Hedgehogs tryst with the cell cycle

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Summary

Hedgehog proteins play an essential role during pattern formation in animal development and, increasingly, much of our appreciation of their modes of action is emanating from studies of their signalling mechanisms at the cellular level. Recent work has provided insights into how Hedgehog controls the cell cycle in a variety of circumstances. The data suggest that this influence may be direct and operates through interaction of the signalling pathway with cell cycle regulators at multiple points within the cell cycle. These new findings have profound implications in the context of clinical conditions – especially cancers – that arise from de-regulated cell proliferation in response to aberrant Hedgehog signalling activity.

Key words: Sonic Hedgehog, Patched, Cyclin, Proliferation, Cell cycle

Introduction

Controlled cell proliferation is a predominant theme in normal embryonic and post-embryonic development, and, in many instances, cell-type specification and cell proliferation are intimately coupled. Several secreted intercellular signalling proteins that behave as morphogens during pattern formation have also been implicated in the regulation of the cell cycle. Hedgehogs (HHs) are one such class of morphogen that have attracted a great deal of attention in recent years (see Fig. 1 for a summary of the signalling pathway) (for a review, see Ingham and McMahon, 2001). Although the requirement for these proteins in diverse events of early embryonic patterning, organogenesis, as well as post-embryonic development and physiology in a variety of organisms has now been extensively described, our understanding of how they influence these processes remains fragmentary. A primary effect in the multitude of functions that HH activity fulfils during development is the specification of cell fate either through short- or long-range inductive signalling. In the ventral epidermis of the developing *Drosophila* embryo for instance, where the function of HH was first discovered and has been most extensively investigated, cells that respond to the signal are the immediate neighbours of those that secrete it. In the vertebrate neural tube, however, induction of a variety of ventral neuronal cell types appears to occur through the concentration-dependent effects of Sonic HH (SHH), the founding member of the vertebrate family of HH proteins, acting over a distance.

Apart from its influence on the fate of cells, in many developmental contexts, HH signalling has been associated with proliferative responses in target cells. For example, SHH has been implicated as a crucial regulator of growth and patterning of the cerebellum (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Here, the signal is produced and secreted by the Purkinje neurons and appears to have a definitive mitogenic influence on the proliferation of cerebellar granule neuron precursors (CGNPs) in the outer granule cell layer. Similarly, during hair follicle morphogenesis in mammals, loss of SHH, which is normally expressed at the tip of the developing epidermal placode, dramatically reduces cell proliferation in the follicular rudiment (Bitgood and McMahon, 1995; Chiang et al., 1999; St-Jacques et al., 1998). Furthermore, another vertebrate Hh paralogue, Indian HH (IHH), has a central role in cartilage formation (St-Jacques et al., 1999), and recent investigations suggest that the signalling pathway is required autonomously in the precursor chondrocytes for their proliferation (Long et al., 2001).

In *Drosophila*, as in vertebrates, HH plays an important role in patterning the appendage primordia. During wing development, HH induces the expression of a another morphogen, a transforming growth factor β (TGFβ) homologue, Decapentaplegic (DPP), and is believed to regulate pattern as well as cell proliferation largely through the activity of this secondary signal (Burke and Basler, 1996; Martin-Castellanos and Edgar, 2002; Nellen et al., 1996). DPP is also a critical proliferative cue for cell division in the germ line of the *Drosophila* ovary (Xie and Spradling, 1998), a process in which the role, if any, of HH is unclear. By contrast, several lines of evidence indicate that HH signalling is essential for the proliferation of the ovarian somatic stem cells and, as in the case of CGNPs and chondrocytes in vertebrates, this effect could indeed be direct (Forbes et al., 1996; Zhang and Kalderon, 2001).

