[Frontiers in Bioscience 5, d493-503, May 1, 2000]

CELL CYCLE CONTROL IN GROWTH PLATE CHONDROCYTES

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1. ABSTRACT

Growth of endochondral bones is regulated by the coordinated proliferation and differentiation of chondrocytes in the epiphyseal growth plates. Many skeletal diseases are caused by pathogenic disruptions of these two processes. While the intracellular mechanisms regulating chondrocyte proliferation and differentiation are poorly understood, recent evidence from studies using genetically altered mice and from experiments in cultured chondrocytes point to a prominent role of cell cycle proteins in this context. This article summarizes our current understanding of the expression, regulation, and function of cell cycle genes in chondrocytes.

2. INTRODUCTION

The majority of our bones (such as the ribs, vertebrae, and the long bones of the limbs) are formed through a process termed endochondral ossification, in which the later bones are first laid down as cartilage precursors before cartilage tissue becomes replaced by bone tissue and bone marrow in a second step (reviewed in (1), 2). The growth plate is a dynamic area of cells that is responsible for longitudinal growth of these bones as well as the transition from cartilage to bone. It consists of resting chondrocytes that have been stimulated by growth hormone-induced IGF-1 (3) to rapidly proliferate in a clonal manner in order to produce columns of chondrocytes. These secrete significant amounts of extracellular matrix consisting of type II collagen and the large proteoglycan aggrecan, as well as other minor components. After several proliferative cycles, the chondrocytes exit the cell cycle to form a maturation zone in which Indian hedgehog (Ihh) and Bone Morphogenetic Protein 6 (BMP-6) are upregulated. The chondrocytes in this zone gradually become enlarged, and at the time when type X collagen expression is evident, are considered to be hypertrophic chondrocytes located in the hypertrophic zone. Mineralization of the cartilage matrix occurs within this zone. Angiogenesis is also stimulated here, introducing both marrow and osteoprogenitor cells as vascular invasion occurs. The osteoprogenitor cells differentiate into osteoblasts and secrete a bone matrix that overlays the mineralized cartilage matrix. These cells also secrete proteases that break down the cartilage matrix. At the same time, the hypertrophic chondrocytes undergo apoptosis, leaving behind bony trabeculae. Control of growth plate chondrocyte progression is essential for proper bone and marrow formation, as demonstrated by a number of dominant-negative and null mutations that have been introduced in mice. Therefore, the chondrocytes of the epiphyseal growth plate control the longitudinal growth of endochondral bone through two distinct, but highly interconnected mechanisms: a) cell proliferation; and b) cell differentiation to hypertrophic chondrocytes, which is accompanied by a large increase in cell diameter and volume. Without these two mechanisms, longitudinal growth will not occur. Numerous skeletal diseases are caused by disregulation of these processes, in particular the large number of skeletal dysplasias which are in general characterized by dwarfism, skeletal deformities, and frequently by early-onset osteoarthritis. Many hormones and growth factors are involved in the control of

