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p16^{INK4A} – Connecting Cell Cycle Control to Cell Death Regulation in Human Leukemia

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1. Introduction

To expand without limitation cancerous cells have to acquire the ability to proliferate without external or internal restrictions. Whereas almost all normal cells within a body will not proliferate without external mitogenic stimuli cancer cells gain defects in growth factor signaling control that mimic external stimulation and thus allow them to divide. However, this unrestricted proliferation also activates an internal barrier to transformation, i.e. it induces the expression of so called cell cycle brakes that stop unrestricted growth at the very basal level of the cell cycle. These cell cycle inhibitors prevent increased cycling by controlling the activity of cyclin/CDK holoenzyms which are the central promoters of cell cycle progression. These cell cycle inhibitors or "brakes" are divided into two families, the INK4 inhibitors and the Cip/Kip inhibitor family. In this chapter we will mainly focus on the INK4 inhibitor protein p16INK4A which is encoded by the INK4A gene locus.

Inactivation of the INK4A gene locus occurs frequently in primary tumor cells of T-cell acute lymphoblastic leukemia (T-ALL), suggesting a critical role of this locus in disease development. Its deletion predicts relapse in childhood acute lymphoblastic leukemia (ALL). Interestingly, this gene locus also represents a main barrier to the generation of induced progenitor stem (iPS) cells and evidence has been provided that downregulation of p16INK4A is associated with enhanced self-renewal and proliferative capacity of hematopoietic stem cells. This suggests that the inactivation of this tumor suppressor in immature pre-cancerous cells might allow them to overcome replicative senescence. Importantly, p16INK4A exerts its effects not only on cell cycle control but changes cell death sensitivity of T-ALL cells by affecting glucocorticoid sensitivity, death receptor-mediated- and intrinsic programmed cell death pathways that are controlled at the level of BCL2 proteins. These effects of p16INK4A on the cell death machinery in ALL cells support the notion that loss of the INK4A gene locus has a two-sided effect during the development of ALL, on one hand it allows unrestricted proliferation of ALL progenitor cells and on the other hand this deletion rescues leukemic cells from cell death. Thus, the effects of p16INK4A in hematopoietic cells and during leukemia development seem to be much more than "just inhibiting cyclin-dependent kinases".

2. Stop or go: A general overview on cell cycle entry

Proliferating eukaryotic cells pass through a complex cell division cycle that is divided into four phases: the gap-phase before DNA replication (G1-phase), the phase of DNA synthesis (S-phase), the gap after DNA synthesis (G2-phase) and mitosis (M-phase). Whereas the length of DNA-synthesis, G2 and M-phase are relatively constant within different cell types, the greatest variation is observed in the duration of G1. Highly proliferative cells pass through this phase within a few hours; differentiated cells, on the other hand, may stay in this cell cycle stage for months, years, or even life time. This situation of final differentiation is called G0. The G0/G1 cell cycle state is usually considered as the time window, in which a cell decides whether to proliferate or to arrest.

Progression through the cell cycle is governed by a family of serine-threonine kinases called cyclin-dependent kinases (CDKs) that associate with different cyclins in distinct phases of the cell cycle (Murray and Hunt 1993; Sherr and Roberts 2004). Whereas the cellular concentration of CDK proteins does not vary significantly during cell cycle, the levels of cyclins undergo dramatic changes during the different phases. They reach characteristic peak levels in specific cell cycle phases and are degraded in other phases.

When quiescent cells re-enter the cell cycle, D-type and E-type cyclins are synthesized sequentially during the G1 interval; both types are rate-limiting for S-phase entry (Quelle et al, 1993). Cyclin Ds assemble with CDK4 (Matsushime et al, 1992) and CDK6 (Meyerson and Harlow 1994) and these complexes are activated by phosphorylation via the so called cyclin activating kinase (Murray and Hunt 1993). Critical substrates of G1 cyclin/CDK complexes are the retinoblastoma gene product pRB and its family members p107 and p130, which are transcriptional repressors that are bound to transcription factors essential for S-phase entry (Cobrinik 2005; Grana et al, 1998). Upon phosphorylation on distinct sites by both, cyclin D/CDK4/6 and cyclin E/CDK2 complexes pRB and its family members lose their function as transcriptional repressors thereby activating the transcription of genes essential for S-phase progression. The activity of CDK4, CDK6 and CDK2 is regulated by mitogenic hormones and by binding of CDK inhibitors (CDKI) of the INK4A family, like p16INK4A, and of the Cip/Kip family, i.e. p21Cip1, p27Kip1 and p57Kip2 (Sherr and Roberts 1999; Ekholm and Reed 2000) (see Figure 1).

3. The strange case of INK4A: One gene locus that codes for two unrelated tumor suppressors

The INK4A gene locus on chromosome 9p21 codes for the two functionally fully unrelated tumor suppressor genes p16INK4A and ARF (known as p14ARF in human and p19ARF in mouse). As outlined above, p16INK4A/CDKN2A was originally identified as a cyclin-dependent kinase inhibitor that, like its family members p15INK4B, p18INK4C and p19INK4D, binds to the kinase subunits CDK4 and CDK6 of D-type cyclins. Cyclin D1, D2 and D3 govern the decision of cell cycle entry from quiescent G0 state. In contrast to other cyclins the activity of cyclin Ds/CDK4 and CDK6 holoenzymes is controlled by various survival signaling pathways e.g. by Ras/Raf signaling, by the PI3K/PKB pathway or by the Wnt signaling pathway which are frequently perturbed in cancer cells thereby leading to aberrant cell cycle entry and cell proliferation. p16INK4A and other inhibitors of the INK4-family (INK4 means "inhibitor of kinase 4") thereby represent a main barrier to increased proliferation of cells with defects in growth factor signaling control. Human tumor cells that

