C. hominis will stimulate progress in research on this organism and its pathogenicity, and strategies for intervention in the diseases it causes. □

Methods

A modified whole-genome shotgun strategy was used to sequence the ~9.2-Mb genome of *C. hominis* isolate TU502, which was derived from an infected child from Uganda. DNA was purified from surface-sterilized oocysts, shotgun and BAC clones were constructed, and end sequences were generated. About 220,000 sequence reads from small insert clones, and end sequences from ~2,000 BAC clones averaging ~35 kbp in size, were generated. The data represents a ~12-fold shotgun clone coverage of the genome with a quality score of Phred 20, and a 7–8-fold coverage with BAC clones. The sequences were assembled with Phrap, yielding a ~9.16-Mb assembly, which was structurally and functionally analysed with a variety of available software programs and in-house scripts (see Supplementary Text 1 and 2 for further details and references).

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MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer

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Hepatocellular carcinoma is generally refractory to clinical treatment[1]. Here, we report that inactivation of the MYC oncogene is sufficient to induce sustained regression of invasive liver cancers. MYC inactivation resulted *en masse* in tumour cells differentiating into hepatocytes and biliary cells forming bile duct structures, and this was associated with rapid loss of expression of the tumour marker α-fetoprotein, the increase in expression of liver cell markers cytokeratin 8 and carcinoembryonic antigen, and in some cells the liver stem cell marker cytokeratin 19. Using *in vivo* bioluminescence imaging we found that many of these tumour cells remained dormant as long as MYC remain inactivated; however, MYC reactivation immediately restored their neoplastic features. Using array comparative genomic hybridization we confirmed that these dormant liver cells and the restored tumour retained the identical molecular signature and hence were clonally derived from the tumour cells. Our results show how oncogene inactivation may reverse tumorigenesis in the most clinically difficult cancers. Oncogene inactivation uncovers the pluripotent capacity of tumours to differentiate into normal cellular lineages and tissue structures, while retaining their latent potential to become cancerous, and hence existing in a state of tumour dormancy.

Cancer is largely caused by genomic catastrophes that result in the activation of proto-oncogenes and/or inactivation of tumour-suppressor genes[2]. Even brief inactivation of a single oncogene can

be sufficient to induce sustained tumour regression[3], indicating that, at least in some cases, oncogene inactivation may result in the permanent loss of a neoplastic phenotype[4]. Liver cancer is one of the most common solid malignancies in the world[5], with no effective treatment for most of the individuals who succumb to this neoplasm[1]. One of the most commonly activated oncogenes associated with the pathogenesis of liver tumours is the MYC oncogene. Animal models have confirmed that overexpression of MYC can induce hepatocellular carcinoma[6–8], whereas inhibition of MYC expression results in a loss of the carcinoma's neoplastic properties[9]. To address the possibility that MYC inactivation may be effective in treating liver cancer, we have developed a conditional transgenic model whereby we can regulate expression of human MYC in murine liver cells.

We used the Tet system[10] to generate transgenic mice that conditionally express the MYC proto-oncogene in liver cells. We crossed TRE-MYC[11] mice with a transgenic line, LAP-tTA[10], where the liver activator protein (LAP) promoter drives expression of the

