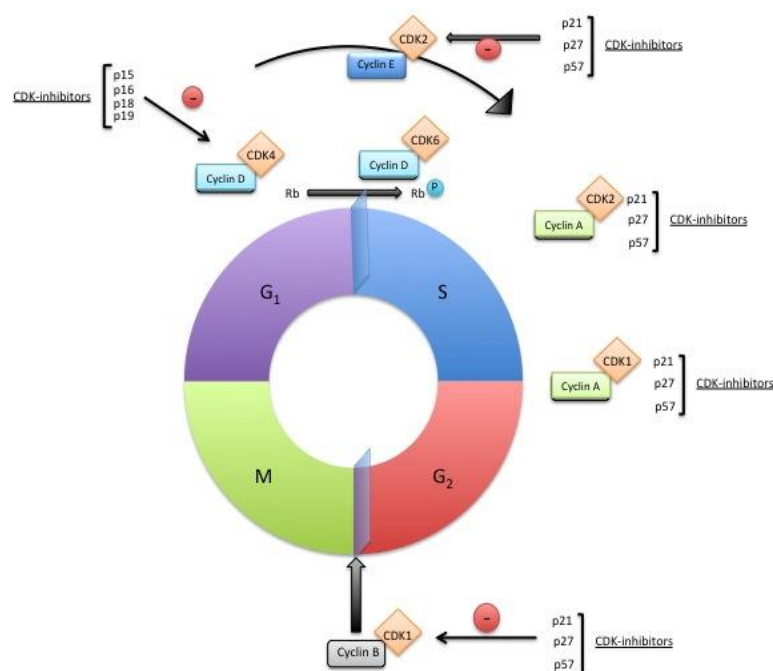




Sveriges lantbruksuniversitet
Fakulteten för veterinärmedicin och husdjursvetenskap

The role of cell cycle control mechanisms in regulated and sustained cell proliferation

Tove Hultman



Självständigt arbete i veterinärmedicin, 15 hp

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Hur cellcykelns kontrollmekanismer verkar i normala och transformerade celler

The role of cell cycle mechanisms in regulated and sustained cell proliferation

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SAMMANFATTNING

Cellcykeln benämns den tid som en cell tillbringar mellan två celldelningar. Cellcykeln innefattar flera parallella processer som alla måste slutföras innan en cell är mogen för delning. För det första måste alla cellens strukturella komponenter (RNA, proteiner och membranlipider) fördubblas och detta sker kontinuerligt genom cellcykeln.

Dessutom måste arvsmassan och några av de kromosomala proteinerna fördubblas och detta sker under ett avgränsat intervall ungefär mitt i cellcykeln (S-fasen).

På ömse sidor om S-fasen finns två ”gaps” som brukar kallas G_1 och G_2 . När celler har passerat en punkt i G_1 -fasen är de oåterkalleligen programmerade för att progrediera genom återstoden av cellcykeln och kommer genomgå nästa celldelning. Ungefär fyra timmar efter mitos befinner sig normalcellen i ett stadium av obeslutsamhet. Under denna fas (G_{1pm}) påverkas cellen av yttre faktorer som exempelvis tillgång till tillväxtfaktorer och närhet till andra celler att fatta ett beslut om den skall fortsätta mot S-fas eller utträda ur cellcykeln och inträda i ett reversibelt vilostadium – G_0 . G_{1pm} -fasen är alltid av konstant längd (fyra timmar) och åtföljs av en variabel del, G_{1ps} , under vilken cellen bygger upp sina strukturkomponenter med varierande hastighet. En liten cell tillbringar en relativt längre tid i G_{1ps} än en större. Tanken är att alla storleksskillnader mellan celler skall vara utjämnade när S-fasen initieras. Till skillnad från normala celler saknar transformerade celler förmågan att utträda i G_0 under suboptimala förhållanden.

Denna artikel syftar till att sammanfatta hur normala och transformerade celler förhåller sig till G_0 -blockeringen samt hur enskilda faktorer styr de båda cellslagets förhållande till proliferationshämmning och "sustained proliferation".

SUMMARY

The cell cycle is the time a cell spends between two cell divisions. The cell cycle 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) need to double in quantity and this occurs continuously through the cell cycle. Furthermore, the genome and some chromosomal proteins must double and this take place during a limited interval in the middle of the cell cycle (S-phase). On either side of the S-phase are two "gaps" which is called G_1 and G_2 .