lack functional pRB, either by mutation or due to viral proteins that target and inactivate pRB, usually show high levels of p16INK4A (Aagaard et al, 1995). In such cells CDK4 and CDK6 do not interact with D-type cyclins but form stable, long half-life, binary complexes with p16INK4A (Parry et al, 1995) demonstrating that the cell cycle inhibitory effect of p16INK4A depends on the functionality of the tumor suppressor pRB.
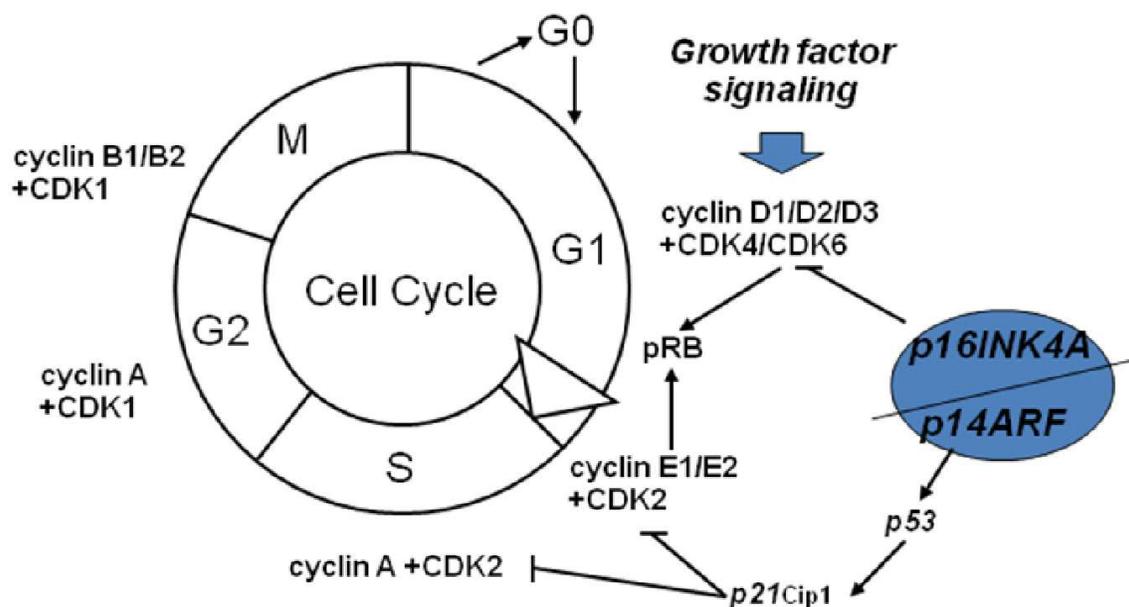

Fig. 1. Dynamic accumulation and assembly of cyclin/CDK complexes in different phases of the cell cycle. Schematic presentation of the cell cycle with emphasis on the G1/S checkpoint regulation by CDK inhibitors, detailed explanation in the text.

However nature has found an elegant way of redundancy to compensate for the loss of p16INK4A function in pRB-deficient tumors by an alternative protein. Adjacent to the INK4A gene locus on chromosome 9p21 an additional promoter region exists that produces a transcript which includes exon 2 and exon 3 of p16INK4A but has an alternate exon 1 (exon1β) (Figure 2). Since the exon 2 is translated in an alternative reading frame (ARF) the resulting protein is completely unrelated to the gene product of INK4A although it shares parts of the mRNA sequence (Quelle et al, 1995). p16INK4A and ARF have no similarities in amino acid composition and are two completely different proteins with distinct functions – but both act as efficient tumor suppressors (Figure 2). This is demonstrated by the fact that mice that are deficient for either ARF or p16INK4A have increased susceptibility to spontaneous or carcinogen-induced cancers (Sharpless et al, 2001; Kamijo et al, 1997). p16INK4A-deficient mice show a much less spontaneous tumor rate than ARF null mice. p16INK4A ablation leads to spontaneous sarcomas and lymphomas within 17 months (Sharpless et al, 2001), whereas the onset of spontaneous sarcomas, carcinomas and lymphomas in ARF-null mice is already observed at the age of 9 months (Kamijo et al, 1997). However, it is difficult to compare results from ARF null mice with the situation in human cells since human (p14ARF) and mouse p19ARF share only about 50% sequence homology. In addition there are apparent regulatory and functional differences between human and mouse ARF. Whereas in senescent mouse fibroblasts p19ARF accumulates and is critical for senescence-induced growth arrest, the human p14ARF seems not involved and is dispensable for this process (Sharpless et al, 2004). In human cells, the senescence process is

mainly controlled by the unrelated twin p16INK4A and the relevance of p14ARF seems also limited for other processes such as prevention of "stemness" in normal human fibroblasts by the expression of specific transcription factors (see below).

Human or murine ARF proteins do not contain any recognizable structural motifs and probably need to interact with other proteins to form functional complexes. The first discovered and best-defined function of ARF is the induction of p53 via inhibition of the p53-degrading E3-ubiquitine ligase MDM2 (mouse) or HDM2 (human) (Sherr 2006). In situations of increased cell cycle progression, e.g. when oncogenic signaling stimulates cell cycle entry or loss of pRB function, ARF is transcriptionally induced via E2F1/DP1 and binds to and inhibits MDM2 or HDM2, respectively. This leads to accumulation of p53 and induction of p53-induced response genes, such as p21Cip1 that interferes with cell cycle progression, or the proapoptotic BCL-2 proteins BBC3/PUMA and PMAIP1/Noxa that induce programmed cell death (Villunger et al, 2003). More recently other functions of ARF have been discovered: in response to oncogenic stress ARF enters the nucleolus and retards rRNA transcription thereby inhibiting ribosome biosynthesis (Itahana et al, 2003) and ARF influences the ATM/ATR kinases during the DNA-damage response in a p53-independent manner (Rocha et al, 2005). Moreover, it was shown that independent of p53 ARF antagonizes the activity of two other critical factors for cell cycle entry, namely E2F and Myc (Sherr 2006). The detailed investigation of how p16INK4A and ARF control cell survival and proliferation in distinct cell types will remain a highly interesting field of research in the next years, in particular the physiologic role of these two unrelated twins in non-transformed somatic cells.

