# MODELLING THE MOLECULAR CIRCUITRY OF CANCER

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Cancer arises from a stepwise accumulation of genetic changes that liberates neoplastic cells from the homeostatic mechanisms that govern normal cell proliferation. In humans, at least four to six mutations are required to reach this state, but fewer seem to be required in mice. By rationalizing the shared and unique elements of human and mouse models of cancer, we should be able to identify the molecular circuits that function differently in humans and mice, and use this knowledge to improve existing models of cancer.

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doi:10.1038/nrc795

The cancer phenotype encompasses a broad collection of characteristics that together create the clinical entity of cancer. By comparing cells and tissues that are derived from cancer patients with those from normal individuals or, in some cases, with histologically normal tissue from the same individuals, it is possible to catalogue their many differences in molecular, cellular and biological properties. Indeed, the recent application of transcriptional profiling to cancer has documented changes in the expression of thousands of genes, as normal cells undergo transformation into their neoplastic derivatives[1–6]. Some of these changes in expression are shared by several types of cancer cell, whereas others seem to be specific to one or a small subset of the cancer-cell types that are encountered in the oncology clinic. Observations such as these have led some to propose that cancer is too complex a disease to rationalize in terms of a small number of underlying principles that govern the countless changes in cancer-cell genotype and phenotype[7].

In contrast to this apparently impenetrable thicket of complexity, several lines of investigation indicate that the emergence of all cancers from normal precursor tissues is governed by a common set of mechanisms that are limited in number[8]. Many of these mechanisms are now being unravelled in human and mouse models of cancer but, significantly, studies of human and mouse cancer biology yield conflicting conclusions about the number of distinct cellular alterations that are required to generate malignancies. Repeated observations indicate that the induction of tumours in mouse models of cancer depends on fewer genetic changes than are required in comparable tissue sites in humans. Understanding precisely how human and mouse models of cancer differ in molecular terms should lead to a clearer understanding of the complex nature of neoplastic transformation. What is our current understanding of the molecular circuitry that programmes malignant-cell transformation, and how can we reconcile the differences between human cell systems and animal models to develop models that truly reflect the molecular circuitry of human cancer?

## Multiple steps to cancer: transformation

Despite the apparent complexity of the cancer phenotype, early studies indicated that cancer might be the result of very few changes — perhaps as few as one — in the genome[9]. The manipulation of transforming retroviruses that infect mice and birds showed that the neoplastic phenotype could be conferred on mouse and avian cells by the introduction of viral genomes that consist of only a few genes, only one of which — a virus-borne oncogene — was directly involved in cell transformation[10]. Similarly, transfection of genomic DNA that was derived from human tumours[11–14] or from chemically transformed rodent cells[15–18] led to the identification of single oncogenes that could transform NIH-3T3 cells — a cell line of immortal mouse fibroblasts.

Further studies, however, soon revealed that a single gene mutation was rarely, if ever, sufficient to accomplish the entire process of transformation.

## Summary

- The process of malignant transformation occurs in discrete histopathological steps, many of which correlate with specific genetic alterations. Several lines of evidence implicate a limited number of molecular pathways, the disruption of which contributes to most, if not all, cancers.
- Rodent and human experimental models of cancer have contributed to our understanding of specific cancer-associated mutations. Although these cancer models share many essential components, several important signalling pathways seem to function differently in human and rodent models of transformation.
- Immortalization is an essential prerequisite for the formation of a tumour cell. Human cells must circumvent two barriers — replicative senescence and cellular crisis — that limit cell lifespan to achieve immortalization. These barriers are regulated by telomere shortening and by the RB and p53 tumour-suppressor pathways.
- Ablation of the ARF–p53 pathway suffices to immortalize many mouse cells. Telomere shortening does not seem to limit the lifespan of cells that are derived from inbred mice.
- In parallel with these differences in immortalization, pairs of introduced oncogenes will transform mouse cells, whereas the transformation of human cells requires additional introduced genes.
- Identifying and characterizing these species-specific differences will allow the construction of human and rodent models of cancer that increasingly phenocopy human cancer. Such models will revolutionize the screening and testing of candidate chemical and biological anticancer therapies.


When similar studies were carried out with rodent cells that lacked an immortalized phenotype, in primary culture, single oncogenes were found to be insufficient for transformation. Instead, pairs of cooperating oncogenic mutations, such as *Ras* and *Myc*[19], or *Ras* and the adenovirus E1A protein[20], were necessary for the transformation of these cells. In retrospect, it became clear that the creation of established cell lines *in vitro* selected for cells that had acquired some of the alterations that occur commonly as normal cells progress towards malignancy *in vivo*; so, cells such as NIH-3T3 fibroblasts had sustained premalignant changes even before the introduction of oncogenes. At the same time, similar conclusions were drawn from work with the avian erythroblastosis virus, in which two cooperating oncogenes — v-erbA and v-erbB — were shown to be essential for leukaemogenesis[21].

Subsequent studies using transgenic mice showed that pairs of oncogenes that are capable of collaborating to transform cells *in vitro* could also drive tumour formation with far more rapid kinetics than those observed in mice that carry only single oncogenes in their germ line[22,23]. These observations indicated that the *in vitro* transformation experiments had direct relevance to mechanisms that operate *in vivo* during cancer pathogenesis and that the number of changes needed to complete tumour progression *in vivo* was not much larger than the number of distinct transgenes that were used to initiate these tumours. But the kinetics of tumour formation in these mice indicated that the introduced transgenes, on their own, were insufficient to trigger tumorigenesis, and that one or two additional stochastic changes — ostensibly somatic mutations in the genomes of target cells — were needed to complete the process[23].

