REVIEW

The potential for chemical mixtures from the environment to enable the cancer hallmark of sustained proliferative signalling

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Abstract

The aim of this work is to review current knowledge relating the established cancer hallmark, sustained cell proliferation to the existence of chemicals present as low dose mixtures in the environment. Normal cell proliferation is under tight control, i.e. cells respond to a signal to proliferate, and although most cells continue to proliferate into adult life, the multiplication ceases once the stimulatory signal disappears or if the cells are exposed to growth inhibitory signals. Under such circumstances, normal cells remain quiescent until they are stimulated to resume further proliferation. In contrast, tumour cells are unable to halt proliferation, either when subjected to growth inhibitory signals or in the absence of growth stimulatory signals. Environmental chemicals with carcinogenic potential may cause sustained cell proliferation by interfering with some cell proliferation control mechanisms committing cells to an indefinite proliferative span.

Abbreviations

AhR	aryl hydrocarbon receptor
AP-1	activator protein 1
AR	androgen receptor
CDK	cyclin-dependent kinase
DHT	dihydrotestosterone
EGF	epidermal growth factor
ER	oestrogen receptor
IL	interleukin
MAPK	mitogen-activated protein kinase
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
NP	nanoparticle
PAH	polycyclic aromatic hydrocarbon
PI	phosphatidylinositol
PI3K	PI-3-kinase
PKC	protein kinase C
PP2A	protein phosphatase 2A
PPAR	peroxisome proliferator-activated receptor
PTEN	phosphatase and tensin homolog
Rb	retinoblastoma
siRNA	small interfering RNA
SRD5A	steroid 5-alpha reductase
TGF-β	transforming growth factor-β

Introduction

In two classical articles, Hanahan *et al.* (1,2) introduced the term 'Hallmarks of Cancer' to constitute an organizing principle that provides a logical framework for understanding 'the remarkable diversity of neoplastic diseases'. The basis for this new concept was the idea that as normal cells undergo step-by-step transformation towards neoplasia, they acquire a succession of hallmark capabilities. Hanahan *et al.* argued that tumours are more than insular masses of proliferating malignant cells. Instead, they are complex tissues composed of multiple distinct cell types that participate in heterotypic interactions with one another. Recruited normal cells, which build up the surrounding stroma, play an active role in tumourigenesis rather than act as passive bystanders. Thus, stromal cells contribute to the action of certain hallmark capabilities.

The hallmarks of cancer include six core attributes, namely sustained proliferative signalling, evading growth suppression, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death. Underlying these hallmarks are genomic instability and inflammation. Finally, two enabling characteristics (also referred to as emerging hallmarks) have been added to this list: reprogramming of energy metabolism and evading immune destruction (2).

This article has aimed at scrutinizing the hallmark of sustained proliferative signalling with respect to the disruptive potential of mixtures of chemicals in the environment. But in order to fully grasp the impact of this hallmark of cancer, the proliferative characteristics of the normal complex organism will be briefly summarized.

In normal adult tissues, the size of cell population is determined by the rates of cell proliferation, differentiation and cell death. As a general rule, increased cell numbers may result from either increased proliferation or decreased cell death. The impact of differentiation depends on the circumstances under which it occurs. Skeletal and cardiac muscle cells and (sometimes) neurons are considered terminally differentiated cells; that is, they are at an end stage of differentiation and are not capable of proliferating. Such non-dividing cells have left the cell cycle and cannot undergo mitotic division in postnatal life. However, recent results demonstrate that although neurons and skeletal muscle have some regenerative capacity, cardiac muscle has very limited, if any, regenerative capacity (3).

In some adult tissues, such as liver, kidney and pancreas; mesenchymal cells, such as fibroblasts and smooth muscle; vascular endothelial cells and resting lymphocytes and other leukocytes, the differentiated cells are normally quiescent but are able to proliferate when needed in response to stimuli and are thus capable of reconstituting the tissue of origin. The regenerative capacity of stable cells is best exemplified by the ability of the liver to regenerate after partial hepatectomy and after acute chemical injury.

In proliferative or continuously dividing tissues (also called labile tissues), cells proliferate throughout life, replacing those that are destroyed. These tissues include surface epithelia, such as stratified squamous surfaces of the skin, oral cavity, vagina and cervix; the lining mucosa of all the excretory ducts of the glands of the body (e.g. salivary glands, pancreas, biliary tract); the columnar epithelium of the gastrointestinal tract and uterus; the transitional epithelium of the urinary tract and cells of the bone marrow and hematopoietic tissues. In most of these tissues, mature cells are terminally differentiated, short-lived and incapable of proliferation, but they may be replaced by new cells,

arising from stem cells. Thus, in such tissues, there is a homeostatic equilibrium between the proliferation of stem cells, their differentiation and death of mature (differentiated) cells. Active proliferation of normal cells can be stimulated by physiologic and pathologic conditions. The proliferation of endometrial cells under oestrogen stimulation during the menstrual cycle and the thyroid-stimulating hormone-mediated replication of cells of the thyroid that enlarges the gland during pregnancy are examples of physiologic proliferation. Many pathologic conditions such as injury, cell death and mechanical alterations of tissues also stimulate cell proliferation. Physiologic stimuli may become excessive, creating pathologic conditions such as nodular prostatic hyperplasia resulting from dihydrotestosterone (DHT) stimulation and the development of nodular goiters in the thyroid as a consequence of increased serum levels of thyroid-stimulating hormone.

Cell proliferation is largely controlled by signals (soluble or contact-dependent) from the microenvironment that either stimulate or inhibit cell proliferation. An excess of stimulators or a deficiency of inhibitors leads to net growth and, in the case of cancer, sustained cell proliferation. Although accelerated growth can be accomplished by shortening the cell cycle, the most important mechanism of growth is the conversion of resting or quiescent cells into proliferating cells by making the cells enter the cell cycle. Both the recruitment of quiescent cells into the cycle and cell cycle progression require stimulatory signals to overcome the physiologic inhibition of cell proliferation (4,5).

In many cancers, an inter- and intra-tumour heterogeneity makes it even more challenging to define proliferative signatures and growth phenotypes. Not only is the tumour of one patient different from the tumour of another patient but also primary and metastatic lesions from different sites in the same patient exhibit vast genomic variations (6). Even within a given biopsy, there is cellular and genetic heterogeneity, which is likely associated with cellular differences in proliferation, survival mechanisms, invasiveness and drug resistance (7). Many tumours, therefore, show varying degrees of differentiation, and some can virtually not be distinguished from the parental tissue. Thus, focusing on differences between tumours and normal tissues frequently occurs in a twilight zone where it is virtually impossible to end up with a yes/no answer. Furthermore, in most tumours, it is only a small minority of the tumour cells that actually proliferate. The percentage traversing the cell cycle is generally higher in poorly than highly differentiated tumours (8,9). Very rarely, all cells in a tumour can be shown to undergo

active proliferation. It has even been shown that in some neoplasms, tumour cells actually proliferate at a slower rate than the cells in their parental tissue.

However, irrespective of which perspective one chooses, one of the fundamental differences between a non-neoplastic and a transformed cell is that tumour cells cannot halt proliferation when subjected to growth inhibitory signals or in the absence of growth stimulatory signals (1,2). This indicates that the hallmark of sustained cell proliferation is foundationally imbedded in the control of the cell cycle. In other words, without proliferation, a tumour cannot exercise its neoplastic characteristics (10).

The cell cycle is the time a cell spends between two cell divisions and includes several parallel processes, all of which must be completed before a cell is mature for dividing.

In the first place, all subcomponents (RNA, protein and membrane lipids) generally need to double in quantity and this occurs continuously throughout the cell cycle (11). Furthermore, the genome and some chromosomal proteins must double, and this takes place during a limited interval in the middle of the cell cycle (S phase). On either side of the S phase are two 'gaps' (G_1 and G_2) (12) (Figure 1).

When the cell has passed a critical point in the G_1 phase termed the 'restriction' point (R), it is irreversibly programmed to progress through the remainder of the cell cycle and most cells will undergo the next cell division (13). However until this point, and in the stage of the cell cycle that represents ~4h following mitosis (G_1 post-mitosis or G_{1pm}), a normal cultured cell is in a state of 'indecision' (14). During this time, cells can be affected by several external factors (such as the presence of certain growth factors and proximity to other cells), which will prove decisive as to whether the cell continues towards S phase or exits the cell cycle to enter a reversible resting stage (G_0) (14). Alternatively, cells at this point can enter an irreversible state of senescence.

G_{1pm} phase is always of relatively constant length (3.5–4h) but is followed by a variable phase (G_1 presynthesis or G_{1ps}) during which time the cell builds its structural components with varying speed (14,15).

Generally, a small cell spends a relatively longer time in G_{1ps} than a larger one, the idea being that any size differences between cells will be adjusted before S phase is initiated (16) (Figure 2).

Whereas normal cells retain the ability to withdraw to the resting state (G_0) under suboptimal conditions for cell proliferation or become senescent (typically following differentiation),
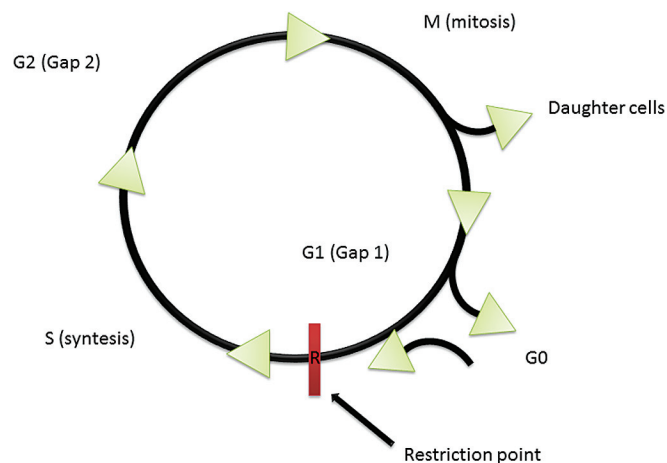

Figure. 1. Overview of the eukaryote cell cycle. For abbreviations and explanations, see text (12,13).
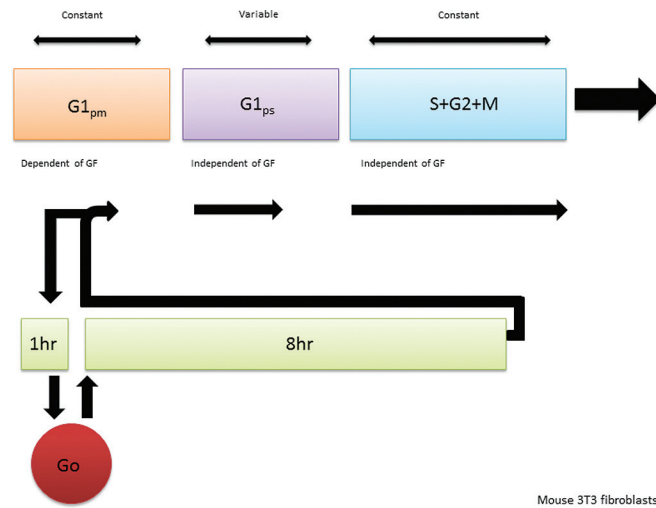
Figure. 2. Exit from and re-entry into the cell cycle. This is according to a model based on cultured mouse 3T3 fibroblasts as described in detail elsewhere (15,16). For abbreviations and explanations, see text.

transformed cells lack this ability, and this can initiate a state of sustained proliferation. Additionally, there are a number of substances that can potentially intervene and short circuit the operational cell cycle controls outlined above (14–17). This article aims to clarify the regulatory pathways acting on the cell cycle and to identify how damaging substances present as chemical mixtures in the environment can affect the transition from a normal cell to a tumour cell with respect to sustained proliferation capacity. Other contributions will deal in greater detail with alternative cellular fates that can be overridden by tumour cell transformation such as apoptosis, differentiation and senescence.