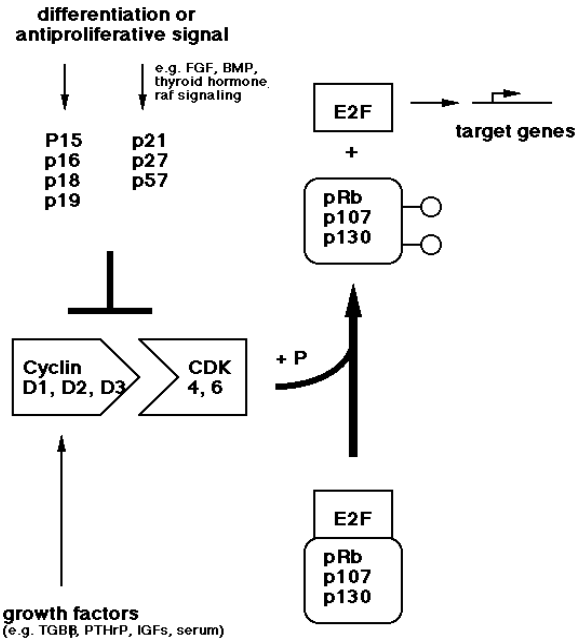

Figure 1: Control of progression through the G_1 phase of the cell cycle. Progression through the G1 phase of the cell cycle is regulated mainly by complexes of D-type cyclins with Cdk4 or Cdk6, followed first by cyclin E/Cdk2 and later cyclin A/Cdk2 complexes. Phosphorylation of the pocket proteins pRb, p107, and p130 by these complexes causes the release of E2F transcription factors which then can activate the transcription of target genes necessary for cell cycle progression and DNA replication. The expression of D-type cyclins is highly responsive to mitogenic stimulation; for example, cyclin D1 expression in chondrocytes can be induced by TGFβ, PTHrP, IGF1 and 2, and serum. The activity of cyclin/Cdk complexes can be inhibited by two different groups of proteins, the INK group (p15, p16, p18, and p19), and the CIP/KIP group (p21, p27, and p57). Expression of these genes is often regulated by extracellular modulators of proliferation and differentiation; for example, the p21 gene is induced by BMPs, FGF, thyroid hormone, and the c-Raf pathway in chondrocytes.

chondrocyte proliferation and differentiation. The last few years have seen significant progress in the identification and characterization of several of these signals. For example, the Parathyroid Hormone Related Peptide (PTHrP) has been identified as a key factor necessary for proliferation of chondrocytes (recently reviewed in (2). Genetic inactivation of the genes encoding PTHrP or its receptor cause a premature differentiation of chondrocytes and severe skeletal deformities, leading to perinatal death (3, 4). Additional extracellular factors important for growth plate physiology include Indian hedgehog (5, 6), BMP receptors (7), Transforming Growth Factor ß (TGFß) family (8) , Fibroblast Growth Factor receptor 3 (9), Insulin-Like Growth Factors (10-13), and many others. However, the molecular mechanisms of action of these factors, and in particular the target genes regulated by these factors, remain poorly understood.

Gene disruption studies in mice have suggested a role for cell cycle genes in the control of chondrocyte proliferation and differentiation. Disruption of several cell cycle genes cause direct or indirect skeletal defects (see section 5. for details), while in other cases mutant mice show altered growth (either gigantism or dwarfism) suggesting altered control of growth plate chondrocyte proliferation and/or differentiation, as seen, for example, in Bcl-2-deficient mice, which display shortened long bones and accelerated chondrocyte maturation (14). These changes in skeletal size are often accompanied by changes in the sizes of other body organs, since growth factors, receptors, and signaling molecules may have multiple targets. In parallel, studies in other tissues and cell types, in particular in skeletal muscle, have revealed a close connection between the cell cycle apparatus and factors that control cell differentiation.

The cell cycle apparatus regulates cellular proliferation. Progression through the cell cycle is controlled by specific complexes composed of a kinase (cyclin-dependent kinase, CDK) and a regulatory subunit, called cyclin (reviewed by Weinberg; (15). The activities of these complexes are tightly regulated by at least three different mechanisms: a) the abundance of the cyclin subunit; b) the phosphorylation status of the CDK subunits; and c) the presence of inhibitor proteins (cyclin dependent kinase inhibitors; CKIs) of two different families, the INK and the CIP/KIP families (figure 1). In physiological situations (for example during development), cells respond to mitogenic or antimitogenic stimuli during the G1 (or Gap1) phase of the cell cycle, which follows immediately after mitosis (15). Mitogenic stimulation in G1 causes the induction of cyclin gene expression, in particular the expression of D-type cyclins. The D-type cyclins form complexes with the CDKs 4 and 6 and control progression through G_1 via the phosphorylation of the Retinoblastoma Protein (pRb) and the closely related p107 and p130 proteins. In their hypo-phosphorylated forms, these proteins bind to transcription factors of the E2F family, and the resulting complexes act as transcriptional repressors. Once phosphorylated by CDKs, the pRb proteins can no longer bind to E2F, and free E2F activates the transcription of target genes, for example genes that are necessary for DNA replication or for progression through later phases of the cell cycle. The activity of the D-type cyclins and their associated CDKs can be counteracted by at least seven different inhibitors, including four members of the INK family (p15, p16, p18, and p19), and three members of the CIP/KIP family (p21, p27, and p57). In spite of this, active cyclin D-CDK4 or CDK6 complexes facilitate mitogen-independent cyclin E-CDK2 activation by associate with a CIP/KIP CKIs, thus lowering inhibitor levels and allowing cyclin E-CDK2 complexes to trigger degradation of CKIs by phosphorylation of specific residues (reviewed by (16)).