4. The role of the INK4A locus for the generation of induced pluripotent stem cells (iPS)

Tissue repair and permanent replacement of damaged or aged cells are essential for the life of complex organisms and usually depend on a distinct, unspecialized stem cell population with almost unlimited proliferative capacity. With age, the capacity of these stem cells to proliferate and generate progenitors declines, which may also contribute for many age-related symptoms. The reprogramming of normal, differentiated cells by the three transcription factors Oct4, Klf4, and Sox2 has opened a completely new field of research and raised the hope to regenerate almost all cell types and tissues within the human body by generating stem cells from somatic cells of a patient. However the process that is initiated by these three transcription factors works at low efficiency and remains poorly understood. In embryonal stem cells and in induced progenitor stem (iPS) cells the INK4A gene locus is completely silenced and neither p16INK4A nor ARF are expressed. The lack of INK4A proteins seems to be a hallmark of different kinds of stem cells. Interestingly, this epigenetic silencing is not due to enhanced DNA-methylation of the INK4A promoters but results from so called "bivalent chromatin" which is present at the INK4A gene locus (Ohm et al, 2007). "Bivalent" means that repressive methylation marks (H3K27me3) and activating methylation marks (H3K4me3) are present on the same histone molecule, leading to a chromatin state that silences gene expression but can be reversed during differentiation of a cell. Such domains are characteristics of embryonal stem cells and are frequently associated with binding sites for Oct4 and Sox2. Although such binding sites are absent in the INK4A gene locus the presence of these bivalent domains suggests that the INK4A gene locus adopts a silent configuration in stem cells, but, in contrast to DNA-methylation-induced

silencing, retains the ability to be re-activated during differentiation processes (Li et al, 2009). It is well established that the rate of induced pluripotent stem cells generated from somatic cells significantly drops with the age of the organism they are obtained from. In humans also the cellular levels of p16INK4A increase with age. Back to back several different groups showed in 2009 that the INK4A gene locus critically impairs successful reprogramming to pluripotent stem cells and that it represents a main barrier to iPS cell programming (Utikal et al, 2009; Marion et al, 2009; Banito et al, 2009; Li et al, 2009). Also in these papers the differences of p16INK4A and ARF between murine and human cells become evident: In mouse cells, the ARF-p53 pathway has more impact on preventing the generation of pluripotent stem cells from somatic cells, whereas p16INK4A seems to play a minor role in the mouse. In human fibroblasts, knockdown of ARF does not affect at all the generation of iPS cells whereas knockdown of p16INK4A significantly improved reprogramming efficiency. This suggests that depending on the species, either p16INK4A or ARF represent a barrier to "back-differentiation" of normal somatic cells and prevent the induction of "stemness" in cells that have differentiated into a certain lineage. This makes this gene locus so important for the medical application of induced progenitor cells to e.g. replace damaged tissue of a patient, but also underlines that p16INK4A and/or ARF may be critical for maintaining tissue architecture and function in complex organisms by preventing uncontrolled expansion or "development" of somatic cells with stem cell-like abilities.

5. Regulation of INK4A in hematopoietic stem cells and their progenitors

As discussed before the INK4A gene locus represents a main barrier to the generation of iPS cells. In hematopoietic stem cells and many other stem cell types e.g. neuronal stem cells (Molofsky et al, 2006), the INK4A gene locus is not active. In particular the downregulation and silencing of p16INK4A seems to be essential for the enhanced self-renewal and proliferative capacity of human hematopoietic stem cells (Janzen et al, 2006). At the transcriptional level the INK4A expression is modulated by three main regulators, beta lymphoma Mo-MLV insertion region (BMI1), ETS1 and inhibitor of DNA binding 1 (Id1) whereas age-related induction of p16INK4A and ARF in human cells is mainly related to the balance between ETS1 and Id1 proteins (Ohtani et al, 2001). The reversible silencing of this gene locus in hematopoietic stem cells can be ascribed to the activity of the BMI1 protein. BMI1 belongs to the polycomb group genes, which are transcriptional repressors that control gene expression patterns during differentiation and development (Simon and Kingston 2009). The polycomb group genes fall into two subgroups that are either part of polycomb repression complex 1 (PRC1) or polycomb-repression complex 2 (PRC2). PRC2 is the so called "initiation complex" that functions as a histone-methyltransferase which specifically methylates histone H3 on lysine 27 causing gene silencing. As outlined above methylation of histone H3 on lysine 27 (H3K27me3) and on lysine 4 (H3K4me3) are hallmarks of "bivalent" chromatin that is silenced but retains the ability to be reactivated upon cell differentiation processes. BMI1 is part of PRC1 which is the so called "maintenance complex" that in a second step recognizes trimethylated H3K27. BMI1 directly associates with the INK4A locus and it was demonstrated that repression of the INK4A gene locus depends on the continuous presence of the PRC2 complex (Bracken et al, 2007). Several lines of evidence suggest that BMI1 is critical for maintaining "stemness" at least in human hematopoietic stem cells (Figure 2). In cord blood hematopoietic cells BMI1 expression is highest expressed in the hematopoietic stem cell population and gradually

