G1 Phase Control and Cell Differentiation

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Abstract

Cell differentiation is usually accompanied by irreversible cell cycle exit. The G1 regulatory molecules have been shown to be exquisitely regulated during the differentiation process and in many models they have been shown to play a pivotal role in differentiation. The cell cycle exit concomitant with the onset of differentiation occurs in G1 phase and it is mediated and maintained by (i) up-regulation of CDK inhibitor proteins, (ii) activation of the RB protein family (pRB, p107 and p130) and (iii) subsequent inactivation of E2F proteins. Among these G1 regulatory molecules, $p21^{WAF1-}$, $p27^{KIP1}$, p130 and E2F4 have been most predominantly involved as differentiation inducers. Studies in cell culture models as well as in vivo models through transgenic and knockout mice demonstrate that $p21^{WAF1}$ and $p27^{KIP1}$ play important but distinct roles in differentiation and that the cell cycle arrest and differentiation inducing functions can be genetically separated. Also, p130, rather than pRB, functions more frequently as the pocket protein regulating cell cycle exit during differentiation. Despite these broad generalizations, there is a large variation in the roles of these regulators depending on the model under study. Therefore, we have reviewed separately the regulation and functions of G1 phase regulatory proteins in the main differentiation models.

Introduction: The Switch Differentiation-Proliferation

With a few exceptions, cell differentiation involves exit from cell cycle and an irreversible proliferative arrest. During differentiation, each committed cell triggers the expression of number of tissue-specific genes coordinately with cell cycle exit. It is conceivable that the high expression levels of many tissue-specific genes required to bring about the tissue functions are incompatible with the transient switch-off of transcription that occurs during mitosis.

Thus, the cell committed to differentiation must take two decisions:the decision to irreversibly arrest cell cycle progression and enter the G0 state and the decision to trigger the expression of differentiation transcription factors and tissue-specific genes. The maintenance of the state of irreversible cell cycle arrest is a common feature to mature of cells from very different tissues and the general hypothesis is that common mechanisms for cell cycle arrest operate during or prior differentiation.

The data gathered over the last years aims towards the idea that the cell cycle exit concomitant with the onset of differentiation or differentiation commitment occurs in G1 phase and it is mediated and maintained by (i) up-regulation of CDK inhibitor (CKIs) proteins, particularly $p21^{WAF1}$ and $p27^{KIP1}$; (ii) activation of the RB protein family (pRB, p107 and p130) and subsequent inactivation of E2F proteins.

Although it is clear that CKIs and RB proteins are involved in the cell cycle exit associated to differentiation, there are two questions to be answered:i) Is the cell cycle arrest provoked by these proteins sufficient to trigger differentiation? ii) Do these proteins have a role in differentiation control independent from the cell cycle arrest function?

Given the redundancy of regulatory circuits that control G1 progression, and the intricate network of interactions between the regulatory proteins, it has been difficult to dissect the

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contribution of individual proteins to differentiation. Moreover, the data indicate that the roles of each protein may differ depending on the cell type.

CKIs in Differentiation

As a general rule, the expression of ^{p21WAF1} and $p27^{KIP1}$ increases during differentiation. The up-regulation of other CKIs as p57-KIP2, $p16^{INKa}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ has also been reported in the differentiation response, although the involvement of these CKIs has been much less studied. In models where the issue has been studied in detail, it has been found that the onset of the up-regulation varies with the CKI in each particular model and in many cases the up-regulation of $p21^{WAF1}$ is rapid and occurs later and is transient while that of $p27^{KIP1}$ is more maintained. This is the case, for example, in intestinal cells,[1,2] keratinocytes,[3] preadipocytes,[4] muscle cells,[5,6] and myeloid cells (ref. 7 and Muñoz-Alonso and León, unpublished) it has been found that the induction of $p21^{WAF1}$ is rapid and transient while induction of $p27^{KIP1}$ occurs later and it is maintained. However, there are exceptions to this general rule, as in P19 neuronal differentiation.[8] In the models where it has been studied in detail, it has been found that $p21^{WAF1}$ up-regulation occurs at the transcription levels and depends on the Sp1 and Sp3 binding sites in the proximal $p21^{WAF1}$ promoter, as in keratinocytic,[9] neuronal[10] and intestinal cell[11] differentiation.

While it is clear that differentiation is usually accompanied by up-regulation of CKIs, it is less clear whether CKI up-regulation is a consequence or, on the contrary, triggers the differentiation process. In many models, the forced expression of $p21^{WAF1}$ and $p27^{KIP1}$ genes after transfection or viral infection is sufficient to induce differentiation, but this result is not always found. Finally, in the cases where enforced expression of CKI results in differentiation, it is not clear whether this is a result of the cell cycle arrest brought about by the CKI or whether the CKI has a pro-differentiating function unrelated to cell cycle arrest.

However, as a general conclusion it can be stated that $p21^{WAF1}$ and $p27^{KIP1}$ play nonequivalent roles in differentiation. In several models, as intestinal cell differentiation, $p27^{KIP1}$ is a more efficient differentiation inducer than $p21^{WAF1}$, while in neuronal differentiation $p27^{KIP1}$ serves to arrest cell cycle without differentiation induction (see below). Mice deficient in $p21^{WAF1}$ show abnormal keratinocytic differentiation (reviewed in 12). In the case of $p27^{KIP1}$ in glial differentiation of Xenopus retinal cells, investigators have been able to separate the differentiation-promoting region, in a N-terminal domain different from that required for its cell-cycle inhibitory function.[13]

A similar conclusion can be drawn from CKI-deficient mice models. $p21^{WAF1}$, $p27^{KIP1}$ and p57-KIP2 deficient mice are viable. Mice deficient in $p21^{WAF1}$ develop normally and harbor no detectable abnormalities in all their organs.[14] Mice deficient in $p27^{KIP1}$ show higher growth rate and are 20-30% larger than wild-type animals without other developmental abnormalities.[15-17] Mice lacking p57-KIP2 show growth retardation and defects in chondrocyte, muscle and kidney differentiation, without major effects in proliferation, and most animals die within a few hours after birth.[18-20]

Mirroring their differences in differentiation and development, $p21^{WAF1}$ and $p27^{KIP1}$ are differently involved in carcinogenesis. There are no consistent and significant changes in expression and mutations of $p21^{WAF1}$ in human cancer. In contrast, low levels of $p27^{KIP1}$ are frequent in some tumor types and associated to malignant progression in epithelial derived tumors (breast, prostate, stomach and particularly colorectal cancer).[21-24] Moreover, $p27^{KIP1}$ nullizygous and heterozygous mice are more predisposed to radiation- or chemically-induced tumors than $p21^{WAF1}$-deficient mice. The cancer-related phenotypes of CKI- and RB-deficient mice have been reviewed elsewhere.[25,26]

Finally, there is an important variability in the regulation of cyclins among the differentiation models under study, but the over-expression of cyclin D3 emerges as a common feature in some of them, as muscular,[27,28] hematopoietic[29,30] and adipocytic[4] differentiation.

pRB and E2F in Differentiation

The elucidation of the role of E2F and pRB in differentiation have been hampered by the existence of several proteins composing both the RB family and the E2F family. pRB, p107 and p130 constitute the "pocket protein family ". pRB was the first described and best known member of the family, and is one of the tumor suppressor genes most frequently inactivated in human cancer.[31] The three pocket proteins are structurally very similar, although p107 and p130 are more closely related to one another than they are to pRB. The three proteins associate with members of the E2F family and can be phosphorylated by CDKs, although they differ on the E2F partner and phosphorylation kinetics. pRB and p107 show similar phosphorylation patterns, being hyperphosphorylated during G1 progression. In contrast, complexes of p130-E2F are predominant in G0 phase.[32] p130 is already phosphorylated in G0 cells, although it undergoes additional phosphorylation in other sites upon mitogenic stimulation of the cell.[33-35] As described below, different pocket protein-E2F complexes are formed depending on the particular differentiation model or differentiation lineage.

Disruption of both Rb alleles results in embryonic lethality. In contrast, mice deficient in p107 or p130 develop normally. However, embryos deficient in pRB/p107 or pRB/p130 show a similar phenotype than RB-deficient embryos but they die two days earlier, thus revealing some functional overlap between the pocket proteins. Interestingly, in a different mouse strain (Balb/c) deficiency in p130 results in embryonic lethality and deficiency in p107 results in severe postnatal growth impairment.[35-38]

The involvement of pRB in differentiation has been demonstrated in several differentiation models as keratinocytes, adipocytes and particularly muscle cells (recent reviews include:34,35,37-39).