Since these proteins perform the ultimate control of cell proliferation and differentiation, it is very likely that all extracellular factors controlling growth plate activity directly or indirectly regulate some aspect of the cell cycle machinery. However, neither the detailed expression, nor the regulation and function of most cell cycle genes are

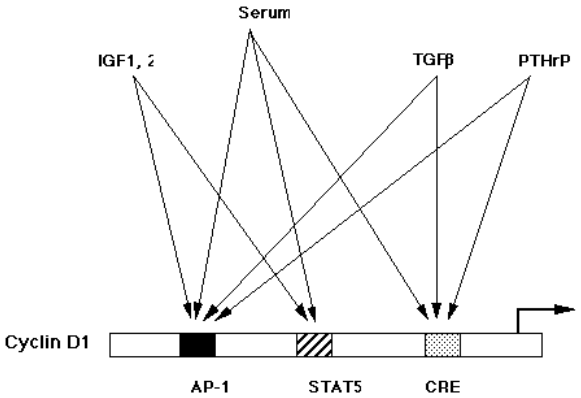

Figure 2: Integration of proliferative signals on the cyclin D1 promoter. We have identified three cis-active sites in the human cyclin D1 promoter that are necessary for maximal promoter activity in chondrocytes: a CRE (cAMP response element), an AP-1 site, and a binding site for STAT5 transcription factors. All three elements are activated by serum. IGF-induction of the cyclin D1 promoter requires the AP-1 and STAT5 sites, whereas the effects of TGFβ and PTHrP are mediated by both the AP-1 and CRE elements. It is possible that additional mitogenic and anti-mitogenic signals converge on the cyclin D1 promoter in chondrocytes.

known in chondrocytes. This review attempts to summarize our current knowledge in this field and to point to future directions. In view of the many diseases associated with irregular growth plate function, a better understanding of these processes is extremely important and promises to be highly beneficial.

3. EXPRESSION OF CELL CYCLE GENES IN CHONDROCYTES

Only few studies have addressed the expression patterns of cell cycle genes during chondrocyte differentiation. Most literature is available for the cyclin-dependent kinase inhibitors. The $p21^{Waf1/Cip1}$ gene is upregulated during chondrocyte differentiation *in vivo* (17) and *in vitro* (18). A similar expression pattern has been described for the $p57^{Kip2}$ gene (19, 20). In addition, expression of the $p27^{Kip1}$ gene has been described in chondrocytes *in vivo* (21). The same authors reported expression of cyclin E and CDKs 4 and 6 in chondrocytes, but hardly detectable levels of cyclin D1 protein in chondrocytes of wild type mice (whereas strong cyclin D1 expression was observed in transgenic mice overexpressing the proto-oncogenic transcription factor c-Fos). In contrast, cyclin D1 can be easily detected in primary mouse and rat wild type chondrocytes, as well as in several chondrogenic cell lines (12). This discrepancy is likely due to the generally low expression levels of cyclin D1 *in vivo* (22), which makes it difficult to detect this protein with immunohistochemistry (in particular in the paraformaldehyde-fixed, paraffin-embedded sections used by the authors).

In addition, we have observed expression of all three D-type cyclins, cyclins A and E, pRb, p107, p130, and CDKs 2, 4, and 6 in chondrogenic cell lines and/or primary chondrocytes (F. Beier, A. Taylor, Z. Ali, D. Mok, and P. LuValle, unpublished observations). However, detailed analyses of the expression of most cell cycle genes during chondrocyte differentiation, particularly *in vivo*, have not been performed.