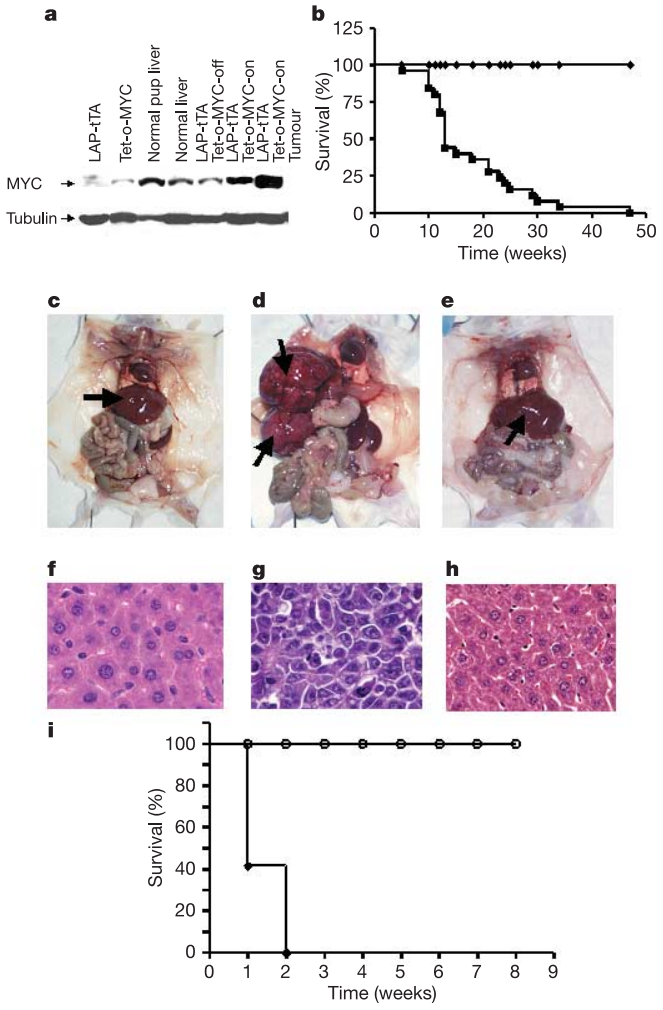

Figure 1 Conditional MYC overexpression in the liver induces hepatocellular cancer whereas MYC inactivation results in sustained tumour regression. **a**, Western analysis for MYC transgene expression. Mice transgenic for both LAP-tTA and tet-o-MYC overexpress MYC in the liver, but mice transgenic for LAP-tTA alone, or where MYC is inactivated, do not. Normal adult and pup livers were used as controls. **b**, Kaplan–Meyer survival curve comparing survival of transgenic mice in the presence (squares) or absence (diamonds) of MYC transgene expression. MYC transgene expression was induced in 3-week-old mice by removing doxycycline treatment. Each cohort consists of 25 mice. **c**, **f**, Mice transgenic for both LAP-tTA and tet-o-MYC and treated with doxycycline exhibited a normal liver grossly (**c**) and histologically (**f**). **d**, **g**, Mice transgenic for both LAP-tTA and tet-o-MYC that have not been treated with doxycycline succumb to multi-focal liver tumours (**d**) that histologically (**g**) are hepatocellular cancers. **e**, **h**, Mice with liver tumours that are subsequently treated with doxycycline to suppress the MYC transgene undergo complete regression of their tumours (**e**) and histologically (**h**) the liver appears normal. **i**, Kaplan–Meyer survival curve of mice with liver tumours that are either not treated (diamonds) or treated with doxycycline to suppress MYC transgene expression (circles). Each cohort consisted of ten mice.