decreases when these cells mature into more differentiated progenitor cells. Overexpression of BMI1 enhances the self-renewal of hematopoietic stem cells, increases the engraftment potential and results in stem cell maintenance. Knockdown of BMI1 in cord blood CD34+ and in acute myeloid leukemia (AML) CD34+ cells reduces progenitor-forming capacity, stem cell marker expression and long-term culture-initiating cell frequencies significantly suggesting that loss of BMI impairs the maintenance of stem cells and progenitor cells. In parallel, because of the gene-silencing effect of the BMI1 containing PRC1 complex on the INK4A gene locus, loss of BMI1 in C34+ cord blood and AML cells causes the induction of p14ARF and p16INK4A, significantly increased apoptosis and the production of cellular reactive oxygen species (Rizo et al, 2009). Lack of BMI1 in hematopoietic cells from BMI1 knockout mice also resulted in an increased expression of p16INK4A and p19ARF. The fact that the deletion of INK4A/ARF in the BMI1-/-background partly restored the self-renewal capacity of hematopoietic stem cells demonstrates the importance of silencing of the INK4A gene locus by BMI1 for the maintenance of hematopoietic stem cells (Oguro et al, 2006).
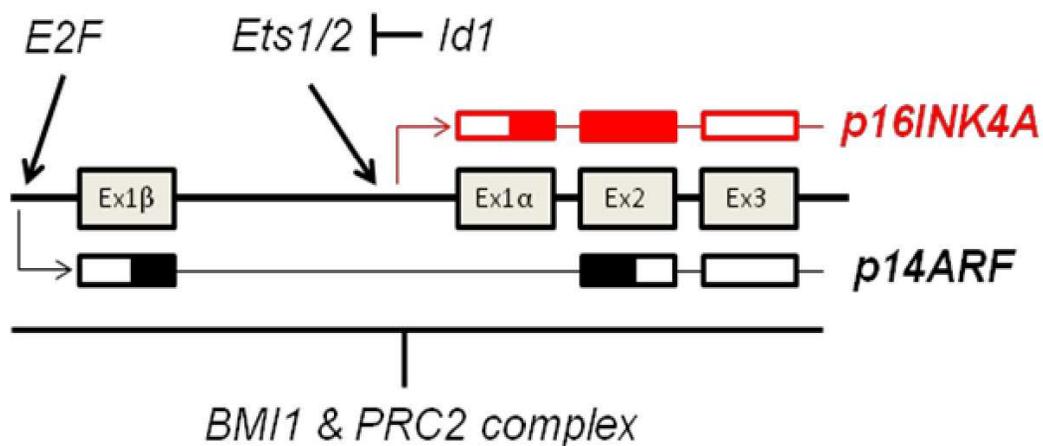

Fig. 2. The INK4A gene locus generates the two unrelated tumor suppressor proteins p16INK4A and ARF by alternative promoter usage and splicing, which are subjected to a complex regulation in hematopoietic stem cells and progenitor cells. Stress and senescence activate p16INK4A via Ets1/2 transcription factors and accelerated cell cycle entry triggers ARF expression. Id1 heterodimerizes and blocks Ets1/2 in young unstressed cells or stem cells. In stem cells BMI1 as part of the polycomb complex PRC2 causes epigenetic gene silencing of the entire locus by bivalent histone methylation (see text).

6. Is p16INK4A critical for the development of hematologic malignances?

The almost unlimited replicative capacity of stem cells and efficient generation of progenitors may be a double edged sword for a multicellular organism. On one hand this proliferative capacity allows efficient repair, regeneration and plasticity of tissues, on the other hand it increases the risk of acquiring genetic defects in this stem cell population that may result in hyperproliferative diseases, among them malignant transformation and cancer. Considering the fact that the INK4A gene locus codes for two tumor suppressors with completely different functions which either critically control the pRB or the p53 gene network, it becomes clear that genetic abnormalities of this gene locus may have a dramatic impact on progression of a damaged hematopoietic stem cell into precancerous cells that

give rise to leukemia. Due to the unusual structure of this gene locus, mutations often affect both, p16INK4A and p14ARF gene products, thereby deleting the gatekeepers of two essential check points. This may explain why mutations of the INK4A gene locus are observed in almost all human cancers. However, the effects of the INK4A gene products are also cell lineage specific, which might explain why some malignancies, for example T-ALL, show very high frequencies of homozygous deletion of the INK4A gene locus. Deletions frequently affect exon 2, thereby destroying both, p16INK4A and p14ARF, but there are also patients with alterations in either exon 1α or exon 1β which only affect one of the tumor suppressors (Cayuela et al, 1996; Cayuela et al, 1997; Hebert et al, 1994; Quelle et al, 1995). In addition methylation of the p16INK4A promoter was reported in T-ALL patients, leading to permanent silencing of the INK4A gene (Gardie et al, 1998; Drexler 1998). From a prognostic point of view inactivation of the INK4A locus seems also highly important for human lymphoblastic leukemia. Loss of INK4A predicts relaps in children with acute lymphoblastic leukemia suggesting a critical role of this locus in disease development and also highlighting the need for additional therapies to treat this subgroup of T-ALL patients (Kees et al, 1997); (Okuda et al, 1995). Several attempts have been undertaken to better understand, why inactivation of the INK4A gene locus is critical in particular for the development of hematopoietic malignancies. One oncogene that is thought to initiate T-cell leukemia is the TAL1/SCL oncogene. TAL1/SCL expressing T-ALL patients have a high incidence (up to 90%) of deletion of exon 2 in the INK4A gene locus. Although TAL1/SCL overexpression induces leukemia in transgenic mice, leukemia by this oncogene is characterized by a long latency suggesting that additional genetic events are required (Condorelli et al, 1996). To elucidate the contribution of the INK4A gene locus to leukemogenesis Shank-Calvo and colleagues (Shank-Calvo et al, 2006) mated TAL1 transgenic mice with single knockout mice for either p16INK4A or p19ARF. Of note, each of these mice developed T-cell leukemia rapidly, indicating that loss of either p16INK4A or p19ARF accelerates TAL1-induced leukemia in mice.

The fact that the INK4A genes are inactivated at high incidence in hematopoietic malignancies might be ascribed to the specific roles of these two proteins in slowing down the proliferation of hematopoietic progenitor cells, as discussed above. INK4A-/- mice possess increased thymus size and cellularity suggesting involvement of p16INK4A in the control of thymocyte proliferation. These animals exhibit increased numbers of CD4 and CD8 T lymphocytes in thymus and spleen (Bianchi et al, 2006) which also reflects increased proliferative potential. By using somatic, tissue specific ablation of p16INK4A in the T- or the B-lymphoid progenitor cells it was recently demonstrated that in the T-cell lineage loss of p16INK4A attenuated age-dependent thymic involution or increased production of naive T-cells. In the B-cell lineage p16INK4A inactivation significantly accelerated lymphoid tumorigenesis. Interestingly, the animals mainly suffered from tumors that manifested in the central nervous system but still expressed CD45 leukocyte-common antigen. These tumor cells were negative for a neuromeningeal marker, proving that they are not brain tumors and expressed B-lymphocyte markers demonstrating their B-cell origin (Liu et al, 2011). In this paper the authors argued therefore that in the T-cell linage p16INK4A merely regulates cell senescence in the mouse, whereas in the B-cell lineage loss of p16INK4A contributes to lymphoid cancer. These results are contradictory to the high prevalence of p16INK4A loss in human T-cell leukemia progenitor cells and may be ascribed to the already above discussed differences of the role of p16INK4A and ARF between mice and men. Senescence, cell cycle arrest and cell death are three possible physiologic fates of a cell

that may be triggered by the two tumor suppressors of the INK4A gene locus. In the next chapter we will discuss how p16INK4A affects programmed cell death.