Consistent with these observations in animal models, epidemiological studies of the kinetics of tumour appearance in human populations indicated that 4–6 rate-limiting events — thought to be distinct somatic mutations — were required for the formation of human tumours[24]. In agreement with these numbers, the study of colon carcinoma pathogenesis implicated at least 4–6 distinct histopathological stages during the development of colon cancer[25]. These stages include the formation of HYPERPLASTIC epithelium, DYSPLASTIC epithelium, several types of ADENOMA and, finally, true CARCINOMAS. Specific genetic mutations frequently accompany these histopathological transitions, each involving the protein component of a specific regulatory pathway, perturbation of which would seem to be necessary to effect the specific stage of malignant-cell transformation and therefore tumour progression[26]. This model of colon cancer progression has stood as a paradigm for the developmental programme of other cancer types. Regrettably, in the decade since this scheme was first proposed, the genetic biographies of other human cancers have not been charted in comparable detail. Nevertheless, the transitions that are associated with human colon cancer development provide support for the idea that the steps of oncogenesis and tumour progression are relatively few in number.

Still, extrapolations from cancer epidemiology and histopathology can hardly be expected to provide accurate measures of the number of genetic changes that are required to convert normal human cells into cancer cells. More compelling observations might derive, instead, from direct manipulation of human cells and their transformation into tumour cells. Until recently, such experimental transformation had proved extremely difficult, because of the limited lifespan of normal primary human cells[27]. The rare successes, which used chemical or physical mutagens to aid the process, by necessity yielded cells of undefined genetic constitution[28,29]. However, the recent elucidation of the role of telomere function in human-cell physiology[30,31], and the isolation of the genes that encode the essential subunits of the telomerase holoenzyme[32–35], have now made possible the direct transformation of human cells[36–38]. This type of cell transformation is achieved by introducing a limited set of genetic elements into previously normal human cells. Direct comparisons of rodent- and human-cell transformation have yet to be undertaken, but enough information is already at hand to discern common and distinct behaviours of these two types of cell.

*Differences in cell immortalization.* It has long been known that rodent cells are easily transformed *in vitro*, whereas human cells have been extremely difficult to transform[28,39]. These differences in transformability parallel species-specific differences in the frequency with which the two classes of cells undergo spontaneous immortalization following extended passage *in vitro*[40]. As unlimited replicative lifespan seems to be an essential component of the neoplastic phenotype[41], some of the differences in the transformability of human versus rodent cells might well be traced to the fundamentally

HYPERPLASIA
An increase in the number of cells in a tissue or organ without gross morphological changes.

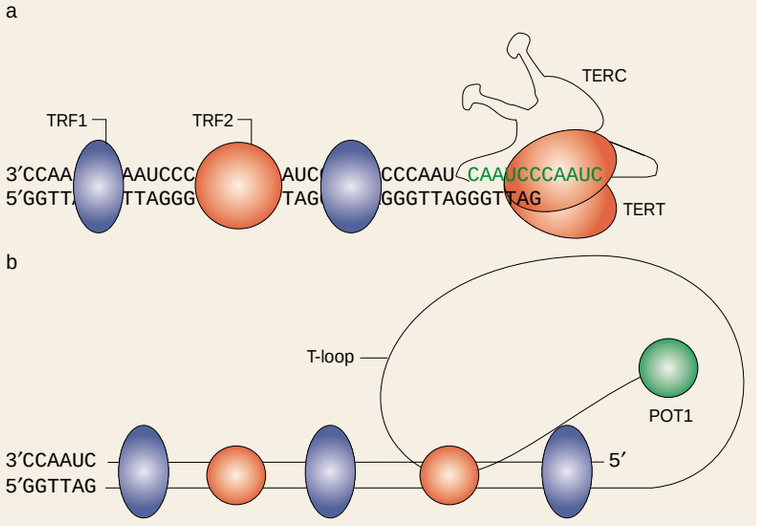
DYSPLASIA
The disordered growth that is characterized by changes in size, shape or differentiation programmes of cells in a tissue, often leading to architectural changes to the tissue or organ and generally representing a premalignant state.

ADENOMA
An ordinarily benign neoplasm of epithelial tissue in which the tumour cells form glands or gland-like structures.

CARCINOMA
A malignant neoplasm of epithelial cells that is characterized by dysplasia, hyperplasia and invasion of surrounding tissues.

### Box 1 | Molecular structure of the telomere

The telomere is composed of G-rich repetitive elements (TTAGGG in mammals), as well as several DNA-binding proteins[119] (shown in panel **a**). Recent evidence indicates that mammalian telomeres form a folded structure, termed a T-loop[120] (shown in panel **b**). Telomerase is composed of two essential subunits — TERT and TERC. The RNA subunit is expressed ubiquitously and provides the RNA template for the telomere polymerization reaction. TERT is the protein catalytic subunit and is a member of the reverse transcriptase family of enzymes[121]. In addition to telomerase, several other telomere-specific binding proteins, including the TRF1 and TRF2 proteins that bind double-stranded telomeric sequences[122–124], and POT1, which binds single-stranded telomeric sequences[125], populate the telomere and are important in telomere maintenance.




different ways in which the replicative lifespan of rodent- and human-cell lineages is controlled. Recent work indicates that human and rodent cells differ in at least two molecular mechanisms that are important determinants of this trait.

*Immortalization: the role of telomeres.* Telomeres — the repetitive DNA elements at the ends of eukaryotic chromosomes — protect the ends of chromosomes from degradation and from end-to-end fusion with other chromosomes[42,43] (BOX 1). In addition to preventing the chromosome ends from being recognized as products of DNA fragmentation, telomeres are important in regulating the lifespan of human cell lineages[30]. In most normal human cell types, chromosomes progressively lose telomeric DNA sequences during successive rounds of cell growth and division in culture[44] (BOX 2). This makes the length of telomeric DNA a useful molecular marker of the number of cell divisions through which a cell lineage has passed since it originated in the embryo. Indeed, a strong correlation exists between the further replicative capacity of a cell population *in vitro* and the existing length of the telomeres in its cells[44,45]. After erosion below a threshold length, the telomeric DNA and the nucleoprotein complex that constitutes the telomere lose their ability to protect chromosomal ends. At least two cell-physiological responses — senescence and crisis — have been postulated to be triggered as a result[30].