In general, p130 is highly expressed in quiescent [32] and in differentiated cells. This is found in several lineages, such as muscular,[40-45] keratinocytic,[46] intestinal,[47] neuronal[48] and hematopoietic[49] lineages. Interestingly, embryonic stem cells deficient in the three pocket proteins (pRB, p107 and p130), show a deregulated G1 and impaired differentiation, as assayed by limited capacity to form differentiated teratocarcinomas.[50]

pRB -deficient mice show defects in differentiation of neuronal, lens and erythroid precursor cells,[51-53] whereas muscular differentiation cannot be properly evaluated because of the embryonic lethality of these mice. In contrast, mice deficient for p107 or p130 show no clear defects. Mice defective for both pocket proteins show neonatal lethality with deregulated chondrocyte growth and impaired bone development.[40,54] Thus these genetic disruption experiments demonstrate nonredundant roles of pocket proteins during development, and the same conclusion can be drawn from the differentiation models. The characterization of a pRB mutant that retains the ability to induce differentiation of Saos-2 cells but cannot bind E2Fs and induce G1 arrest demonstrates a critical role for pRB in regulating differentiation.[55] The ability to regulate differentiation may explain why pRB has tumor-suppressor properties lacking in p107 and p130 (reviewed in 39).

E2F is a six-member family,[56,57] although E2F5 and 6 have not been studied in relation with differentiation. Recent studies with oligonucleotide microarrays have identified a number of genes related to differentiation and cell fate, including homeobox genes and genes related to signal transduction by factors from the transforming growth factor (TGF) family. The study also revealed differences in expression among E2F members.[58] E2F1 is a paradoxical gene as it can function as an oncogene or tumor suppressor gene depending on the tissue type.[39] Induc-

tion of apoptosis by E2F1 is a fundamental property not shared by its siblings E2F2, 3, 4, or 5. The involvement of E2Fs in differentiation has been addressed in several studies, analyzing the formation of complexes of these transcription factors with pocket proteins. In most models, the E2F protein usually found in the complexes is E2F4. E2F4 is involved in keratinocyte[59] and neuronal[60] differentiation, whereas E2F1[38,39] and E2F3[61] function has been related to cell cycle progression rather than to differentiation.

Although there are a few common facts to differentiation models, summarized above, it is clear that each tissue differentiates with its own molecular peculiarities. Actually, an important conclusion that emerges from the published studies is the variety of pathways controlling the G1 arrest in differentiation depending on the cell type. Moreover, it has recently been shown that some transcription factors that induce differentiation of particular tissues control not only tissue-specific proteins but also cell cycle regulatory proteins. The clearest example is the muscular differentiation transcription factor MyoD, which up-regulates $p21^{WAF1}$, cooperate with pRB to arrest growth and binds CDK4 (revised in 62,63). Given the number of G1 regulatory proteins involved (CKIs, CDKs, pocket proteins and E2Fs) and their interrelations it is difficult to obtain a complete picture of their expression in a particular model, and dissect which change determines differentiation or is a consequence of differentiation. Although cell culture differentiation models may be not physiological, it allows the study of molecular changes during differentiation under controlled conditions, and a lot of information on the molecular biology of differentiation has been generated by these models.

We summarize below the involvement of G1 phase control proteins in the major differentiation models. We will first review the differentiation of epithelial-derived cell types (epidermal, neuronal, intestinal) and secondly the differentiation of mesenchymal-derived cell types (muscular and hematopoietic). The most relevant data on models of nonhematopoietic differentiation are summarized in (Tables 1 and 2).

Keratinocytic Differentiation

Up-regulation of CKIs has been shown in several models of keratinocyte differentiation. Normal human epidermal keratinocytes undergo differentiation by suspension culture, with concomitant up-regulation of $p21^{WAF1}$,[64,65,46] $p27^{KIP1}$ and $p16^{INKa}$.[46] Primary mouse keratinocytes are induced to differentiate in response to raised calcium concentration in the medium. In this model, differentiation and withdrawal from the cell cycle also correlates with induction of $p21^{WAF1}$, $p27^{KIP1}$ and p57-KIP2 and their association with Cdk2.[66-68] It has been demonstrated that $p21^{WAF1}$ binds to calmodulin in a calcium-dependent manner[69] thus providing a direct link between the differentiation agent and the effector molecule for growth arrest. In a different model, $p21^{WAF1}$ and $p27^{KIP1}$ are induced concomitantly with differentiation of hair follicle cells in rats.[70]

The available data on $p21^{WAF1}$ involvement in keratinocyte differentiation are somewhat contradictory. Mice deficient in $p21^{WAF1}$ have no alterations in epidermis,[59,71] but primary keratinocytes derived from these mice show impaired calcium-induced differentiation in culture, with a drastic down-regulation of differentiation markers linked with late stages of keratinocyte differentiation.[66] In contrast, keratinocytes derived from $p27^{KIP1}$ deficient mice differentiate normally.[66] Mice deficient in $p21^{WAF1}$ show a reduced self-renewal potential of keratinocyte stem cell populations[72] and double $p21^{WAF1}/p16^{INKa}$ null mice show more profound alterations.[59]

In murine epidermal differentiation, maximal expression of $p21^{WAF1}$ occurs in postmitotic cells, while it is low in proliferating stem cells and mature mouse keratinocytes. Murine keratinocytes can be differentiated in culture by high calcium concentrations. This process is accompanied by a rapid and transient up-regulation of $p21^{WAF1}$, which returns to basal levels

Table 1. Regulation and effects of G1 regulatory proteins in some non-hematopoietic differentiation models. It is also indicated when the lack of effect of a particular protein was found.

Cell Model (Species)	Differentiation	Cell Cycle Regulator Expression	Differentiation Induced or Increased by	References
Prim. keratinocytes (hum)	Keratinocytic	↑p21 ↑p27 ↑p16	p16 (not p21, p27)	46,64,65
Prim. keratynocytes (mouse)	Keratinocytic	↑p21 ↑p27	(Inhibited by p21)	3
HaCAT (hum)	Keratinocytic	↑p21 ↑p27	E2F4	59
HIEC6 (human)	Enterocytic	↑p21 ↑p27	p21, p27 (not p16)	78
TsFHI (human)	Enterocytic	↑p21 ↑p27	p27	1,78
HT-29 (human)	Enterocytic	↑p21 ↑p27	p27	2,78
CaCo-2 (human)	Enterocytic	↑p21 ↑p27 ↑p130/E2F4		1,47
Oligodendrocytes (mouse)	Neuronal	↑p21 ↑p27 (not p16)	p21	93-96
Retinoblasts (Xenopus)	Glial	↑p27	p27	13
Ectodermal cells (Xenopus)	Neuronal	↑p27	p27	103
NTera (human)	Neuronal	↑p21	p21	80-82
N1E-115 neuroblastoma (mouse)	Neuronal	p27	p27	105
NT2/D1 embryonal carcinoma (human)	Neuronal	↑p27 (not p21)	p27 (not p21)	8
PC12 (rat)	Neuronal	↑p21 ↑E2F4	p21, E2F4	60,80-82
P19 (mouse)	Neuronal	↑p21 ↑p27 ↑p130 ↓E2F1, ↓E2F2, ↓E2F4		8,85
Retina photoreceptors (mouse)	Neuronal	↑p27 ↑p57	Not p27	98
Corti neurons (mouse)	Neuronal	↑27	Not p27	102
TSU-Pr1 (hum)	Neuronal		p21	104
ATDC5 (mouse)	Chondrocytic	↑p21 ↑p27. (not p15, p18, p19)		227
C2C12 (mouse)	Muscular	↑p18 ↑p21 ↑p27 ↑p57 (not p19) ↑p130	p16, p21, p57	41,116,117,127
C2 (mouse)	Muscular	↑D3 ↑p130		43,128
L6 (rat)	Muscular	↑p130 ↓p107		44,127
Saos-2 (human)	Osteocytic		Rb	55
3T3-L1 (mouse)	Adipocytic	↑p21 ↑p27 ↑p18 ↑p130	Rb	230,233

after 24 h.[3] Enforced expression of $p21^{WAF1}$, but not of $p16^{INKa}$, in post-mitotic cells (using adenoviral vectors) inhibits differentiation.[3] This differentiation inhibition is not reproduced in human keratinocytes induced to differentiate in suspension culture. In this system, differentiation is associated to elevation of $p21^{WAF1}$, $p27^{KIP1}$ and $p16^{INKa}$, but enforced expression of

$p21^{WAF1}$ does not induce differentiation. Overexpression of $p16^{INKa}$ and $p27^{KIP1}$ also fails to induce differentiation.[46] However, expression of antisense $p27^{KIP1}$ prevents the differentiation of primary mouse keratinocytes induced by suspension culture, but did not prevent growth arrest.[73] Thus, involvement of $p21^{WAF1}$ and $p27^{KIP1}$ in this differentiation system is still under discussion.