4. REGULATION OF CELL CYCLE GENE EXPRESSION IN CHONDROCYTES

Over the last few years, numerous studies have addressed the regulation of cell cycle gene expression in many different cell types. One of the major conclusions of these studies is that key cell cycle genes, such as the cyclin D1 and p21 genes, can be regulated by a large number of different transcription factors and signaling pathways, and that the control of these genes in a particular cell type is performed by cell type-, organ-, and differentiation-specific mechanisms (reviewed in (23). Therefore it is very likely that chondrocyte-specific signals contribute to the regulation of cell cycle gene expression in cartilage, and that detailed analyses of these pathways are necessary for a complete understanding of chondrocyte proliferation and differentiation.

4.1. The cyclin D1 gene

The D-type cyclins (cyclins D1, D2, and D3) genes are necessary for the progression through the G_1 phase of the cell cycle (23). Their expression is in general rapidly induced in response to mitogenic stimulation of cells, and the levels of D-type cyclins have been suggested to form the molecular mechanism controlling progression through the restriction point in late G_1 (15, 24). Because of the growth-retarded phenotype of cyclin D1-deficient mice (see below), some of our own research has focused on the mechanisms controlling cyclin D1 expression in chondrocytes. In serum-starved chondrocytes, cyclin D1 promoter activity and protein expression is rapidly induced by a variety of mitogenic stimuli. Cyclin D1 antisense oligonucleotides inhibit the proliferation of rat chondrosarcoma cells and primary rat chondrocytes (Beier, Taylor, and LuValle, unpublished) supporting the idea that cyclin D1 is necessary for chondrocyte cell multiplication. The growth plate morphology of cyclin D1 deficient mice (see section 5) also supports this role**.** We have previously demonstrated that a CRE (cyclic AMP response element) in the cyclin D1 promoter is necessary for maximal activity of the promoter in chondrocytes through the binding of the transcription factors ATF-2 (Activating Transcription Factor 2) and CREB (CRE-binding protein; (25). This corresponds to reduced proliferation (26) and cyclin D1 protein expression (25) in chondrocytes of ATF-2-deficient mice. More recently, we have shown that binding sites for Stat5 (signal transducer and activator of transcription 5) and AP -1 (activating protein 1) transcription factors are also necessary for maximal activity of the cyclin D1 promoter in chondrocytes (Beier, Pestell, and LuValle, unpublished observations). All three sites are involved in cyclin D1 promoter induction by serum. Furthermore, these sites are also necessary for the transcriptional response to specific growth factors (summarized in figure 2). Whereas the AP-1 and CRE elements confer responsiveness to PTHrP and TGFß stimulation, IGF treatment results in activation of the

AP-1 and Stat5 sites. A role for the AP-1 site is also suggested by the observation that overexpression of c-Fos in transgenic mice causes the formation of chondrosarcomas characterized by high levels of cyclin D1 protein (21). In summary, our data suggest that multiple extracellular factors and intracellular signaling pathways contribute to the regulation of the cyclin D1 gene in chondrocytes. This gene may therefore play an important role in the integration of multiple mitogenic (and possibly anti-mitogenic) stimuli by chondrocytes.

4.2. The cyclin A gene

Cyclin A forms active kinase complexes with CDK 2 and CDK1 (Cdc2) and controls progression into S-phase, as well as through later phases of the cell cycle. The cyclin A gene is an E2F-responsive gene and therefore induced downstream of D-type cyclins and cyclin E (27). In addition, however, the cyclin A gene is directly regulated by a number of other transcription factors, including factors binding to a CRE in the cyclin A promoter (28-30). We have shown that, as in the case of the cyclin D1 gene, ATF-2 and CREB are necessary for the induction of the cyclin A promoter by serum (48). Ectopic expression of dominant-negative versions of ATF-2 or CREB causes delayed and reduced induction of the promoter. Similarly, chondrocytes isolated from ATF-2-deficient mice display lower levels of cyclin A protein and inhibited induction of cyclin A expression by serum. The majority of these effects are mediated by the CRE; however, promoter mutants in which the CRE is destroyed still show some reactivity to overexpression of wild type or dominant-negative ATF-2. This effect is likely due to altered levels of cyclin D1 in these cells, leading to increased activity of E2F. In contrast to many other cell types, Jun or Fos proteins do not appear to play a role in the activity of the cyclin A CRE in chondrocytes, but seem to regulate the cyclin A gene through (a) different element(s).