In contrast to the behaviour of telomeres in normal, non-immortalized cells, those present in experimentally immortalized human cells and, more importantly, in cancer cells that are derived from patients, are maintained at stable lengths through repeated cell-division cycles[46]. Most (85–90%) of these tumour cells succeed in doing so by acquiring expression of telomerase, which is a reverse transcriptase that extends telomeric DNA[47–49] (BOX 1). Ectopic expression of TERT — the catalytic subunit of the telomerase holoenzyme — in mortal, telomerase-negative human cells confers telomerase activity on these cells and enables them to maintain stable telomere lengths[50,51]. For many human cell types, fibroblasts being a typical example, this stabilization of telomere length immortalizes early-passage cultures[50,51], whereas other types, such as mammary epithelial cells and keratinocytes, often require genetic changes in addition to activation of telomerase for immortalization[52,53]. Although other factors, such as the culture conditions, might also affect the immortalization process[54], these observations show that telomere maintenance controls the replicative lifespan of various types of human cell. Moreover, these results indicate that acquisition of replicative immortality can be a relatively simple process at the molecular level, requiring only the derepression of a single gene, *TERT*, and the resulting expression of its product.

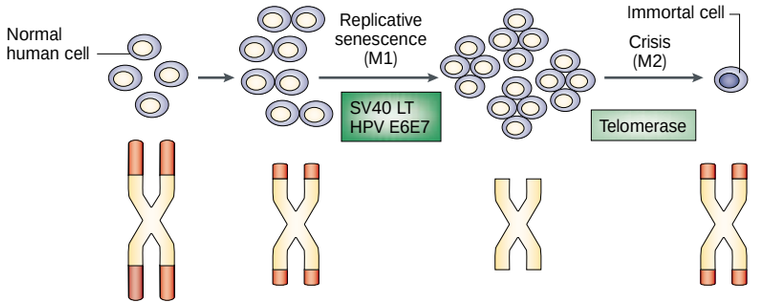
In stark contrast, telomerase is constitutively expressed in most somatic tissues of inbred mice because efficient repression of telomerase expression, which occurs during the development of most post-embryonic human cell lineages, is absent in most tissues of inbred mice[55]. Moreover, mouse telomeres are maintained at lengths that are 3–10 times greater than in comparable human cells[56]. In effect, telomeres do not seem to limit the replicative lifespan of mouse cells for two reasons: first, mouse cells begin their replication *ex vivo* with extremely long telomeres; and, second, the tendency for progressive telomere erosion might effectively be countered by the basal telomerase activity that is constitutively present in these cells[40].

A striking demonstration of the substantial endowment of telomeric DNA in normal mouse cells has come from observations of mice that have had their telomerase activity eliminated by germ-line deletion of the gene that encodes Terc — the essential RNA component of the telomerase holoenzyme[57]. Telomerase-negative mice must undergo six generations before their cells contain telomeres of lengths that are routinely observed in human cells. The lineages of these cells are estimated to have passed through hundreds of growth-and-division cycles (~125 cell divisions for female mice and ~310 cell divisions for male mice) since the inactivation of the telomerase enzyme[57], and only at this point do cells from tissues with high proliferative rates begin to show loss of replicative potential[58].

Such experiments indicate that the endowment of telomeric DNA in most mouse cells is far greater than would be required by any cell lineage during the lifespan

### Box 2 | Telomere length, telomerase activity and replicative lifespan

Most normal human cells are mortal, lack telomerase activity and show telomere shortening with passage in culture. By contrast, most cells that are derived from human cancer patients are immortal, express telomerase and have stable telomere lengths. Experimentally, human cells have a limited replicative lifespan and enter an irreversible growth arrest, which is termed replicative senescence or M1, after extended passage[72]. Introduction of viral oncoproteins, such as simian virus 40 (SV40) large T antigen (LT) or human papillomavirus (HPV) E6 and E7, into human cells before M1 allows continued proliferation with further shortening of telomeres[75,76]. Eventually, such post-senescent cells enter a second proliferative barrier, termed crisis or M2, which is characterized by widespread apoptosis and extremely short telomeres[46,126]. These shortened telomeres fail to protect the chromosome from degradation and, in the setting of p53 loss, drive genomic instability, which is manifested by aneuploidy and non-reciprocal translocations[127,128]. Rare surviving cells ($1 \times 10^{-7}$)[74] have extensive karyotypic alterations, telomerase enzyme activity and stable telomere lengths[46]. Experimental activation of telomerase through introduction of the catalytic subunit of telomerase, TERT (BOX 1), allows some human cells to avoid replicative senescence[50,51,129] and immortalizes all post-senescent human cells[130–132]. Although most human cancer cells maintain stable telomere lengths by the action of telomerase[47,48], a significant minority (10–15%) lack telomerase activity and harbour long heterogeneous telomeres[133]. This second mechanism to maintain telomere length has been termed alternative lengthening of telomeres (ALT). The dual role of telomeres in protecting chromosomal integrity and in limiting replicative lifespan explains how telomere shortening might serve as a factor that both restricts and promotes malignant transformation. Under most circumstances, short telomeres limit replicative lifespan, inducing replicative senescence or crisis, depending on the status of the RB and p53 pathways. This greatly reduces the pool of premalignant cells. However, when telomere erosion reaches the point when chromosomes are no longer protected, such as at crisis, increased aneuploidy and translocations are observed— as seen in mice that lack both functional telomerase and p53 (REFS 46,118,128). This might promote further malignant evolution in the rare cells that survive this process. One of the genes that is often activated during this process is *TERT*, leading to telomerase activation and stabilization of telomeres.




| | | | | | | |
|---|---|---|---|---|---|---|
| **Telomerase** | OFF | OFF | OFF | OFF | OFF | ON |
| **Immortal** | NO | NO | NO | NO | NO | YES |
| **Genome stable** | YES | YES | ? | ? | NO | ? |

of the whole organism. These observations would seem to explain why, unlike human-cell transformation *in vitro*, the transformation of cultured rodent cells is not constrained by the absence of telomerase and by relatively short telomeres. A different situation governs human-cell transformation *in vitro*: ectopic expression of *TERT* is essential for high efficiency of cell transformation[36]. Taken together, these observations indicate that differences in telomere biology between humans and mice account for part of the observed difference in immortalization frequency.