When the cell has passed a point in the G-phase they are irreversibly programmed to progress through the remaining of the cell cycle and will undergo the next cell division. When a cell have passed a point in the G_1 phase, approximately four hours after mitosis, a normal cell is in a state of indecision. During this phase (G_{1pm}), the cell is affected by external factors, such as presence of certain growth factors and proximity to other cells to make a decision whether to continue toward S-phase or exit the cell cycle and enter a reversible resting stage- G_0 .

G_{1pm} phase is always of constant length (four hours), followed by a variable phase of G_1 (G_{1ps}) during which the cell builds its structural components with varying speed.

A small cell spend a relatively longer time in G_{1ps} than a larger one. The idea is that any size differences between cells will be adjusted before S-phase is initiated. Unlike normal cells, the transformed cells lack the ability to withdraw the G_0 under suboptimal conditions.

This article aims to summarize how normal and transformed cells relate to the G_0 -blocking and how individual factors determine the both cells relation to cell proliferation inhibition and "sustained proliferation."

INTRODUCTION

Normal cells differ from tumour cells, in one fundamental capacity. Whereas normal cells are subject to a proliferation control, tumour cells can escape from this control mechanism and display an inherent capacity to unlimited proliferation. There is a fundamentally logical explanation for the existence of a control mechanism in normal cells. Such cells proliferate when they receive a signal to do so. This is the case during embryogenesis as well as in adult life. Basically cells proliferate to fulfil a need. When the task is completed, the cells will cease growing until they are stimulated to resume proliferation.

Tumour cells have somehow acquired a capacity to escape from this important control mechanism. And this ability to overcome growth inhibitory signals is what makes tumour cells deleterious to the organism. Even though these fundamental differences were pinpointed some time ago (Dulbecco & Elkington 1971, Stoker 1973, Holley et. al. 1975) the key issue remains; How can the finely tuned control mechanisms that operate in a narrow interval in the cell cycle be overcome during transformation. And more importantly; can these control mechanisms be reinstated in tumour cells in order to reverse the transformation process.

Cancer issues in general have stimulated an enormous interest in the scientific community. It is believed that two million scientific articles with some bearing on cancer are published every year. It is an impossible task to even attempt to systemise this information. So this paper attempts instead to focus on one key-issue, namely exit from and re-entry into the cell cycle, and moreover, how tumour cells act to avoid this gate control. Emphasis will be on the activation of key oncogenes that occur in tumour cells during the transformation process.

MATERIAL AND METHODS

Most articles were found in the databases; Google Scholar, PubMed and Web of knowledge. I used search-terms like "sustained proliferation", "eukaryote cell cycle", growth factor overexpression" and "Cyclin overexpression". The articles along with some papers kindly provided from my supervisor have formed the basis for my literature review.

LITERATURE REVIEW

The normal cell cycle

The current description of the cell cycle was formulated by JM Mitchison 1971: “The cell cycle of a growing cell is the period between the formation of the cell by the division of its mother and the time when the cell itself divides to form two daughters.” (Mitchison, 1971)

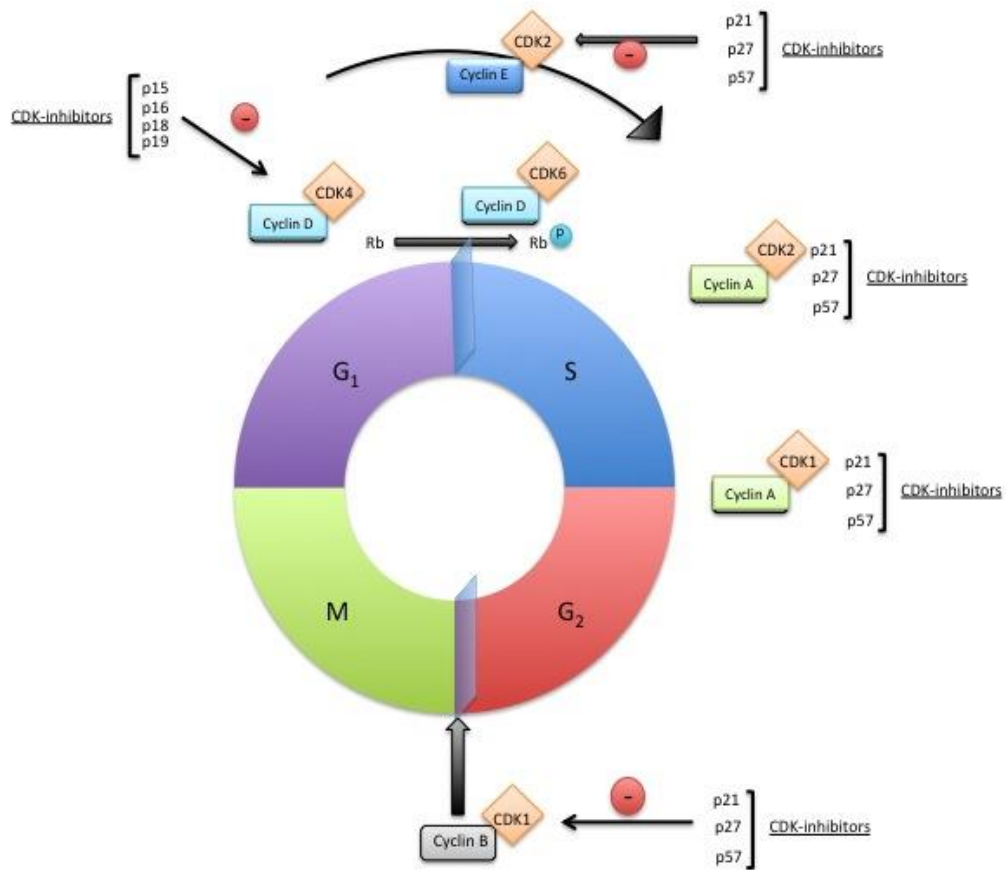
The intermitotic cell cycle consists of four different phases, the G_1 (*presynthetic phase*), S (*synthesis of DNA*), G_2 (*premitotic phase*) and M (*mitotic phase*). This was proposed by Howard and Pelc after studying onion roots cells over time. (Howard and Pelc, 1953) Normal cells are also capable of leaving the cell cycle and enter a state of quiescence (G_0). From which they can be restimulated to enter the cell cycle.