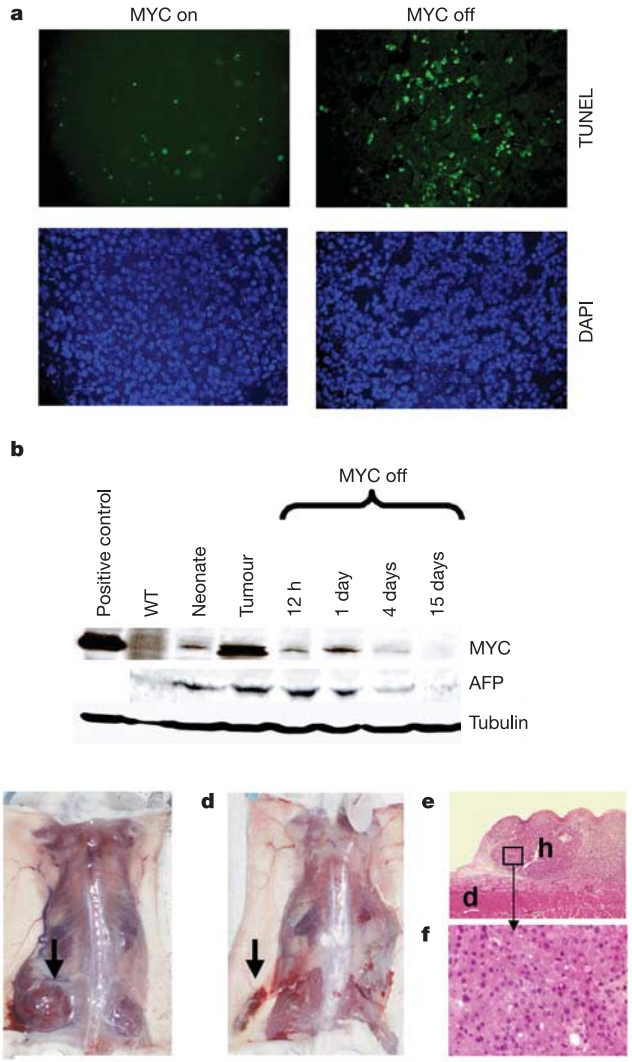

Figure 2 MYC inactivation in liver tumours results in rapid tumour regression associated with loss of expression of tumour markers, differentiation and apoptosis. **a**, TUNEL assay of a liver tumour before and after MYC inactivation. Upper panels show TUNEL staining and lower panels show 4,6-diamidino-2-phenylindole (DAPI) staining of nuclei. Representative data from one of four experiments is shown. MYC inactivation is associated with the differentiation of liver tumour cells into normal hepatocytes. **b**, Western blot analysis for expression of MYC and AFP in normal wild-type (WT) mouse liver, liver of neonatal mice, liver tumour with MYC overexpression and liver tumour where MYC has been inactivated for the indicated periods of time. **c**, Liver tumour cells were transplanted subcutaneously into a SCID mouse (arrow). **d**, MYC inactivation results in tumour regression of the transplanted tumour. **e**, Histological analysis of the tumour site reveals normal-looking hepatocytes (marked as h) within the epidermis (marked as d). **f**, Higher magnification of the differentiated hepatocytes. The experiment was performed five times in six different transgenic tumours in five mice per cohort.

tetracycline trans-activating protein (tTA) in liver cells. Progeny possessing both transgenes expressed MYC, whereas mice with either transgene alone or mice with both transgenes and treated with doxycycline did not express MYC (Fig. 1a). We activated MYC transgene expression in 3-week-old mice by discontinuing doxycycline treatment. Subsequently, all transgenic mice that overexpressed MYC succumbed to liver tumours (Fig. 1b, d, e) with a mean latency of tumour onset of 12 weeks, whereas transgenic mice continuously treated with doxycycline remained free of disease (Fig. 1b, c, f). High levels of MYC expression were detected in tumours but not in liver cells from mice continuously treated with doxycycline, as measured by western analysis and immunohistochemistry (Fig. 1a; see also Supplementary Fig. 1a). Thus in this LAP-tTA/tet-off MYC conditional transgenic mouse model overexpression of MYC in adult mice can reproducibly induce liver cancer. Our results are consistent with previous reports that a MYC transgene can induce liver cancer[6–8].

Upon histological analysis the MYC-induced tumours resembled hepatocellular carcinomas and/or hepatoblastomas. The transgenic tumours were locally invasive throughout the liver, frequently associated with malignant peritoneal effusions that spread via metastasis into the thoracic cavity with invasion into the parenchyma of the lungs. Tumours were readily transplantable into SCID (severe combined immunodeficient) mice, as described below. Liver cancer is particularly refractory to therapeutic intervention[1,5,12], therefore, we anticipated that oncogene inactivation in a liver tumour would be even less effective in causing tumour regression than in other types of cancer. Surprisingly, all transgenic mice ($n = 50$) that were moribund with liver tumours exhibited rapid and sustained tumour regression when treated with doxycycline to inactivate MYC transgene expression (Fig. 1i). Upon gross examination 4 weeks after MYC inactivation there was no evidence of tumour persistence, where gross and microscopic liver morphology had been restored to normal (Fig. 1e, h). Thus, the targeted inactivation of MYC alone may effectively induce sustained regression of MYC-induced hepatocellular neoplasia.

To examine the mechanism by which MYC inactivation induces the regression of liver tumours, we treated moribund mice with doxycycline. Within 4 days of MYC inactivation the liver tumours differentiated into normal liver cells accompanied by apoptosis, as