7. p16INK4A regulates programmed cell death and death sensitivity in leukemia cells

When a cell is hit by a genotoxic insult it will either try to repair the genetic damage or, if not possible, undergo programmed cell death. Otherwise the mutation will be inherited to the daughter cells, which may give rise to precancerous cells that slowly proceed into malignancy. Slowing down cell cycle progression upon genotoxic stress may therefore help cells to get time for efficient damage repair and thereby prevent programmed cell death. On the other hand cell cycle arrest in the presence of oncogenic signaling may constitute a death signal per se since apoptosis may be the only way for a precancerous cell to respond to this "conflicting signaling" situation. INK4A gene products are not expressed in hematopoietic stem cells and therefore may not play an essential role for the quiescence state that every stem cell has to enter after division to preserve its replicatory potential. Instead, INK4A proteins accumulate in the progenitor cell population and seem to limit their proliferation.

As a consequence of p16INK4A deficiency rapid movement through the cell cycle may sensitize leukemia cells to genotoxic stress, which has been shown in different types of cancer cells. In p16INK4A-deficient MEFs and U2OS osteosarkoma cells the lack of p16INK4A sensitizes these cells to cell death induction by UV irradiation, whereas p16INK4A-proficient cells are largely resistant. The authors also observed that UV-induced apoptosis in p16INK4A-deficient cells coincided with decreased levels of pro-survival BCL2 and increased levels of pro-apoptotic BAX proteins (Al Mohanna et al, 2004). Moreover, in p16INK4A-deficient mice increased numbers of CD4 and CD8 T-cells are found in thymus and spleen and these increased cell numbers correlated with reduced T-cell apoptosis in the thymus rather than increased proliferation rates (Bianchi et al, 2006). The increased rates of DNA-synthesis in p16INK4A-deficient cells may expose an "archilles heel" to DNA-damage-induced cell death, but there are also data that highlight that p16INK4A reconstitution sensitizes T-ALL cells to certain forms of apoptosis. This suggests that p16INK4A may exert also additional effects beyond cell cycle inhibition, such as the regulation of proteins critical for cell death initiation and cell death decision in mammalian cells. We demonstrated already ten years ago that tetracycline-regulated p16INK4A reconstitution in p16INK4A-deficient CCRF-CEM cells, a human T-ALL cell line, sensitizes these leukemia cells to physiologic levels of cortisol, a glucocorticoid that also plays a significant role during T-cell selection and T-cell maturation in the thymus. In this early study we were able to show that p16INK4A significantly induces the expression of endogenous glucocorticoid receptor thereby markedly lowering the threshold for glucocorticoid-induced apoptosis (Ausserlechner et al, 2001). In this paper we discussed that loss of p16INK4A in precancerous hematopoietic cells may render them resistant to glucocorticoid-induced cell death by physiologic levels of cortisol. Since the strikingly increased sensitivity was difficult to explain by increases in receptor levels alone we sought for additional mechanisms further downstream of the glucocorticoid receptor that may contribute to p16INK4A-regulated death sensitivity. Indeed, also in T-ALL cells activation of p16INK4A affects the balance of death inducers and death protectors at the level of mitochondria, but also activates death ligands. For studying p16INK4A effects in different T-ALL cell lines we applied a tightly controlled tetracycline-regulated expression system,

based on different tetracycline-activated transactivators and repressors (Ausserlechner et al, 2006). In Molt4 T-ALL cells, for example, p16INK4A causes increased sensitivity to UV-irradiation, which is associated with induction of the pro-apoptotic BH3-only protein BBC3/Puma (Obexer et al, 2009a).

In principle programmed cell death can be initiated by a number of different signals originating either from outside of the cell (extrinsic pathway) or from intrinsic signals (Strasser 2005). Soluble or cell bound death ligands such as FASLG/FAS ligand or TRAIL bind to their cognate receptors, thereby inducing the formation of the so called death-inducing signaling complex (DISC) which contains the adaptor molecule FADD and procaspase-8. The autocatalytic cleavage and activation of procaspase-8 triggers the downstream caspase cascade. Mitochondria are central decision makers of apoptosis that integrate death signals originating from DNA-damage, growth factor withdrawal, glucocorticoid-treatment and anoikis. These stimuli trigger cell death either by directly regulating cell survival/cell death genes, or by deregulating cellular networks, which leads to apoptosis. Pro- and anti-apoptotic BCL2 proteins, referred to as the "BCL2-rheostat", are involved in this cell death decision either as direct targets or as sensors for cellular stress. BCL2-proteins can be divided into three groups. The prosurvival multi-domain BCL2-proteins BCL2, BCL2L2/Bcl-w, BCL2L1/Bcl-x_L, BCL2A1/A1 and MCL1 share four BCL2-homology (BH) domains, whereas multi-domain proapoptotic proteins BAX and BAK1/Bak are characterized by three BH-domains (Strasser 2005). The third group is the family of BH3-only proteins which contain only the BH3-domain and heterodimerize with prosurvival BCL2-proteins. The two models that have been proposed for apoptosis
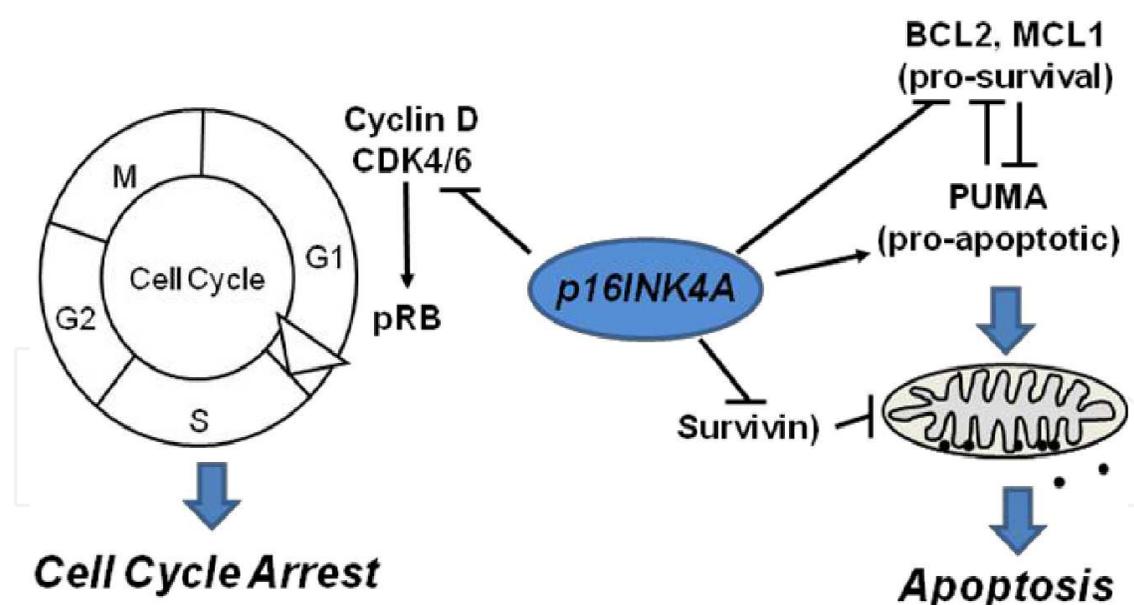