Moreover in 1971 Mitchison suggested the existence of two parallel processes that build up the cell cycle. He performed his studies on growing cells from fission yeast *Schizosaccharomyces pombe* culture. One cycle includes DNA synthesis and mitosis (*chromosome cycle*), whereas the other includes cell enlargement, in which the cell doubles its components, (*the growth or cytoplasmic cycle*). (Mitchison,1971)

The relationship between the two cycles had prevent been highlighted by Prescott who by studying *Amoeba proteus*, concluded that a cell couldn't divide until it has reached a critical size, and not until then initiate S-phase. (Prescott,1955)

In the normal cell cycle different cyclins, cyclin-dependent kinases (CDKs) and their inhibitors regulate the cellular progress through the different phases in the cycle. In tumour cells the regulation is often deranged, and they can easier be triggered to proceed towards division. Another feature is that tumour cells can continue to cycle even with fundamental aberrations.

For the cell to continue towards the cell cycle, CDKs are required to phosphorylate critical target proteins. CDKs are continuously expressed, in contrast to cyclins that are synthesized at different phases of the cell cycle, subject to need. The CDKs are activated by phosphorylation upon binding to cyclins. The cyclin protein-family consists of, at least 15 discovered proteins, four of them, cyclin D, E, A, and B are active in different parts of the cell cycle. (Hunter & Pines, 1994)



The first cyclin to accumulate in the cell cycle, in mid G₁, is cyclin D and plays an important role by binding and activating CDK4. Cyclin D and CDK4 form a complex, which phosphorylates the retinoblastoma susceptibility protein (Rb).

When cyclin D phosphorylates Rb, it releases the transcription factor, E2F, which in hypophosphorylated form is inactive. When the cell enters mitosis cellular phosphatases remove phosphate group from Rb and thus reconstructs hypophosphorylated form of Rb. (Hunter & Pines, 1994)