Overview of the field

The control of the cell cycle

Many genetic alterations that contribute to tumour development in humans encode proteins that regulate progression through the G_1 phase of the cell cycle. Tumourigenic transformation of normal human epithelial and fibroblastic cells can be achieved by expressing activated Ras oncogene and Simian Virus 40 early region genes encoding both large and small T antigens (18). These genes have been widely suggested to affect progression through G_1. Ras can be activated in response to a wide variety of growth factors, and therefore, Ras-activating mutations are now considered to be a constitutive proliferation signal in the absence of growth factors (19). Simian Virus 40 large T antigen binds and inactivates the p53 and Retinoblastoma (Rb) tumour suppressor gene products, both of which prevent progression beyond G_1 (20).

The tumour suppressor p53 monitors genomic integrity and stops the transition from G_1 to S if the DNA is damaged (21). Rb is active during a large part of G_1 and is a target of G_1 cyclin-dependent kinases (CDKs) that promote G_1 cell cycle transition. Simian Virus 40 small t antigen exerts an inhibitory effect on protein phosphatase 2A (PP2A), which has also been considered a tumour suppressor. PP2A is a serine/threonine phosphatase that is involved in the regulation of G_1 transit (22). The finding of a mechanism that is able to compensate for the loss of PP2A provides further insight into the requirements for transforming human cells since the many and complex roles of PP2A have to date obscured its impact on tumourigenesis (23).

The Ras/Raf protein kinase cascade normally elevates cyclin D alongside phosphatase and tensin homolog (PTEN) and Myc expression (Figure 3) (24,25). PTEN acts as a tumour suppressor that promotes G_1/S transition, whereas Myc is a key transcription factor and proto-oncogene acting at the G_1 restriction point (24,25). All the above form part of a complex signalling pathway that includes the protein kinase mTOR (the mammalian target of rapamycin).

Ultimately, signals that increase mTOR kinase activity increase the expression of Myc and activate cyclin D. PTEN removes a phosphate group from phosphatidylinositol (PI)-3,4,5-tris-phosphate (PIP3) to generate PI-4,5-bis-phosphate (PIP2), and the reverse reaction, catalysed by PI-3-kinase (PI3K), has been suggested to play a key role in cell cycle progression (26).

Consequently, the loss of PTEN, a phenomenon common in human cancer, results in an elevation of PIP3 levels, which in turn recruits specific proteins with plextrin homology domains, such as phosphatinositide-dependent kinase 1 (27) and Akt (also known as protein kinase B). Phosphatinositide-dependent kinase 1 then phosphorylates Akt (another plextrin homology domain kinase), which subsequently influences cellular progression through G_1, driving it toward cell proliferation via enabling it to commit to cell division (28) (Figure 3).

Akt also suppresses TSC1/2 (the tuberous sclerosis complex), a GTPase-activating protein that inhibits the GTPase, Rheb (28). Rheb in turn contributes to the activation of the mTOR complex 1 (mTORC1), reportedly by stimulating the dissociation of the inhibitory factor, FKBP38, from mTORC1 (29–31). Moreover, Rheb activates phospholipase D1, which generates the phosphatidic acid necessary for the assembly of mTORC1. Rheb's role in activating mTOR is vital, since mTOR is generally implicated in tumour cell proliferation and survival (32). Thus, the activation of mTOR appears to act in concert with Ras signalling in the transformation process of normal cells.

It is obvious that there are several other specific non-redundant signals controlling G_1 progression that need to be dysregulated in order to obtain sustained tumour cell proliferation. If cells are deprived of growth factors in the first part of G_1, they exit the cell cycle and enter a reversible state of quiescence, G_0. The evidence for the existence of a specific G_0 state is that it takes more time to transition from G_0 to S phase than it does to transition from the end of mitosis (start of G_1) to S phase (14–17).
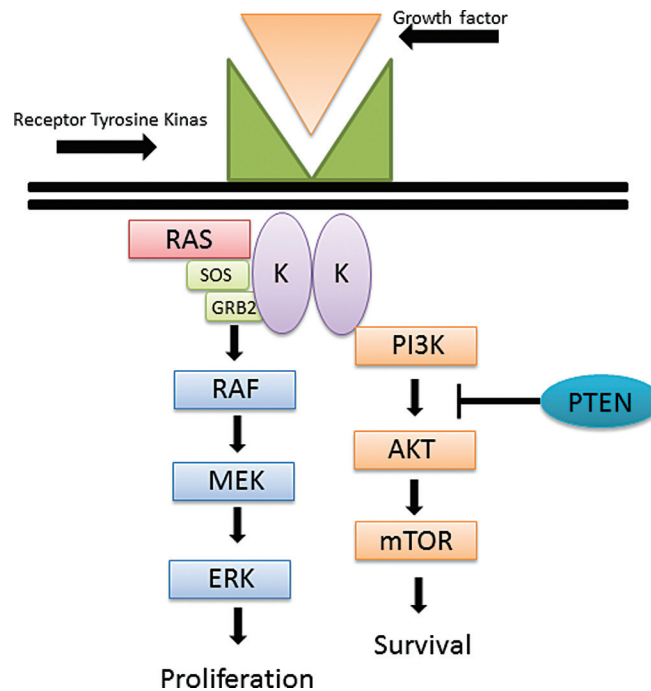
Figure. 3. A simplified overview of key routes in intracellular signalling commencing with receptor activation of Ras (26–28).

The time for post-mitotic cells to reach the restriction point, R, is ~3–4 h, and this time course was observed to be remarkably constant for virtually all cell lines examined (14–17).

G_1, therefore, could be divided into two portions as shown in Figure 2: G_{1pm} for G_1-post-mitotic and G_{1ps} for G_1-pre-synthesis, whereby the length of G_{1pm} was relatively constant, whereas the length of G_{1ps} was, in contrast, variable (6–9). Importantly, it is this variability in the duration of G_{1ps} that contributes to almost all variability in the total inter-mitotic time. This suggests that there are two independent decisions made by the cell in G_1. In G_{1pm}, the cell decides *if* it is going to progress towards mitosis or exit into G_0. In G_{1ps}, the cell is committed to DNA replication and mitosis but decides *when* it will enter S phase (16).

Key regulators of cell cycle progression are the cyclins that interact with and activate specific CDKs. There are 2 major classes of cyclins active in the G_1 phase of the cell cycle: cyclin D and cyclin E. Cyclin D binds either CDK4 or CDK6, and cyclin E complexes with CDK2. Cyclin D, together with either CDK4 or CDK6, phosphorylates Rb to generate hypo-phosphorylated Rb. Hypo-phosphorylated Rb binds with the E2F family of transcription factors that are required for the transition from G_1 to S phase (21).

The association of E2F with Rb is dependent on the hypo-phosphorylation provided by the cyclin D:CDK4/6 complex, and therefore, the suppression of E2F by Rb depends on cyclin D:CDK4/6 (33). However, phosphorylation of Rb by cyclin D:CDK4/6 also results in a dissociation of histone deacetylases from Rb. These enzymes invoke the derepression of cyclin E gene expression (34) (Figure 4).

Cyclin E:CDK2 is inhibited by the CDK inhibitor p27Kip1, but p27Kip1 also interacts with cyclin D:CDK4/6. As cyclin D levels increase, more p27Kip1 is sequestered, and as a consequence, cyclin E:CDK2 releases its inhibitory p27Kip1 to act on the more abundant cyclin D:CDK4/6. It has been proposed that the sequestration of p27Kip1 by cyclin D:CDK4/6 activates cyclin E:CDK2 (21,33).

The location of the restriction point at the transition from G_{1pm} to G_{1ps} is ~3.5 h following mitosis, a point where cyclin D levels increase. This is because growth factors that facilitate passage through R do so by stimulating increases in cyclin D levels. Activated Ras, which mimics growth factor signals, also stimulates an increase in cyclin D levels. In a tumour context, it is notable that transformation of cells by Ras is dependent on cyclin D (34,36).

In summary, there appears to be a significant correlation between cyclin D levels and progression from G_{1pm} to G_{1ps}, but whether the hypo-phosphorylation of Rb by cyclin D–CDK4/6 has any role in this transition is unclear, since hypo-phosphorylation of Rb occurs also in G_{1ps}. However, in the absence of Rb, cells apparently do not leave the cell cycle under suboptimal growth conditions, indicating that inactivation of Rb may still be necessary to enter quiescence.

After progression through the cyclin D-dependent phase, cyclin E becomes activated and Rb becomes hyper-phosphorylated by the cyclin E/CDK2 complex. At this point, Rb no longer binds E2F, and the released E2F can then activate the transcription of many genes needed for transition into the S phase. Another substrate for cyclin E/CDK2 is its inhibitor, p27Kip1. Phosphorylation of p27Kip1 makes it susceptible to ubiquitination and degradation by the proteasome. Here, cyclin D is no longer required for sequestering p27Kip1 to activate cyclin E/CDK2. Hence there appears to be a feedback loop whereby cyclin E–CDK2 suppresses Rb and liberates E2F, which in turn increases cyclin E levels so that cyclin E/CDK2 can continue to suppress Rb and keep p27Kip1 targeted for degradation. Such cells can now progress through G_{1ps} into S phase (35,37,38).