Despite the prospect that, at least in certain circumstances, HH can directly trigger cell proliferation, the underlying mechanism had remained enigmatic. Recent discoveries using molecular and biochemical approaches in cultured vertebrate cells, in conjunction with genetic analysis in *Drosophila*, have now provided evidence that the activities of HH signalling components can indeed interface with core cell cycle regulators and modulate their expression and/or activity. We discuss new findings from disparate lines of investigation and their significance in extending our perception of the association between HH signalling and the cell cycle.

HH signalling controls the expression cell cycle regulators

The first clear indication that HH signalling can somehow directly act at the heart of the cell cycle machinery comes from studies on the effects of SHH on the proliferative capacity of cultures of human keratinocytes (Fan and Khavari, 1999). These showed that exposure to SHH can not only promote proliferative responses in keratinocytes but also abrogate their cell cycle arrest induced by overexpression of the cyclin-dependent kinase (CDK) inhibitor p21. In a more systematic study, the molecular details of the proliferative role of SHH were analysed in primary cultures of CGNPs (Kenney and Rowitch, 2000). Aspects of the influence of SHH on CGNPs can be recapitulated in culture on exogenous administration of the protein (Dahmane and Ruiz-i-Altaba, 1999; Wechsler-Reya and Scott, 1999). In line with previous reports, Kenny and Rowitch observed significantly increased cell proliferation, which was concomitant with the induction of canonical targets of vertebrate HHs (see Fig. 1).

A hallmark of classic mitogens is that they are capable of eliciting a proliferative response even from mitotically quiescent cells that are arrested in G0 phase. Interestingly, in the above experiments, SHH was unable to make quiescent granule cell precursors re-enter the cell cycle. Thus, at least in this context, SHH activity cannot be equated entirely with that of a typical mitogen. Thus, in normal development, SHH signalling might be necessary to maintain rather than initiate CGNP proliferation in the cerebellum. It is also notable that this effect of SHH on CGNP proliferation appears to occur independently of the mitogen-activated protein (MAP) kinase activation.

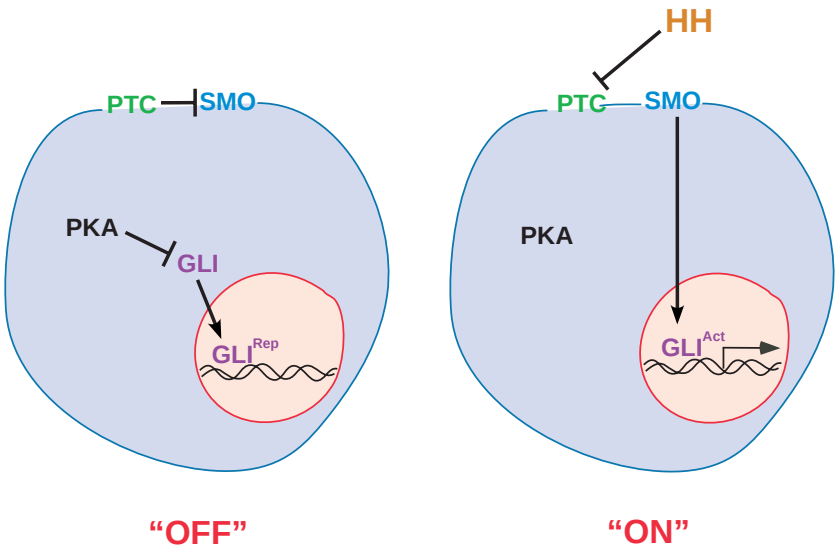

Fig. 1. A highly simplified schematic illustrating the central components and the general mechanism of HH signal transduction. In cells not responding to HH (the "OFF" state), PTC – a twelve-transmembrane-domain-containing protein and the receptor for HH ligands – represses the activity of SMO, a G-protein-coupled receptor-like seven-transmembrane-domain-containing protein. The intracellular consequence of this repression is the PKA-mediated inactivation or conversion of the GLI family of transcription factors (CI in *Drosophila*) into repressors (GLI^{Rep}) and constitutive repression of HH target genes. On reception of HH through its binding with PTC (the "ON" state), SMO inhibition is somehow relieved and this results in the nuclear accumulation of activated forms of GLIs (GLI^{Act}) that induce HH target gene transcription. A conserved target is *ptc* itself, as upregulation of PTC by HH serves to restrict its signalling range. In vertebrates, *Gli1*, like *ptc*, also appears to be transcriptionally regulated by HH through the activities of other GLI proteins. For further details and modulations of the pathway see Ingham and McMahon (Ingham and McMahon, 2001).