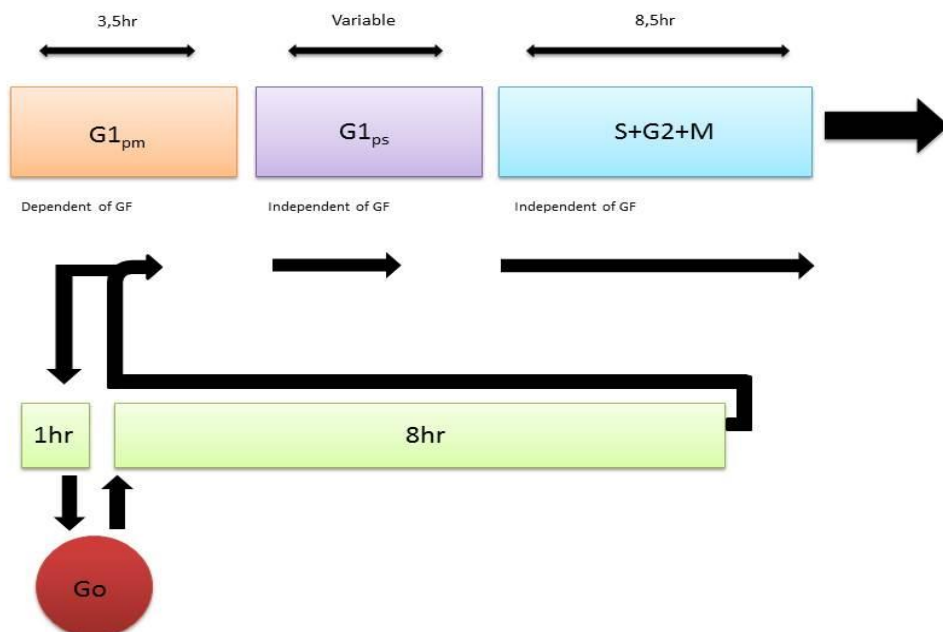
The concentrations of cyclin D and E will also rise when quiescent cells are stimulated to re-enter the cell cycle by appropriate stimulus. This results in the activation of cyclin D-CDK4 and cyclin E-CDK2 at G₁/S transition point. It causes phosphorylation of Rb and it will dissociate itself from the complex with E2F, and hence activate the target genes, such as DNA polymerase, cyclin E and thymidine kinase, and in this way stimulate the G₁-progression. To provide stability through the G₁-S progression cyclin E-CDK2 also phosphorylates p27 and p21, that belong to the Cip/Kip protein family, these proteins inhibit cyclin E and hence function as tumour suppressors. Cyclin E also induces the up regulation of histone gene transcription by phosphorylation of p220npat, which also contributes to the shift from G₁ to S. Cyclins are unstable proteins that are degraded in at least two different ways. One way involves the BCR ubiquitin ligase. The other is mediated through the Cul-3-mediated ubiquitin proteasome pathway. (Tat Siu et. al., 2012)

The “If” and “when” decisions

25 years ago Engström and Larsson proposed that when a cell progresses through the G_1 it has to make two independent decisions. The first decision is made in early G_1 (G_{1pm} post mitosis) whether or not to continue its progression towards DNA-replication and mitosis. This is a strict yes/no decision, and it depends on the presence of certain growth factors. If there is a lack of growth factors, the cell will leave the cell cycle and enter the state of quiescence, G_0 .

The next decision is a “when” decision, and is made in late G_1 , after the restriction point, (G_{1ps} pre-synthesis). In contrast to the “if-decision” this is only a question of when to initiate the DNA-synthesis and the decision itself is made irrespective whether or not the cell has been exposed to suboptimal conditions. This decision accounts for most of the variability of the cell cycle. Larsson et al, provided evidence that cells that are younger than 3-4 hour, and thus located in the postmitotic phase of G_1 , prolonged their cell cycle with 8 hours after a serum-deprivation of only one hour. It was also shown that cells older than four hours, and thereby located in presynthetic phase of G_1 , S or G_2 did undergo mitosis irrespective whether or not serum was present. (Larsson, 1984) Whereas S, G_2 and M-phases have a constant duration, G_1 varied in length, and could be influenced by the presence of certain growth factors. (Dafgård, 1990)

To be able to prove this Engström and Larsson used *time lapse cinematography*. A bottle containing exponentially growing cells was placed in an inverted microscope. A video camera system was attached for time elapse cinematography. In the starvation experiment the serum-containing medium was removed, the cells washed in saline and serum free medium added. After the experiment lapsed serum was added. By subsequent development and analysis of the cells the intermitotic time position of each cell could be determined and hence the cellular age at the onset of the serum-starvation be estimated. (Larsson, 1984)



Sustained proliferative signalling

A cell that sustains proliferative signalling can continue to divide even in a suboptimal environment, when it otherwise should be forced into cell arrest. Thus the tumour cell is equipped with a mechanism to avoid being captured in G_0 . The important question is how such cells succeed to avoid the “if”-decision in G_{1pm} .

In normal cells, the production of the proliferative signals are strictly controlled, thus they switch on relevant genes when a growth factor is added. To circumvent this, tumour cells, can produce their own growth factors and overexpress the receptors. Another way to sustain proliferation is to send signals to the normal cells in the stroma and they will answer by producing growth factors that will operate in a paracrine fashion. They can also increase the amount of receptors expressed on the cell surface or activate protein in the downstream signalling pathway (Hanahan & Weinberg, 2011).