The involvement of E2Fs in keratinocyte differentiation has also been studied. Squamous differentiation of keratinocytes is associated with down-regulation of E2F1, and it is upregulated in squamous cell carcinomas with respect to healthy epidermis. Consistently, E2F1 overexpression inhibits differentiation, although suppression of E2F1 activity (with dominant negative mutants) does not induce differentiation.[74] In another study, E2F1 expression is constitutive during differentiation of human epidermal keratinocytes induced in culture, whereas E2F4 is predominantly expressed at the onset of differentiation. HaCaT cells transfected with E2F1 are unable to differentiate, but cells transfected with E2F4 show an increased differentiation rate compared to vector-transfected cells.[59] So, E2F1 and E2F4 appear to have opposite functions in human keratinocyte differentiation. Finally, it has been reported recently that pRB up-regulates $p21^{WAF1}$ in epithelial cells, but not in fibroblasts. This effect is transcriptional, and depends on Sp1 and Sp3 binding sites in the proximal $p21^{WAF1}$ promoter.[9]

Enterocytic Differentiation

$p21^{WAF1}$, $p27^{KIP1}$ and p57-KIP2 are accumulated, as determined by immunohistochemistry, during differentiation of human enterocytes in intestinal crypts.[75] During spontaneous differentiation of the human intestinal cell line CaCo2 cells, a well-known model of human colon cell differentiation, there is an up-regulation of $p27^{KIP1}$ and a rapid and transient elevation of $p21^{WAF1}$ levels.[65,76,77] This differentiation is also accompanied by increased expression of p107 and p130. The predominant complex that accumulated during differentiation was p130/E2F4.[47]

Differentiation of human HT-29 colon cancer cells by sodium butyrate is accompanied by transient up-regulation of $p21^{WAF1}$ and more sustained elevation of $p27^{KIP1}$. Ectopic expression of $p27^{KIP1}$ in human colon cancer derived cells increases the sensitivity of the cells to induction of differentiation, whereas enforced expression of $p21^{WAF1}$ shows the opposite result.[2,78]

The other cell line that has provided important information on the role of CKIs in intestinal cell differentiation is the human intestinal cell line tsFHI. While CaCo2 and HT-29 are tumor-derived cell lines, tsFHI cells are conditionally immortalized by the thermosensitive SV40 T-antigen mutant. At the permissive temperature (e.g., 32°C), tsFHI cells proliferate displaying crypt cell markers. When shifted to the nonpermissive temperature (39°C) the cells undergo irreversible growth arrest and differentiation into brush border cells. With differentiation, $p21^{WAF1}$ and $p27^{KIP1}$ were strongly induced, but with different kinetics:the $p21^{WAF1}$ increase was rapid but transient and the $p27^{KIP1}$ increase was delayed but sustained, i.e., a similar pattern than colon cancer cell lines reviewed above.[1] In this cell line, forced expression of $p21^{WAF1}$ and $p27^{KIP1}$ led the cells to expression of differentiation markers. This differentiation was temporally dissociated from inhibition of pRB phosphorylation, and $p27^{KIP1}$ was more efficient inducing differentiation than $p21^{WAF1}$. A striking result is that $p27^{KIP1}$ failed to complex with cyclins and CDKs, despite its fivefold increase in differentiating cells.[1] Thus, in this cell line $p21^{WAF1}$ is the main CKI involved in irreversible growth arrest during the early stages of cell differentiation, whereas $p27^{KIP1}$ may induce or stabilize expression of differentiated traits, in a function independent from cell cycle arrest. A similar result has been reported for HT29 intestinal cells (see above). Therefore $p27^{KIP1}$ seems to be a critical protein in intestinal cell differentiation. Also, ectopic expression of $p21^{WAF1}$ and $p27^{KIP1}$ (but not $p16^{INKa}$)

induce differentiation in a normal human intestinal cell line (HIEC6) and the authors suggest that $p21^{WAF1}$ may act indirectly by elevating $p27^{KIP1}$ levels.[78] A similar regulatory cross-talk between $p21^{WAF1}$ and $p27^{KIP1}$ has been reported recently for K562 myeloid leukemia cells[79] (see below).

It is noteworthy that low expression of $p27^{KIP1}$ has been linked to poor prognosis in colorectal cancer.[21,23] Thus it is conceivable that the inhibition of differentiation in tumor cells devoid of $p27^{KIP1}$ is one of the mechanisms contributing to malignant progression in these tumors.[2]

Neuronal Differentiation

Up-regulation of CKIs has been reported during differentiation induced in several neuronal cell lines. One of the most broadly used is the rat pheochromocytoma PC12, which undergoes neuronal differentiation (with neurite formation) in response to nerve growth factor (NGF). PC12 cell differentiation induced by NGF is accompanied by $p21^{WAF1}$ induction, and enforced expression of $p21^{WAF1}$ induces growth arrest and increased sensitivity to the differentiation induced by NGF, but it does not directly lead to a differentiated phenotype (i.e., neurite extensions).[80-82] Unexpectedly, cyclin D1 also increases with PC12 differentiation.[80] In the PC12 model it has been shown that up-regulation of $p21^{WAF1}$ after NGF addition is dependent on a Sp1/Sp3 binding site next to the TATA box.[10] A similar result was found in the differentiation of the intestinal cell line CaCo2.[11] It is noteworthy that a low $p21^{WAF1}$ expression in embryonic brain and spinal cord was observed during mouse embryogenesis.[83]

Murine P19 cells treated with retinoic acid differentiate into neuroectoderm, with mixed populations of postmitotic neurons, astrocyte-like cells and oligodendrocyte-like cells (reviewed in 84). Neuronal differentiation of P19 cells occurs with concomitant up-regulation of $p21^{WAF1}$ and $p27^{KIP1}$, but $p27^{KIP1}$ induction is rapid while $p21^{WAF1}$ expression remains low until neurites are formed. Consistently, inhibition of $p27^{KIP1}$ expression by antisense oligonucleotides results in differentiation inhibition.[8] Increase in $p27^{KIP1}$ is also detected in P19 cells differentiated by ectopic expression of neuroD2 or MASH1 genes.[85] Interestingly, differentiated P19 cells display high levels of cyclins D1 (as in PC12 cells, see above) and D2[86] and consistently endogenous CDK4 activity is high while CDK2 activity is low during neural differentiation of P19 cells.[48,86,87]

Neuronal differentiation of other cell lines such human embryonal carcinoma NT2/D1 cells,[8] neuroblastoma N2aβ cells differentiated by T3 hormone [88] or NTera2[8] are associated by elevation of $p27^{KIP1}$. Differentiation of NTera2 is also accompanied with $p15^{INK4b}$ and $p16^{INKa}$ induction.[89]

Differentiation of neurons during embryogenesis is a highly regulated process in which neuronal precursor cells exit the cell cycle and differentiate in a tightly coupled process. Involvement of pRB and E2F in neurogenesis has been recently reviewed.[84,90] Several in vivo models of neuronal differentiation have been studied. Differentiation of oligodendrocytes has provided interesting clues on the role of CKIs in differentiation. Oligodendrocytes originate from multipotent cells in proliferative ventricular areas of the brain. Primary cultures of progenitors can be isolated and induced to differentiate by serum deprivation.[91] During this differentiation, both $p21^{WAF1}$ and $p27^{KIP1}$ are induced, while $p16^{INKa}$ expression is maintained.[92,93] Ectopic expression of $p27^{KIP1}$ efficiently inhibits cell cycle progression, but is not sufficient to induce rodent oligodendrocyte differentiation, as assessed by expression of differentiation markers.[94,95] Consistently, oligodendrocyte precursors derived from $p21^{WAF1}$- and $p27^{KIP1}$-deficient mice continue to proliferate and show delayed differentiation, demonstrating that both CKIs are required for proper oligodendrocyte differentiation.[93,96] Interestingly, work with these mice models show that $p27^{KIP1}$ is required for withdrawal from the cell cycle while $p21^{WAF1}$ is not.