Examination of the status of D-type cyclins, central regulators of G1 phase progression, revealed that, upon incubation with SHH, the CGNPs preferentially upregulate *cyclin D1* and *D2* RNA and cyclin D1 protein. The induction of cyclin D2 protein seemed to be much more restricted in this situation, possibly owing to some kind of post-transcriptional regulation. A similar upregulation of cyclin D1 has also been attributed to IHH activity in proliferating chondrocytes during cartilage development (Long et al., 2001). Complexes of D-type cyclins with their cognate CDKs regulate the activity of the retinoblastoma (RB) protein, such that hyperphosphorylated RB is no longer able to antagonise the E2F transcription factors, which results in the expression of S-phase-promoting factors, such as cyclin E. Consistent with the observation that SHH promotes upregulation of D-type cyclins is the accumulation of hyperphosphorylated RB under these conditions, an effect that can be specifically blocked by activating protein kinase A (PKA), a potent intracellular inhibitor of HH signal transduction (see Fig. 1). Notably, however, primary cultures of CGNPs derived from mice lacking D1 or D2 cyclins show wild-type proliferative responses to SHH stimulation. While this would suggest that the D-type cyclins are functionally redundant, it is striking that animals lacking cyclin D2 (but not cyclin D1) are characterised by reduced numbers of granule cells in their cerebella (Fantl et al., 1995; Huard et al., 1999; Sicinski et al., 1995). Such a discrepancy between the in vivo effects and the in vitro properties of mutant cells may indicate an additional role for cyclin D2 that is distinct from its promotion of G1 phase progression. However, the non-physiological conditions of cell culture systems should perhaps also be taken into account. In this context, analysis of the effects of SHH on *cyclin D1 D2* double-mutant CGNP cells, as well as exploration of the proliferative responses of controlled SHH misexpression in the cerebella of intact *cyclin D2* mutant mice, may be particularly revealing.

An independent line of evidence for the transcriptional effects of HH signalling on D-type cyclins has come from gene expression profiling using microarrays to probe a rat kidney cell line stably transformed with human GLI1 (Yoon et al., 2002), a transcriptional activator of HH target genes in vertebrates (Fig. 1). Under these conditions, there was specific upregulation of *cyclin D2* RNA, an effect that was further confirmed through northern hybridisations. Scanning of the genomic region upstream of the human *cyclin D2* gene revealed a consensus binding site for GLI1 within the core promoter that can be retarded in gel shift assays in the presence of recombinant human GLI1. GLI1 is itself a direct target of

HH signalling and is induced by the activities of other GLI proteins (see Fig. 1). This could partly explain the requirement of protein synthesis for SHH-mediated *cyclin* gene expression in cultured CGNPs (Kenny and Rowitch, 2000). Clearly, further work will be required to provide a better understanding of whether HH influences *cyclin* gene transcription only through GLI1 or additional modes of regulation, through other GLIs or intermediate steps.

Control of cell proliferation and cellular growth by HH in *Drosophila*: insights from genetic analysis