Over the last two decades a large number of proto-oncogenes have been discovered. The proto-oncogenes play an important role in the generation of cell growth and proliferation. For instance, proteins encoded by proto-oncogenes are often *growth factor ligands* and *receptors*, *transcription factors*, *signal transducers* and *cell cycle regulators*. These oncogenes often have similar functions, and contribute to tumour cell self-sufficiency for growth, division and survival. (Adamson, 1987)

Growth factors as oncogene products

It has generally been assumed that Growth Factors (GF) contribute to tumour formation, by binding to cell membrane receptors and thus stimulate cell proliferation.

It has been shown that transformed cells grown *in vitro* require less serum. Moreover, in many types of cancer, a loss of requirement for specific GF's *in vivo* has been established.

In 1960, Cohen and Levi-Montalcini became the first persons to describe the Epidermal Growth Factor (EGF). EGF is structurally related to TGF- α (Transforming Growth Factor), and both binds to the same receptor. Some oncogene products have been found to be homologous to the Fibroblast Growth Factors (FGF). The first oncogene to be studied in this sense was *hst* (human stomach tumour) which is now official known as FGF-3.

In several tumours the proto-oncogene *sis* is overexpressed which codes for the beta-chain, of an important growth factor, Platelet Derived Growth Factor (PDGF). PDGF is a potent mitogen, and binds to two cell-surface receptors, PDGFr α and β . Activation of PDGF receptors causes migration and proliferation of several cells, for instance fibroblasts, smooth muscle cells and monocytes. It's assumed that a PDGF or PDGF-like molecule is produced in most transformed mesenchyme cells.

Fibroblast Growth Factor and Epidermal Growth Factor both increase the early transcription of proto-oncogene *fos*, whereas PDGF induce *c-myc*. (Goustin et. al., 1986)

Cell surface receptors as oncogene products

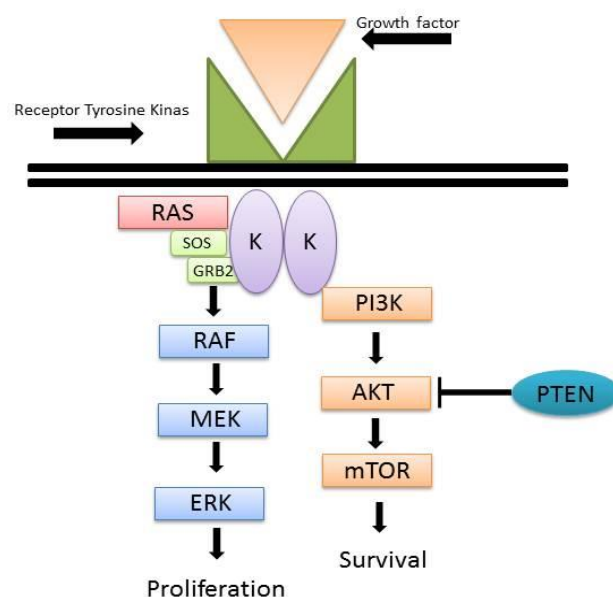
Receptors with tyrosine kinase activity consist of three different parts, one extracellular domain, one trans-membrane hydrophobic part and one intracellular tail that have a tyrosine kinase activity. When a growth factor binds, it causes dimerization of the receptor, tyrosine phosphorylation and activation of the receptor tyrosine kinase. The tyrosine kinase then activates a lot of different effector molecules, several of them involved in the cell cycle. (Adamson, 1987)

When an oncogene encodes growth factor receptors, it results in a constitutive dimerization and activation without binding a growth factor, which in turn leads to self-sufficiency. Several oncogenes that encode growth factor receptors have been found, for instance; *erbB2*, which is homologous to the EGF-receptor gene. Moreover, *erbB* encode a truncated growth factor receptor, which seems to be activated without presence of growth factors. (Goustin, et. al., 1986)

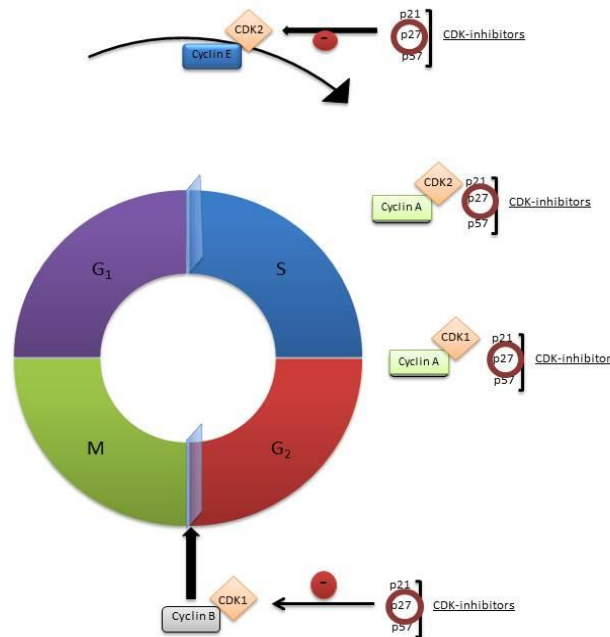
The Ras oncogene

Alterations in proteins belonging to Ras gene-family are the most commonly occurring in human tumours. The mutated version of Ras occurs in about 30% of all human tumours and 95% of human pancreatic cancer. (Barbacid, 1990). Ras plays an important role as signal transducer of mitogenic signals, and as an on/off switch for variety of critical cellular activities, such as proliferation and differentiation.