Instead, $p21^{WAF1}$ is required for the establishment of the differentiation program of oligodendrocyte progenitors following growth arrest. Also, $p21^{WAF1}$-deficient mice display delayed myelinization, which is consistent with the impaired differentiation of oligodendrocytes (which are myelinating cells of the central nervous system). Thus, the two CKIs serve nonredundant roles in this program of differentiation, with $p27^{KIP1}$ being responsible for arrest and $p21^{WAF1}$ having a function independent of its ability to control cell cycle exit.[96]

Development of retina photoreceptor cells constitutes an useful model of neuronal differentiation (photoreceptor cells) in vivo. Interestingly, $p27^{KIP1}$ and p57-KIP2 are expressed in different subpopulations of retinal precursor cells. $p27^{KIP1}$ is up-regulated in a pattern coincident with the onset of differentiation of most retinal cells in the mouse developing eye.[97] Mice deficient for $p27^{KIP1}$ have an increased fraction of mitotic cells through retina development as well as extensive apoptosis. Enforced expression of $p27^{KIP1}$ (by adenovirus) led to premature cell cycle exit but had no dramatic effects on differentiation.[98] Interestingly, the concomitant deficiency in cyclin D1 rescues the low cellularity of the retina of $p27^{KIP1}$ deficient mice.[99] In the developing retina model, it has also been shown that p57-KIP2 plays distinct roles, acting first as a cell cycle inhibitor in mitotic progenitor cells and then controlling differentiation of a subpopulation of postmitotic neuronal cells (amacrine cells) during postnatal development of the retina. Interestingly, in retina of p57-KIP2 –deficient mice apoptosis compensates for increased cell division.[100] It has also been proposed that an increase in CDK activity mediated by CKI depletion is not sufficient to cause cell-cycle defects but it is nevertheless able to perturb differentiation.[101]

A parallel example is the differentiation of hair cells in the developing organ of Corti of the inner ear. These cells undergo their terminal division at embryonic day 13-14 with concomitant induction of $p27^{KIP1}$ expression. In $p27^{KIP1}$ null mice, cell proliferation continues after day 14.[102] Thus, in sensory neurons from retina and ear, $p27^{KIP1}$ maintains the cell in a quiescent state and allows differentiation.

Another in vivo model is the neuroectodermal differentiation of vertebrates. During gastrulation of *Xenopus*, parts of early ectodermic cells differentiate into neuroectodermic cells, and $p27^{KIP1}$ expression is restricted to postmitotic cells from neural plate. In ectodermic cells, enforced expression of $p27^{KIP1}$ arrests cell cycle progression but did not induce neural differentiation.[103] Retinoblasts in *Xenopus* differentiate into glial cells. In these cells $p27^{KIP1}$ induces both growth and differentiation.[13] Overall, from these in vivo models it can be concluded that, in neural differentiation, growth arrest mediated by $p27^{KIP1}$ is not sufficient to trigger differentiation.

Human prostate cancer cell line TSU-Pr1 can differentiate into microglia-like cells by phorbol ester (TPA) treatment, with $p21^{WAF1}$ induction, and enforced expression of $p21^{WAF1}$ results in differentiation.[104] In contrast, differentiation of mouse N1E-115 neuroblastoma cells can be induced by overexpression of $p27^{KIP1}$ or pRB.[105]

Members of the pRB family are differentially expressed during development of nervous system. In the central nervous system, p107 expression was restricted to proliferating cells (i.e., cells of ventricular zone of developing mammalian neocortex), while pRB was expressed in areas of both proliferating and differentiating cells. In contrast to pRB and p107, expression of p130 was low throughout embryogenesis.[90,106] The involvement of pRB in neuronal differentiation has been studied in pRB -deficient mice. These mice are embryonic lethal, and analysis of both central and peripheral nervous systems in $Rb^{-/+}$ revealed numerous abnormalities, particularly in hindbrain, dicencephalon, spinal cord and dorsal root ganglia. These abnormalities include ectopic mitoses, decreased neuronal cell survival and neurite outgrowth, and were accompanied by decreased expression of the neurotrophins receptors TrkA and TrkB.[51-53,107] However, despite the severe neuronal phenotype of pRB deficient mice, dorsal root ganglia and cortical progenitor cells from E12.5 Rb null embryos were able to differentiate in culture.[53,108]

pRB expression also increases dramatically during neuronal differentiation of P19 cells, while no such increase occurs in mutant cells that fail to respond to retinoic acid.[48,86,87] PC12 differentiation is associated with accumulation of hypophosphorylated pRB, and microinjection of a monoclonal antibody specific for the hypophosphorylated form of pRB blocked the neurite outgrowth initiated by NGF.[109] Thus, pRB hypophosphorylation plays a crucial role in PC12 neuronal differentiation.

In developing brain, E2F1 and E2F2 expression is high in proliferating cells (ventricular zone) and suppressed in postmitotic neurons from the marginal zone, and the opposite regulation is observed for p130.[110] E2F4 is up-regulated during PC12 neuronal differentiation elicited by NGF, while E2F1, E2F3 and E2F5 are down-regulated. Moreover, ectopic expression of E2F4 enhanced the NGF-mediated differentiation of PC12 and lowered the rate at which cells lost their neuronal phenotype following NGF removal. Consistent with this role in the PC12 model, E2F4 expression also increases in the developing rat cerebral cortex and cerebellum, concomitantly with the onset of neuronal terminal differentiation.[60] Furthermore, retinoic acid-induced differentiation of P19 cells is associated with loss of expression of E2F1, E2F3 and E2F4, while E2F2 remains high. pRB and p130 also increases with differentiation.[48] E2F1 levels are very low in undifferentiated cells and increase upon RA-mediated differentiation.[111]

In other in vivo models, E2F1 is down-regulated in the developing quail neural retina between embryonic days E8-E10, just after the arrest of neuroretina division.[112] In the mouse retina, cyclin D1 protein decreases as photoreceptor matures, and transgenic mice with enforced expression of cyclin D1 disrupt photoreceptor differentiation and retina development.[113]

Muscular Differentiation

Muscular differentiation is probably the differentiation phenotype where the involvement of CKIs and pocket proteins has been studied in more detail. During differentiation, skeletal muscle cells withdraw from the cell cycle and fuse into multinucleated myotubes. This process can be reproduced in cell culture with some skeletal muscle-derived cell lines. Two of the most used cell lines are C2 and its derivative C2C12. These cells can differentiate with concomitant increase of $p21^{WAF1}$, $p27^{KIP1}$, $p18^{INK4c}$ and p57-KIP2. $p21^{WAF1}$ undergoes an initial increase but decreases when the cells become terminally differentiated. In contrast, $p27^{KIP1}$ and $p18^{INK4c}$ gradually increase, being $p18^{INK4c}$ the CKI that undergoes the greatest induction.[6,114,115] Actually, all of the CDK6 and half of the CDK4 are complexed with $p18^{INK4c}$ in differentiated C2C12 cells.[116] Interestingly, there is a switch of $p18^{INK4c}$ transcripts so the large mRNA predominant in proliferating cells disappears during differentiation while a smaller $p18^{INK4c}$ mRNA is predominant in differentiated cells. In contrast, expression of $p19^{INK4d}$ decreases with differentiation.[4,116] Enforced expression of $p21^{WAF1}$, $p16^{INKa}$ and p57-KIP2,[6,117] stimulates muscular differentiation in C2C12 cells as well as in 10T1/2, another cell line with muscular differentiation potential.

Cyclins D1, E and A are down-regulated during C2 or C2C12 muscular differentiation whereas cyclin D3 is greatly induced.[27,28,118] Cyclin D3 mediates the interaction of CDK4 and $p21^{WAF1}$ with pRB in differentiated C2 cells and critically contributes to the irreversible exit from the cell cycle.[118] Ectopic expression of cyclin D1 blocks C2C12 differentiation and this can be reversed by coexpression of $p21^{WAF1}$.[6,119] Consistently, expression of cyclin D1, but not cyclins A, B, D3 and pRB, inhibits the expression of MyoD.[28]

pRB is induced during muscular differentiation of murine myoblasts and ectopic expression of pRB induces differentiation or restores differentiation capability of murine myoblasts.[120-123] Also, repression of pRB by antisense RNA[122] or inactivation of pRB by SV40 large T antigen[120,124] inhibits differentiation. Moreover, pRB is required for muscle development (see below). As $p21^{WAF1}$ is induced during differentiation, it is expected that the the

growth suppressor function of pRB is activated. The adenovirus E1A protein binds and inactivates both pRB and $p21^{WAF1}$, and is able to reactivate DNA synthesis in differentiated muscle cells. However, this reactivation is abolished by a mutant E1A gene that binds pRB, but not $p21^{WAF1}$, indicating that $p21^{WAF1}$ function is dominant over pRB in maintaining the cell cycle arrest of C2C12 differentiated cells.[125] Conversely to vertebrate myotubes, pRB is expressed in newt myotubes, an observation that can be related with the regeneration capability of urodele limbs, which requires cell cycle reentry and local reversal of differentiation.[126]