While the studies alluded to above begin to provide a reasonable framework for the molecular mechanisms that link HH and the cell cycle, more direct evidence comes from studies by Duman-Scheel et al. (Duman-Scheel et al., 2002) using genetic analysis in *Drosophila*. The compound eyes of flies are highly organised fields of specialised neurons and accessory cells, the assembly of which requires coordinated cell proliferation and HH signalling. One of the targets of HH in the developing eye, as in the wing primordium, is *dpp*. Although in the eye, cell proliferation is also affected by DPP, it seems less clear whether it exerts a stimulatory or inhibitory influence in this context (e.g. Horsfield et al., 1998; Penton et al., 1997). Notwithstanding this uncertainty, there is a striking juxtaposition of differentiating photoreceptors that express HH with a distinct group of eye precursor cells that undergo a highly synchronous S phase; this hints at a role of HH signalling in instigating this event (Fig. 2). Inhibition of this so-called 'second mitotic wave' limits the numbers of progenitors available for generating all of the differentiated cell types in the eye (de Nooij and Hariharan, 1995). Given this intimate association between HH secreting and proliferating cells, it is unsurprising that a screen for genes interacting with RB function in eye development should uncover *ptc*, which encodes the receptor for HH and negatively regulates signalling in its absence (see Fig. 1).

Duman-Scheel and colleagues showed that overexpression of the *Drosophila* RB homologue in these proliferating eye cells delays their entry into S phase and that the effect can be suppressed by loss of one copy of the wild-type *ptc* gene. This link between HH signalling and a primary cell cycle regulator is reinforced by observations that photoreceptor precursors in the eye primordium that lack *smoothened* (*smo*) gene function and are therefore incapable of transducing the HH signal (see Fig. 1) fail to enter the second mitotic wave. In addition, ectopic activation of HH signalling can induce cells normally arrested in G1 to enter S phase precociously. These effects of modulated HH signalling on the proliferation patterns of eye cells are mirrored by corresponding alterations in the levels of *cyclin D* and *cyclin E* transcripts and proteins. Thus, the control of cell cycle in the eye by HH must be mediated in part through its regulation of these cell cycle mediators and is consistent with previous reports describing the ability of ectopic cyclin E to drive premature S phase entry in this tissue (Crack et al., 2002; Richardson et al., 1995). Intriguingly, Duman-Scheel et al. provide further evidence that, in this instance, HH in fact induces cell proliferation through two independent influences on the levels of cyclins. HH signalling not only promotes S phase entry through the induction of *cyclin D*, which suppresses RB function (and thereby activates E2F targets such as *cyclin E*), but also directly stimulates transcription of *cyclin E* itself through Cubitus interruptus (CI), the GLI family protein that activates HH target genes in flies.

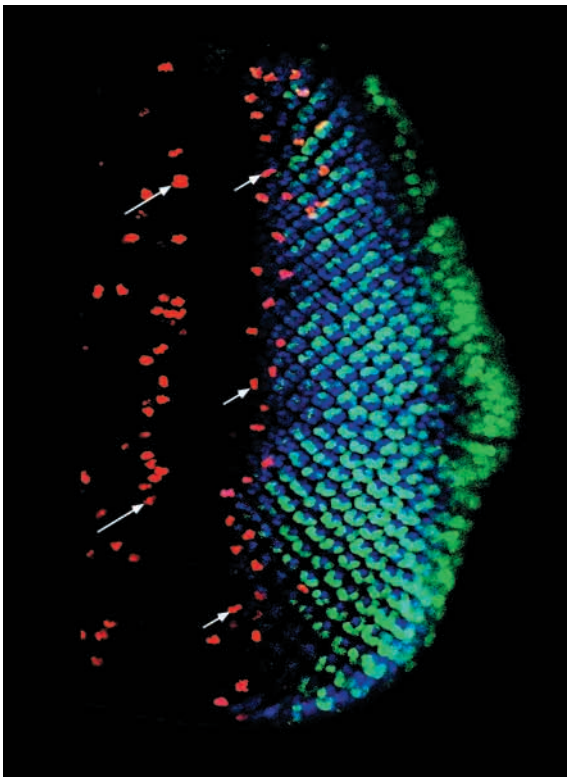

Fig. 2. The developing *Drosophila* eye primordium: A paradigm for studying how HH controls the cell cycle? Differentiating photoreceptor cells, labelled with antibodies that recognise a neuron-specific protein (BLUE), express and secrete HH (GREEN), as revealed by GFP expression from a *hh* reporter transgene. Cells immediately anterior to these differentiating photoreceptors enter a synchronised S phase followed by mitosis (small arrows) in response to this source of HH activity. These mitotic cells are marked with antibodies to phospho-histone (RED), which also labels randomly dividing cells anteriorly in the developing primordium (long arrows).