4.3. The $p21^{Waf1/Cip1}$ gene

The $p21^{Waf1/Cip1}$ gene encodes a CDK inhibitor and is upregulated during chondrocyte differentiation *in vivo* (17). Similar to the cyclin D1 gene, the promoter of the p21 gene is regulated by multiple cell-type specific pathways and transcription factors. In chondrocytes, p21 expression has been shown to be controlled by FGFs (31) , thyroxin, BMPs (R.T. Ballock, personal communication), and the c-Raf pathway (18). The first hint for a role of FGFs in the control of p21 expression in chondrocytes was the observation that patients suffering from thanatophoric Dysplasia type 2 (TD2) display increased levels of p21 in chondrocytes (32). TD2 is a skeletal dysplasia caused by activating mutations of the FGF-Receptor 3 gene. Activation of the receptor leads to stimulation of the p21 promoter via activation of the transcription factor STAT-1. More recently, it was shown that FGF-1 also induces p21 expression in chondrocytes during normal skeletal development, dependent on STAT-1 (31).

In addition, Thyroid hormone and BMPs (Bone Morphogenetic Proteins) have also been shown to induce the expression of p21 in primary rat chondrocytes (R.T. Ballock, personal communication). Finally, we have shown that the c-Raf/MEK/ERK pathway is necessary for maximal expression of the p21 gene in chondrogenic cell lines and primary mouse chondrocytes (18). This effect is likely mediated, at least in part, by one or more binding sites for Ets-family transcription factors in the p21 promoter. The same pathway is also necessary for expression of the collagen X gene, the classical marker of differentiated chondrocytes (33), and appears to play a crucial role in the coordination of cell-cycle withdrawal and differentiation-specific gene expression during chondrocyte differentiation. In agreement with such a role, the c-Raf/MEK/ERK pathway does not appear to be necessary for proliferation of chondrocytes, in contrast to most other cell types (F. Beier, Z. Ali, and P. LuValle, unpublished data). A role for c-Raf in chondrocyte maturation is further supported by the phenotype of c-Raf-deficient mice which display delayed ossification (34).

In summary, these data suggest that the p21 gene, similar to the cyclin D1 gene, is regulated by multiple stimuli and might act as an integrator of different signals. However, it should be pointed out that the described signals and pathways are also able to interact upstream of the p21 gene. For example, c-Raf can be stimulated by BMPs (35), and intensive cross-talk between the c-Raf cascade and JAK/STAT factors has been described (see for example (36-38). Whether such relations exist in chondrocytes, and whether they play a role in the control of p21 expression, remains to be shown.

5. CELL CYCLE FUNCTION IN THE ENDOCHONDRAL GROWTH PLATE

5.1. *In vivo* models

Aberrant growth phenotypes resulting from disruption or inappropriate expression of a variety of genes have been documented in recent years. Unfortunately, very few of the intracellular components involved in the signaling pathways associated with these gene products have been identified. Given the unequivocal association between skeletal growth and chondrocyte proliferation/differentiation, it is reasonable to suggest that cell cycle genes are possible direct or indirect targets of these pathways. For example, overexpression of Growth Hormone (GH) results in enhanced body growth (39), as does overexpression of Insulin-like Growth Factor-1 (IGF-1) (40), although to a lesser extent. The similarity in phenotypes is not surprising, because IGF-1 mediates many of the effects of GH (41). Mice lacking the IGF-1 gene demonstrate significant dwarfism at birth and depending on genetic background, may have a low survival rate (42, 43). In contrast, mice lacking the paternal allele of the imprinted IGF-2 gene are viable, although also significantly dwarfed (10, 44). IGF-2 can increase cyclin D1 levels , thus promoting progression through G1 to S (45), suggesting that it's absence may inhibit cell proliferation. Mice lacking either of the IGF receptors have obvious growth phenotypes as well. The IGF-1 receptor mediates the biological effects of both IGF-1 and IGF-2 (46)(34, 35). Mice that are null for the IGF-1 receptor gene (*Igf1-r*) have severe growth deficiency, delayed ossification, and die at birth (42). The imprinted IGF-2 receptor (also known as

the cation-independent mannose-6-phosphate receptor, IGF-2R) plays a role in IGF-2 turnover, thus effectively regulating the amount of IGF-2 available to the IGF-1 receptor. Mouse embryos that have null mutations in the maternal allele of the IGF-2R gene (*Igf2-r*) show fetal overgrowth, premature ossification and skeletal anomalies, and are perinatally lethal due to cardiac abnormalities and the inability to initiate or sustain respiration (12)(36, 37).