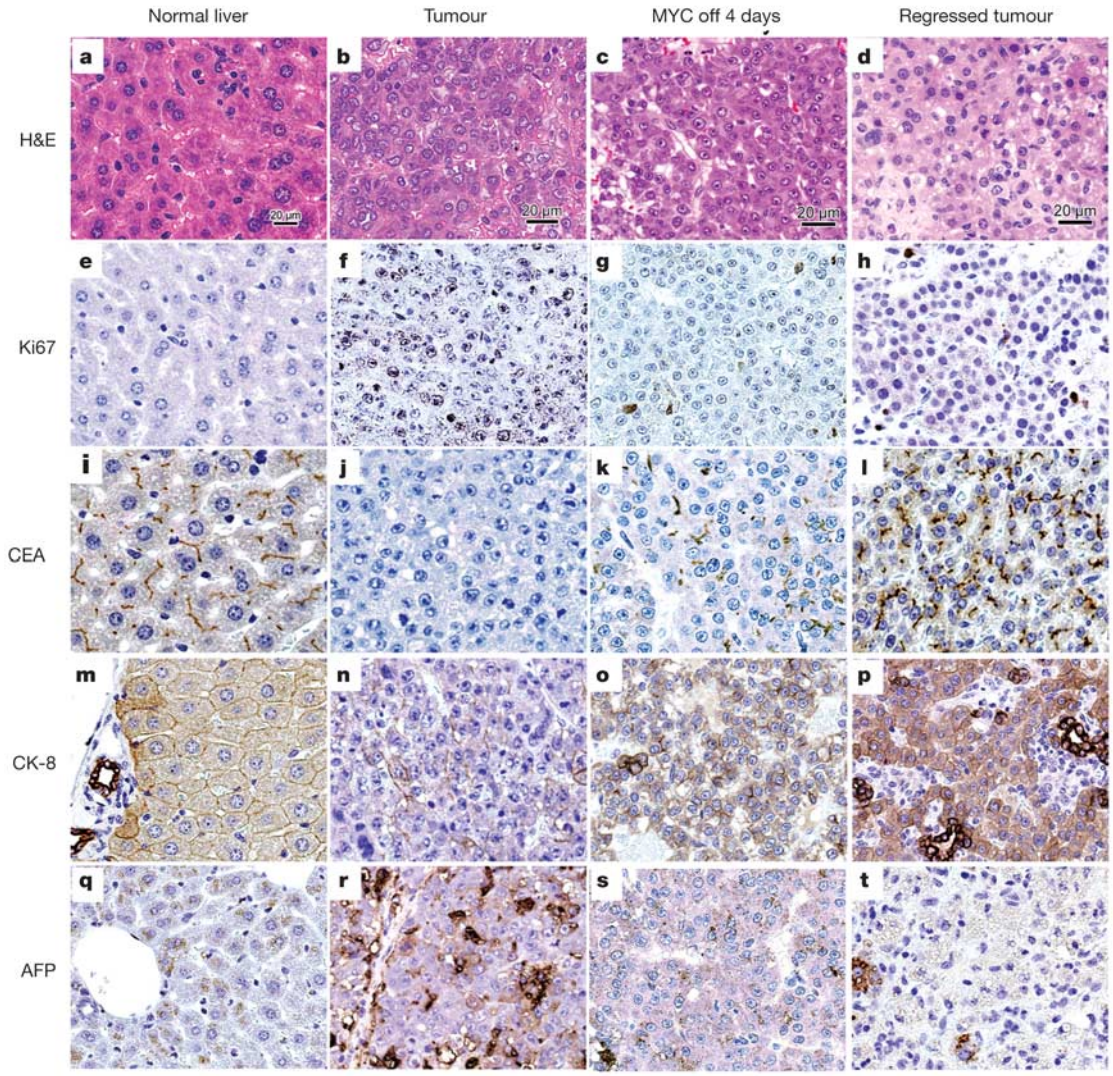

Figure 3 MYC inactivation in liver tumours results in the formation of normal hepatic structures. **a**–**t**, Normal liver (**a**, **e**, **i**, **m**, **q**), MYC-overexpressing tumour (**b**, **f**, **j**, **n**, **r**), tumour where MYC has been inactivated for 4 days (**c**, **g**, **k**, **o**, **s**) and regressed tumour (**d**, **h**, **l**, **p**, **t**). Serial sections were stained with haematoxylin and eosin (H&E; **a–d**). Immunohistochemical analysis for markers as indicated: **e**–**h**, Ki67; **i**–**l**, CEA; **m**–**p**, CK-8; and **q**–**t**, AFP. Representative data are shown from one of three experiments. See also http://imagearchive.compmed.ucdavis.edu/publications/Shachaf.

determined by TdT-mediated dUTP nick end labelling (TUNEL) assay (Fig. 2a). Within 2 weeks most of the tumour had grossly regressed, demonstrating that MYC inactivation results in the rapid elimination of most of the tumour cells.