Transit through the cell cycle is affected in an unusual way by transforming growth factor-β (TGF-β) and related growth factors, which inhibit cell cycle progression in late G_1 and concomitantly increase cyclin E/CDK2 inhibitor p27Kip1 levels (39,40). TGF-β can consequently inhibit transit through the cell cycle at the cyclin E-dependent point late in G_{1ps}. Defects in p27Kip1 expression
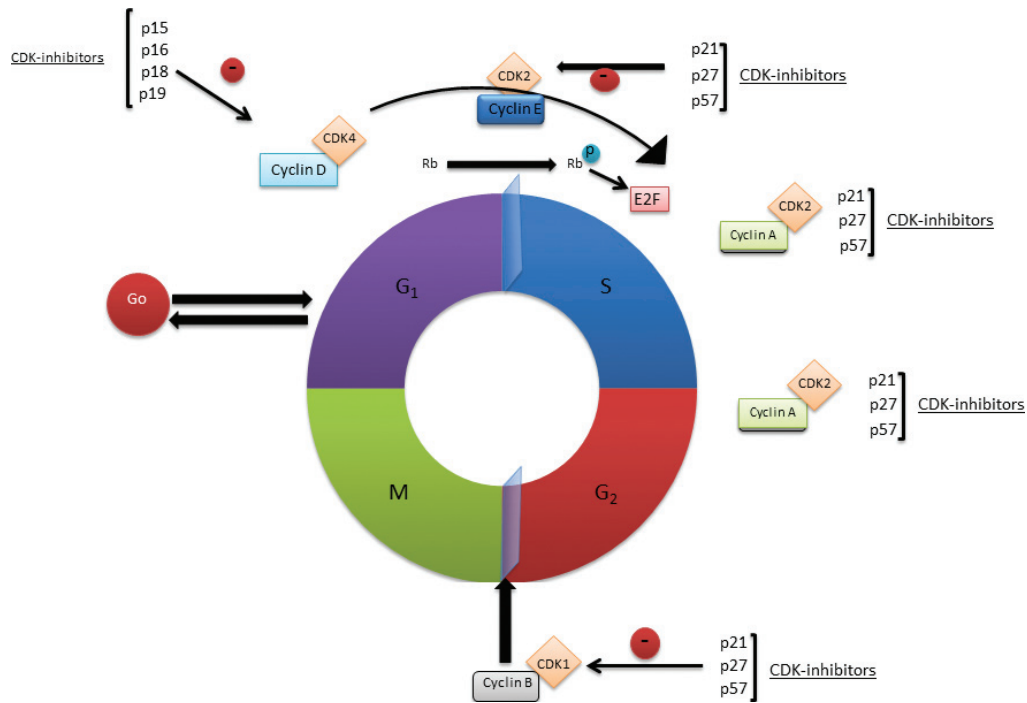
Figure. 4. The role of cyclins in the transition through various stages of the cell cycle. Cyclins associate with different cdks and their combined activities drive proliferating cells through checkpoints en route to mitosis in a concentration-dependent fashion. Each of the depicted cyclin–cdk complexes has antagonistic factors that will inhibit their action on cell cycle transit (reviewed in ref. 35).

are common in human tumours. TGF-β-induced cell cycle arrest occurs together with inhibition of Myc gene transcription. This is in accordance with the role of Myc as a downstream target of mTOR and phosphoinositide-specific phospholipase C signals, both of which inhibit TGF-β signalling. There is further evidence for a late G_{1ps} checkpoint mediated by TGF-β. One group of tumour promoters—phorbol esters—cooperate with activated Ras, but not elevated Myc, to transform primary rodent fibroblasts. This suggests that phorbol esters can act as progression factors for transit through G_{1ps} (41). The tumour-promoting effects of phorbol esters are thus the result of inhibiting protein kinase Cδ (PKCδ) synthesis. PKCδ is required for the TGF-β signals that induce cell cycle arrest. Phorbol esters thus facilitate passage through the G_{1ps} checkpoint mediated by TGF-β (42,43).

The connection between cyclin E and mTOR via TGF-β signalling provides us with an important link to cell nutrition. mTOR is activated by amino acids and is suppressed by low adenosine triphosphate levels. Earlier studies showed that Swiss 3T3 cells are sensitive to selective amino acid starvation and could be inhibited or activated according to the amino acid balance in the culture medium. The lack of essential amino acids, similar to treatment with the mTOR inhibitor rapamycin, results in G_1 cell cycle arrest. However, re-entry of cells into the cell cycle after amino acid addition is significantly faster (2 h) than when cells have entered quiescence after growth factor depletion (12 h). The time difference between recovery from amino acid starvation versus serum deprivation distinguishes the classical restriction point from an mTOR-dependent checkpoint (44,45).

Signals activated by insulin and insulin-like growth factor-1 (IGF-1) also provide a response to nutritional sufficiency. One model has described commitment and progression of cells from G_0 into S phase whereby transient exposure to platelet-derived growth factor was sufficient to get cells to 'commit' to cell cycle entry, but 'progression' through the rest of G_1 required

continuous treatment with IGF-1 (46). This early study is consistent with the model where platelet-derived growth factor is needed for re-entry into the cell cycle and IGF-1 required for the subsequent transit through G_{1ps} (Hultman,T. and Engström,W., submitted). Platelet-derived growth factor increases cyclin D expression, whereas IGF-1 activates PI3K, mTORC1 and mTORC2 (47). Thus, the commitment/progression model for passage through the G_1 phase of the cell cycle is consistent with both a growth factor cyclin D-dependent restriction point and an mTOR and cyclin E-dependent cell growth checkpoint.

The AMP-dependent kinase phosphorylates the Regulatory Protein of mTOR (Raptor). Therefore, it is required for inhibition of mTORC1 (48). As AMP-dependent kinase has been widely implicated as a sensor of metabolic capability of the cell, a 'metabolic checkpoint' regulated by AMP-dependent kinase and mTORC1 in G_{1ps} has been hypothesized. Moreover, PI3K signalling, which contributes to mTOR activation, is involved in the control of growth and metabolism. Thus, a significant body of evidence is emerging that implicates mTOR as a critical mediator of cell cycle progression, through G_{1ps}, that can be distinguished both functionally and temporally from G_{1pm} (49).

Activator protein 1 (AP-1) collectively refers to a class of functionally related transcription factors that are characterized by a basic leucine-zipper region. It comprises members of the Jun protein family (c-Jun, JunB and JunD) and Fos protein family. The Fos family includes c-Fos, FosB, Fos-related antigen-1 and Fos-related antigen-2 as well as smaller FosB splice variants FosB2 and DeltaFosB2. All these proteins, after dimerization, bind to the so-called 12-O-tetradecanoylphorbol-13-acetate response elements in the promoter and enhancer regions of target genes. Additionally, some members of the ATF (ATFa, ATF-2 and ATF-3) and JDP (JDP-1 and JDP-2) subfamilies, which share structural similarities and form heterodimeric complexes with AP-1 proteins (predominantly Jun proteins), can bind to

12-O-tetradecanoylphorbol-13-acetate response element-like sequences. In contrast to Jun proteins, Fos family members are not able to form homodimers but heterodimerize with Jun partners, giving rise to various trans-activating or trans-repressing complexes with different biochemical properties.

It has been suggested that one AP-1-regulated gene might be preferentially induced by Jun-Fos dimers. Experimental data have also shown that single characteristics of a transformed phenotype are triggered by specific Jun-Fos protein dimers. Generally, AP-1 proteins have both overlapping and unique roles and function in a tissue- and/or cell-specific mode. Using the analysis of expression and/or activity of all Jun and Fos family members, it was shown in several experimental systems that malignant transformation and progression is accompanied by a cell type-specific shift in the AP-1 dimer (50).

AP-1 converts extracellular signals of evolutionary conserved signalling pathways like mitogen-activated protein kinase (MAPK) TGF-β and Wnt into changes in the expression of specific target genes that harbour AP-1-binding sites. Growth factors, neurotransmitters, polypeptide hormones, bacterial and viral infections, as well as a variety of physical and chemical stresses, employ AP-1 to translate external stimuli both into short-term and long-term changes of gene expression. However, it should be noted that AP-1 proteins are capable of recruiting different transcription co-factors, depending on cell type or physiologic/pathologic context. Although AP-1 proteins are primarily associated with the regulation of cellular proliferation, it seems that one of their main features is their ability to cross-interact with various other crucial signal transduction pathways, thus affecting important cellular events (51).

Much of the dysregulation in human neoplasia hinges on defects in the control of G_1 cell cycle progression. The defence against unwarranted progression through G_1 into S phase is programmed cell death, cell senescence or cell differentiation. Therefore, suppression of oncogenic signals, in principle, can resurrect these defences and result in apoptosis or senescence. Although many genetic alterations have been discovered in human cancers and cancer cell lines, and a major effort to identify still more genetic alterations in cancer cells is ongoing, these mutations may affect a relatively small number of regulatory points in G_1 of the cell cycle. Understanding these key regulatory sites may allow for rational targeting of relatively few signals that override the key regulatory points in G_1 that apparently are necessary in most, if not all, forms of neoplasia.

However, there are still unresolved issues that constitute a challenge for the future. First of these are the overlapping actions of signals that facilitate passage transition from G_{1pm} to G_{1ps}. Ras activates multiple downstream targets in addition to the Raf/MEK/MAP kinase pathway. And significantly, Ras can activate PI3K, which feeds into the mTOR pathway. Ras also activates Ral-GDS, which leads to elevated PLD activity and similarly feeds into the mTOR pathway. The activation of RalA has been reported to be important for the transformation of human cells by activated Ras. Human cancer cells with activating mutations to Ras are dependent on RalA and PLD activity for survival, indicating that RalA and PLD are important Ras targets for maintaining cell transformation. Studies also reveal that in addition to activating Raf/MEK/MAP kinase signals and elevating cyclin D expression, Ras is capable of activating signals that target mTOR. Ras can also stimulate Myc expression, which is also a target of mTOR signals (36) (Hultman,T. and Engström,W., submitted).

Thus, logically, Ras should be able to dysregulate both the restriction point and the cell growth checkpoint. However,

many studies have found that introduction of both Ras and Myc genes is required for the transformation of either mouse or human cells. Therefore, although Ras is able to induce Myc expression under some circumstances, Ras could not transform primary cells without Myc. In addition, cyclin D-null cells have been shown to be resistant to transformation by Neu and Ras but not to transformation by Myc. Therefore, although Ras can activate multiple signalling pathways in different cell contexts, it is likely that the most critical target of Ras is the Raf/MAP kinase pathway to stimulate cyclin D and passage through the growth factor—G_{1pm} (Figure 5) (36) (Hultman,T. and Engström,W., submitted).

Another troublesome issue is the surprising finding that cyclin E knockout mice develop almost normally. However, although the cyclin E-deficient cells are apparently capable of dividing during development, cells from the cyclin E-null mice are resistant to transformation, supporting the premise that cyclin E is a critical target of signals activated during tumourigenesis. It is possible that cyclin E represents a mechanism for promoting cell cycle progression not utilized during development but is able to drive G_1 cell cycle progression under oncogenic stimulation (35) (Hultman,T. and Engström,W., submitted). However, this article will argue that environmental chemicals with carcinogenic potential contribute to sustained cell proliferation by interfering with some basic cell control mechanisms.

Membrane receptors and sustained proliferation

The ERBB/HER receptors are type I growth factor receptors with tyrosine kinase activity corresponding to epidermal growth factor (EGF) stimuli. Their main function is flow of information from the extracellular environment to the cell's nucleus. EGF receptors promote proliferation when stimulated, providing a survival advantage in those cells. The members of this family are four proteins that integrate the membrane: EGF receptor 1 (also called EGFR, ERBB1 or HER-1), HER-2 (also called ERBB2 or Neu), HER-3 (also called ERBB3) and HER-4 (also called ERBB4) (Figure 6). The ERBB receptors belong to the greater family of receptor tyrosine kinases and are cell surface allosteric enzymes. These enzymes consist of a trans-membrane hydrophobic domain that separates an extracellular ligand-binding domain and an intracellular kinase domain. In order to activate their tyrosine kinase activity, ligands that contain an EGF-like domain bind to ERBB receptors. Different EGF-like ligands activate different receptors of the ERBB family, except ERBB2, which has no identified ligand yet (52).

Dimer formation between the four receptors occurs to activate the tyrosine kinase domain. Upon activation, ERBB receptors activate downstream intracellular pathways, including PI-3K/Akt, Ras/MAPK, PLCγ1/PKC, STAT and Par6-atypical PKC pathways (53). These pathways are involved in different cellular functions such as inhibition of apoptosis, progression of proliferation, differentiation, angiogenesis, metastasis, epithelial–mesenchymal transition and cell motility. Proper regulation of these signalling networks is a prerequisite for cell homeostasis. Deregulation and subsequent aberrant signalling due to mutation, amplification or presence of autocrine loops contributes in the development of carcinomas.