Cell proliferation and cell growth are important determinants of the size and shape of developing embryos, organs and tissues. The extent to which these two processes are connected and coordinated is one of the central focuses in developmental biology (for a review, see Tapon et al., 2001). Evidence for such a link comes from the finding that cyclin D has the capacity to drive both cell division and cellular growth (Datar et al., 2000; Meyer et al., 2000). Likewise, HH signalling is known to shape growth and pattern of a variety of tissues during development. Consistent with this scenario is the fact that clones of cells in the proliferating wing primordium exhibit enhanced growth in the presence of unabated HH signalling, as opposed to decreased growth when the pathway is constitutively repressed (Duman-Scheel et al., 2002). Furthermore, in line with the ability of HH signalling to induce *cyclin D* expression, these effects of HH on the growth of developing wing cells are critically dependent on the activity of this cyclin and its associated kinase, CDK4.

PTC1 binds the M-phase promoting factor (MPF) and regulates its activity

A fascinating twist to the cyclin-HH connection comes from biochemical analyses that suggest a direct physical interaction between a vertebrate PTC paralogue, PTC1, and regulators of the cell cycle (Barnes et al., 2001). Cylin B1 and the associated kinase CDK1/CDC2 are central components of the MPF whose function is thought to be critical for the G2/M transition of the cell cycle. In fact, nuclear-cytoplasmic shuttling of cyclin B1 appears to be a pivotal event controlling its activity and is thought to be regulated by phosphorylation-dependent modulation of its nuclear export signal (NES) (Yang and Kornbluth, 1999). Thus, phosphorylation of a set of serine residues, beginning in late G2, inactivates the NES, allowing nuclear accumulation of cyclin B1 and consequently M-phase progression. In a yeast two-hybrid screen for proteins that participate in trafficking and localisation of cyclin B1, Barnes and colleagues unexpectedly identified PTC1 amongst prey that associate with a bait mimicking phosphorylated cyclin B1. The relevance of this interaction, which is mediated by the large intracellular loop linking the two extracellular domains of PTC1, was substantiated by further experiments that showed productive interactions between PTC1 and cyclin B1 in cells grown in culture. Indeed, PTC1 can associate with an active MPF complex and also sequester a nuclear-targeted variant of cyclin B1 that resembles its phosphorylated state and predominantly retains it in the cytoplasm or plasma membrane – an interaction, which Barnes et al. go on to show, is mitigated by exposure to SHH. Furthermore, they found that overexpression of PTC1 in cultured cells, as in developing tissues of the fly, prevents cellular growth and proliferation and that this effect can be specifically suppressed by a form of cyclin B1 mutated to mimic its dephosphorylated state.

Taken together, this study suggests that apart from regulating the expression of cyclins, HH may have a more immediate influence on the cell cycle through regulation of the PTC-mediated subcellular localisation of the MPF. Although the data supporting this idea are biochemically robust, they are based to a large extent on overexpression studies in cell culture and not only beg corroboration in an in vivo developmental context but also provoke further challenging questions about the cellular basis of this interaction. Perhaps the most perplexing of them involves the subcellular compartment in which the interaction is likely to occur, especially given the mixed opinion in the literature about the distribution of PTC within the cell. Studies of *Drosophila* PTC in vivo, as well as in cultured cells that express the endogenous protein, have revealed that it has a largely intracellular localisation in multivesicular bodies (Capdevila et al., 1994; Denef et al., 2000; Strutt et al., 2001). On the other hand, overexpression of mammalian PTC1 in cell culture, including the data presented by Barnes and colleagues, have shown that PTC1 ‘atypically’ decorates the plasma membrane (Carpenter et al., 1998; Stone et al., 1996) or, in common with *Drosophila* PTC, is present in cytoplasmic vesicles (Incardona et al., 2002). Furthermore, given the caveat that even conserved regions of the PTC molecule can have distinct behaviours in different species (Johnson et al., 2002), it will be important to determine the integrity of this interaction in other systems.