The three Ras-genes (*K-Ras*, *N-Ras* and *H-Ras*) are tightly regulated in normal cells, and a mutation in the Ras-genes can create constitutively active proteins, which in turn, can result in uncontrolled proliferation. (Takashima & Faller, 2013).



Active Ras is able to induce degradation of p27, a cyclin-Cdk-complex inhibitor. If Ras then is mutated and constitutively activated, it is possible that it causes an overexpression of cyclin E by down-regulating its inhibitor (Philipp-Staheli, et. al., 2000). When Ras oncogene results in a p21 product, may activate the growth factor pathway, without binding from a growth factor, or presence of its receptor (Goustin, et. al., 1986).



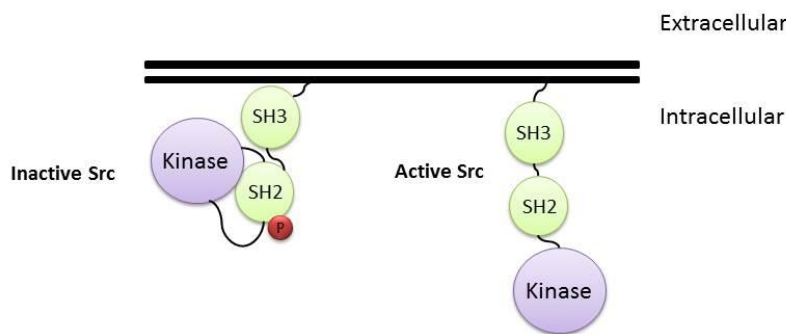
The Src oncogene

In 1996 Brown and Cooper identified C-src as a cellular form of V-src, which was first traced in avian tumour virus (Rous sarcoma virus). Src is a non-receptor tyrosine kinase and function as a cellular transduction pathway in numerous cellular processes, including; differentiation, proliferation, survival, motility, cell to cell-adhesion and angiogenesis. To be able to regulate these cell processes Src has to interact with several factors, such as; cell surface receptors (PDGF, EGF and FGF-receptors), steroid receptor hormones and many others. (Ishizawa & Parsons, 2004)

An increased level of Src mRNA has been shown in many types of cancer, including pancreatic, hepatocellular and gastric cancer. Although it has been known for long that C-src plays an important role the in development and progression of cancer, we still don't know its ultimate function.

When cellular signals that promote proliferation, motility or survival are mediated intracellular Src-family kinases are activated. These functions along with effects on cell-cell adhesions promotes the metastatic potential of the cells. (Yeatman, 2004) Src activation is very carefully controlled via phosphorylation. Inhibition of Src's catalytic activity is achieved by phosphorylation upon Tyr-527 residue, thus it promotes an intermolecular interaction with the SH2 domain. In contrast, activation of Src is achieved by phosphorylating the Tyr-416 residue.

The v-Src which lacks this sequence, and are no longer capable of being regulated. The result is a constitutively active signal transduction pathway. (Xu et. al., 2012)



Jun, Fos and AP1 transcription factor

Jun and Fos together form a transcription factor called *Activating Protein-1* (AP-1). It consists of a mixture of hetero- and homo-dimer, between proteins in jun-family (c-jun, JunB and JunD) and fos-family (C-fos, FosB, Fra1 and Fra-2). The AP-1 formation transduces the growth factors messages to the nucleus, which leads to secondary gene expression. AP-1 is involved in proliferation, differentiation and cell death. (Mechta-Grigoriou et al., 2001).

Development of tumours is a multistep process, and AP-1 is probably involved in the control of cell proliferation via the signals to the nucleus. But, it should be noted that AP-1 also can have an anti-proliferative activity. It is not until c-JUN is activated by JUN amino-terminal kinases (JNK) that AP-1 completely promotes the cell-cycle progression. Consequently, the AP-1 complex induces transcription of the regulators that promotes the progression of the cell cycle factors, such as cyclin D, or represses the cell cycle inhibitor.

