould SE, Scales SJ, et al. A paracrine requirement for hedgehog signalling in cancer. Nature 2008;455(7211):406-10
47. Daniel VC, Marchionni L, Hierman JS, et al. A primary xenograft model of small-cell lung cancer reveals irreversible changes in gene expression imposed by culture in vitro. Cancer Res 2009;69(8):3364-73
48. De Witt Hamer PC, Van Tilborg AA, Eijk PP, et al. The genomic profile of human malignant glioma is altered early in primary cell culture and preserved in spheroids. Oncogene 2008;27(14):2091-6
49. Ruiz i Altaba A. Therapeutic inhibition of Hedgehog-GLI signaling in cancer: epithelial, stromal, or stem cell targets? Cancer Cell 2008;14(4):281-3
50. Kasper M, Jaks V, Fiaschi M, Toftgard R. Hedgehog signalling in breast cancer. Carcinogenesis 2009;30(6):903-11
51. Lewis MT, Ross S, Strickland P, et al. Defects in mouse mammary gland development cuased by conditional haploinsufficiency of Patched-1. Development 1999;126:5181-93
52. Moraes RC, Zhang X, Harrington N, et al. Constitutive activation of smoothened (SMO) in mammary glands of transgenic mice leads to increased proliferation, altered differentiation and ductal dysplasia. Development 2007;134(6):1231-42
53. Lewis MT, Ross S, Strickland PA, et al. The Gli2 transcription factor is required for normal mouse mammary gland development. Dev Biol 2001;238(1):133-44
54. O'Toole S, Swarbrick A, Millar E, et al. Aberrant hedgehog signaling is an early event in breast cancer development. Cancer Res 2009;69:70s
55. Wicking C, Evans T, Henk B, et al. No evidence for the H133Y mutation in Sonic Hedgehog in a collection of common tumour types. Oncogene 1998;16(8):1091-3
56. Vorechovsky I, Benediktsson KP, Toftgard R. The patched/hedgehog/smoothened signalling pathway in human breast cancer: no evidence for H133Y SHH, PTCH and SMO mutations. Eur J Cancer 1999;35(5):711-3
57. Kubo M, Nakamura M, Tasaki A, et al. Hedgehog signaling pathway is a new therapeutic target for patients with breast cancer. Cancer Res 2004;64(17):6071-4
58. Sinha S, Singh RK, Alam N, et al. Alterations in candidate genes PHF2, FANCC, PTCH1 and XPA at chromosomal 9q22.3 region: pathological significance in early- and late-onset breast carcinoma. Mol Cancer 2008;7:84. Published online 6 November 2008, doi:10.1186/1476-4598-7-84
59. Nessling M, Richter K, Schwaenen C, et al. Candidate genes in breast cancer revealed by microarray-based comparative genomic hybridization of archived tissue. Cancer Res 2005;65(2):439-47
60. Wolf I, Bose S, Desmond JC, et al. Unmasking of epigenetically silenced genes reveals DNA promoter methylation and reduced expression of PTCH in breast cancer. Breast Cancer Res Treat 2007;105(2):139-55
61. Zhang X, Harrington N, Moraes RC, et al. Cyclopamine inhibition of human breast cancer cell growth independent of Smoothened (Smo). Breast Cancer Res Treat 2009;115(3):505-21
62. Zhao J, Chen G, Cao D, et al. Expression of Gli1 correlates with the transition of breast cancer cells to estrogen-independent growth. Breast Cancer Res Treat 2009;115:505-21

Affiliation

Sandra A O'Toole[1,2,3,4] Alexander Swarbrick[1,3] & Robert L Sutherland[†1,3]
[†]Author for correspondence
[1]Garvan Institute of Medical Research,
Cancer Research Program,
Darlinghurst 2010,
NSW, Australia
Tel: +61 2 9295 8322; Fax: +61 2 9295 8321;
E-mail: r.sutherland@garvan.org.au
[2]Royal Prince Alfred Hospital,
Department of Anatomical Pathology,
Camperdown, 2050, NSW, Australia
[3]Faculty of Medicine,
University of NSW,
St Vincent's Clinical School,
Australia
[4]University of Sydney,
School of Medicine,
NSW, Australia