There are multiple potential ligands for the ERBB receptors. The ectodomain of ERBB proteins is highly conserved and ligand interaction promotes a conformational change. The extracellular domain in the 'ligand-free' scenario obtains a close/'tethered' composition, masking the dimerization-binding sites of the protein. This extracellular region has four distinct domains, two
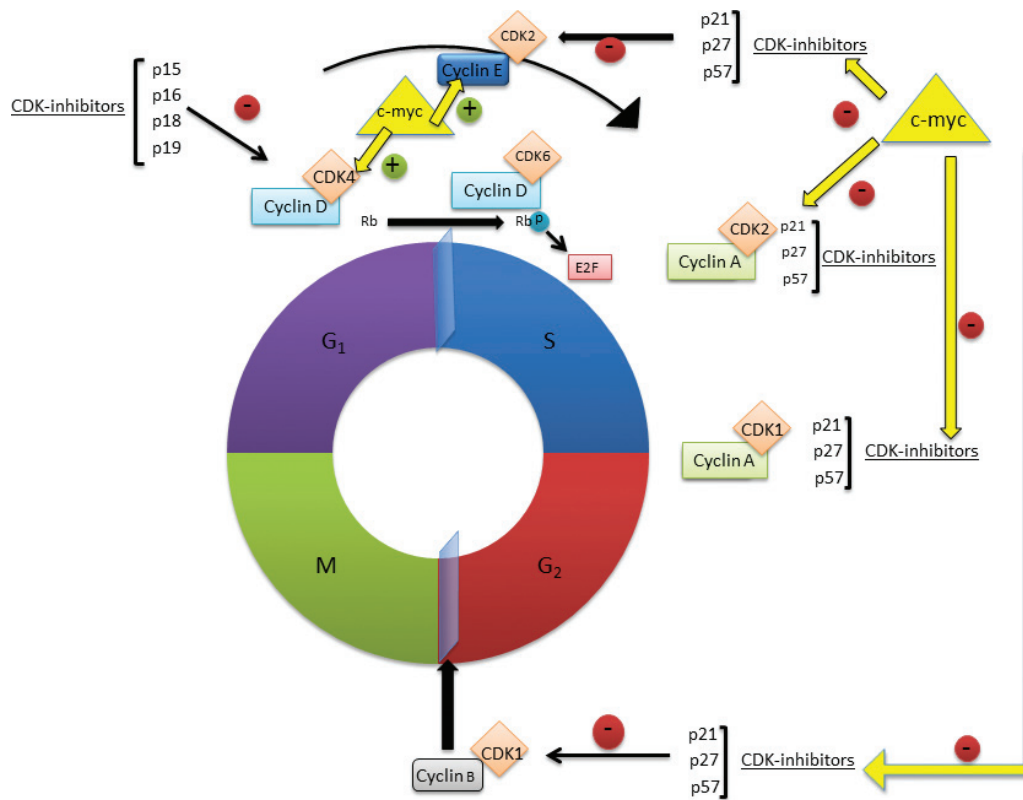
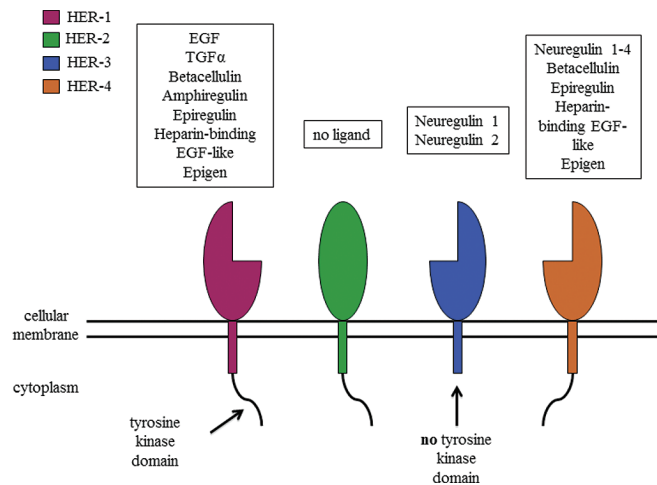**Figure. 5.** The involvement of Myc in sustained cell proliferation.

Fig. 6. The different human EGF receptors with respect to structure and function. For detailed explanation, see text (reviewed in ref. 52).

of which are leucine rich and are responsible for ligand binding. After ligand binding, the conformational change results in open composition of the extracellular domain and exposure of dimerization interfaces, leading to subsequent dimerization of the ERBB proteins.

Although ERBB receptors are membrane proteins, there is increasing evidence of nuclear translocation and function (54). All four receptors have been reported to be located in the nucleus of cancer and normal cells. Full-length nuclear EGFR is implicated in transcriptional regulation, DNA replication and DNA repair. In several tumours, EGFR has been found in the cancer cell's nucleus, and these patients have a remarkably poor outcome (55). In mouse type II epithelial cells, EGFR and ERBB-2 have been shown to be mainly localized to the nucleus,

and to a lesser extent the cytoplasm, whereas ERBB-3 was found almost exclusively in nucleoli, and ERBB-4 shuttled between the nucleolus and the cytoplasm (56,57). ERBB-3 has been found to be present in the nucleus of human mammary epithelial cells, and when nuclear export inhibitor is used, accumulation of ERBB-3 in the nucleus occurs. Heregulin β1 stimulation can shift ERBB-3 from the nucleolus to the nucleoplasm and then to the cytoplasm, demonstrating an additional role for ERBB-3 in the nucleolus.

Nuclear and cytosolic receptors and sustained cell proliferation

In the context of sustained cell proliferation, the aberrant function of a number of nuclear receptors has been implicated in

progression of hormone-associated cancers such as those of breast, prostate and endometrium (58,59). In particular, the sex steroid hormone receptors for oestrogens (ER) and androgens (AR) are being extensively studied given their critical roles in the growth of hormone-dependent breast and prostate cancers. Other nuclear receptors such as the peroxisome proliferator-activated receptors (PPARs) and those for thyroid hormones, progesterone and glucocorticoids and the cytosolic aryl hydrocarbon receptor (AhR) are also known to modulate cell proliferation.

Sex hormone signalling is initiated by interaction of oestrogens and androgens with their respective receptors. These receptors, once bound in the cytoplasm and nucleus, dimerise and translocate to the nucleus where they attach to their respective oestrogen and androgen responsive elements to recruit transcription factors, resulting in sex hormone-specific gene regulation. In hormone-dependent cancer cells, these genes invariably result in the synthesis of critical proteins involved in stimulation of cell proliferation. Beside the genomic pathway of sex hormone receptor-mediated signalling, sex hormone receptors may also activate cytoplasmic signalling pathways involved in cell survival and proliferation (58–66).

Oestrogens, oestrogen receptors and their relationship to growth factors

Oestrogen's effects are diverse, influencing proliferation, apoptosis, differentiation and metabolism. Direct effects are mediated by ER. Increasing knowledge of ER has expanded understanding of both its subcellular location and activity involving a variety of mechanisms. Similar to other nuclear receptors, the oestrogen receptor contains an N-terminal domain, DNA-binding domain, a hinge region and a ligand-binding domain. As with other nuclear receptors such as the androgen receptor, the ER acts as a transcription factor to mediate many of these proliferative events. Two main forms of ER have been identified, ERα (60) and ERβ (61). Both traditionally belong to the nuclear receptor family of transcription factors, each having splice variants. The two share a high degree of sequence homology in the DNA-binding domain but have differing binding affinities for ligands at the ligand-binding domain and have distinct functions relating to proliferation and apoptosis. Whereas activation of ERα causes cellular proliferation, ERβ has recently been identified as a novel tumour suppressor gene (67). Moreover, a decline in the amount of the ERβ relative to ERα is associated with cellular proliferation in both normal and tumourigenic ovarian surface epithelium at both messenger RNA and protein levels (67,68) and has been implicated in the ability of epithelial cells of both prostate and ovary to resist undergoing apoptosis (69). ERβ has been shown to achieve control over both normal and oestrogen-induced proliferation by decreasing total amount of Rb, phosphorylated Rb and phospho-AKT as well as cyclins D1 and A2. The combined effect is one of reducing the frequency of cells in S phase and increasing the frequency of cells seen in the G₂/M phase of the cell cycle. ERβ additionally directly suppresses the activity of ERα. In MCF-7 breast cancer cells, adenovirus-introduced expression of ERβ leads to repression of c-myc, cyclin D1 and cyclin E transcription while increasing messenger RNA expression of p21 and p27, having the combined effect of arresting cells in G₂ (70). Decreased levels of ERβ may therefore act to drive immortalization in breast and ovarian epithelial cells. Oestradiol exposure has been shown to suppress ERβ messenger RNA and protein levels both *in vitro* and *in vivo* (67,68,71).

Oestrogen can also, however, drive transcription of target genes by indirect mechanisms that involve ligand-bound oestrogen receptor binding to other transcription factors that in turn bind their cognate ligands. Examples include oestrogen's modulation of AP-1 (62), stimulatory protein-1 (63,64) and nuclear factor kappa B (65). Additionally, oestrogen exerts rapid (non-genomic) actions by its interactions with a number of signal transduction pathways that include ERK/MAPK, PLC/PKC, p38/MAPK and P13/AKT (66).

17β-Estradiol (E2) and progesterone, along with growth factors such as EGF or IGF-1, play a pivotal role in the development of the mammary gland (72). E2 and EGF support the proliferation of ductal epithelial cells. Similarly, E2 and IGF-1 stimulate proliferation of uterine epithelial cells. Both tissues require E2 and growth factors, and treatment with E2 and EGF or IGF-1 is synergistic. Dysregulation of these signalling molecules results in uncontrolled proliferation and survival. E2 can enhance EGF and IGF-1 ligand and receptor expression, and EGF may also regulate ER and receptor coregulator protein expression. EGFR or IGF-1R knockout mice display a decreased or abolished E2 proliferative response. Conversely, inhibition of EGFR activity or introduction of mutant EGFR decreases E2-stimulated signalling and proliferation in breast cancer cells. Forced down-regulation of ER in breast cancer cells by small interfering RNA (siRNA), or inhibition by anti-oestrogens, abrogates E2 and EGF stimulation of DNA synthesis (73,74).

There is a population of ER molecules localized to the cytoplasmic and membrane compartments. This population is especially important in ER-positive breast cancer, where ERα protein is overexpressed up to 10-fold. ER can associate directly with the cytoplasmic membrane via a palmitic acid covalently associated with a specific cysteine in the ligand-binding domain (75). E2 binding to ER in this respect is fundamental to the initiation of ER activation and rapid ER signalling. GPR30, an E2-binding G-protein coupled receptor, acts via G protein βγ subunits and can potentially modulate E2-stimulated changes in protein kinase A or MAPK activity. GPR30 is expressed in many tissues and cell types and is present in a subset of breast cancer cells that do not express ERα and ERβ. It can often be stimulated by anti-oestrogens such as tamoxifen as well as pure antagonists. Currently, it appears that although GPR30 may play a role in certain biological processes or even proliferation of certain cell types, E2-stimulated proliferation of breast cancer cells has, however, not been associated with this protein (76).