Fig. 3. Expression of p16^INK4A induces growth arrest in the G1 phase of the cell cycle and shifts the balance of pro- and anti-apoptotic BCL2 proteins towards the edge of cell death (detailed explanations in the text).

induction by BH3-only proteins suggest that either strong BH3-only proteins such as BCL2L11/Bim, truncated Bid or BBC3/Puma directly activate BAX or BAK1 ("direct activator/de-repressor model") (Kim et al, 2006) or that these BH3-only proteins neutralize the pro-survival function of anti-apoptotic BCL2-proteins ("displacement model") (Labi et

al, 2006; Willis et al, 2007). Upon cell death decision BAX or BAK1 oligomerize in the mitochondrial outer membrane which causes cytochrome *c* release from mitochondria and activation of the further downstream apoptosome complex.

Interestingly, the apoptosis-regulatory effect of conditional reconstitution of p16INK4A in T-ALL leukemia cells depends on the therapeutic agent that is applied to the cells: p16INK4A markedly protects against programmed cell death induced by doxorubicin, etoposide and vinblastine (Figure 4), but in parallel sensitizes these cells to FAS- and GC-induced apoptosis (Figure 4 and (Obexer et al, 2009a)). Since p16INK4A induces an almost complete cell cycle arrest in these cells and uncouples growth from cell cycle arrest (Ausserlechner et al, 2005) we believe that this protective effect is merely a consequence of accumulation of cells in the G1 phase of the cell cycle. DNA-damaging agents such as doxorubicin or etoposide and tubulin-destabilzing compounds such as vinblastine exert their effects mainly on proliferating cells in S-phase or mitosis, respectively. Slowly proliferating cells or cells that are arrested in the G1-phase of the cell cycle may be therefore more resistant (Bacher et al, 2006). However, conditional p16INK4A also protects T-ALL cells against bortezomib-induced cell death (Figure 4). Although, the proteasome-inhibitor bortezomib/Velcade™ may perturb the correct degradation of cell cycle regulators such as cyclins, it has been shown that bortezomib is highly effective on slowly proliferating cancer cells such as chronic myeloid leukemia. This suggests that p16INK4A has a direct or indirect affect on cell death regulators at the level of death receptors and/or BCL2-proteins. When analyzing the expression of death receptors and their ligands it became evident that p16INK4A induces FAS-ligand mRNA expression in these cells, but does not change the FAS-receptor expression. Since CCRF-CEM cells are FAS-sensitive, this might cause an autocrine death signal that lowers death resistance in general since also the FAS downstream effectors Caspase-8 and Bid showed accelerated cleavage in p16INK4A expressing cells upon FAS-induced apoptosis. However, by introducing a dominant negative FADD mutant that blocks death receptor signaling we provided evidence that increased death sensitivity in p16INK4A expressing T-ALL cells can be ascribed to distinct changes in the composition of pro- and anti-apoptotic BCL2-proteins at the level of mitochondria (Obexer et al, 2009a). Whereas the potent pro-apoptotic protein BBC3/Puma accumulated in p16INK4A expressing CCRF-CEM and Molt4 cells, the pro-survival proteins BCL2 and MCL1 were downregulated after 24 hours and completely lost after 48 hours of p16INK4A expression. Although p16INK4A-expressing T-ALL cells did not undergo programmed cell death spontaneously within 48 hours, these significant changes in the balance of pro- and antiapoptotic BCL2-proteins reduce the capacity of T-ALL cells to cope with additional apoptotic signals such as binding of death-ligands or glucocorticoid-induced cell death (Figure 3). We demonstrated the relevance of Puma, BCL2 and MCL1 by retroviral overexpression and knock down, but did not further investigate other forms of cell death (Obexer et al, 2009a). Concerning the observation that p16INK4A expression renders T-ALL cells less sensitive to bortezomib, we found that p16INK4A reconstitution induced rapid loss of the BH3-only protein PMAIP1/Noxa in both CCRF-CEM T-ALL and Molt4 T-ALL cells (Obexer et al, 2009a). Noxa is a "weak" BH3-only protein that does not neutralize all pro-survival BCL2-proteins, but preferentially binds to MCL1, BCL2A1/A1 (Willis et al, 2007) and, as recently shown by our group also to BCL2L1/Bcl-x$_L$. Noxa acts as a sensitizer that critically regulates the sensitivity to e.g. proteasome-inhibition-induced cell death (Hagenbuchner et al, 2010). The rapid loss of Noxa upon p16INK4A reconstitution might therefore explain, why p16INK4A-expressing T-ALL cells show reduced sensitivity to bortezomib-induced apoptosis.
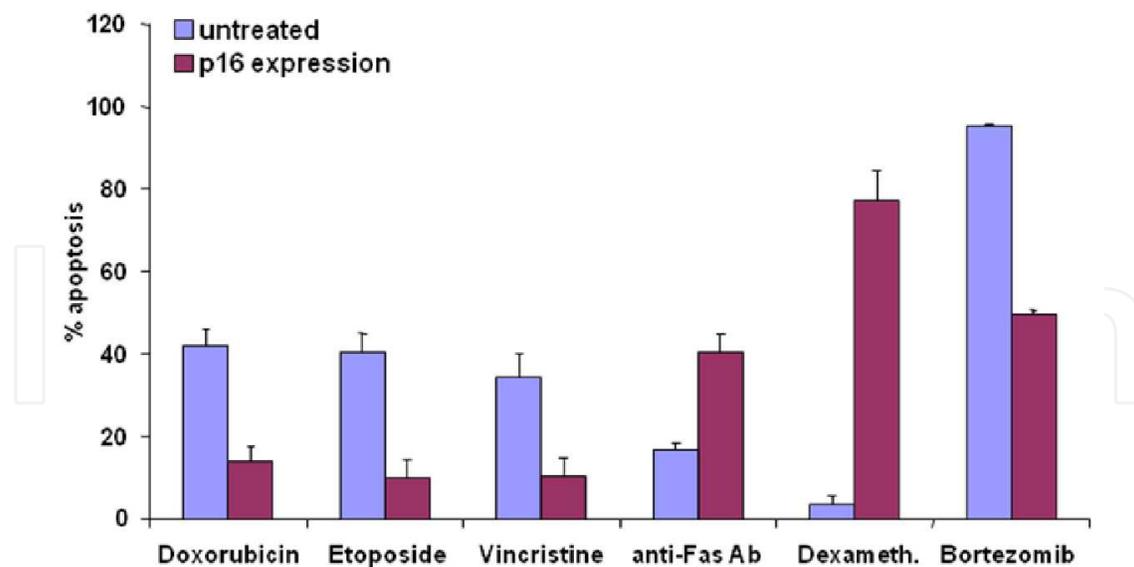
Fig. 4. p16INK4A expression differentially affects the sensitivity of T-ALL cells to various death-inducing agents. T-ALL cells expressing p16INK4A in a tetracycline-regulated manner were cultured for 24 hours in the presence of doxycycline (200 ng/ml) to switch on p16INK4A expression and were then treated with the therapeutic agents doxorubicin (8 µM), etoposide (8 µM), vincristine (8 µM), dexamethasone (10 nM) and bortezomib (10 nM) for additional 24 hours. To activate death receptor signaling an anti-Fas-receptor antibody (clone CH11, 0.1 µg/ml) was applied for 4 hours. Cells were resuspended in hypotonic propidium-iodide containing buffer according Nicoletti et al (Nicoletti et al, 1991) and the percentage of apoptotic cells was assessed in a Beckman-Coulter FC500 flow cytometer. Shown is the mean of three independent experiments.