In proliferating murine C2 and C2C12 and rat L6 myoblasts, p107 is the predominant pocket protein and its levels decrease during differentiation. In contrast, p130 increases with differentiation[41,43,127] and during differentiation of C2C12 there is an early accumulation of p130/E2F4 complexes.[41,42] Additional data using differentiation-defective cell lines indicate that p130/E2F accumulation is a necessary event in terminal differentiation of C2 cells, but not for cell growth arrest.[43] The involvement of p130 in muscular differentiation is stressed by findings in myoblasts acting as reserve cells that renew the muscular tissue in adult animals. In these cells p130 but neither pRB nor p107 accumulates during muscle differentiation.[45] In cultured myoblasts, hyperphosphorylated and hypophosphorylated forms of p107 are down-regulated to the same extent, while most of the p130 that is up-regulated during differentiation corresponds to the hyperphosphorylated form.[127]

Another example of the role of pocket proteins concerns the muscular differentiation of L6 cells. In these cells, p107 levels are down-regulated during differentiation, while p130 protein levels are up-regulated. Despite both p107 and p130 become phosphorylated during myogenesis, the E2F-site DNA binding complexes containing p107 detected in undifferentiated growing cells, are replaced in myotubes with complexes containing only p130.[27]

Data obtained with cells in vitro and with knockout mice (see below) indicate that proper regulation of E2F and pocket proteins is crucial for the coupling between cell cycle arrest and differentiation onset in skeletal myocytes. For example, C2 myoblasts transfected with E2F1 are still able to fuse into myotubes, express muscle specific proteins and up-regulate $p21^{WAF1}$. However, unlike wild-type cells, these E2F1-differentiated myocytes did not switch off proliferation, indicating that the primary role of E2F1 in these cells is to maintain proliferation.[128]

MyoD is a HLH transcription factor that induces muscular differentiation (for a recent review see 62). There is cross-talk between MyoD and G1-controlling proteins as demonstrated by a number of findings.[62,63]

a) MyoD induces $p21^{WAF1}$ expression during differentiation of murine C2C12 or 10T1/2 myocytes and prevents reassociation of CDK2-cyclin A to E2F4. By these mechanisms MyoD maintains cell cycle arrest during differentiation.[42,83,115,129,130] However, $p21^{WAF1}$ is expressed in myogenic cells of MyoD- or myogenin-deficient mice,[83] demonstrating that $p21^{WAF1}$ expression is not strictly dependent on these transcription factors.

b) p57-KIP2 increases MyoD expression by stabilization of the protein in C2C12 cells. This depends on a direct interaction between the basic region of the bHLH region of MyoD and the N-terminal domain of p57-KIP2. However, there is no MyoD/$p16^{INKa}$ interaction despite that $p16^{INKa}$ also up-regulates MyoD.[114,117]

c) Myo D activates the expression of cyclin D3 in the absence of new protein synthesis in differentiating C2 cells.[118] As mentioned above, D3 is the only cyclin upregulated during myogenic differentiation.[27,131]

d) MyoD induces the expression of Rb activating its promoter.[132] Activation of Rb (as well as $p21^{WAF1}$ and cyclin D3 genes) by MyoD occurs in the absence of new protein synthesis.[118] Interestingly, MyoD also up-regulates E2F1 at early stages of differentiation. This presumably contributes to increase the concentration of pRB -E2F1 transcription-repressing complexes in differentiating myocytes.[133]

e) MyoD cooperates with pRB to activate MEF2 (a muscle-specific bHLH transcription factor that cooperates with MyoD for myogenic differentiation).[134] A direct interaction between pRB and MyoD has been described in vitro[124] but has not been reproduced in vivo.[135] In fibroblasts lacking pRB, MyoD induces an aberrant skeletal muscle differentiation program, with normal expression of early differentiation markers such as myogenin and $p21^{WAF1}$, but attenuated expression of late differentiation markers such as myosin heavy chain. Similar defects were not observed in cells lacking either p107 or p130, indicating that the defect is specific to the loss of pRB.[136]

f) Overexpression of cyclin D1 inhibits myogenesis and Myo D transcriptional activity.[6,28,119] This inhibition correlates with phosphorylation of MyoD.[137]

g) CDK4 binds, phosphorylates and inhibits MyoD in 10T1/2 cells, thus explaining the effects of cyclin D1 described above.[138] MyoD interacts with CDK4 through a conserved 15 amino acid domain in C-terminus of MyoD.[135,138] In contrast to cyclin D1 and CDK4, overexpression of cyclin E and CDK2 in differentiated myotubes cannot reactivate DNA synthesis, despite pRB phosphorylation.[139]

h) MyoD is down-regulated in G1 phase after phosphorylation on Ser200 and subsequent degradation. This phosphorylation is carried out by CDK2-cyclin E, a process reminiscent of the phosphorylation/degradation of $p27^{KIP1}$.[140]

Muscular Differentiation in vivo

p57-KIP2 deficient mice show defects in many tissues (bones, lens, kidney) but exhibits normal muscle development.[18,20] Also, mice deficient in $p21^{WAF1}$ show no alteration of muscle development.[14,141] Consistently, p57-KIP2 is the only CKI expressed in adult skeletal muscle,[142] although $p27^{KIP1}$ is expressed transiently in developing myotomes of the mouse embryo.[143,144] However, double knockout mice lacking $p21^{WAF1}$ and p57-KIP2 fail to develop myotubes, and show increased proliferation and endoreplication.[130] This phenotype resembles that of myogenin-null mice,[145] but myogenin is expressed in the $p21^{WAF1}$/ p57-KIP2 double mutant mice.

Disruption of both Rb alleles results in embryonic lethality but partially rescued Rb mutant fetuses (with a Rb minigene that allows low expression of pRB, mgRb:$Rb^{-/-}$) survive birth. These animals express the transgenic pRB in brain but not in muscles or other tissues,[121] and the muscular differentiation is severely impaired, with increased apoptosis, shorter myotubes, giant nuclei, endoreduplication and failure to express late muscle markers. The importance of pRB in muscle differentiation is underlined by the fact that mice deficient in both pRB and Id2 (an antagonist of pRB antiproliferative activity) survive to term with minimal defects in neurogenesis and hematopoiesis (see below), but they died from severe reduction of muscle tissue.[146] In composite mutant fetus mgRb:$Rb^{-/-}$/$p21^{-/-}$ these defects are further augmented, demonstrating that $p21^{WAF1}$ also contributes to myogenesis in vivo. In contrast, E2F1 and p53 are dispensable during aberrant myogenesis in Rb-deficient fetuses.[147] The muscular phenotype of mice with low or no expression of pRB in muscle precursors is explained because pRB is required for expression of muscle-differentiation markers. Interestingly, development of the myogenic phenotype in $Rb^{-/-}$ cells correlated with increased expression of p107. However, these cells were induced by serum to reenter the cell cycle, demonstrating that p107 cannot maintain the terminally differentiated state in $Rb^{-/-}$ myotubes.[148]

Hematopoietic Differentiation

The study of the molecular mechanisms of hematopoietic differentiation is particularly challenging because this is multilineage differentiation, in which a single population of stem cells generates at least nine distinct mature cell types, with functions ranging from immune

Table 2. Regulation and effects of G1 regulatory proteins in some hematopoietic differentiation models

