EMBO Molecular Medicine

Mouse models and mouse supermodels

Varnat et al, EMBO Mol Med, 1, 338–351, report that Hedgehog (HH) signalling is essential for growth of human colon carcinoma (CC) (Varnat et al, 2009). In the accompanying Closeup article (Gulino et al, 2009) state that while participation of the HH pathway in CC has been controversial because of conflicting and contradictory data (Varnat et al, 2009) resolve this controversy. Although I agree the study resolves the controversy, it is not for the reasons stated by Varnat et al and it is not resolved in their favour. As often the case in scientific disputes, the devil is buried deep in the methodological details.

Based on genetic studies, the HH pathway clearly plays a critical role in basal cell carcinoma (BCC) and in a subset of medulloblastoma (MB) (Rubin & de Sauvage, 2006). Preclinical studies, in a spontaneous mouse MB model, demonstrated that oral delivery of a small molecule inhibitor (HhAntag) of Smoothened (SMO) eliminated even large MB very efficiently (Romer et al, 2004). Recent clinical trials of another inhibitor, GDC-0449, validated this approach, reporting remarkable successes in a Phase I setting (Rudin et al, 2009; Von Hoff et al, 2009). As discussed in Varnat et al, in contrast to BCC and MB, mixed preclinical results have been reported for a range of human tumours, including CC, melanoma, prostate cancer, pancreatic tumours, glioma, etc. that do not harbour HH pathway mutations, yet exhibit elevated pathway activity. The majority of these studies rely on the use of tumour cell lines and xenografts treated with SMO inhibitors, particularly the naturally occurring teratogen, cyclopamine. The most extensive investigation of a broad range tumour cell lines and xenografts to date, including CC, failed to confirm these reports, although it did identify a few transplanted tumours in which the HH pathway activity was elevated in mouse stromal cells (Yauch et al, 2008). These lines exhibited a modest growth inhibition when treated with a SMO inhibitor due to a paracrine response of stromal cells to HH ligands secreted by the tumour cells.

The conclusions reported by Varnat et al depend on data obtained using cyclopamine to treat CC cells cultured *in vitro* and in xenograft transplants. However, cyclopamine is known to be a highly toxic compound, which is why it was not a good candidate for development as a pharmaceutical. In culture, it exhibits non-specific cytotoxic affects at concentrations above 1 μM (Romer et al, 2004; Yauch et al, 2008), yet Varnat et al use doses up to 10 μM to show growth inhibition of CC cells. Furthermore, as previously pointed out, placing MB tumour cells into monolayer culture results in suppression of the HH pathway (Sasai et al, 2006). This could be an issue for all cultured tumour cells, but the use of highly sensitive assays to monitor expression levels of HH pathway target genes may give the false impression that the pathway remains active and responsive to cyclopamine. Thus, the CC studies in cell culture reported by Varnat et al utilized cyclopamine at a level associated with non-specific growth inhibition and, in any case, the HH pathway may not have been active.

Cyclopamine also displays a narrow therapeutic index *in vivo*. Previously, we were unable to reach a systemic level of cyclopamine that completely suppressed the HH pathway, in a mouse functional imaging model, due to toxic side effects (Kimura et al, 2008). In contrast, Varnat et al report dramatic effects of cyclopamine on tumour regression in xenograft models (Fig 4). Xenograft tumour models have a venerable but checkered history in the development of anticancer drugs. Nevertheless, they play a central role in the ongoing pediatric preclinical testing programme (PPTP). This programme uses defined xenograft models, following procedures approved by the National Cancer Institute, for drug testing (Houghton et al, 2007). These published procedures differ in substantial ways from the approach used by Varnat et al to test the effect of cyclopamine on MB.

The standard recommended xenograft approach involves propagating tumours subcutaneously in immunosuppressed mice until they reach a size of 200–500 mm^3 before treating them systemically, usually by intraperitoneal or oral delivery, of the drug being tested. This contrasts with the approach stated by Varnat et al, 'As soon as the tumour was palpable (1–2 mm), cyclodextrin-conjugated cyclopamine or cyclodextrin alone (Sigma) was injected intra- and/or peritumourally (sic) at 10 mg/kg once daily'. Thus, Varnat et al treated small tumours of less than 10 mm^3, which means the tumours were not yet fully established, by repeated injection directly into the tumour mass. Under these circumstances, it is impossible to determine the actual concentration of cyclopamine delivered to tumour cells, but it would clearly be much higher than the non-specific toxic dose. Furthermore, the tumour tissue would receive extensive damage from the multiple needle injections and the hydrostatic pressure from the inoculums volume. These effects explain why Varnat et al observed a response to cyclopamine in CC xenografts, whereas other investigators, who delivered SMO inhibitors systemically, did not. This flaw in the use of xenograft models may be prevalent in the scientific community, as many groups have published positive results from the use of cyclopamine in xenografts. Based on such results it has been estimated that HH pathway signaling is responsible for 25% of all human cancer deaths (Lum & Beachy, 2004). These hyperbolic calculations raised revenue expectations for HH pathway inhibitors to giddy heights and prompted many pharmaceutical companies to invest in development of SMO inhibitors for the treatment of cancer. Therefore, I feel it is important to draw attention to this issue and to request investigators to include the details of their mouse model treatment regimes in each publication.