However, additional levels of apoptosis modulation by p16INK4A in T-ALL cells exist. One protein that is highly expressed in many cancers e.g. in up to 65% of acute lymphoblastic B-cell leukemia is the anti-apoptotic protein BIRC5/Survivin (Troeger et al, 2007). Survivin belongs to the family of Inhibitor of Apoptosis Proteins (IAPs) that are characterized by so called BIR domains that allow them to interact with caspases and other molecules involved in cell death signaling. Survivin has also additional functions since it acts as a chromosomal passenger protein and blocks apoptosis induction at the level of mitochondria (Obexer et al, 2009b). Recently, it was shown that mice overexpressing Survivin in hematopoietic stem cells show a high incidence of hematologic tumors. This pro-oncogenic effect of Survivin was not due to increased proliferative potential but to increased death resistance of hematologic cells (Small et al, 2010).

Interestingly, the loss of p16INK4A during leukemogenesis apparently contributes to high levels of Survivin in T-ALL cells: in leukemia cells engineered to express p16INK4A the Survivin steady state expression levels are completely repressed upon p16INK4A induction (Figure 5A). To directly assess the relevance of Survivin-repression for changes in apoptosis sensitivity we retrovirally transduced human Survivin into T-ALL cells with conditional p16INK4A expression. As shown in Figure 5A this ectopic Survivin compensates for the loss of the endogenous protein during p16INK4A expression. Interestingly, ectopic Survivin prevented FAS-induced death sensitization (Figure 5B), but did not affect the increased sensitivity of p16INK4A-expressing T-ALL cells to glucocorticoid-induced cell death (Figure 5C). These results suggest that in T-ALL cells Survivin interferes with death-receptor-