Hematopoietic Cell Models (Species)	Hematopoietic Lineage (Inducer-Agent)	Cell-Cycle Regulator Expression	Differentiaton Induced or Increased by	References
$CD34^+$ (human)	Myeloide (SCF,IL-6,GM-CSF)	↑p21,↑p15,↑D1		7,29,158,180,197,239
	Monocyte (FL, IL-3)	↑pRb		187
	Megakaryocyte (PPP,Tpo)	↑p21,↑D3,↑p15, ↑p27↑p16,↑E		29,180,198,202
	Erythrocyte (IL-3,SCF,IL-6, Epo)	↑p21,↑p27		29,197
Progenitor cells (human)	Erythrocyte (GM-CSF, IL-3, Epo)	↑pRb		188
FVA erythroblast (murine)	Erythrocyte (Epo)	↑p21,↑p27		214
Bone marrow macrophages (murine)	Osteoclasts (ODF/RANKL,TNFa)	↑p21,↑p27		178
B cells (human)	Activated mature B cells	↓p27,↑p18,↑pRb		218
T cells and thymocytes (human)	Activated mature T cells	↓p27		223,224
HL-60 (human)	Monocyte/macrophage (TPA, 1,25$(OH)_2D_3$, butyrate)	↑p21,↑p27,↑D1,↓A, ↓E,↓B,↓cdk2	p21, p27	156-158,160,184-186
	Granulocyte (DMSO, RA)	↑p21,↑p27,↓D1,↓D2		157,158,160,186
U937 (human)	Monocyte/macrophage (TPA, 1,25$(OH)_2D_3$, butyrate)	↑p21,↑p27, ↑p15,↑p16,↑p18,↓A	p21, p27	156,157,169 158,163,168,170,174
M1 (murine)	Monocyte/macrophage (IL-6)	↑p21,↑p19,↓D1, ↓E2F-1		158,181,195
32Dcl3 (murine)	Granulocyte (G-CSF)	↑p27,↑p130	p19, p130	165,182,193
NB4 (human)	Granulocyte (ATRA)	↑p15		180
UF-1 (human)	Granulocyte (1,25$(OH)_2D_3$)	↑p21,↑p27	p27	164
K562 (human)	Megakaryocyte (TPA)	↑p21,↑p27,↑D1,↓B, ↓cdc2,↓A,↓E	p21	158,171,200,205,212, Muñoz and León
CMK (human)	Megakaryocyte (Tpo)	↑p21,↑D1	p21, p27	184,199
HEL (human)	Megakaryocyte (TPA)	↑D1,↓E,↓A		184,205
MEG-01 (human)	Megakaryocyte (TPA)	↑p21,↑p27		201
MegT (murine)	Megakaryocyte	↓B		209
Dami (human)	Megakaryocyte (TPA)	↑D1	D1	205
UT-7 (human)	Megakaryocyte (TPA)	↑p21	p21	198
F-36P-*mpl* (human)	Megakaryocyte (Tpo)	↑D1,↑D2 ↑D3,↓A,↓B	D+↓cdc2 activity	207
HB60-5 (murine)	Erythrocyte (Epo)	↑p27		215
MEL (murine)	Erythrocyte (HMBA)	↑p21,↑p27,↑p15, ↑p18,↓CDK6	p21	216,217

response to oxygen transport. The involvement of the molecules controlling G1 phase in hematopoietic cell differentiation is summarized in Tables 2 and 3.

Table 3. Hematopoiesis-related phenotypes of knockout mice or transgenic mice for G1-regulatory proteins

In Vivo Model	Hematopoietic Phenotype	References
$Rb^{-/-}$ mice	Impaired erythropoiesis	51,52,107
$p107^{-/-}$ mice (Balb/cJ background)	Myeloproliferative disorders	36
E2F-$4^{-/-}$ mice	Abnormalities in hematopoietic lineage development	149,150
C/EBP$\alpha^{-/-}$ mice (E2F repression-deficient)	Dysplasia of neutrophil granulocytes	196
$p15^{-/-}$ mice	Lymphoproliferative disorders	240
$p16^{-/-}$/p19Arf $^{-/-}$ mice	Abnormal extramedullary hematopoiesis Increased proliferation of myeloid progenitors	241 154
$p16^{-/-}$ mice	Extended life span of bone marrow macrophages Enhanced mitogenic responsiveness of T cells	242 243
$p18^{-/-}$ mice	Lymphoproliferative disorders	240
$p27^{-/-}$ mice progenitor cells	Increased proliferation of myeloid progenitors and reduced differentiation in response G-CSF	165
$p21^{-/-}$ mice	Increased proliferation and impaired self-renewal of hematopoietic stem cells, Decreased myeloid colony formation, Increased proliferation of T lymphocytes	152 244 245
E2F-1-transgenic megakaryocytes	Blocked terminal differentiation, severe thrombocytopenia	213
D3-transgenic megakaryocytes	Enhanced ploidy, increased differentiation	204
D1-transgenic megakaryocytes	Enhanced ploidy, not increased differentiation	20
p27-transgenic T cells	Impairment development and function of T cells	225

Analyses of mice lacking selectively one of G1-phase cell-cycle regulator genes have suggested that most of them may be dispensable for hematopoietic development. One exception is pRB, whose disruption causes embryonic lethality and the mutant embryos exhibit a marked increase of immature nucleated erythrocytes.[51,52,107] Recently, it has been indicated that the $Rb^{-/-}$ phenotype is a to the consequence of uncontrolled Id2 functions, as Id2-Rb double knockout mice survive to term with no defects in hematopoiesis.[146] Also, loss of other cell-cycle regulator genes provokes altered hematopoiesis to a lesser extent. In this way, for example, targeted inactivation of E2F4 leads to a deficiency of various mature hematopoietic cell types together with an increased number of immature cells in several lineages.[149,150]

However, the functions of many cell-cycle regulators are partially redundant and, alternatively, family members are able to substitute for one another during development when one of them is target inactivated.[37] Indeed, in various instances the combined loss of two functionally similar regulators has given rise to failure of hematopoiesis. Thus, for example, E2F1 and E2F2 double-knockout mice display impaired B-cell differentiation, reduced threshold for antigen

activation of T cells and, in general, increased hematopoietic progenitor proliferation.[151] Moreover, in spite of the absence of abnormality in the hematopoietic phenotype of knockout mice for one individual cell-cycle regulator, studies carried out on cells derived from such animals have demonstrated that some of these proteins play essential functions in hematopoietic differentiation. For example, the absence of $p21^{WAF1}$ promotes the entry of hematopoietic stem cells into the cell cycle, which lead to stem cell exhaustion,[152] whereas loss of $p27^{KIP1}$ or $p16^{INKa}$ induces the increase of lineage committed progenitor proliferation, indicating a dominance of $p21^{WAF1}$ in bone marrow stem cell self-renewal and of $p27^{KIP1}$ and $p16^{INKa}$ in progenitor cell kinetics.[153,154] In addition, extensive studies in a great variety of hematopoietic model systems and the fact that the expression of some cell-cycle regulators, such as $p15^{INK4b}$ and $p16^{INKa}$, is frequently lost in leukemogenesis (reviewed in 155), have proposed an involvement of these proteins in the regulation of hematopoiesis, as reviewed below.

Myeloid Differentiation

During normal myeloid differentiation, $p21^{WAF1}$ and $p27^{KIP1}$ are expressed, but with different kinetics and subcellular localization. In $CD34^+$ cells differentiating towards myeloid lineage, the expression of $p21^{WAF1}$ is nuclear and transient, and, interestingly, concurrent with cellular proliferation, suggesting that the primary role for $p21^{WAF1}$ could be in coordinating the transition into differentiation rather than in maintaining the differentiated state. In contrast, the $p27^{KIP1}$ protein level is relatively constant but its subcellular localization changes from nuclear to cytoplasmatic at progressive stages of differentiation, indicating that $p27^{KIP1}$ might serve different functions at stages-specific of myeloid maturation.[7]

Likewise, expression of both proteins are increased along myeloid lineage by multiple differentiation-inducing agents in a variety of hematopoietic cell lines, such as HL-60,[156-162] U937,[156,158,163] K562 (Muñoz-Alonso and León, unpublished), M1,[158] UF-1[164] and 32D.[165] The up-regulation of $p21^{WAF1}$ is an immediate early response to differentiation-inducers and precedes terminal differentiation, indicating that induction of this protein is a primary mediator of differentiation rather that a consequence of growth suppression.[157,158,160] In this line, in retinoic acid-induced differentiation of acute promyelocytic leukemia cells, $p21^{WAF1}$ has been shown to play a crucial role during commitment to differentiation, independently of CDK inhibition and cell cycle arrest.[166] On the other hand, it has been suggested that $p27^{KIP1}$ leads to differentiation by causing cell cycle arrest. Consistent with this idea, several reports have shown that differentiation is accompanied with an increase of CDK2-bound $p27^{KIP1}$, concomitant with CDK2 inactivation, in ML-1 and U937 cells.[167,168] Furthermore, a direct stabilization of $p27^{KIP1}$ by $p21^{WAF1}$ in myeloid derived K562 cells has been observed recently, which might help to coordinate the differentiation-specific functions of these CKIs.[79]

Ectopic overexpression of $p21^{WAF1}$ and/or $p27^{KIP1}$ in U937 cells, in the absence of hormone, results in an induction of the expression of monocyte/macrophage-specific markers[163,169,170] and in HL-60 both proteins accelerate the differentiation triggered by TPA.[160] Also, exogenous $p21^{WAF1}$ initiates differentiation of K562 cells[171] and exogenous $p27^{KIP1}$ enhances maturation of UF-1 cells[164] (Table 2).