Early activation by E2 in breast cancer cells, leading up to cell division, appears to be associated with the cognate nuclear ERs. E2 stimulation of MAPK in breast cancer cells occurs within 3–15 min and can be inhibited with anti-oestrogens or knockdown of ERα. Cells from ERα/ERβ-knockout mice cannot support rapid E2 cytoplasmic signalling, and introduction of ERα or ERβ into ER-negative breast cancer or other cell lines reintroduces a functional E2 signalling. Introduction of siRNAs for ERα and/or ERβ abrogates rapid E2 signalling, whereas introduction of GPR30 siRNA and knockdown of this protein do not. However, these results may be cell specific, and GPR30 may well play a significant role in non-genomic E2 effects and steroid-regulated proliferation in other cell types (77,78).

Since ERα and ERβ lack kinase activity, it seems reasonable to assume that other molecules transduce information from E2–ER binding to stimulate cytoplasmic signalling. An intracellular tyrosine kinase c-Src, which links many growth factor receptors and signalling molecules, plays a pivotal role in E2 cytoplasmic signalling. E2-stimulated proliferation and activation of intracellular pathways such as MAPK and signal transducer and activator of transcription are inhibited in the presence of c-Src inhibitors or a mutated kinase-deficient c-Src. It has been difficult to demonstrate direct association of purified ER and c-Src,

although ER directly associates with the p85 subunit of PI3K and several adaptor or scaffold proteins have now been proposed to serve a role in enabling or stabilizing ER-c-Src associations and signalling, including Shc, MNAR and Cas. Because c-Src activity is restricted by intramolecular interactions, association with scaffold proteins may serve to stimulate c-Src activity and activate signalling through a number of intracellular signalling pathways, including PI3K and Shc-Grb/Sos/Raf/Ras to MAPK (79,80).

The IGF-1R has been shown to be a docking protein for ER–Shc complexes on membranes. Activated Shc and IGF-1R are required for E2-stimulated activation of MAPK. A third adaptor/scaffold protein, p130Cas (Cas), can also be found in cellular complexes containing ER, c-Src and PI3K, and siRNA for Cas dilutes E2-stimulated c-Src and MAPK activity in breast cancer cells. Additional mechanisms for E2 action via the EGFR also exist in breast cancer cells. Transactivation/phosphorylation of EGFR by E2 in MCF7 cells can also involve the rapid liberation of heparin-binding EGF via matrix metalloproteinases; this protein binds to EGFR and sparks off cytoplasmic pathways that can be blocked by antibodies to this ligand for EGFR (81).

The role of cytokines in sustained proliferation and their relationship to oestrogen

Oestrogen has been shown to exert inhibitory effects on selected cytokine production and activity, with most research in this area centred on the effects of the interleukins (IL-1, IL-6) and tumour necrosis factor alpha. Following menopause, there is evidence for a spontaneous increase in these pro-inflammatory cytokines (82) that have also been shown to independently cause cell proliferation (83–86). Recently, it has been proposed that increases in such cytokine production and activity may play a role in resistance to tamoxifen in sporadic ovarian epithelial cancer, a cancer mainly occurring in women above the age of 60 (UK, 2013). In an elegant series of experiments, Wang *et al.* (87) have shown that tamoxifen resistance is initiated by IL-6 in ovarian cancer cells and is associated with increases in ERα, decreases in ERβ, increased interaction between ERα and steroid receptor coactivator-1, but not steroid receptor coactivator-1 and ERβ, and the blockade of oestrogen-induced ER nuclear translocation.

In an independent study looking at cytokine ability to sustain proliferation *per se*, the IL-3-dependent myeloid progenitor cell line was used as a model system to trial the effect of cytokine withdrawal on cell cycle progression and on apoptosis. The authors concluded that cytokine withdrawal resulted in downregulation of cdk2/cyclin E/A and cdk4/cyclin D2-associated kinase activity and subsequent proliferation arrest (88).

Androgen receptor-mediated cell proliferation

The actions of androgens run remarkably in parallel to those of oestrogens, with the marked exception that there appears to be only one form of androgen receptor. The AR gene (formally named NR3C4) is located on chromosome Xq11–12 and contains eight exons coding for a protein with ~919 amino acids dependent on the number of polyglutamine and polyglycine repeats. Similar to other nuclear receptors, the androgen receptor contains an N-terminal domain, DNA-binding domain, a hinge region and a ligand-binding domain. The proliferative effects of androgens are most well understood in prostate cancer, of which the growth is strongly androgen dependent in early stages of the disease. As with other nuclear receptors such as ER, the androgen receptor acts as a transcription factor to mediate many of these proliferative events. The main driver

of androgen-dependent prostate cancer cell proliferation is DHT that is formed locally in high concentration from conversion of testosterone by steroid 5-alpha reductase (SRD5A). Once androgen bound, the AR dissociates from heat-shock proteins, homodimerises and is translocated to the nucleus where it binds specific androgen receptor-responsive elements on DNA. It then recruits other transcriptional coregulators (coactivators, repressors) to allow for increased expression of specific genes involved in (cancer) cell proliferation. A well-known example of such a gene is that coding for the protein prostate-specific antigen. AR activation is implicated in the transcriptional upregulation of EGF (89) and vascular endothelial growth factor (90) and in turn may be activated androgen-independently by various growth factors, including IGF, keratinocyte growth factor and fibroblast growth factor (91). Some of these effects are non-genomic and, similarly to oestrogens, androgens induce a direct association of cytoplasmic AR with c-Src (92,93), resulting in MAPK activation and increased DNA synthesis.

Key mechanisms involved in AR-mediated sustained cell proliferation include (i) over expression of AR, (ii) increased constitutive ligand-independent activation of AR, (iii) decreased degradation of AR, (iv) increased intracellular synthesis of androgens, in particular DHT through local overexpression of SRD5A and (v) mutations in AR. Each of these mechanisms has complex causes that are far from well understood. Androgen receptor over expression occurs in 80% of prostate cancer patients that no longer respond to antiandrogen treatment (94,95) and this occurs through a process of gene amplification in response to androgen-deprivation therapy (96,97).

In LNCaP cells, a single point mutation T877A in the ligand-binding domain increases its promiscuity for ligands other than androgens, thus allowing other steroid hormones to increase cell proliferation (98). In another cell line 22Rv1, several AR splice variants are present, including an AR protein that is the product of exon 3 tandem duplication as well as splice variants of C-terminally truncated AR protein (AR3) that lacks the ligand-binding site and contains an aberrant exon 3 (exon 3b) and is constitutively active (99–101). The truncated AR3 variant is also commonly found in prostate cancer tissues (102).

Altered steroid metabolism is another key mechanism that may result in sex hormone receptor-mediated sustained cell proliferation. Key enzymes in the final step of biosynthesis of androgens and oestrogens are 3-oxo-5-alpha-steroid 4-dehydrogenases (SRD5A1/2) and the cytochrome P450 family 19 protein, CYP19, respectively, and their upregulation has been implicated in various proliferative diseases such as breast cancer, benign prostate hyperplasia and prostate cancer. Inhibitors of SRD5A1/2+, such as finasteride and dutasteride, are effective in the treatment of the prostatic diseases (103) and inhibitors of CYP19 are effective in the treatment of breast cancer (104). Increased activity of SRD5A in prostate tissue results in increased local DHT levels, which activates AR-mediated cell proliferation. In normal prostate tissue, steroid 5a-reductase activity is mostly catalysed by the SRD5A2 isoform, whereas in prostate cancer, the SRD5A1 gene becomes active resulting in an additional contribution to the biosynthesis of DHT from precursor androgens. The mechanism of SRD5A1 activation is not clear, but SRD5A1 expression is dependent on the transcription factor SP1 (105), which is overexpressed in a number of cancers, including prostate cancer (106).

Altered coactivator function also plays a role in AR-mediated sustained cell proliferation. Ligand-bound AR once bound to androgen responsive elements will recruit coactivators or corepressors, which results in increased or decreased gene

transcription, respectively (107,108). Although a large number of cofactors exist for the AR, the receptor has specific cofactor preferences. It has, for example, less affinity for the p160 (src) family of cofactors that other nuclear receptors prefer to attract, but a preference for the AR-associated protein cofactors. Dependent on the type of ligand bound to the AR, there may be preferential recruitment of either activating cofactors over repressors or *vice versa*. Many AR antagonists appear to act by producing a ligand-bound form of AR that preferentially recruits the nuclear receptor co-repressor and silencing mediator for retinoid and thyroid hormone receptors corepressors that in turn recruit histone deacetylases that perform the opposite task of histone acetylases and prevent chromatin opening and transcription of target genes (109). One can thus imagine that overexpression of certain AR coactivators or mutations in AR that facilitate coactivator recruitment could result in sustained cell proliferation in AR-responsive tissues such as prostate.

The aryl hydrocarbon receptor

The AhR is a cytosolic ligand-activated transcription factor of the basic helix-loop-helix/period AhR nuclear translocator single-minded family of transcription factors and is ubiquitously expressed (110). Tryptophan metabolite and indole derivatives have been proposed as possible physiological ligands for AhR; however, it is established that AhR is strongly activated by binding of polychlorinated biphenyls, polychlorinated dibenzodioxins (dioxins), polychlorinated dibenzofurans and polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene. In the cytosol, the AhR is chaperoned by repressor proteins but ligand binding results in conformational change, dissociation of the repressor proteins and heterodimerization with the AhR nuclear translocator. The dimer translocates to the nucleus where it binds to specific response elements in the DNA termed dioxin response elements or xenobiotic response elements located near the promoter of target genes, and recruitment of coactivating transcription factors leads to transactivation of gene expression (110). The major gene targets are phase I and phase II drug-metabolizing cytochrome P450 enzymes, which suggests that the AhR functions as a sensor of foreign compounds leading to upregulation of enzymes necessary for their clearance from the body. These enzymes are also involved in a range of metabolic reactions of which one example would be the synthesis of 17β-estradiol (E2) by aromatase (CYP19A1) and metabolites of E2 by CYP1A2 and CYP1B1. Pollutant chemicals, therefore, may act to cause sustained proliferation in cells possessing oestrogen receptors through increasing levels of endogenous oestradiol at inappropriate times.

Some studies report that elevated and constitutively active levels of AhR have been found in advanced human breast tumours and breast cancer cell lines, with a strong correlation between expression of AhR and the degree of the tumour malignancy (111). Reducing AhR expression by knockdown attenuates cell proliferation, anchorage independent growth, migration and apoptosis, *in vitro*, and reduced orthotopic xenograft tumour growth and pulmonary metastasis *in vivo*.

Approaching a conceptual challenge

One of the key remits of the Halifax project was to formulate a holistic view of environmental chemical carcinogenesis in relation to the hallmarks of cancer (2). This article has examined mechanisms that affect the control of cell proliferation and have been shown to be affected in cells that have relaxed their proliferation control mechanisms. It is known that a wide range of chemicals can induce proliferation in cells under experimental

conditions, some generally and some conditionally upon the expression of appropriate receptors. However, the controversy arises when the experimental exposure under controlled conditions is compared to real tissue levels of the chemicals *in vivo*, which are usually lower than those that are needed to induce cell proliferation *in vitro*. Therefore, the Halifax project has aimed at examining the effects of low dose mixtures of potential environmental chemical carcinogens. It was shown some years ago that mixtures of xenoestrogens could induce proliferation at concentrations where each individual would have had no measurable effect in the so-called 'something from nothing effect' (112–114). A more specific example has been published recently where it was shown that a low dose mixture of five paraben esters can increase cell proliferation at concentrations where each individually did not (115). Furthermore, the additive effects of parabens were studied at the actual concentrations measured in human breast tissue (116) and it was found that some breast tissues contained a concentration of one paraben sufficient to stimulate cell proliferation in human breast cancer cells *in vitro*, but other tissue samples had only sufficient if all five paraben esters were mixed and in some cases left for a longer time frame (117). [This is particularly an interesting example since oestrogen in itself has been shown to be a carcinogen, but at higher doses (114)]. Moreover, other analyses using xenoestrogens reach similar conclusions (115–118). Thus, it seems reasonable to assume that there are other combinations of potentially carcinogenic chemicals present in low doses that in the environment can contribute to carcinogenesis via the sustained proliferation hallmark. This provides a basis for further analysis.