REPLICATIVE SENESCENCE
Normal human cells that are propagated serially in culture eventually reach a growth arrest that is characterized by a flattened cell morphology and continued metabolic activity without widespread cell death.

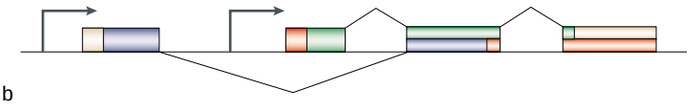
*Immortalization: the role of RB and p53 pathways.* In addition to telomeres and telomerase, a second vital regulatory mechanism that controls replicative lifespan differs between mouse and human cells. Through alternative splicing, the *CDKN2A* locus encodes two proteins in both mouse and human cells[59] (FIG. 1). One of these proteins is INK4A (also known as p16), which is a cyclin-dependent kinase inhibitor that specifically blocks the activity of cyclin-dependent kinase 4 (CDK4) and CDK6 by preventing their interaction with D-type cyclins. These proteins act together to govern the phosphorylation status of the retinoblastoma protein (RB)[60]. The other product of this locus is ARF (also known as p14 in humans and p19 in mice), which is a positive regulator of p53 levels[61]. So, the protein products of the *CDKN2A* locus regulate the activities of two tumour-suppressor pathways (RB and p53) that are disrupted in most human cancers and have important functions in regulating cell proliferation and stress-induced apoptosis[62,63].

In mouse embryo fibroblasts (MEFs), genetic inactivation of either the Arf- or the p53-encoding locus seems to suffice for cell immortalization. As primary MEFs are passaged in culture, they eventually reach a growth arrest — sometimes also referred to as senescence — in which levels of Arf and, therefore, p53 are elevated[64]. The spontaneous inactivation of either of these loci therefore allows the outgrowth of clones of immortalized MEFs[65,66]. Consistent with this observation, in the presence of an antisense RNA that attenuates Arf synthesis, MEFs avoid the senescence that normally stands in the way of their immortalization[67]. As Arf positively regulates p53 levels, the alteration of this single pathway might suffice for immortalization of MEFs *in vitro*.

The Ink4a protein also accumulates as primary MEFs approach this growth arrest[68,69], but mutations that result in the elimination of Ink4a production are not consistently observed, and genetic ablation of the transcript that encodes Ink4a, leaving the transcript that encodes Arf intact, does not allow immortalization[70,71]. This growth arrest in mouse cells differs qualitatively from the REPLICATIVE SENESCENCE that was first described by Leonard Hayflick and Paul Morehead in human cells[72]; ARF does not seem to be elevated in human fibroblast cultures at replicative senescence[73], and human cultures rarely, if ever, spontaneously escape from the growth arrest that is associated with replicative senescence[74].

The lack of success in deriving spontaneously immortalized human cells is explained in part by the fact that, at the genetic level, escape from senescence is more complex in human fibroblasts than it is in their rodent counterparts. Several lines of evidence show that both the RB and the p53 tumour-suppressor pathways must be inactivated to bypass replicative senescence[46,74]. In particular, introduction of viral oncoproteins that inactivate both RB and p53, such as simian virus 40 (SV40) LARGE T ANTIGEN (LT) or HUMAN PAPILLOMAVIRUS (HPV) E6 AND E7, allow human fibroblasts to bypass senescence; consequently, introduction of LT mutants that lack the ability to bind either RB or p53 (REF. 75), or introduction of either HPV E6 or E7 oncoproteins alone[76], fails to allow such cells to bypass senescence. Moreover, expression of dominantly

a ***CDKN2A***



b

| Gene product | | | | | Pathway | | | Enforces growth arrest secondary to: Proliferation | RAS |
|---|---|---|---|---|---|---|---|---|---|
| **Human** | | | | | | | | | |
| | INK4A | ⊣ | D-type cyclins CDK4/6 | ⊣ | RB | | | + | + |
| | ARF | ⊣ | MDM2 | ⊣ | p53 | | | – | – |
| **Mouse** | | | | | | | | | |
| | Ink4a | ⊣ | D-type cyclins Cdk4/6 | ⊣ | Rb | | | +/– | – |
| | Arf | ⊣ | Mdm2 | ⊣ | p53 | | | + | + |

Figure 1 | **The *CDKN2A* locus and proliferation control in human and mouse cells. a** | The INK4A and ARF proteins are encoded by a single genetic locus (*CDKN2A*) in both human and mouse cells[60,61]. ARF is produced by alternative splicing and translation in an alternative reading frame. **b** | INK4A inhibits the action of cyclin-D–CDK4/6 (cyclin-dependent kinase 4/6) complexes, which regulate RB. ARF inhibits the ubiquitin ligase MDM2, leading to elevated levels of p53. A plus sign indicates that ablation of this pathway is required for immortalization either during extended passage in culture or after expression of high levels of oncogenic HRAS.

interfering alleles of p53 (REF. 77), inactivation of p53 through the expression of HPV E6 (REF. 76) or genetic ablation of p53 in primary human fibroblasts extends cell lifespan[78], but the resulting cells are still not immortalized. By contrast, expression in MEFs of LT mutants that cannot bind RB but retain the ability to bind p53 results in immortalization[79–81].

As human cells approach replicative senescence, the levels of INK4A increase, whereas ARF levels remain stable[73,82]. In some human epithelial cells, such as mammary epithelial cells and keratinocytes, loss of INK4A function — caused by mutation or promoter methylation — cooperates with the expression of telomerase to allow immortalization[52,53]. However, cells that are immortalized in this way invariably also accumulate mutations of p53 (REF. 83). These observations implicate a dominant function for INK4A, rather than ARF, in human-cell replicative senescence. Although telomere shortening might be one signal that triggers a p53-dependent growth arrest[84], three pathways — those regulated by telomere shortening, p53 and RB — seem to regulate replicative senescence in human cells, whereas only one — the Arf–p53 pathway — regulates MEF replicative lifespan (FIG. 1). At present, it remains unclear whether these three pathways act in concert to enforce replicative senescence in human cells, or whether they form successive barriers to immortalization[85].