Moreover, suppression of $p21^{WAF1}$ by antisense techniques results in decreased expression of maturation markers by differentiation agents in HL-60 and U937 cells[166,172-175] and increases sensitivity to induced apoptosis, presumably by facilitating activation of the apoptotic protease cascade.[173,174,176] In line with this last observation, in U937 promyelocytic cells, $p21^{WAF1}$ confers cell survival from monocyte/macrophage differentiation-induced cell death.[175,177] Likewise, in ODF/RANKL-treated macrophages from mouse bone marrow cells, a mixture of $p21^{WAF1}$ and $p27^{KIP1}$ antisense oligonucleotides inhibits osteoclast differentiation.[178]

$p15^{INK4b}$ expression is up-regulated during differentiation into myeloid lineage of normal $CD34^+$ progenitor, of blasts isolated from patients of acute promyelocytic leukemia and of the NB4 promyelocytic cell line.[29,179,180] Interestingly, blasts from patients developing ATRA syndrome display high levels of $p15^{INK4b}$ and ATRA treatment does not increase or even downmodulate this protein, providing new insights into understanding the pathogenesis of this syndrome.[180] Also, $p19^{INK4d}$ is induced during macrophage differentiation of M1 cells[181] and its overexpression, and the resulting inhibition of cyclin D-dependent kinase activity, leads to this lineage in 32Dcl3 cells.[182] Cyclin-dependent kinases could act during G1 phase to interfere with differentiation-specific programs, which might be executed in noncycling cells. In agreement with this concept, it has been shown that during granulocytic maturation of ML-1 cells there is an increase in CDK4-bound $p18^{INK4c}$ and CDK2-bound $p27^{KIP1}$, as well as a decrease in CDK6-bound cyclin D3, showing a complex regulation of CDKs during differentiation.[167] Furthermore, the overexpression of cyclin D2 or D3 in 32Dcl3 myeloid cells prevents their ability to differentiate to granulocytes in response a G-CSF.[183] Interestingly, cyclin D1 is up-regulated during TPA-induced macrophage differentiation of HL-60 cell line,[184-186] whereas it is down-regulated in DMSO-induced granulocytic pathway of these cells,[186] and in IL-6-induced macrophage differentiation of M1 cells.[181] In addition, overexpression of cyclin D1 inhibits induced macrophagic differentiation in M1 cells[181] but no granulocytic maturation in 32Dcl3 cells,[183] suggesting that this cyclin plays an additional role other than regulation of cell cycle progression.

Among the pRB family proteins, p130 seems be responsible for maintaining cells in G0 state of the cell cycle. In $CD34^+$ cells, p130/E2F4/DP-1 complex predominates and when the cells proliferate in response to cytokines, p130 is phosphorylated and replaced by p107 as the main E2F binding partner.[49] Similar to other systems, when hematopoietic stem cells undergo differentiation and exit the cell cycle, levels of p107 decline, while p130 increases. Likewise, changes of pRB have also observed along myeloid lineage commitment of hematopoietic progenitor cells, but whereas levels of hypophosphorylated pRB are upregulated during monocytic maturation, they are low during granulocytic pathway, and only the monocytic differentiation is inhibited by antisense Rb oligonucleotides.[187,188] In addition, induced monocytic differentiation of leukemia cell lines has been correlated with activation of pRB by hypophosphorylation[189,190] and both suppression of this protein by antisense techniques and its overexpression reduces induction of differentiation in U937 cells, but no G1-accumulation.[191] Therefore, it has been suggested that pRB plays a critical role in the monocytic lineage pathway by mechanisms that are not strictly related to control of the cell cycle. In fact, it has been proposed that pRB may promote cell differentiation through its interaction with transcription factors different from E2F. For example, during induced differentiation of U937 cells, pRB interacts with and activates the transcription factor NF-IL6.[192] However, p130 has been shown to play an important role in granulocytic differentiation,[127,193] as its enforced expression, but not of pRB, inhibits induced maturation of 32Dcl3.[193]

Also, E2F family proteins are involved in myeloid differentiation. Deregulated E2F1, in conjunction with ectopic expression of Bcl-2 to delay apoptosis, prevents granulocytic differentiation of 32Dcl3, whereas E2F3 has no effect.[194] Also, overexpression of E2F1 blocks the induced differentiation of the M1 myeloblastic cell line by promoting cell cycle progression, but surprisingly it does prevent the induction of $p16^{INKa}$ and $p15^{INK4b}$ inhibition of CDK4 activity, and subsequent hypophosphorylation of pRB, indicating that deregulated E2F1 uncouples p15/p16- pRB pathway from growth arrest.[195] Recently, a study has demonstrated in vivo that E2F repression by C/EBFα is required for granulopoiesis, as mice harboring E2F repression-deficient C/EBFα alleles exhibit dysplasia of neutrophil granulocytes.[196]

Megakaryocytic Differentiation

The terminal process of megakaryocytic differentiation is different from that of other hematopoietic lineages, as the cells undergo endomitosis during the late phase of maturation, which causes polyploidization. Although several groups have identified a number of cell-cycle regulators implicated in endomitosis, the results of these investigations are contradictory and the precise roles of these molecules in megakaryocytopoiesis are not fully understood.

Human megakaryocytes derived from $CD34^+$ cells display high levels of $p21^{WAF1}$ and $p27^{KIP1}$. $p21^{WAF1}$ is expressed in cycling megakaryoblasts whereas $p27^{KIP1}$ is only detected in cell-cycle arrested megakaryocytes.[197] The expression of $p21^{WAF1}$ is an early event and precedes polyploidization, suggesting that it might be implicated in this process.[198] Indeed, $p21^{WAF1}$ is upregulated during induced megakaryocytic differentiation in some hematopoietic cell lines, such as CMK,[199] UT-7,[198] K562[200] (Muñoz-Alonso and León, unpublished), and MEG-01,[201] and its overexpression in two cell lines with a megakaryocytic phenotype leads to nucleus polylobulation.[198,199] In addition, thrombopoietin, the hematopoietic factor that regulates megakaryocytic differentiation and platelet production, increases $p21^{WAF1}$ transcription by the transcription factor STAT5.[199]

However, megakaryocytopoiesis in $p21^{WAF1}$ deficient mice is normal and overexpression of $p21^{WAF1}$ in $p21^{-/-}$ or normal megakaryocytes inhibits ploidization, suggesting that $p21^{WAF1}$ is not essential for the determination of the ploidy profile, but probably plays an important role in the exit from the endomitotic cell cycle.[202]

Also, $p27^{KIP1}$ is up-regulated in induced megakaryocytic differentiation of K562 (Muñoz-Alonso and León, unpublished) and of MEG-01 cells.[201] Ectopic expression of $p27^{KIP1}$ lead to megakaryocytic differentiation of CMK cells,[199] but, like $p21^{WAF1}$, its overexpression in normal megakaryocytes also induces an endomitotic cell-cycle arrest.[202] Moreover, high levels of other CKIs, p16-INK4a and $p15^{INK4b}$, are found in megakaryocytic lineage, associated to hypophosphorylated pRB.[180,202] Recently it has demonstrated that $p15^{INK4b}$ mediates, at least in part, the stimulation of megakaryocytic differentiation by autocrine TGF-β1.[203]

D-type cyclins are critically important for cell cycle progression and, because their expressions are high in megakaryocytes and the endomitotic process requires DNA replication, have been supposed to participate in polyploid formation. Indeed, cyclin D3 is upregulated in $CD34^+$ undergoing megakaryocytic lineage and the treatment of these cells with cyclin D3 antisense oligonucleotide inhibits their maturation, while abrogation of cyclin D1 or cyclin D2 have little effect.[29,30] Moreover, transgenic mice overexpressing cyclin D3 have megakaryocytes of higher ploidy than the control animals and exhibit an increased number of differentiated cells of this lineage,[204] demonstrating that this cyclin is involved in polyploidization. Cyclin D1 is increased in induced megakaryocytic differentiation of several human cell lines as Dami, K562 and HEL,[200,205,206] although regulation of cyclin D3 in these systems has not been reported. The overexpression of cyclin D1 alone induces growth arrest but fails to increase ploidy in Dami megakaryocytic cell line, and it enhances polyploidization during TPA-induced differentiation.[205] A recent study has shown that transgenic mice in which cyclin D1 is overexpressed in megakaryocytes display a moderate increased ploidy in these cells, with no increase in the number of differentiated cells, suggesting that this cyclin also may promote polyploidization.[206] However, a different study shows that in F-36P-*mpl* cells overexpression of D-type cyclins alone does not induce differentiation, but together with the expression of a dominant negative form of CDC2 includes megakaryocytic maturation, indicating that decreased CDC2 activity may contribute to endomitosis.[207] This observation is consistent with reports demonstrating that endomitosis is accompanied by low CDC2 activity due to down-regulation of CDC25C phosphatase or the decreased expression of cyclin B.[208-210] As well, cyclin E has been shown to be actively complexed with CDK2 during polyploidization of HEL cells[211] and the mainte-

nance of cyclin E in G2/M cells determines cyclin A expression and the entrance of K562 cells into re-replication cycles.[212] Similar to its role in myeloid differentiation, deregulated E2F1 also affects megakaryocytopoiesis, as overexpression of this protein blocks terminal differentiation and causes proliferation in transgenic megakaryocytes.[213]