Environmental substances that interfere with proliferative signalling

One result of the 20th century chemical revolution has been the accumulation in the environment of synthetic chemicals that mimic the action of naturally occurring signal molecules. Some of these molecules were developed to withstand natural degradation (e.g. polychlorinated biphenyl congeners), whereas others engender more harmful metabolites (e.g. DDT). Two consequences have been their magnification of concentration to trophic levels and their global spread. The legacy of certain chemical exposures has permanently altered the present and future health of man and wildlife. These effects can be viewed in two different modes. Firstly, chemicals can act via direct exposure or 'context-dependent' modifications. Alternatively, epigenetic modification can be 'germ-line dependent' being manifest in each generation in the absence of the causative agent. Since the change in the epigenome is permanently incorporated in the germ line, such environmental factors have the potential to redirect the future course of tumour development. In accord with the major aim of this article, we have chosen to concentrate on some important environmental substances that can be assumed to contribute to carcinogenesis by affecting some of the pathways described here.

Environmental oestrogenic chemicals

Over the past century, many environmental chemicals have been found to have the ability to interfere with oestrogen signalling either through binding to cellular oestrogen receptors or through altering endogenous oestrogen production (Figure 7) (119). Such receptor-mediated mechanisms of toxicity have challenged long-held concepts in toxicology, because effects can be found at lower concentrations of chemicals, and actions can be specifically targeted by the receptors within the cell.

Furthermore, contrary to the basic premise of toxicology 'the dose makes the poison', receptor-mediated mechanisms can often display non-monotonic responses (120). Most oestrogenic chemicals are organic molecules where binding to the ligand-binding pocket of the oestrogen receptor is facilitated by at least one *para*-hydroxy phenyl grouping that is present either intrinsically or following metabolic conversion (121). However, several metal ions have also been reported as capable of binding to oestrogen receptors and influencing their subsequent actions (122).

Many of these oestrogenic compounds have been identified as having proliferative effects in breast cancer cell lines that are dependent or responsive to oestrogen for proliferation, and the proliferative effects have been shown to be mediated through the oestrogen receptor (123). Environmental oestrogens may enter the human body from exposure through diet, occupational exposure, the domestic environment and use of personal care products (123).

Since hundreds of environmental chemicals with oestrogenic properties have been measured in the human body, it is clear that these chemicals are present as mixtures in the long term in body tissues although often at what are considered low doses in the p.p.m. range (123). All environmental oestrogenic compounds (with the exception of diethylstilboestrol and ethynyloestradiol) bind to the oestrogen receptors with lower affinity than the main physiological oestrogen 17β-oestradiol (124). As a consequence, many studies (but not all) suggest that the compounds are present in human tissues individually at concentrations that are lower than would be required for an effect on cell proliferation in cell culture models. However, the functionality of these compounds needs to now be investigated, not individually, but reflecting the environmental reality of their presence as complex mixtures (125). Cell culture studies show that low levels of mixtures of oestrogenic chemicals can combine to increase the magnitude of the oestrogenic response on proliferation and

can do so even at concentrations where each, individually, might have no effect on proliferation (112). Furthermore, cell culture models have shown that increasing the length of a proliferation assay can enable responses to be measured at later times for lower doses, which reflects the environmental reality of long-term exposure (115).

Other chemical disruptors

More recently, a greater understanding of the interaction of environmental and endogenous chemicals with the nuclear PPARs (126) and/or cytosolic AhR (110) has further revealed the ability of these chemicals to interfere with biological and disease processes including development, differentiation, inflammation and tumour formation. PPARs are activated by both endogenous (e.g. fatty acids and eicosanoids) and exogenous ligands. Exogenous PPAR ligands include hypolipidemic drugs (e.g. fenofibrate) and persistent environmental pollutants including food contaminants such as perfluorooctanoic acid and perfluorooctane sulfonate, phthalate esters and herbicides (e.g. lactofen). Current evidence has shown antiproliferative-, proapoptotic- and differentiation-promoting activities displayed by PPAR ligands. PPAR ligands further affect the expression of different growth-related genes through both PPAR-dependent and PPAR-independent mechanisms (127).

Nanoparticles

Nanoparticles (NPs), by virtue of their size (formally <100 nm), have the ability to traverse lipid membranes and then to translocate to both cytoplasmic and nuclear compartments. Their ability to penetrate the more porous nuclear membrane after endocytic plasma membrane transfer is well recognized and a host of NP constructs have been shown to locate to the nucleus after relatively short cell exposure times. This unique materials
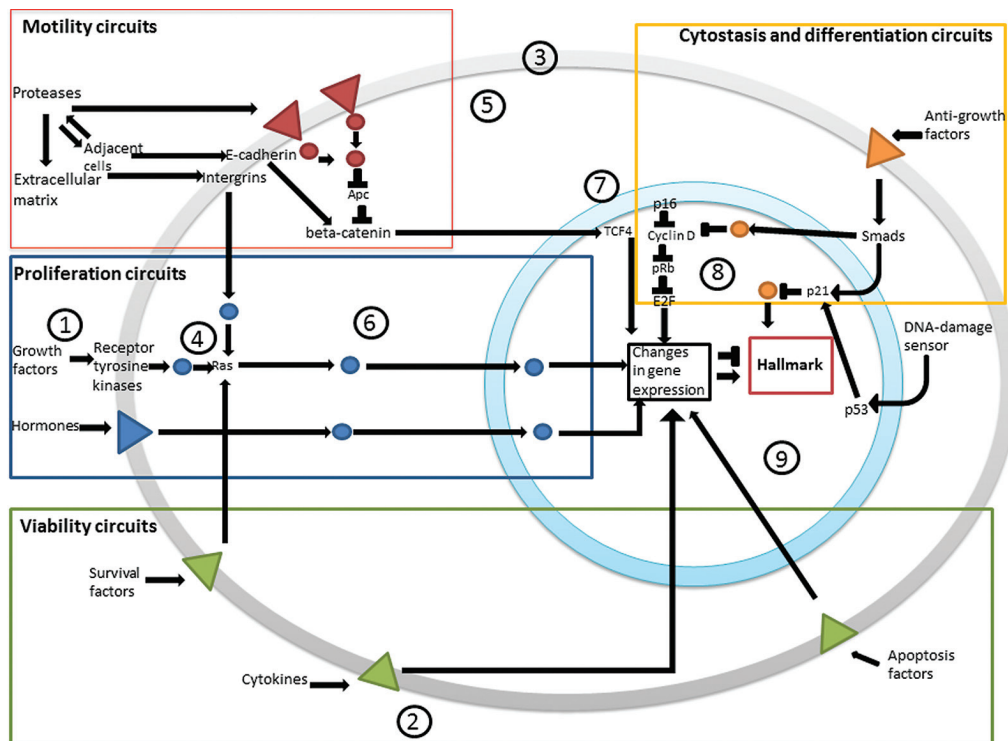

Fig. 7. Topographical and functional distribution of disruptive chemicals. Numbers 1–9 correspond to headings in Table 1 and Supplementary Table 1. Redrawn from (1).

facility can be attributed as much to their surface properties as to their size. Even with the same materials, mutagenicity extends well beyond that of their micron scale counterparts, and relates in part, though not totally, to this ability to enter the cell nucleus (128). NPs can be broadly classified into inorganic and polymeric; but for biological effects, this classification is probably of less importance than the ultimate physicochemical nature of the constituent components, which in any case overlap.

For biological interactions, the key drivers are, respectively, surface profile, chemistry, charge and energy, with overall size a determinant of mobility within the cell. Bioactivity is determined by the inherent solid state nature of NPs, in nearly all cases, and is not an outcome of particle dissolution, even though the high surface area/volume ratio of NPs facilitates this process. The release of soluble components certainly can occur, and it is here that the action of polymeric and inorganic particles may be different. However, dissolution processes can also confound our understanding of toxicity mechanisms. Thus, in the case of CuO, DNA toxicity is only seen through the release of copper ions; however, external copper ion exposure of the cell generates no toxic effects because the ion does not cross the cell membrane. Alternatively, although Ag NPs show little cell toxicity because of a limited release of silver ion from the Ag surface, direct Ag ion exposure of the cell shows clear toxic effects (128). Although such observations are considered in the framework of toxicity, other biological effects leading to cell proliferation may be similarly complicated.

The manifestation with regard to the cell cycle is that with minor adenosine triphosphate reduction, there is G_1 accumulation and with major adenosine triphosphate reduction, an excess G_2/M (129). A specific example of ROS-mediated proliferation is that seen with tungsten carbide/cobalt NPs (130). Here, free radical generation has been linked to the proliferative stimulus via AP-1, enabled by the MAP kinase pathway (ERKs, p38 kinase and JNKs). ROS damage to DNA will take specific reaction pathways, though these have not been delineated. One pathway that has been characterized is that due to peroxynitrite ion, which is found to generate DNA deletion mutations (131). Damage by other mechanisms may be more NP location related and likely to be exaggerated by direct NP access to the nucleus (132).

A further effect that has been seen is that of actual cell ROS lowering in the case of mesoporous (2–50 nm pore) silica NPs. Two mechanisms are considered here, one is ROS scavenging by formation of surface partial-charge transfer complexes, and the other is biochemical signalling. The latter appears to lead to Bcl-2 overexpression and resulting increase in antioxidant enzymes (e.g. the glutathione peroxidase, catalase) with an accumulation of glutathione. The cell cycle consequences are a prolongation of S and M phases and thereby the promotion of cell proliferation (133).

Categorization and prioritization of disrupted targets and mechanisms most relevant for promoting sustained proliferative signalling as a contributing mechanism to tumour development

Up to this point in the present review, our team of researchers has presented a comprehensive overview of the cellular and molecular mechanisms involved in the cancer hallmark termed 'sustained proliferative signalling'. In doing so, we have revealed the many potential targets and pathways that environmental chemicals either alone or in combination with other chemicals can disrupt, so as to promote and/or maintain unrestrained cell proliferation.

The next logical step is to table what we propose is a list of candidate chemicals and the targets they disrupt to facilitate sustained cell proliferation. Tabled also is the source of the chemical in the environment, the route(s) of exposure and any known adverse health effects. The source of the current available evidence is also supplied (Table 1 and Supplementary Table 1). Some of the information pertaining to commonly used herbicides and pesticides was from the only available source, the 2009 Environmental Protection Agency ToxCast report, using high-throughput assay. The Environmental Protection Agency-screened chemicals included in the table carried the highest scores for the ToxCast Growth signalling counts and were indicative of the number of growth-associated genes that were activated and in how many assays. Published findings provided the information for the remaining high priority disruptors listed (Table 1 and Supplementary Table 1).