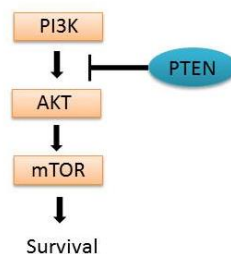
In contrast overexpression of JunB appears to be a antagonizing factor to the induction of cyclin D, and in this way has an inherent anti-proliferative activity. (Shaulian, E & Karin, M. 2002)

PTEN

PI3-Kinase and PTEN are two of the most frequent mutated proteins in cancer. PTEN encodes a phosphatase. PTENs particularly target is phosphatidylinositol-3,4,5-trisphosphate (PIP3) and phosphatidylinositol-3,4-biphosphate, which are specialized plasma membrane lipids. When the PI3K cellular signalling chain is active it produces these lipids. PTEN can then act like an off-switch for PI3K, by removing phosphate from the D3-position.

It may be hypothesised that PTEN functions as a tumour suppressor. Receptor Tyrosine Kinase (RTK) on the cell surface membrane senses survival and cell growth signals and consequently activates PI3K via an enzyme, that generates PIP3 as well. If PTEN is mutated it might result in an unregulated activation of PI3K. The PI3K-pathway controls cell growth, survival and proliferation of the cell. When PIP3 is active it will require several kinases, including Phosphoinositide-Dependent Kinase 1 (PDK1) and the Protein Kinase b/Akt family. Eventually Akt-targets will get phosphorylated partially by phosphorylation by PDK1, and these will play an important role in regulating; apoptosis, proliferation, nutrient response glucose homeostasis, cell size and DNA-damage.

In conclusion, inactivation of PTEN can lead to a constitutive activation of the Akt signalling pathway. (Sansal & Sellers, 2004).



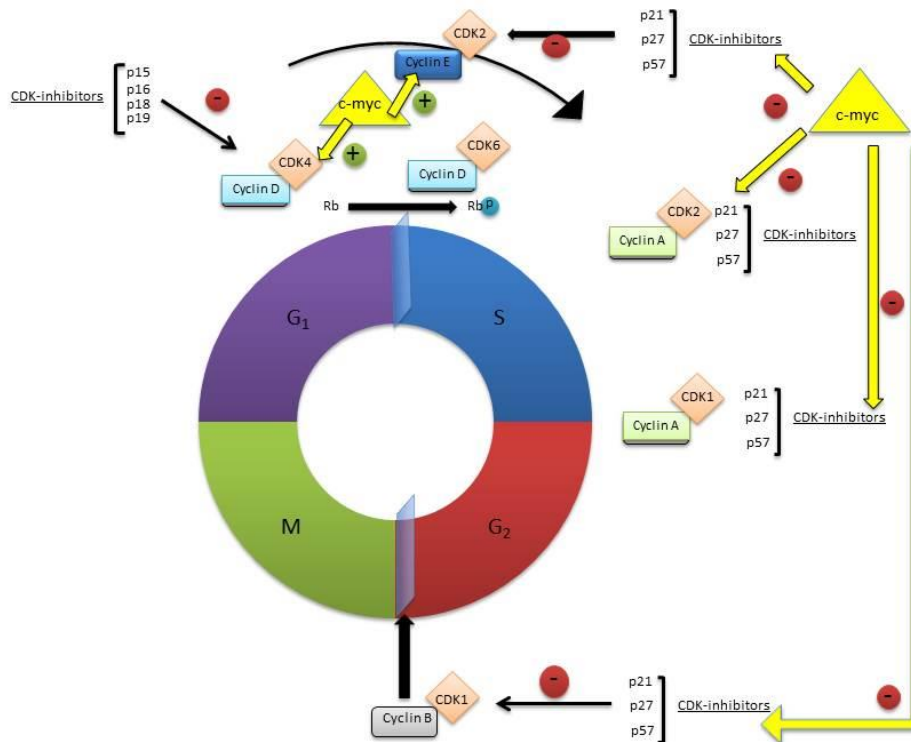
The Myc oncogene

Like other proto-oncogenes the expression of C-myc in normal tissue is carefully regulated by external and internal signals. C-myc is known to regulate proliferation, apoptosis, differentiation and metabolism.

It has been shown that resting cells only express a small amount of C-myc, while growing cells, stimulated by growth factors transiently express an increased amount of C-myc. The activation of C-myc in cancer can have different causes, for example; chromosomal translocation or gene amplification – which increases the expression of Myc.

The role of C-myc in the cell cycle progression is not clearly known, but one model suggests that C-myc accelerates the progression through G₁ and S and hence promoting cell proliferation.