Erythroid Differentiation

During erythropoietin-dependent terminal erythroid differentiation of primary erythroblasts from spleens of mice infected with the anemia-inducing strain of Friend virus, both $p21^{WAF1}$ and $p27^{KIP1}$ are induced, but only $p27^{KIP1}$ associates with the G1 CDKs (CDK4, CDK6 and CDK2). Binding of $p27^{KIP1}$ to CDK2 (but not CDK4 or CDK6) correlates with pRB hypophosphorylation and growth arrest.[214] In the HB60-5 cell line $p27^{KIP1}$ is also up-regulated during erythroid differentiation, with inhibition of CDK2 activity.[215] However, although $p27^{KIP1}$ overexpression causes G1 arrest, it does not promote terminal erythroid differentiation.[215,216] Studies carried out on murine erythroleukemia (MEL) cells have provided important information on the complex regulation of CDKs by CKIs during differentiation program.[216,217] In this model, terminal cell division is mediated by induction of $p15^{INK4b}$, $p18^{INK4c}$, $p21^{WAF1}$ and $p27^{KIP1}$, thereby leading to sequential inhibition of the G1 CDKs. A specific order of the combined inactivation of CDK2 and CDK6 is essential to trigger differentiation, the inhibition of CDK2 being required first. Among the CKIs, only $p21^{WAF1}$ is able to inhibit both CDKs (CDK2 and 6) and its ectopic expression induces cell differentiation, but not overexpression of other CKIs. Importantly, these investigations have also shown that differentiation decisions occur only in the G1 phase and that CDK4 and CDK6 play different roles at different stages of differentiation. On the other hand, and consistently with the phenotype of Rb-null mice, pRB phosphorylation is induced and sustained during erythroid maturation and the suppression of this protein by antisense techniques inhibits erythropoiesis in stimulated differentiation of human hematopoietic progenitors cells and leukemic cell lines.[188,191]

Lymphoid Differentiation

In resting B cells, $p27^{KIP1}$ is strongly expressed and its expression decreases during activation leading to final differentiation of normal B cells into Ig-secreting cells.[218-220] Conversely, $p18^{INK4c}$ is upregulated in this process, concomitant with inhibition of pRB phosphorylation by cyclin D3/CDK6, indicating that $p18^{INK4c}$ is involved in the subsequent early G1 arrest necessary for terminal B lymphocyte differentiation.[218,221] Also, the expression of p57-KIP2 varies as a function of the stage of B-cell differentiation, nonetheless the role of p57-KIP2 in this hematopoietic differentiation has not been examined.[222] Similarly, in the T cell lineage, $p27^{KIP1}$ is also abundant in thymocytes and peripheral T lymphocytes and its expression is down-regulated both during development when $CD4^-$ $CD8^-$ thymocytes differentiate into $CD4^+$ $CD8^+$ cells, as well as on mitogenic stimulation of peripheral T lymphocytes.[223,224] Recently, it has been demonstrated that enforced expression of $p27^{KIP1}$ in transgenic T cells resulted in differentiation arrest of these cells and impairment of T cell-dependent immune response, indicating that down-regulation of $p27^{KIP1}$ is required for the normal development of T cells.[225] In addition, p130 seems be the principal member of pRB family proteins responsible for the lymphoid cell cycle control, as ectopic expression of p130 blocks the growth of F7 pro-B cell line, but not the high levels of phosphorylated pRB or phosphorylation-resistant pRB mutants.[226]

Other Differentiation Lineages

Chondrocytic and Osteogenic Differentiation

Chondrogenic differentiation of mouse ATDC5 prechondrocytes is accompanied by $p21^{WAF1}$ and $p27^{KIP1}$ up-regulation, while $p15^{INK4b}$, p18-INKc and $p19^{INK4d}$ did not change. Consistently, reduction of endogenous $p21^{WAF1}$ by antisense RNA inhibits early differentiation.[227]

Vitamin D3 or vitamin K induces differentiation in MG-63 osteosarcoma cells, accompanied by $p21^{WAF1}$ up-regulation. Ectopic expression (by adenoviral infection) of $p21^{WAF1}$ results in differentiation.[228] In another Rb-null osteosarcoma cell line, Saos-2, ectopic expression of pRB induces differentiation,[55] suggesting that pRB may be required for osteoblast differentiation. This hypothesis has been confirmed in knockout mice. As mentioned above in the muscular differentiation paragraph, mgRb:$Rb^{-/-}$ mice (i.e., mice with lower than normal expression of pRB) show shortened and brittle bones, although the authors warn that these defects may be an indirect consequence of muscle degeneration, as they are also observed in myogenin deficient mice.[147] Mice deficient for both p107 and p130 display deregulated chondrocyte growth with increased chondrocyte density, defective bone development, shortened limbs and neonatal lethality. Thus these pocket proteins play an important role in limb development through their abilities to control chondrocyte proliferation.[40] In contrast, although $p27^{KIP1}$ is induced in osteoblast differentiation and osteoblasts from $p27^{KIP1}$ null mice proliferate faster, they retain competence for proliferation.[229]

Adipocytic Differentiation

The murine cell line 3T3-L1 undergoes adipocytic differentiation in response to hormonal stimulation (insulin, dexamethasone and isobutylmethylxanthine). During induced adipocytic differentiation of 3T3-L1 cells there is an initial up-regulation of $p18^{INK4c}$, $p21^{WAF1}$ and $p27^{KIP1}$, and later, concomitant with irreversible growth arrest and terminal differentiation, the level of $p21^{WAF1}$ declines with a concomitant increase of $p18^{INK4c}$.[4,230] During 3T3-L1 differentiation, cyclin D1 expression is repressed, cyclin D2 levels are transiently elevated and cyclin D3 is highly and persistently up-regulated. Moreover, differentiated cells contain active CDK4-cyclin D3 complexes.[4] Thus, the pattern of $p18^{INK4c}$ and cyclin D3 is similar compared to in muscular differentiation (see above).

pRB is required for adipocytic differentiation of fibroblasts. Fibroblasts derived from pRB -deficient mice cannot differentiate into adipocytes, and ectopic expression of wild-type pRB (but not mutant pRB) enabled $Rb^{-/-}$ fibroblasts to differentiate.[231] The transcription factors C/EBPα and C/EBPβ, are induced during adipocytic differentiation and are required for differentiation. pRB induces adipocyte differentiation through direct interaction with c/EBPα that stimulates its activity.[231,232]

In sharp contrast, fibroblasts derived from deficient for p107 and p130 can differentiate into adipocytes. Moreover, over-expression of pRB in wild-type cells promotes differentiation whereas over-expression of p107 antagonizes differentiation.[233] This difference can be in part explained for the requirement of pRB in maintaining cell cycle exit as well as potentiating the activity of the differentiation-associated transcription factor C/EBPα, as p107 does not affect C/EBPα transcriptional activity.[233] Terminal differentiated of 3T3-L1 cells contain high levels of p130 and low p107,[234,235] although shortly after the addition of the differentiation inducers there is a DNA synthesis burst accompanied by elevation of p107, that is later repressed.[234] Interestingly, in the 3T3-L1 model there is a reciprocal effect of C/EBPα, in differentiation. C/EBPα mediates disruption of E2F/p107 complexes and induces formation of p130/E2F complexes.[235]

Lens Cells Differentiation

Ocular lens arises from a sphere of epithelial cells, and it is already formed by mouse embryonic day 11.5. pRB deficient mice display impaired lens development, with inappropriate apoptosis in lens fiber cells.[236] Lens develops normally in p57-KIP2 deficient and $p27^{KIP1}$ deficient mice, but lens of mice deficient in both $p27^{KIP1}$ and p57-KIP2 are grossly abnormal.[20,237]

Luteal Cell Differentiation

$p27^{KIP1}$-deficient mice, besides hypercellularity in many tissues, display female infertility because luteal cells fail to withdraw from the cell cycle after hormonal stimulation, although the cells complete the differentiation program. Thus, the absence of $p27^{KIP1}$ uncouples differentiation and growth arrest during the hormone-induced differentiation of granulosa into luteal cells.[238] Interestingly, absence of cyclin D1 does not rescue the $p27^{KIP1}$ null phenotype.[99] This model constitutes another example of $p27^{KIP1}$ exerting a differentiation function independent from cell cycle arrest.

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