To evaluate unambiguously the fate of tumour cells upon MYC inactivation, we transplanted tumour cell suspensions into SCID mice. After tumours were engrafted into these animals MYC was inactivated. One day after MYC inactivation MYC expression had decreased to 30% of previous levels, and after 4 days it was almost undetectable (Fig. 2b). Similarly, protein expression of the embryonic tumour cell marker characteristic for liver cancer, α-fetoprotein (AFP), was reduced to 50% of previous levels after 1 day and virtually abolished after 4 days. Tumours began to regress within the first 3 days after MYC inactivation and completely regressed within 30 days, with a residual scar persisting at the site of initial transplantation (Fig. 2d). Notably, histological examination of scar tissue present after MYC inactivation at the site of tumour transplantation in the skin of SCID mice revealed normal liver cells (Fig. 2e, f) resembling hepatic lobules (see also below and Fig. 3). We inferred that MYC inactivation might induce the differentiation of liver cancers into normal liver cells. A trivial possible explanation for our results is that normal liver cells were being transplanted along with cancer cells. This seems to be unlikely as MYC inactivation was observed to induce the differentiation of tumours even when they had been serially transplanted into SCID mice for five passages over a 2-yr time span, beyond the expected duration of proliferative capacity for any normal cells. Tumour cells derived from a distant lung metastasis were also able to differentiate into