Thus, it is clear that not all mouse models are created equal. While it may be appropriate to compare results from

studies carried out in models that test compounds by direct inoculation of small xenograft tumours, it is not appropriate to compare these data with results obtained from supermodels in which large tumour volumes were challenged by physiological, systemic drug delivery. The question remains which model is more predictive of clinical outcome? Like the fashion industry, I think I will place my money on the supermodels.

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DOI 10.1002/emmm.201000090

On hedgehogs and human cancer

Ariel Ruiz i Altaba and Frédéric Varnat thank the editor for inviting a response to Dr. Curran's letter, which questions the specificity of cyclopamine and the xenografts used in our recent work on human sporadic colon carcinomas (CCs) (Varnat et al, 2009). To respond, in detail, we address five issues.

First, a key issue is when and where is the pathway active in order to know if effects of HH-GLI antagonists can be specific. We and others established that *GLI1* transcription is, so far, the only reliable and general HEDGEHOG-GLI (HH-GLI) pathway activation marker (*e.g.* Lee et al, 1997), and that it is expressed in many kinds of sporadic human tumours. Antisense ribonucleic acid (RNA) *in situ* hybridizations on fresh patient-derived tissues have unambiguously shown the tumour cells themselves, not the surrounding stroma, express *GLI1* and other HH-GLI pathway components in basal cell carcinomas, melanomas, gliomas, medulloblastomas, prostate and colon cancers (Clement et al, 2007; Dahmane et al, 1997; Sanchez et al, 2004; Stecca et al, 2007; Varnat et al, 2009). Recent *in situ* hybridization images of pancreatic cancer are undecisive (see Lauth et al, 2010). In cases where antibodies to pathway components have been used, strong labelling is found in the tumour cells themselves (*e.g.* Varnat et al, 2009; see also www.proteinatlas.org). So far there is no evidence of exclusive pathway activity in human stromal cells. In contrast, mice display paracrine Hh signalling in several organs but the use of human xenografts to determine mode of action appears inappropriate as these are chimeras. The epithelial tumor cells of human carcinomas are thus main targets of HH-GLI antagonists.

Second, is cyclopamine specific? The plant alkaloid cyclopamine, discovered by R. Keeler over 40 years ago has been extensively used (with over 400 entries in PubMed) to specifically block HH signalling. Cyclopamine induces cyclopia in newborns from mothers of several species that eat the producing plant *Veratrum californicum*, the mouse Shh KO mimics this phenotype and the labs of Roelink and Beachy showed that cyclopamine acts on cells that receive the Shh signal by blocking Smoothened (Smo; SMOH in humans) a key transducer of Hh signals (*e.g.* Chen et al, 2002).

Cyclopamine can block HH-GLI signalling in human sporadic tumour cells *in vitro*, specifically decreasing *GLI1* messenger RNA (mRNA) levels, at concentrations ranging from 1 to 10 μM showing dose-dependent effects, modulated by serum levels. The best tests for specificity rely on mimicry by targeting SMOH with RNA interference (RNAi), the use of insensitive SMOH mutants and pathway epistatic analyses. Targeting SMOH through lentivector-mediated short hairpin RNA (shRNA) silencing mimics the effects of cyclopamine in multiple human tumour cells (Clement et al, 2007; Stecca et al, 2007; Varnat et al, 2009). The effects of 1–10 μM cyclopamine in CCs are rescued by expressing GLI1 or inhibiting suppressor of fused, positive and negative pathway elements, respectively, that act downstream of SMOH (Varnat et al, 2009; Frédéric Varnat and Ariel Ruiz i Altaba in preparation). *SMOH* RNAi is also rescued by GLI1 expression in gliomas and CCs (Clement et al 2007; Varnat et al, 2009). Furthermore, expression of a constitutively active form of Smo (SmoA1) renders cells insensitive to 5 μM cyclopamine (Kim et al, 2010), and expression of N-Myc, a GLI target, in cerebellar cells rescues the effects of cyclopamine (Kessler et al, 2009). Claims that treatments with 1–10 μM cyclopamine are universally toxic or non-specific are thus unfounded.

While Sasai et al (2006) find that HH signalling is repressed in medulloblastoma cells *in vitro*, several labs have proven activity in these cells (*e.g.* Eberhart, Gulino, Watkins). Indeed, results from over 35 labs (including those of Beachy, Dierks, Ingham, Nusslein-Volhard, Matsui, Melton, Reya, Robbins, Roussel, Scott, Tabin and Watkins) on species ranging from humans to fish support the specificity of this drug.

Third, is our conclusion that HH-GLI signalling is essential in human sporadic CCs based *solely* on data with cyclopamine? No. We have provided parallel lines of evidence in favour of a key role of HH-GLI in tumour cells using RNAi: independent, 21-nucleotide siRNAs to *GLI1* block prostate cancer cell proliferation and lentivector-encoded shRNAs to *SMOH* or expression of GLI3R block melanoma, glioma and CC cancer proliferation, promoting apoptosis (Clement et al, 2007; Sanchez et al, 2004; Stecca