Taken together, these observations indicate that, in addition to the important contribution of telomeres to replicative lifespan, different proteins that are encoded by *CDKN2A* are necessary to regulate the cell lifespan in humans and mice. Although some of these differences between mouse and human cells might reflect the particular conditions that are used to culture these various cells[54,86,87], their different sensitivities to stresses that are associated with proliferation *ex vivo*[88], or the particular cells studied[89], the observation remains that human and mouse cells respond differently to these physiological stresses, particularly in the expression of these two proteins that are encoded by *CDKN2A*. So, mouse and human cells seem to differ in two of the regulatory pathways — telomere maintenance and the RB pathway — that are influential in governing immortalization.

*Responses of cells to introduced oncogenes.* The introduction of a cloned oncogene into a mammalian cell will often provoke senescence or apoptosis[68]. These responses are presumably mounted by many cell types to block the proliferation of clones of mutant, oncogene-bearing cells. These responses also explain why the formation of a transformed cell clone often depends on the actions of a second introduced gene, the main function of which is to neutralize the antineoplastic defence mechanism that is triggered by the previously introduced one[90]. Although this function is physiologically quite different from that governing the acquisition of immortalized growth, the same gene products — RB and p53 — are often involved in both processes.

In both mouse and human fibroblasts, expression of high levels of V12 HRAS — a constitutively active (and therefore oncogenic) mutant of HRAS — leads to a premature growth arrest that is similar in many respects to the arrest that is observed when human cells reach replicative senescence[68]. Ablation of Arf or p53 in mouse cells allows them to avoid the growth arrest that is induced by Hras[66,68]; as is the case with the senescence-like arrest of MEFs, the p53 pathway is dominant here.

In stark contrast to the behaviour of mouse cells, inactivation of both the RB and p53 pathways is needed for human cells to tolerate high levels of HRAS[68,91]. This additional requirement for inactivation of the RB pathway exists despite evidence that RAS signalling leads to induction of cyclin D1 expression, which alone might be expected to inactivate RB[92–95]. Although the inactivation of p53 and RB has been accomplished through different experimental means in human and rodent cells[68], these differences in response to high levels of V12 HRAS parallel what is observed during the spontaneous immortalization of human and rodent cells. In addition, although expression of high levels of V12 *HRAS* results in upregulation of ARF in MEFs[66], introduction of HRAS into human fibroblasts fails to elicit ARF expression[73], indicating that human and mouse cells differ in their response to HRAS. These observations reinforce the conclusion, derived previously from immortalization studies described above, that the p53 pathway has a dominant function in the physiology of mouse cells, but both the p53 and RB pathways are involved in the comparable processes in human cells.

A Ras-transformed cell must escape from premature senescence if it is to succeed in spawning a clone of tumorigenic cells[68,90]. This explains why the responses of cells to introduced oncogenes and to extended passage in culture, as described above, help to determine the number of distinct genetic changes that must occur before cells become transformed to a tumorigenic state. These observations also indicate that some of the same

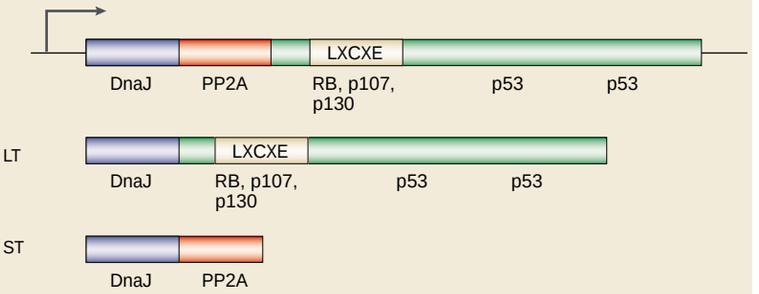
SV40 LARGE T ANTIGEN
(LT). A multifunctional protein product of the simian virus 40 early region that is necessary to establish a permissive host-cell environment for viral replication by interactions with host proteins. Large T antigen binds and functionally inactivates both the RB and p53 tumour-suppressor proteins.

HUMAN PAPILLOMAVIRUS E6 AND E7
Viral oncoproteins that are derived from certain human papillomavirus types that are associated with increased risk of cervical cancer. E6 binds to and targets p53 for ubiquitin-mediated degradation. E7 binds and inactivates RB.

### Box 3 | SV40 early region oncoproteins

The SV40 early region produces several viral proteins by means of alternative splicing, including the large T (LT) and small T (ST) antigens. These proteins have important functions in establishing a permissive environment for viral replication by interactions with several host-cell proteins. LT and ST share the 82-amino-acid amino-terminal end, which contains a domain that is related in sequence to the DnaJ family of molecular chaperones[134]. The LT oncoprotein binds members of the RB-protein family (RB, p107 and p130) by means of a conserved sequence (LXCXE), as well as by binding to p53 using two conserved regions that are not found in ST. ST binds protein phosphatase 2A (PP2A) by means of a unique domain that is formed from an intron in the LT-coding sequence[135]. An additional protein (17 kT) contains only the shared amino-terminal domain plus three additional amino acids, and does not bind RB, p53 or PP2A (not shown).

**SV40 early region**




pathways that trigger growth arrest during extended passage in culture might also be involved in the response to oncogenes. In fact, the phenomenology of transformation is even more complex than indicated by these descriptions of senescence and immortalization, in that three distinct patterns of behaviour have been ascribed to mouse, rat and human cells.