Conclusion: HH, cell cycle and cancer

Much of the current interest and excitement that centres on the biology of HH signalling largely concerns its proven connection with several congenital abnormalities and disease conditions in humans. Perhaps the most menacing of these situations, and those that dramatically underscore the essential role that HH plays in controlling cell proliferation, are some of the commonest forms of cancer. For instance, mutations in PTC that abrogate its function or those in SMO that render it constitutively active, in combination with GLI1 and GLI2, have been abundantly implicated in the genesis of basal cell carcinomas (BCCs) of the skin as well as medulloblastomas of the cerebellum (Ruiz i Altaba et al., 2002). In addition, the fact that HH acts as a stem cell factor in various situations, such as in the *Drosophila* ovary (Zhang and Kalderon, 2001) and during haematopoiesis in vertebrates (Bhardwaj et al., 2001), opens up the possibility that erratically behaving stem cell populations contribute to the generation and growth of HH-signalling-induced tumours. Even though all these findings provide a firm genetic association between anomalous HH signalling and the incidence of cancer, we currently have very little cell biological perception of the underlying mechanisms that actually link these two processes. It is highly likely that the effects of HH in these circumstances, as in situations of developmental cell proliferation, are mediated through its multiple effects directly on the cell cycle machinery or indirect consequences of the misregulation of intermediate mitogenic signals and growth factors.

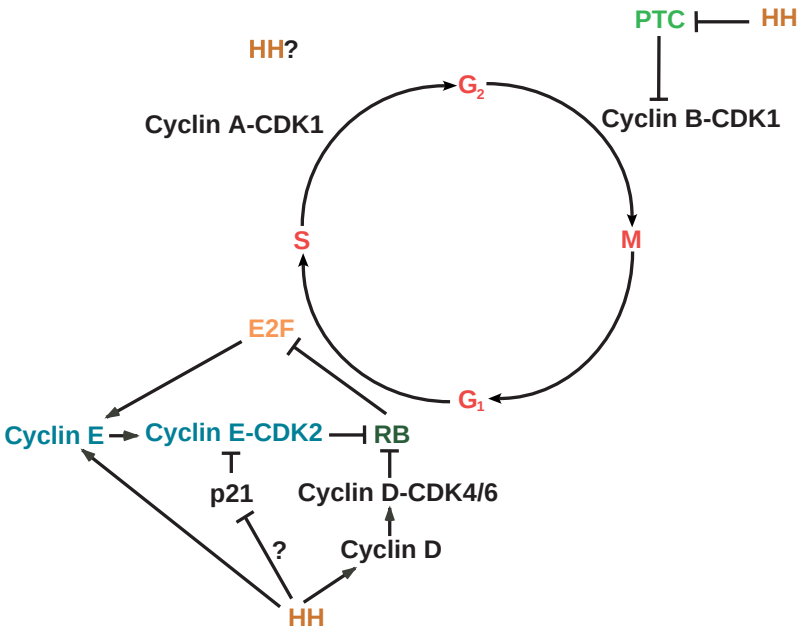

Fig. 3. HH influences the expression and activity of core cell cycle components at multiple points within the cell cycle. This figure summarises our current understanding of this regulation.

Clearly, these are the beginnings of our appreciation of how HH influences the cell cycle and cell growth, and the studies discussed here should serve to reinforce how little we know about this important aspect of HH function. Nevertheless, taking into consideration the morbid effects of unrestrained HH signalling, these new data are particularly significant. We have to wait with eager anticipation for the emergence of a more lucid picture that integrates all these recent findings and for the identification of other ways by which HH can control such processes and contribute to carcinogenesis in situations of inappropriate signalling activity.

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