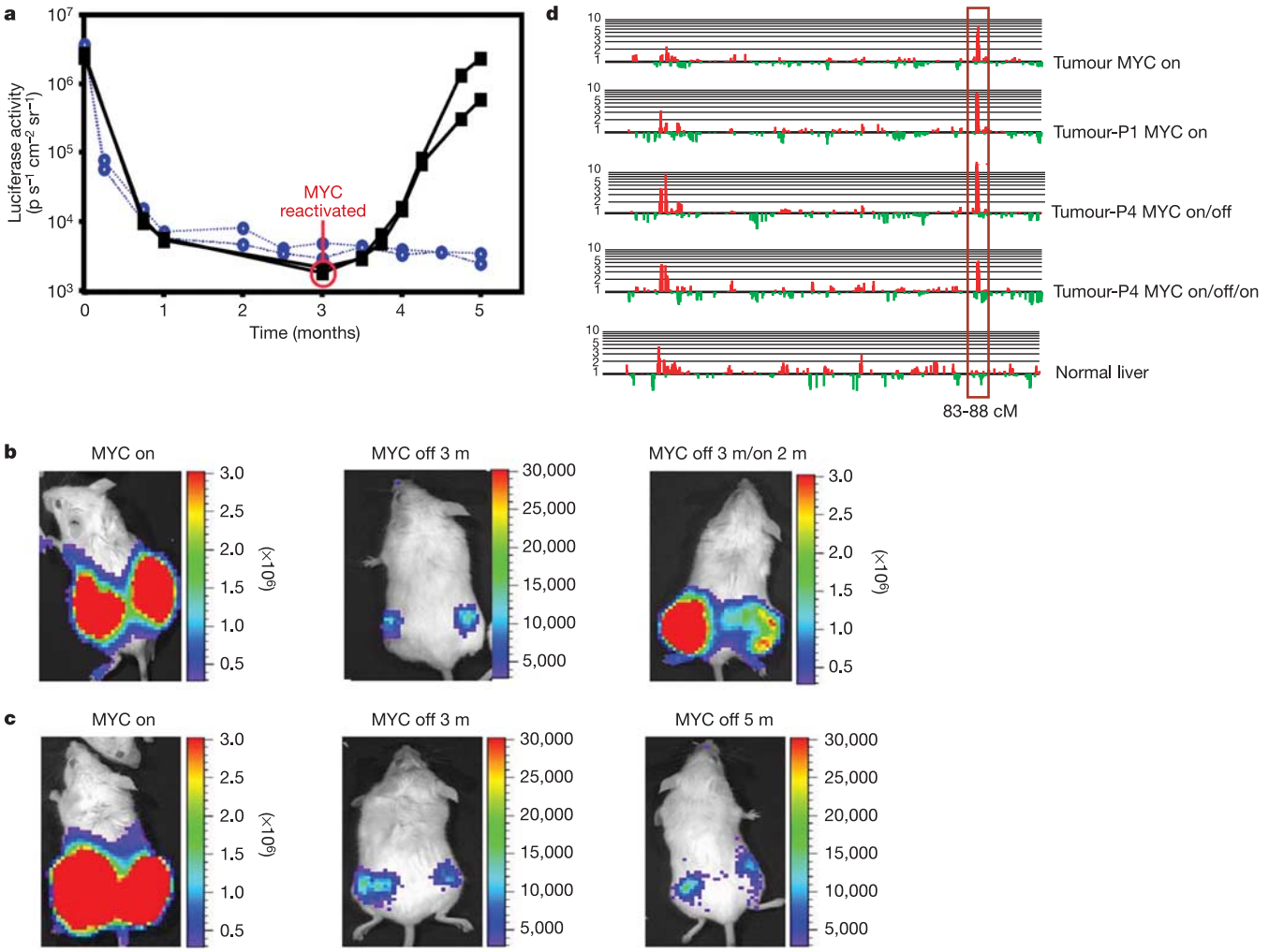

Figure 4 Tumour dormancy observed in liver tumours after MYC inactivation. **a**, Kinetics of tumour regression using *in vivo* bioluminescence imaging of luciferase-labelled liver tumours. Transplanted tumours undergo rapid regression but residual, persisting luciferase activity remains at the site of tumour growth. Upon MYC reactivation tumour growth re-occurred. For visualization of tumour growth, a pseudocolour image representing luciferase light intensity is superimposed over a greyscale reference image of the representative animals in each treatment group: squares, MYC on, then MYC off, and finally MYC on; circles, MYC on then MYC off. Luciferase activity is measured in photons per cm^2 per s per steradian (p cm^{-2} s^{-1} sr^{-1}). **b**, Representative pictures for mouse where MYC is on (left), MYC is on and then off for 3 months (3 m) (centre), and MYC is on, off for 3 months and then reactivated for 2 months (right). **c**, A representative control mouse is represented for the same time points: MYC on (left), MYC on then off for 3 months (centre), and MYC remains off for 5 months (right). Data are representative of five different experiments with 1–10 animals in each group. **d**, Array CGH analysis of tumours before and after MYC inactivation and reactivation. Array data are shown for a primary tumour tissue (tumour MYC on), transplanted tumour (tumour-P1 MYC on), transplanted tumour (passage 4) where MYC is inactivated (tumour-P4 MYC on/off), transplanted tumour (passage 4) where MYC is reactivated (tumour-P4 MYC on/off/on) and normal liver tissue (normal liver). DNA copy number for chromosome 2 genes is displayed graphically as a ratio (tumour/normal) on a $\log_{10}$ scale along the chromosome. The boxed area marks the region of genomic amplification identified in chromosome 2 shared by this tumour. Red represents gain in gene copy number and green represents deletion in gene copy number.

liver tissues when transplanted into SCID mice. Finally, normal hepatocytes were found to be incapable of persisting beyond 5 days when transplanted into SCID mice (see below). We conclude that upon MYC inactivation, tumours can differentiate into normal liver cells.

To validate further that MYC inactivation induces the differentiation of liver tumour cells, we performed a careful kinetic analysis of the effect of MYC inactivation. Within 4 days most of the tumour cells differentiated into normal hepatocytes and formed normal liver structures (Fig. 3). Tumours lost their neoplastic histological features such as high mitotic index, large nucleoli and hyperchromasia, and they now exhibited a normal nuclear/cytoplasmic ratio. The differentiated tumour cells were Ki67-negative, consistent with their reduced rate of cellular proliferation. Most of the tumour cells lost expression of the immature differentiation marker AFP and instead expressed the hepatocyte and biliary cell liver markers carcinoembryonic antigen (CEA) and cytokeratin 8 (CK-8)[13], consistent with the formation of sinusoids, bile canaliculi and bile duct cells. Even after prolonged MYC inactivation for up to 8 months some of these differentiated tumour cells persisted, growing under the skin of a SCID mouse (Fig. 3). Moreover, some cells acquired the liver stem cell marker cytokeratin 19 (CK-19)[13–15] (Supplementary Fig. 2). Thus, upon MYC inactivation most of the liver tumour cells are able to differentiate into hepatocytes and biliary cells, forming bile duct structures.

Next, we examined the consequences of the reactivation of MYC expression. Within 2 weeks of MYC reactivation there was gross evidence for tumour re-growth. Tumours were found to have identical histology to the original transplanted tumour (Supplementary Fig. 1c). These tumours were still dependent on MYC transgene expression, as inactivation of the transgene with doxycycline resulted in tumour regression (Supplementary Fig. 3). Hence, MYC inactivation results in the differentiation of liver tumours, but these tumours retain the latent capacity to regain their neoplastic features upon MYC reactivation.

To examine better the consequences of MYC inactivation and reactivation *in vivo*, we used *in vivo* bioluminescent imaging[16] to visualize tumour growth and response to therapy. We generated liver tumours that were also transgenic for firefly luciferase by crossing the LAP-tTA/tet-o-MYC mice with CMV-GFP-LUC mice[16]. The number of tumour cells transplanted and the size of tumour correlated with the light emitted by luciferase activity, allowing us to quantitatively detect as few as 1,000 tumour cells (Supplementary Fig. 4) and to non-invasively examine tumour cell growth and regression in real time.