Table 1. Overview of exposure routes and targets for 17 candidate environmental carcinogenic chemical disruptors

Chemical disruptor	Exposure route	Target
Bisphenol A	Epoxy resins, plastics	2, 4, 5, 6, 8, 9
Cyprodinil	Fungicide	1, 2, 3, 4, 5, 6
Edible oil adulterants	Food contaminant	4
Etoxazole	Insecticide	1, 2, 3, 6
Imazalil	Fungicide	2, 3, 5, 6, 7
Lactofen	Herbicide	2, 3, 5, 6, 7
Maneb	Fungicide	1, 2, 3, 6, 9
Methoxychlor	Insecticide	1, 2, 3, 4, 6, 9
Perfluorinated octonoid sulphate	Flame retardant	1, 2, 4, 6, 7, 9
Phthalates	Plasticizer	5, 6, 7
Phosalone	Insecticide	1,3,6,7
Polybrominated diphenylethers	Flame retardant	5
Prochloraz	Fungicide	1,3,5,6,7
Pyridaben	Insecticide	1,3,6
Trenbolone acetate	Synthetic anabolic steroid	6

The targets are presented in detail in Supplementary Table 1. Figures in the target column correspond to the targets named in Supplementary Table 1 and represent (1) growth factors and their receptors, (2) cytokines and their receptors, (3) B lymphocyte markers, (4) downstream signalling, (5) AhR, (6) steroid hormone receptors, (7) PPAR, (8) AP-1 proteins/transcription/translation regulators and (9) cell cycle regulators.

For the Environmental Protection Agency-screened chemicals generally, a dose of ~100 µM of each individual chemical was used in each assay. The potency of the chemical in assays giving a positive (gene activation) response was summarized using AC_{50} (i.e. at a concentration of 50% maximum activity), or the lowest effective concentration values, depending on the nature of the dose–response data collected for the assay. It should be stated here that the use of nominal potency in determining hazard identification and prioritization of chemicals for further testing has been challenged, because *in vitro* assay conditions cannot account for the *in vivo* impact of bioavailability, metabolic clearance and exposure characteristics ([134]). More recently, *in vitro* to *in vivo* extrapolation using human dosimetry and exposure information with reverse toxicokinetics alongside *in vitro* toxicity high-throughput assay has been valuable in assessing the validity of high-throughput *in vitro* screening to provide hazard predictions at organismal level ([135–137]). Importantly, a recent analysis using *in vitro* to *in vivo* extrapolation of a subset of ER-activating chemicals for the ToxCast Phase 1 has shown that the estimated human oral doses (OEDs) for high-throughput assay were higher than those reported for actual human exposure. It could therefore be suggested that adverse effects caused by these chemicals would happen only at exposures far in excess of actual exposure. Nevertheless, authors caution that for oestrogenic chemicals such as bisphenol A and cyprodinil, and the methoxychlor metabolite HPTE, the median oral estimated dose for *in vitro* exposure is lower than 1 mg/kg/day and relatively small margins exist (<100-fold) between the OED and the highest human exposure estimate ([138]).

When compiling Supplementary Table 1, it became apparent that many of the listed chemicals simultaneously disrupted a number of the same targets, giving rise to a sustained proliferation target 'signature'. Importantly, every chemical listed with the exception of the edible oil adulterants and possibly PBDEs interacted with oestrogen receptor and/or androgen receptor. This holds some significance in light of comments made by Chang *et al.* ([138]).

We additionally provide a subsequent figure (Figure 7), which aims at presenting the disruptive mechanisms in a cell topographical context.

Crosstalk between sustained proliferative signalling and the other hallmarks of cancer

The carcinogenicity of low dose exposures to chemical mixtures in any given tissue will likely depend on the simultaneous instigation of several important tumour initiation and promotion mechanisms as well as the disruption of several important defence mechanisms. Thus, it was felt that a better way of identifying potential carcinogenic effects of combinations of environmental chemicals would be to conduct a thorough review of each team's prioritized chemicals and their targets.

Our team chose to conduct this cross-validation exercise using five prototype chemicals drawn from an original list of 17 (see Table 1 and Supplementary Table 1). The prototype chemicals chosen were bisphenol A, perfluorooctane sulfonate, lactofen, imazalil and phosalone. These were cross-validated in conjunction with two well-known human carcinogens—benzo(a)pyrene and 12-*O*-tetradecanoylphorbol-13-acetate—both used as a gold standard references. Benzo(a)pyrene is a polycyclic aromatic hydrocarbon that was identified as a human carcinogen a long time ago. Its main sources are residential wood burning, coal tar, automobile exhaust fumes all smoke resulting from the combustion of organic material, including cigarette smoke and charbroiled food. 12-*O*-tetradecanoylphorbol-13-acetate is a diester of phorbol and a potent tumour promoter often used in biochemical research to activate protein kinase C, an intermediary in the signal transduction pathway. 12-*O*-tetradecanoylphorbol-13-acetate was first found in the croton plant, a shrub found in Southeast Asia, that provokes a poison ivy-like rash. For the other (candidate) chemicals, bisphenol A is a carbon-based synthetic compound. It is employed to make certain plastics and epoxy resins. Perfluorooctane sulfonate is a man-made fluorosurfactant and global pollutant. It has been used as a fabric protector, as a fire fighting foam, in the semiconductor industry and in hydraulic fluids used in commercial aviation. Lactofen is a complex ester of acifluorfen and is a nitrophenyl ether selective herbicide. It is used in postemergence applications to certain crops that are resistant to its action. Imazalil (enilconazole) is a fungicide widely used in agriculture, particularly to protect plantations of citrus fruit trees. It is also used in veterinary medicine as a topical antimycotic. Phosalone, finally, is an organophosphate that is commonly used as an insecticide and an acaricide.

The aim was to see if the same disruptive actions that were apparent for these chemicals in the causation of sustained proliferative signalling also occurred across the full range of mechanisms that are known to be relevant in cancer biology, i.e. the other cancer hallmarks. Tables 2 and 3 summarize this cross-validation data, supported by an extensive literature search.

To illustrate how the cross-validation works, we give the example of H-Ras as a prioritized target, and benzo[a]pyrene as a protypical disruptor influencing sustained proliferation during carcinogenesis (Tables 2 and 3). Ras proteins are involved in signalling networks controlling cellular proliferation, and mutations of the *H-ras* gene lead to an imbalance in the proliferative signalling during carcinogenesis ([259]). Benzo[a]pyrene, on the other hand, is an established human environmental carcinogen that favours tumour cell proliferation ([260]).

H-Ras can be seen to be complementary to (i.e. promotes rather than opposes) at least six other hallmarks of cancer, vis-à-vis evasion of anti-growth signalling ([141,142]), angiogenesis ([143]), genetic instability ([144]), tissue invasion and metastasis ([148]), tumour-promoting inflammation ([149,150]) and tumour microenvironment ([151]). This suggests H-Ras holds a multifunctional role as a target involved in the promotion of carcinogenesis. Partial complementarity is also identified for deregulated metabolism ([139,140]) and resistance to cell death ([145,146]), while H-Ras's role in immune system evasion was not able to be established. Interestingly, oncogene H-RasV12-induced senescence is associated with DNA damage response in normal cells, suggesting an inhibitory role in tumourigenesis through replicative immortality pathways ([147]).

Benzo[a]pyrene can be seen as complementary to eight other hallmarks of cancer: deregulated metabolism ([218]), evasion of anti-growth signalling ([219]), genetic instability ([221]), resistance to cell death ([219]), replicative immortality ([219]), tissue invasion and metastasis ([221]), tumour-promoting inflammation ([222]) and tumour microenvironment ([223]). However, benzo[a]pyrene opposes angiogenesis because of the inhibitory effect of the metabolite benzo[a]pyrene-3,6-dione on vascular endothelial growth factor expression through HIF-1α-binding site ([220]). A 'not known' association exists with respect to immune system evasion, similar to H-Ras. Overall, H-Ras and benzo[a]pyrene share a significant degree of synergy across most of the hallmarks in carcinogenesis, including sustained proliferative signalling.

Table 2. Cross-validation of target pathways

Priority targets	Sustained proliferative signalling								
	Deregulated metabolism	Evasion of anti-growth signalling	Angiogenesis	Genetic instability	Resistance to cell death	Replicative immortality	Tissue invasion and metastasis	Tumor promoting inflammation	Tumor microenvironment
H-Ras	± (139,140)	+ (141,142)	+ (143)	+ (144)	± (145,146)	− (147)	+ (148)	+ (149,150)	+ (151)
Jun/Fos/AP1	± (152,153)	− (154–157)	0	+ (158,159)	± (160,161)	± (162,163)	+ (164)	+ (165,166)	+ (151)
Cyclin D, IL8, CXCL	± (167–170)	0	+ (171)	+ (172)	0	± (173,174)	+ (175)	+ (176,177)	0
PPAR	+ (178)	+ (179)	− (180)	0	− (181,182)	+ (183–185)	+ (186)	+ (187)	0
AhR	+ (188)	+ (189)	+ (190)	+ (191)	± (192–194)	+ (195,196)	± (197,198)	+ (199)	+ (200)
EGF receptor	+ (201)	0	+ (202)	+ (203)	± (204,205)	+ (206)	+ (207)	+ (208)	0
Steroid hormone receptors	+ (209)	0	0	+ (210)	0	± (211–213)	+ (214)	+ (215,216)	+ (217)

Target pathways sustained proliferative signalling were cross-validated for effects in other cancer hallmark pathways. Targets that were found to have opposing actions in a particular hallmark (i.e. anti-carcinogenic) were denoted using '−', whereas targets that were found to have promoting actions in a particular hallmark (i.e. carcinogenic) were denoted using '+'. In instances where reports on relevant actions in other hallmarks were mixed (i.e. reports showing both pro-carcinogenic potential and anti-carcinogenic potential), the symbol '±' was used. Finally, in instances where no literature support was found to document the relevance of a target in a particular aspect of cancer's biology were denoted '0'.

Table 3. Cross-validation of disruptors

Prototypical disruptors	Sustained proliferative signalling								
	Deregulated metabolism	Evasion of anti-growth signalling	Angiogenesis	Genetic instability	Resistance to cell death	Replicative immortality	Tissue invasion and metastasis	Tumor promoting inflammation	Tumor microenvironment
Benzo(a)pyrene	+ (218)	+ (219)	− (220)	+ (219)	+ (219)	− (219)	+ (221)	+ (222)	+ (223)
TPA	+ (224)	+ (225,226)	0	0	0	− (227,228)	+ (229)	+ (230,231)	+ (232)
BPA	+ (233,234)	+ (235)	+ (236)	+ (237)	± (235,238–240)	+ (241,242)	0	+ (243)	0
PFOS	0	+ (244)	+ (245)	+ (246)	− (247,248)	0	0	+ (249,250)	0
Lactofen	0	0	0	0	+ (251)	0	0	+ (252)	0
Imazalil	+ (253)	0	− (254)	+ (255)	0	0	0	+ (256)	0
Phosalone	0	0	− (257)	0	0	0	0	+ (258)	0

Disruptors of sustained proliferative signalling were cross-validated for effects in other cancer hallmark pathways. Disruptors that were found to have opposing actions in a particular hallmark (i.e. anti-carcinogenic) were denoted using '−', whereas disruptors that were found to have in a particular hallmark (i.e. carcinogenic) were denoted using '+'. In instances where reports on relevant actions in other hallmarks were mixed (i.e. reports showing both pro-carcinogenic potential and anti-carcinogenic potential), the symbol '±' was used. Finally, in instances where no literature support was found to document the relevance of a chemical in a particular aspect of cancer's biology were denoted using '0'. BPA, bisphenol A; PFOS, perfluorooctane sulfonate; TPA, 12-O-tetradecanoylphorbol-13-acetate.