Moreover, it has been shown that C-myc induces cyclin D, cyclin E, CDK4 and inhibits p27; which is a CDK-inhibitor. Also, C-myc is involved in the cell cycle by its direct target *cdc25A* which product activates both CDK2 and CDK4. Deregulated C-myc also induces cyclin E directly. In a recent study it has been shown that C-myc together with Ras also to induces the CDK1 promoter and that C-myc therefor plays a many sided role in tumorigenesis. (Dang, 1999)



Overexpression of cyclin E

Cyclin E plays an important role in the progress through the G₁ phase of the cell cycle. Along with cyclin D-Cdk2 it phosphorylates the retinoblastoma (Rb), and makes the G₁/S-transition possible.

Lately different types of cancer cells have been shown to express high levels of cyclin E and it has been shown that an overexpression of cyclin E correlates with increased tumour aggression.

One way to derange the normal control of cyclin E expression is via loss-of-function mutation sustained by the gene *FBWX7*. This gene encodes tumour suppressor protein *fbw7*, whose assignment is to provide substrate specificity for an ubiquitin ligase complex, which target several oncoproteins for degradation. This is only one of many ways by which cyclin E can be deregulated in cancer cells. To be able to understand the oncogenic effect of cyclin E, we have to understand the complexity of its function and regulation.

Cyclin E can stimulate its own transcription via a positive feedback and transcription of the cyclin E genes is activated during G₁. This transcription depends on mitogenic input, which includes E2F and Myc activities. If an overexpression of cyclin E occurs, the step that normally would be rate-limiting becomes partially independent of the mitogens.

Cip/Kip proteins, and in particular p21 and p27, inhibit cyclin-Cdk complexes. By activating p53, cyclin can induce p21 expression. It's shown that p21 and p27 is both inhibitors and substrates for cyclin E-Cdk2. Active cyclin E-Cdk2 can phosphorylate p27 and p21 if its Cdk2-bound, resulting in triggering their degradation.

Except from phosphorylating Rb cyclin E phosphorylate CBP/p300 and this stimulates transcription activity and cell cycle progression. (Tat Siu, et. al., 2012)

DISCUSSION

In this article, one of the most fundamental differences between a normal cell and a tumour cell has been reviewed and discussed. Normal cells are under a strict proliferation control. The basis for this mechanism is that a cell should be able to proliferate as long as there is a need for an increased cell number. When this need is fulfilled, normal cells sense the lack of growth stimulatory signals and exit from the cell cycle and enter a reversible state of quiescence termed G_0 . During transformation, cells lose this ability to cease multiplying in response to decreased growth signals and hence continue to proliferate also under suboptimal conditions. This characteristic of the transformed phenotype is the most crucial and fundamental change that the normal cell has to undergo during tumourgenesis. It has therefore been the aim of this article to highlight molecular events directly coupled to this change.

It has been proposed that cells have to make two independent decisions in order to progress towards mitosis. The first decision – “If” which is made during early G_1 , and is clearly influenced by external factors as polypeptide growth factors, cell contacts and surface anchorage. This dependence on external signals is pivotal to the normal phenotype. Thus, it is the inability to make an active “if”-decision that is the key to understanding transformation. (Larsson, 1984)

According to the existing literature it almost a dogma that it is the combination of activated oncogenes that contribute to tumour development. There are two principally different ways by which oncogenes can contribute to the transformation process. Either the proto-oncogene – or the cellular oncogene – can be stimulated to enhanced transcription. The consequence would then be an increased amount of perfectly normal oncogene product that will be available for the transformation process. Examples of this activation can be seen during gene amplification or translocation – N-myc amplification in neuroblastoma is a classic example of the former and C-myc translocation in Burkitts lymphoma is an example of the latter. In each of these cases, increased amounts of N-myc or C-myc protein drives the proliferative circuit and causes cells to acquire a transformed phenotype.

The second alternative is that changes in the coding exons of relevant oncogenes but with an unchanged promoter-enhancer apparatus will yield a normal amount of altered protein product. Classical examples are point mutations as in the Ras-gene or fusion genes as e.g. the abl-bcr fusion gene.

With respect to function, oncogene products can affect different levels in the chain of events leading to cell proliferation. Oncogene products can act as growth factors, membrane receptors (complete or truncated), G-proteins, intracellular tyrosine kinases, nuclear transcription factors etc. It is clear that we have only been scratching the surface of this intricate signalling system.

But it is important that focus remains on the key issue. How is the cells normal control mechanism operating in G_1 effectively overrun by transforming factors? Only when this critical issue is understood, can cancer research take a well-earned leap forward.

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