As described previously, the combination of *Myc* and *Ras* seems sufficient to convert normal rat embryo fibroblasts (REFs) into tumorigenic cells[19]. This pair of oncogenes transforms REFs much more efficiently than MEFs, and fails to transform human fibroblasts. Transformation of MEFs with *Myc* and *Ras*, although inefficient, does occur, but analysis of the karyotype of these cells reveals a high degree of ANEUPLOIDY[96,97], indicating that additional factors contribute to the transformation of these cells. In agreement with these findings, Thompson *et al.* noted that prostate carcinomas that formed in transgenic mice expressing *Myc* and *Ras* were monoclonal, further indicating that one or more additional genetic changes contributed to the formation of these tumours[23]. More detailed studies of the formation of tumorigenic mouse haematopoietic cells indicate that, in addition to introduced *Myc* and *Raf* oncogenes, the inactivation of p53 function is necessary[98]. More recently, the requirement for inactivation of the p53 pathway to facilitate the transformation of MEFs by *Myc* and *Ras* was shown in MEFs that were derived from mice lacking Arf or p53 (REF. 66). Without this additional alteration, mouse cells respond to the introduced oncogenes by undergoing apoptosis or growth arrest. Indeed, expression of *Hras* in both MEFs and mouse skin keratinocytes derived from Arf-null mice leads to full transformation[66,99]. So, the introduction of a *Myc* oncogene into mouse cells that bear the *Ras* oncogene does not solve the problem of Ras-induced senescence: the solution still requires inactivation of the p53 response.

Early experiments indicated that human cells, quite predictably, were even more difficult to transform than either mouse or rat cells, but the precise mechanisms that underlie this difference in behaviour were elusive. Introduction of the combination of SV40 plus *Ras*, or *Myc* and *Ras* oncogenes into human cells rarely yielded transformed cells[39,100], and then only after long periods of selection or further treatment with chemical or physical mutagens. Such experiments indicated that additional changes were required beyond the introduced *Myc* and *Ras* oncogenes, but provided no insight into the nature of these changes.

As immortalization is generally found to be an essential component of the transformed-cell phenotype, we can now predict that these additional changes might involve the p53, RB and telomere pathways, all of which have been found to be necessary for human-cell transformation. Indeed, introduction of the SV40 early region, which includes the SV40 LT oncoprotein, together with *TERT* and *RAS*, induces experimental transformation of many types of human cell[36–38]. Moreover, inactivation of either the RB- or p53-inactivating domains of LT deprives it of its ability to aid in transformation of these cells[101]. Taken together, the long-standing observation that human cells are more difficult to transform derives in part from differences in the functioning of the p53, RB and telomere maintenance pathways in cell immortalization.

The best comparison between the rodent-cell *Myc* plus *Ras* transformation experiments described above is from experiments with human embryonic kidney and mammary epithelial cells in which the combination of the *Myc* plus *Ras* oncogenes required the additional presence of the SV40 early region for successful transformation (R. Beijersbergen, B. Elenbaas, W.C.H. and R.A.W., unpublished observations). Under these conditions, one of the effects of the *Myc* oncogene, although not its only function, is to activate *TERT* expression and therefore telomerase function[102–104]. So, these observations are consistent with the idea that the transformation of human cells with *Myc* and *Ras* requires the additional inactivation of the RB and p53 pathways, as well as the activation of telomerase expression — at least two more changes than are required in mouse cells.

*A third difference between rodents and humans?* A further dimension of complexity is hinted at by a more detailed examination of the sequences in the SV40 early region that are required for rodent- and human-cell transformation (BOX 3). The LT oncoprotein, as mentioned above, inhibits both RB and p53. A second product of the early region is small T (ST), which perturbs one or more isoforms of protein phosphatase 2A (PP2A) — a widely acting serine/threonine phosphatase[105,106]. Mutant SV40 viruses that lack the ability to produce a functional ST protein easily transform mouse cells, but fail to transform human cells[107–109].

ANEUPLOIDY
The state of having an abnormal number of chromosomes. Most human epithelial cancers harbour genomes that are characterized by gross aneuploidy.