induced apoptosis triggered by FAS-ligand. Therefore, the effects of p16INK4A in leukemia cells by far extend the generally described effect as an inhibitor of cell cycle progression.
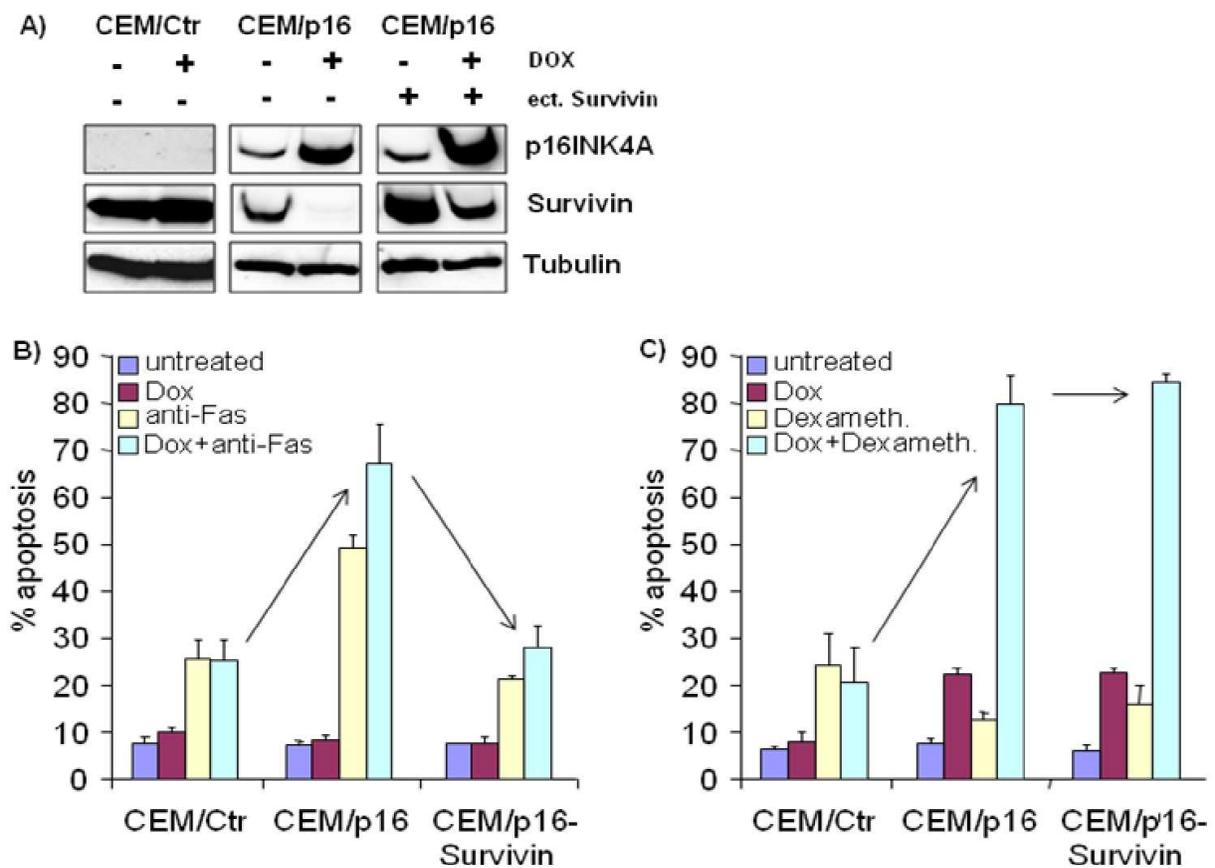

Fig. 5. High levels of p16INK4A in human CCRF-CEM T-ALL cells repress the IAP family member Survivin, which is critical for increased FAS-induced cell death, but not for p16INK4A-induced sensitization to glucocorticoid-induced apoptosis. A) CEM/Ctr cells stably express the reverse tet-transactivator rtTA, CEM/p16 cells are derivatives that contain the rtTA and express human p16INK4A under the control of a tetracycline responsive CMV promoter (Ausserlechner et al, 2001). In presence of 200 ng/ml doxycycline, p16INK4A expression is induced, which causes complete loss of Survivin within 48 hour. Retrovirally transduced Survivin compensates for the loss of the endogenous protein in CEM/p16-Survivin cells. Re-expression of p16INK4A accelerates death-receptor-induced (anti-FAS anibody, 0.1 mg/ml, for four hours) and glucocorticoid-induced apoptosis (10 nM dexamethasone, 24 hours) as shown in B und C (Obexer et al, 2009a). Ectopic expression of Survivin did not change the sensitivity to dexamethasone (C), but prevents increased sensitivity to Fas-induced apoptosis (B). The amount of apoptotic cells was assessed by flow cytometric analysis of propidium-iodide stained nuclei. Each bar represents the mean of three independent experiments.

8. Conclusions

The INK4A genes were discovered in the mid-90s of the last century and intensive studies on their function and regulation have contributed significantly to our current knowledge on

cell cycle regulation (and de-regulation) in normal and malignant cells. Despite all this effort and progress, continuously new aspects of p16INK4A and ARF are discovered, which highlight their diverse functions beyond the inhibition of the cell division cycle. In this chapter we reviewed current findings on how these INK4A-encoded genes are regulated in hematopoietic stem cells and that these proteins also represent a barrier to the artificial generation of pluripotent stem cells from normal differentiated tissue. In addition, both proteins also contribute to death sensitivity: ARF by activating p53 via a well defined pathway, p16INK4A by directly or indirectly affecting the expression and activity of critical death regulators such as BCL2 proteins, death ligands or pro-survival proteins such as Survivin. Under normal, physiologic conditions these significant changes in death sensitivity may determine a deadly barrier for cells that try to return to a less differentiated state and also for precancerous cells that lose proliferative control. These novel findings implicate that INK4A proteins are not "only cell cycle brakes" but serve as gatekeepers that keep the doors closed for those cells that want some piece of "stemness".

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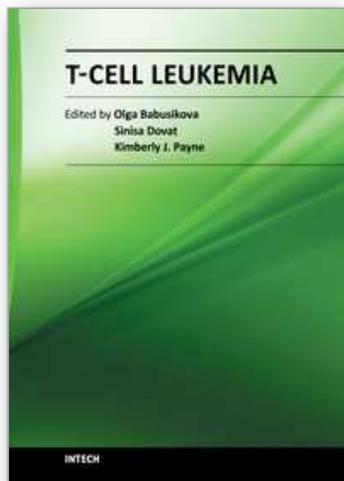
T-Cell Leukemia

Edited by Dr Olga Babusikova

The purpose of this book is to provide a comprehensive review of the scientific advances in T-cell malignancies and to highlight the most relevant findings that will help the reader understand both basic mechanisms of the disease and future directions that are likely to lead to novel therapies. In order to assure a thorough approach to these problems, contributors include basic scientists, translational researchers and clinicians who are experts in this field. Thus, the target audience for this book includes both basic scientists who will use this book as a review of the advances in our fundamental knowledge of the molecular mechanisms of T-cell malignancies, as well as clinicians who will use this book as a tool to understand rationales for the development of novel treatments for these diseases.

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