At 8 months after MYC inactivation luciferase activity was still detectable even when the tumour was not grossly observable (Fig. 4a–c; see also Supplementary Fig. 4). In contrast, normal luciferase-positive liver cells (4×10^7) overexpressing MYC were not detectable 5 days after transplantation into SCID mice. Even when luciferase-positive normal liver cells were transplanted together with luciferase-negative tumour cells, normal hepatocytes were not detectable after 5 days (Supplementary Fig. 5). When MYC expression was restored in the differentiated tumour cells they immediately regained the capacity for proliferation, as demonstrated by an increase in luciferase activity (Fig. 4), and eventually formed grossly visible tumours. Moreover, these tumour cells retained their dependence on MYC expression, as the resumption of doxycycline treatment resulted again in tumour regression (Supplementary Fig. 3). We conclude that MYC inactivation induces a state of tumour dormancy and MYC reactivation is sufficient to restore tumorigenesis.

To confirm further that the liver cells observed upon MYC inactivation and the tumour cells observed upon MYC reactivation were derived from the original tumour cells, array comparative genomic hybridization (CGH) was used to evaluate the presence of genomic signatures. The primary tumour, the serially transplanted tumour, the regressed tumour after MYC inactivation and the restored tumour after MYC reactivation all possessed the identical regional amplification in a region of chromosome 2 (2H1 83–88 cM) (Fig. 4d). These genetic alterations were unique to this tumour. Two other tumours examined were found to have different changes. Hence, the differentiated cells and the restored tumour are all clonally related to the original tumour.

We have demonstrated that highly invasive and malignant liver cancers exhibit rapid and sustained tumour regression upon MYC inactivation. Loss of MYC expression resulted in the differentiation of tumour cells and eventually most of the cells underwent death. However, tumour cells retained the capacity to differentiate and form normal liver. The reactivation of MYC in these cells restored their neoplastic properties. Hence, MYC inactivation in liver tumours can result in the differentiation of tumour cells into normal liver, but some of these apparently normal cells remain in a state of tumour dormancy.

The diagnosis of invasive liver cancer portends a dismal prognosis and is not amenable to existing therapeutic modalities[5,12]. Our results suggest that the targeted inactivation of the MYC oncogene may be an effective strategy for the treatment of some liver cancers. In contrast to what we have observed with haematopoietic tumours[4,11], only after several serial transplantations did the liver tumours rarely relapse after prolonged MYC inactivation. Relapsed tumours were found to express compensatory, increased levels of L- and N-MYC (Supplementary Fig. 6). It remains to be determined whether MYC inactivation will be effective in the treatment of human liver cancers.

When released from the influence of MYC overexpression, liver tumours *en masse* were able to resume a physiological programme and differentiated into normal hepatic lineages including hepatocytes and biliary cells. Thus, the normalization of MYC expression in liver cancers uncovers their pluripotent capacity to differentiate into normal cellular lineages. Although MYC inactivation resulted in the differentiation and sustained tumour regression of tumours, MYC reactivation was capable of immediately restoring neoplastic properties. Hence, MYC inactivation produces a state of tumour dormancy. Many reports indicate that tumour cells can revert to a state of tumour dormancy[17–19]. Clinically, it is frequently observed that after therapy tumours exist in a latent state and even after many years are still capable of reverting back to a neoplastic state[20,21]. Experimentally, tumour dormancy has been induced by means of the suppression of angiogenesis[22,23] or treatment with anti-idiotypic antibodies[17]. We provide here the first report to our knowledge showing that oncogene inactivation can induce tumour dormancy. Liver tumour cells retained the ability to differentiate into multiple hepatic lineages and thus may exist as dormant cancer stem cells[23–25]. In general, tumour dormancy probably reflects changes in epigenetic regulation associated with the differentiation of tumour cells[3,26,27].