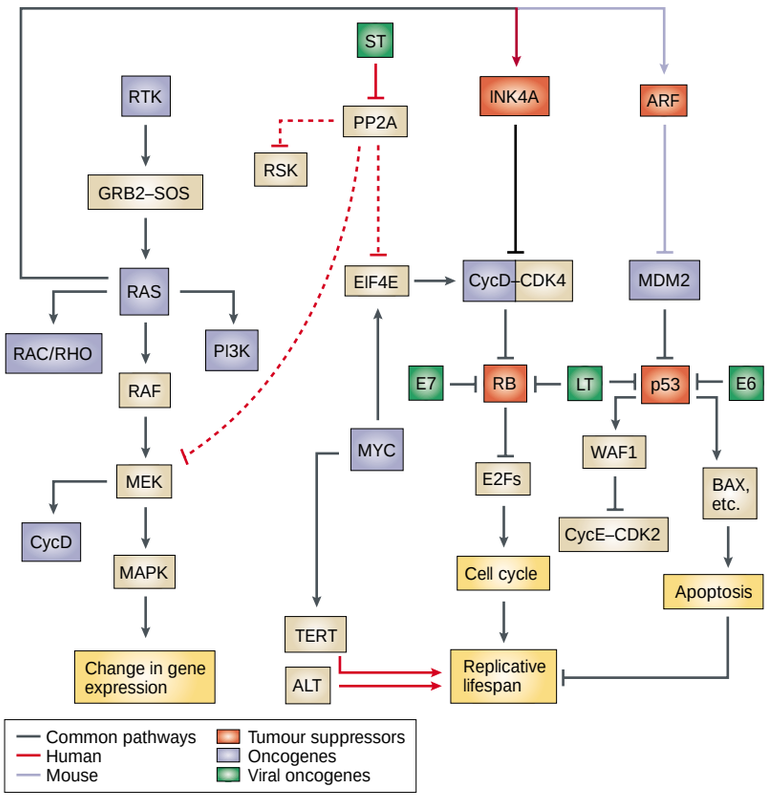



Figure 2 | **The molecular circuitry of cancer.** Although countless differences between normal cells and cancer cells have been documented, much progress in identifying and connecting the fundamental pathways that are responsible for programming malignant cell growth has been made. Most cancer-associated mutations disrupt essential homeostatic mechanisms that regulate cell proliferation and survival. In many cases, particular mutations have been linked to specific biological phenotypes shown by cancer cells (yellow boxes). The cellular machinery that is responsible for controlling mammalian cell physiology is largely shared between human and mouse cells (black lines). Comparisons of human and mouse experimental cancer models identify several pathways that seem to have more prominent roles in human-cell transformation (red lines), as well as other molecular pathways that serve in dominant positions in mouse cancer models (blue lines). Perturbation of these five pathways (RB, p53, telomere maintenance, HRAS and ST–PP2A) allows transformation of human cells. However, significant interactions among these pathways and other molecules that are implicated in the development of particular types of human cancer exist and remain to be characterized in detail. For the purposes of clarity, this representation is simplified and is illustrative rather than comprehensive. For example, clear evidence links RAS signalling to induction of cyclin D1 expression[92–95], and the role of PP2A in most human cancers remains to be elucidated. An alternative method of displaying the molecular circuitry that programmes the cancer phenotype can be found in the poster provided with this issue and at http://www.nature.com/nrc/journal/v2/n5/weinberg_poster/. ALT, alternative lengthening of telomeres; CDK, cyclin-dependent kinase; Cyc, cyclin; E6, human papillomavirus E6 oncoprotein; E7, human papillomavirus E7 oncoprotein; LT, SV40 large T antigen; ST, SV40 small T antigen; TERT, telomerase reverse transcriptase; WAF1, also known as p21 (encoded by *CDKN1A*).

Although the expression of telomerase might be expected to provide a simple explanation for this difference, expression of the HPV proteins E6 and E7 — which also inactivate RB and p53 — together with *TERT* and *RAS*, has failed to transform human fibroblasts[110]. Recent observations indicate that the SV40 ST protein makes an essential contribution to the experimental transformation of human cells that is distinct from those made by SV40 LT, and that ST cooperates with other agents that inactivate RB and p53, such as the HPV E6 and E7 proteins, to transform human cells[101,111]. Although it is clear that the interaction of ST with PP2A leads to increased cell proliferation[112], the plethora of identified PP2A substrates[113] precludes a clear understanding of the molecular pathways that are perturbed by the interaction of ST with PP2A, which is necessary for human-cell transformation.

Taken together, these disparate observations indicate that there are several important differences in the hard-wiring of the growth-controlling circuitry of human and mouse cells (FIG. 2). Although humans and mice share a common set of protein components, the regulation of their function is distinct enough to generate quite different rules governing their transformation. At least two main differences that involve RB and telomerase function are well documented, and a third apparent difference (PP2A) awaits validation. Further differences might be found in the future. The pathways described here are involved in the formation of primary tumours; other pathways that are involved in heterotypic signalling between tumour cells and their environment, and in the processes of invasion and metastasis, are also important in the pathogenesis of human cancer. The discrepant behaviours in the pathways described here indicate that many of the lessons about the molecular bases of cell transformation that have been developed from the study of rodent cells will need to be revisited and re-examined in the context of human cancer biology.

## Implications for modelling cancer

The fundamental differences in the cell-autonomous responses of mouse and human cells to mutant cancer-causing genes cannot fail to exert a great influence on tumour progression as it occurs in these two organisms. So it seems highly likely that the smaller number of genetic changes that are required for rodent-cell transformation *in vitro* is paralleled by a smaller number of steps that are required for tumour progression to reach completion *in vivo*. Independent of these considerations is the fact that the developmental programmes of mice and humans are markedly different; the obvious difference in organism size is only one manifestation of these differences. Consequently, the cellular targets for oncogenic transformation are present in vastly reduced numbers in mouse tissues than in their human counterparts. Conversely, the much shorter lifespan of mice means that the cancers that do appear in these animals must have an accelerated programme of progression compared with human malignancies, which can take 20 or more years to progress.

Despite these fundamental differences at the levels of the cell and the organism, mouse models have provided essential insights into the biology of human cancer. Indeed, these models continue to represent an invaluable tool to understanding not only the cell-autonomous effects of particular cancer-associated mutations, but also the cell-non-autonomous influences on the development of cancer in a physiological context. Use of transgenes to programme ectopic expression of oncogenic alleles both ubiquitously and in specific organ sites has allowed connections to be made between particular oncogenic mutations and

Table 1 | Mouse models of inherited cancer susceptibility syndromes

| Tumour-suppressor gene product | Cancer susceptibility in mouse model | Spectrum of tumours in mice | Spectrum of tumours in humans | References |
|---|---|---|---|---|
| Rb | Yes | Brain, pituitary | Retina, osteosarcoma | 136,137 |
| p53 | Yes | Osteosarcoma, lymphoma, teratoma, soft-tissue sarcomas | Soft-tissue and bone sarcomas, breast, brain, leukaemia, adrenocortical neoplasms | 138,139 |
| Ink4a | Yes | Fibrosarcoma, lymphoma, squamous-cell carcinoma | Familial melanoma, sporadic pancreatic, brain tumours | 140 |
| Arf | Yes | Sarcoma, lymphoma, nervous-system tumours | NA | 66 |
| Pten | Yes | Lymphoma, mammary, adrenal and endometrial neoplasia, GI-tract hamartomas, prostate, colon, skin hyperplasia | Breast, thyroid, brain, prostate, endometrial, melanoma | 141–143 |
| Brca1 | No | NA | Breast, ovary | 144,145 |
| Brca2 | No | NA | Breast, ovary | 146,147 |
| Nf1 | Yes | Phaeochromocytoma, myeloid leukaemia, adrenal medullary tumours | Malignant peripheral nerve-sheath tumours, astrocytoma, glioblastoma, phaeochromocytomas, myeloid leukaemia | 148,149 |
| Nf2 | Yes | Osteosarcoma, fibrosarcoma, lung adenocarcinoma, hepatocellular carcinoma | Acoustic schwannomas, meningiomas, ependymomas, gliomas | 150 |
| Apc | Yes | Min: Multiple GI adenomas, mostly in small intestine APC1638N: few adenocarcinomas in GI tract, desmoid tumours | Multiple GI polyps, mostly in large intestine, progression to adenocarcinoma | 151–154 |
| Vhl | Yes | Cavernous haemangiomas in liver, phaeochromocytoma, ependymoma | Kidney, retina, pancreas | 155 |