As stated previously for endocrine disrupting chemicals, it is generally accepted that chemicals that show a receptor-binding-based mechanism can cause adversity at low doses. For carcinogenic compounds whose mode of action involves their interaction with a receptor, the possibility to identify a threshold may be questionable. Since the biological response depends on both the concentration of chemicals and receptors, as well as on the affinity of the chemicals for the receptors, for those substances acting as receptor agonists, it is generally agreed that in theory one molecule could activate a receptor—leading to adverse effects even at very low doses; benzo[a]pyrene, like many other chemicals, is bioactivated to reactive intermediates that can bind DNA through metabolic steps mediated by CYP1A1 and CYP1B1 enzymes, which in turn are regulated by AhR. Thus, benzo[a]pyrene-induced cell transformation can be due to both genotoxic and non-genotoxic mechanisms, depending on the status of the target cell. In the case of real exposure scenarios to environmental mixtures containing benzo[a]pyrene, it is quite difficult to distinguish which mechanism is predominant. Even if benzo[a]pyrene is classified as a carcinogen and airborne particulate matter has recently been classified as a human carcinogen by International Agency for Research on Cancer (261), the levels of benzo[a]pyrene concentrations commonly measured in urban background do not increase the risk of cancer in cancer risk assessment models. In *in vitro* models resembling the multistep process of carcinogenesis, such as the cell transformation assay, treatment with benzo[a]pyrene leads to a complete transformed phenotype only at certain levels of treatment doses, unless the sensitivity of the test is increased, such as in the BHAS-42 CTA based on the use of H-Ras-transfected fibroblasts. The treatment of non-transfected fibroblasts with airborne samples does not lead to the onset of a complete malignant phenotype but induces the modulation of biological pathways leading to toxicity and inflammation (262). Several scientific reports show that the extent of DNA adducts induced by benzo[a]pyrene as a single chemical is much higher than that induced by mixtures of PAHs, suggesting that 'DNA adduct formation is a poor marker for tumourigenesis induced by complex PAH mixtures' (263). Thus, the carcinogenic effects of environmental mixtures at low doses seems to be related to non-genotoxic effects leading to genetic instability as a consequence of chronic inflammation rather than to initiating events involving the formation of adducts to DNA (263).

Hard evidence was given by Khan *et al.* (264) who reported that dibenzo[a,l]pyrene, which is a kind of PAH derivative, simultaneously induced K-Ras mutation and activation of cell proliferation-related proteins such as cyclin D1 and E. This suggests that DNA adduct-induced mutation leading to altered amino acid sequences of critical oncoproteins also affect cell proliferation at similar dose levels.

The doses used in animal models are not compatible to human exposure data. However, the interesting point is that benzo[a]pyrene doses that can induce cell proliferation and mutate proteins are very similar to human exposure levels implicated in skin carcinogenesis (264) and in liver carcinogenesis (265).

In this article, however, we also nominate oestrogen and androgen receptors as important prototypical targets, in relation to sustained proliferative signalling. It should be remembered that nearly all the chemicals put forward here for cross-validation act as prototypical disruptors bound to ERα and AR. Growth factors (e.g. EGF and IGF) also worked together with ER to promote sustained proliferation. Furthermore, many of these prototypical chemicals simultaneously activated the very same additional priority targets and pathways (growth factor receptors, cytokine receptors, B lymphocytes, AhR, PPAR, downstream signalling pathways and cell cycle regulators) forming what we have termed a 'proliferative signature'.

On cross-validation, oestrogen and androgen hormone receptors were found to complement five other cancer hallmarks: deregulated metabolism, genetic instability, tissue invasion and metastasis, tumour-promoting inflammation and tumour microenvironment. Both complementing and opposing effects were identified for replicative immortality, but the role of steroid receptors in evasion of anti-growth signalling, angiogenesis, resistance to cell death and immune system evasion could not be established.

Environmental steroid hormones as the prototypical disruptor(s) (Table 3) were cross-validated as complementing six other hallmarks of cancer: angiogenesis, genetic instability, immune system evasion, replicative immortality, tissue invasion and metastasis and tumour-promoting inflammation. Both complementing and opposing effects were identified for resistance to cell death, but their role in deregulated metabolism, evasion of anti-growth signalling and tumour microenvironment remains unknown. The examination of steroid hormone receptors and environmental oestrogens and androgens suggests significant synergies between sustained proliferative signalling and many of the hallmarks in carcinogenesis; however, the greater number of unknowns suggest the need for more research in this area.

The critical consideration is that it is probable that many of the proposed prototypical disruptors that have been named in this article, in addition to environmental oestrogens, exist alongside environmental oestrogens, forming variable dose environmental mixtures of chemicals that have the ability to augment one another's carcinogenic arsenal. This review has unveiled the mechanistic links for such a phenomenon.

Implications of using the 'Hallmarks of Cancer' framework to assess for the potential of chemical mixtures from the environment to enable the cancer hallmark of sustained proliferative signalling—towards a unifying hypothesis

Historically, national regulatory agencies have sought to identify and limit our exposures to carcinogens (i.e. individual agents that can cause cancer). However, as the decades have passed, we have come to realize that relatively few chemicals are complete carcinogens (i.e. have the potential to enable all hallmarks to operate on their own). But it is now known that many of the hallmark mechanisms of cancer can be independently enabled by individual chemicals and that realization deserves further consideration in risk assessment.

Although the identification of complete carcinogens will always be an important activity, we now also need to be seriously concerned about the ways in which exposures to combinations of disruptive, but otherwise non-carcinogenic, environmental agents are able to act in concert with one another to instigate the disease. In other words, the hallmarks of cancer framework suggests that we also need to be concerned about cumulative exposures to chemicals that can disrupt the cellular machinery that is associated with any number of these hallmarks, because a multitude of exposures (each enabling a number of hallmarks) could easily instigate cancer. This possibility requires a much more nuanced appreciation of the complexity of the disease.

A serious caveat to overcome is that studies in the past have shown that combinations of low dose carcinogens have—in the systems used at least—not resulted in a cumulative effect on tumour incidence. Nor has the cancer incidence in the population increased in such manner so that an association could be made with the current spread of carcinogens at low doses. In order to square these earlier findings with our hypothesis that it is the combination that matters, one has to consider the dual role of carcinogens, namely direct exposure versus epigenetic modifications. We know that some environmental factors have been linked to aberrant changes in the epigenome in both experimental and epidemiological studies (265–270). Moreover, epigenetic mechanisms can mediate specific mechanisms of toxicity and responses to certain chemicals (266–270). Whereas mechanisms of action of some of these agents are understood, for others, the mode of action remains completely unknown (271–274). Since these epigenetic changes are minute and potentially cumulative, and may well develop over time, it is an uphill struggle to finally establish the cause–effect relationship between low dose environmental carcinogens, epigenetic changes and pathogenesis.

In reality, most environmental exposures involve mixtures. This is the case for indoor as well as outdoor pollutants—PAHs, diesel exhausts, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochlorides, tobacco smoke and smoke from incomplete combustion. It is now obvious that the classical approach that focuses on health effects of environmental agents—one compound at a time and on the exposure period to a particular life stage—needs to be broadened. The concept of defining environmental exposure as an 'exposome' emerged a decade ago (275). The term refers to the summation of all exposures an individual human being experiences over a lifetime—from conception to death. Influences from the external environment constantly modify the internal environment. The combined exposure to both internal and external changes defines the ultimate exposure. It is important to note that the composition and temporal sequence of these exposures are equally important in determining their effects. Thus, the degree of interactions could be infinite and could tend to multiply over time as the individual ages. Moreover, these interactions can be synergistic, antagonistic, combinatorial, attenuating, summative, subtractive or as yet undefined. In other words, the consequences can only be viewed in its entirety and no single component of the exposome can predict the cancer or health outcome (275).

The hallmarks of cancer framework (1), used by the teams of researchers involved in the Halifax Project to assess the individual contributions of disruptive environmental agents for risk assessment purposes, therefore presents a novel approach to identifying chinks in the armour of cancer. The reason for this is that traditionally, researchers have sought to identify elements of cancer causation that may be responsible for one, to at best a few, of cancer's hallmark signatures (e.g. the role of ERβ in opposing cell proliferation, inducing apoptosis and possibly even preventing metastatic spread of ovarian epithelial cancer (69,70). However, cancer presumably has another final signature, one that may be the most difficult of all to deal with, namely that of heterogeneity.

The idea that chemicals persist in the environment is not new, but the idea that these same chemicals may persist in human (and animal) tissues has only recently begun to receive the attention it deserves. It is conceivable that advancing age confers reduced ability of the individual to sulfonate (and therefore inactivate) oestrone and oestradiol obtained from peripheral blood using organic anion-transporting polypeptides (276). This in turn could lead to elevated levels of unconjugated oestradiol

in some tissues (and in tumours). In support of this notion, a recent study reported naturally elevated levels of oestradiol in the ovaries of older (7–10 month old) mice (68,277). Furthermore, a second cohort of mice that received oestradiol treatment continued to exhibit high levels of oestradiol in ovarian tissue a full 2 weeks after cessation of hormone injections. These results strongly suggest that unconjugated oestradiol from both endogenous and exogenous sources can accumulate in ovarian tissue over time. Thus, although a critical window of timing for adverse effects of chemical exposure has been extensively documented over recent years for *in utero* and early life time points, we may also like to think about older age as a potential novel *critical* period where as humans, we are more vulnerable to being exposed to environmental chemicals. This vulnerability may be partly as a consequence of our ageing genome and partly as a consequence of exposure to chemicals that have both cumulative (build up in the body) and additive (combine with other chemicals) adverse effects (278). This might also help to explain why it is that 80% of breast cancers occur over the age of 50 when many of these are oestrogen driven but occur at a time of life when endogenous oestrogen synthesis has waned.

Final remarks

The Halifax Project has aimed at linking exposure to chemicals that exist in our environment as mixtures in individually low (but collectively potentially deleterious) doses to molecular effects on cells that may commit them to the tumourigenic phenotype. The present review is underpinned by the understanding that normal cells differ from tumour cells in one fundamental way, their inherent capacity for unrestrained proliferation. We have endeavoured to provide future researchers with possible mechanisms by which known environmental carcinogens and other chemical mixtures could link to fundamental processes that can explain this change in phenotype.

In closing, this article has aimed at viewing the cancer hallmark of sustained proliferative signalling from the point of the cell cycle and disruption of some basic control mechanisms (279–286). Different pathways have been scrutinized, and the action of a number of potential and known environmental carcinogens discussed with respect to their action on specific routes committing cells to an indefinite proliferative span. We have also focussed on how low dose exposures over a prolonged time span may actually play a more important role than has hitherto been appreciated. The 'Cancer Hallmark' approach taken by the Halifax Project is the first truly holistic approach to tackling cancer's complexity, and it may mark the turning point in the battle of the human race against this deadly disease.

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

Funding

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