The consequences of MYC inactivation apparently depends upon the cellular programming associated with each type of cancer. MYC inactivation in haematopoietic tumours induces differentiation followed by robust apoptosis, which seems to be usually associated with the complete elimination of tumour cells[11]. Haematopoietic cells seem more poised to undergo apoptosis and renewal from the bone marrow compartment of stem cells as part of their normal physiological programme. MYC inactivation in osteogenic sarcoma is associated with the differentiation of tumour cells into mature bone, but is not associated with apoptosis[4]. Bone cells may be more apt to undergo terminal differentiation without apoptosis. Here, MYC inactivation in liver tumours resulted in the differentiation and eventual death of most of the tumour cells, but some of the tumour cells appeared to have retained the potential to differentiate into multiple liver cellular lineages. However, MYC reactivation was capable of restoring the neoplastic properties to some of these differentiated liver cells, revealing that they existed in a state of

tumour dormancy.

Liver cancers may respond differently from other tumours to oncogene inactivation because the liver has the intrinsic ability to regenerate itself, demonstrating that the liver maintains stem cells. Liver tumours may retain stem cell properties, remaining poised upon MYC inactivation to rapidly differentiate into normal-appearing liver parenchymal cells and duct-like structures. Many recent studies report that cancers frequently consist of cellular subpopulations, some of which have retained stem cell properties and are derived from these cells[23–25]. MYC seems to be an example of an oncogene that sustains malignant transformation by transforming cells that retain their capacity for cellular differentiation.

Our results suggest a possible model for how MYC activation induces and sustains tumorigenesis in the liver (Supplementary Fig. 7). MYC seems to result in the malignant expansion of immature liver cells with stem cell features, consistent with previous reports suggesting that liver tumours arise from stem cells[13,14]. Upon MYC inactivation tumour cells began to differentiate and many of them died, but some of the tumour cells showed stem cell properties and differentiated into normal liver. Among these differentiated tumour cells were retained the cancer stem cells, and upon MYC reactivation these cells were a possible source for the re-emergence of the tumour. A less likely possibility for our results is that MYC reactivation resulted in the de-differentiation of the mature hepatocytes that gave rise to tumours. The consequences of oncogene inactivation and reactivation in a given tumour may depend upon the properties of the cellular lineage that has undergone tumorigenesis.

We conclude that there are circumstances when the abatement of oncogene activation is sufficient to resume a normal physiological programme even in cancer cells. Our model system will provide a strategy to interrogate generally how oncogene inactivation uncovers the pluripotent differentiation of cancers and specifically identify the putative liver cancer stem cell. □

Methods

Transgenic mice

Tet-o-MYC transgenic mice have been described previously[4,11]. LAP-tTA mice[10] were provided by H. Bujard. The generation of transgenic CMV-GFP-LUC mice on a FVB background has been described previously[16].

Tumorigenicity assays

To suppress *MYC* transgene expression, mice received doxycycline in their drinking water, changed once per week, at a concentration of 100 μg ml^{-1}. For transplantation experiments, tumours were prepared as single cell suspensions (adapted from ref. 28) by incubating liver tumour pieces in HBSS followed by digestion in 1.5 mg ml^{-1} collagenase in 3 mM KCl, 5 mM NaH_2PO_4, 130 mM NaCl, 10 mM dextrose monohydrate. Cells were washed in PBS twice and re-suspended in PBS, and 10^7 cells were transplanted subcutaneously into SCID mice.

Histology and immunohistochemistry

Tissues were fixed in 10% buffered formalin, paraffin-embedded and 5-μm paraffin sections were stained with haematoxylin and eosin. Staining was performed using conventional methods fully described in Supplementary Methods.

Western blots

Western analysis was performed using conventional techniques. MYC protein expression was detected using the sc-788 antibody (SantaCruz Biotechnology), AFP (sc-15375, SantaCruz Biotechnology) and α-tubulin (CP06, Oncogene).

In vivo bioluminescence imaging

Transgenic mice were anaesthetized either by injection of a ketamine/xylazine solution (50 μl per 10 g) or by inhalation of an isoflurane/oxygen mixture delivered by the Xenogen XGI-8 5-port gas anaesthesia system. Imaging and image analysis were performed as described previously[29] using a cooled CCD camera system (IVIS-100, Xenogen) and LivingImage software (Xenogen).

Array CGH

Genomic DNA labelling, hybridizations and data analysis were performed as described[30], with the following modifications: 4 μg of DNA was labelled in a total volume of 50 μl. DNA from tumours were labelled with Cy5 and hybridized to mouse complementary DNA microarrays containing 20,199 different mouse genes (Unigene clusters). Normal FVB/N mouse spleen DNA labelled with Cy3 was used for reference. For the normal liver sample, normal (FVB/N) male liver DNA was labelled with Cy5 and normal (FVB/N) female DNA was labelled with Cy3 as reference.

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