For further information, the reader is referred to recent reviews on this topic, including REFS 156,157. Apc, adenomatous polyposis coli; Brca1/2, breast cancer 1/2; GI, gastrointestinal; NA, not applicable; Nf1/2, neurofibromatosis 1/2; Pten, phosphatase and tensin homologue; Rb, retinoblastoma; Vhl, von Hippel–Lindau syndrome.

their biological phenotypes in the intact tissue and organism. Furthermore, the tumour predisposition that is observed in mice after the inactivation of candidate tumour-suppressor genes in the mouse germ line has repeatedly validated these genes as elements that are necessary for cancer prevention.

Still, mice that harbour specific germ-line mutations that are known to be associated, in humans, with a specific inborn cancer susceptibility often have a markedly different spectrum of cancers than do humans (TABLE 1). These differences do not invalidate mouse models of cancer as important tools for analysing the process of transformation. They do, however, indicate that we will need to enumerate these species-specific differences to inform the design of future mouse models of human cancer pathogenesis.

Certain elaborations in the design of germ-line inactivations have succeeded in producing mouse PHENOCOPIES of human disease. For example, although germ-line deletion of *Rb* alone did not lead to retinoblastoma in mice, the deletion of both *Rb* and the gene for the related protein, p107, does so[114,115]. Recent work shows that, whereas mice that are heterozygous for the neurofibromatosis tumour suppressor *Nf1* fail to form neurofibromas, chimeric mice that consist in part of *Nf1*$^{-/-}$ cells do form neurofibromas, and co-deletion of the closely linked *Trp53* allele gives rise to malignant peripheral-nerve-sheath tumours — the primary type of malignancy that is observed in the human syndrome[116]. In addition, introduction into the mouse genome of a mutant *Kras2* allele that undergoes stochastic activation results in pronounced and early formation of aggressive lung carcinomas[117]. At present, it remains unclear whether any of these models reveal further differences in human- and mouse-cell physiology. They do, however, indicate that various types of incremental improvements in the experimental strategies that are used to alter the mouse genome in particular tissues, or at particular stages of tumour development, will provide new insights into cancer development.

Detailed knowledge of interspecies differences in the functioning of signalling pathways in mouse and human cells might well be exploited to allow the construction of mouse models that more closely mimic human cancers. Indeed, this approach has already succeeded in providing markedly improved mouse cancer models. For example, homozygous deletion of a telomerase gene when coupled with *Trp53* heterozygosity

PHENOCOPY
A model that recapitulates the clinical and biological characteristics of a specific disease state.

NON-RECIPROCAL TRANSLOCATION
Transposition of two segments between non-homologous chromosomes with loss or gain of genetic material as the result of abnormal breakage and fusion.

in the mouse germ line leads to the development of increased NON-RECIPROCAL TRANSLOCATIONS that are similar to those seen in human epithelial malignancies, and to a shift in the spectrum of tumour types towards increased numbers of epithelial tumours[118]. In addition, although deletion of the entire *Cdkn2a* locus failed to predispose mice to malignant melanoma, mice lacking both Ink4a and Arf on one chromosome and expressing only Arf on the other are, indeed, predisposed to a syndrome that is similar to that seen in patients with familial melanoma[70]. The 'humanization' of the mouse genome, achieved through the replacement of functionally important mouse sequences with their human counterparts, might also yield mouse models that increasingly mimic human cancers. For example, replacement of the sequences that govern Ink4a expression in the mouse genome with their human counterparts might yield mouse cells that more closely approximate human cells.

At the same time, although existing *in vitro* models of human-cell transformation provide an important tool to analyse the function and cooperation of particular cancer-associated mutations, such models cannot fully recapitulate the developmental process of cell transformation that must occur *in vivo*. Moreover, our limited ability to propagate each of the cell types that populate human tissues remains one of the main limitations of these culture-based systems. In the future, improvements in culture systems, as well as the use of telomerase to immortalize human cells, might expand the cell repertoire that is available for the reconstitution *in vitro* of complex human tissues and their use in transformation studies. The further incorporation of specific mutations that are associated with particular types of cancers into these *in vitro* model systems will produce increasingly relevant models of human cancer.

In addition, our ability to create chimeric animals, in which complex human tissues are engrafted in a way that allows their long-term, normal functioning in host mice, might also yield experimental models that prove particularly powerful in addressing specific questions about human pathogenesis. The utility of such xenografts has been limited in part by interspecies incompatibilities in receptor–ligand interactions, which in turn compromise the functionally important heterotypic interactions between engrafted human cells and surrounding mouse tissues. Here, too, re-engineering of the mouse germ line through the humanization of specific ligand or receptor genes might facilitate the modelling of human cancer in rodents.

The development of improved cell- and animal-based models of cancer will continue to provide the tools with which to understand further the complex molecular and cellular interactions that programme the malignant state. At present, both human and mouse experimental models of cancer only partially recapitulate human cancer, but understanding the differences between these models and human cancer, as well as the limitations of each, will lead to improvements in the future. At the same time, such improved experimental models promise to revolutionize the screening and testing of candidate chemical and biological anticancer therapies. The identification of the pathways that are centrally involved in the pathogenesis of each type of human cancer using these models will not only hasten the identification of novel compounds, but also facilitate the rational design of combination therapies that will maximize therapeutic effects and minimize toxicity and the development of resistance. After years of promise and unfulfilled expectation, the further refinement and use of these various experimental models of cancer might finally contribute in substantive ways to improvements in the care of cancer patients.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