Both parathyroid hormone-related peptide (PTHrP) and its receptor (PTHrPR) are required for normal skeletal growth. Null mutations in either one result in reduced zones of proliferation in the growth plate, coupled with premature and irregular endochondral bone formation, resulting in a dwarfed phenotype (3, 47). In contrast, targeted overexpression of PTHrP in chondrocytes of transgenic mice results in delayed endochondral ossification such that at birth, the mice have a cartilaginous endochondral skeleton (48).

Specific point mutations in the human Fibroblast growth factor receptor-3 gene (*fgfr3*) result in achondroplasia, hypochondroplasia, or thanatophoric dysplasias (TD1 and TD2; 41), all of which affect the formation and growth of endochondral bones. Individuals with achondroplasia show reduced chondrocyte proliferation in the growth plates of long bones (49). Thanatophoric dwarfism is a lethal neonatal chondrodysplasia that displays severely shortened and disorganized growth plate chondrocyte columns (50). Mice deficient in FGFR3 have skeletal abnormalities including overgrowth and curvature of long bones, which interestingly resulted in reduced body weight and size compared to wild type littermates (51). Overexpression of FGF-9, a ligand for FGFR3, results in a phenotype in mice that is similar to that of achondroplasia (52).

Disruption of the Indian hedgehog gene (*Ihh*) results in reduced chondrocyte proliferation, inappropriate maturation, and failure of osteoblast development. The phenotype is one of severe dwarfism (6). The Ihh pathway is linked to PTH/PTHrP (5, 47).

The above growth defect phenotypes display alterations in chondrocyte proliferation and/or hypertrophy in growing endochondral bone, suggesting that the cell cycle machinery is somehow affected. For example, chondrocytes from a TD2 fetus with a Lys650Glu mutation in FGFR3 exhibited Stat1 activation and p21 upregulation, when compared to chondrocytes from a normal fetus (32). Interestingly, the corresponding mutation in mouse (a Lys644Glu substitution in FGFR3) causes activation of Stat1, Stat5a, and Stat5b, as well as up-regulation of Ink 4 cell cycle inhibitor family members p16, p18, and p19, and results in an achondroplasia-like dwarfism (53). Another example is mice that are deficient in the transcription factor ATF-2, which display a phenotype similar to hypochondroplasia, dwarfism, and reduced chondrocyte proliferation (26). The expression of cyclins D1 and A are reduced by 70% and 64%, respectively, in ATF-2 -/- chondrocytes (25)(12, 48). These reductions are likely a direct cause of the reduced proliferation of growth plate cells in these mice, corresponding to their dwarfed phenotype.

Disruptions in cell cycle gene expression have also resulted in growth phenotypes affecting the endochondral skeleton. The results of these experiments have contributed to understanding specific temporal and spatial functions of cell cycle components, as well as suggesting detailed mechanisms that may be responsible for some of the genetically-produced phenotypes described above. The following sections review the consequences of disruptions of cell cycle gene expression that affect, directly or indirectly, skeletal development and growth in mice.

5.1.1 The G1 and S cyclins

Mitogen-stimulated D cyclins form complexes with CDK4 and CDK6 for the purpose of phosphorylating Rb or other pocket proteins in order to allow cell cycle progression through G1 in lieu of cell cycle exit. Subsequently, CDK2-cyclin E and CDK2-cyclin A complexes become activated. Activation of all three cyclin-cdk complexes is necessary for S-phase entry (54, 55).

Overexpression of cyclin D1 shortens G_1 phase duration, as well as G_0 to S and G_1 to S transit, while cyclin D2 overexpression reduced G_0 to S duration as well as decreasing serum dependency (56, 57). D cyclins share significant sequence homology (58), although they appear to be somewhat cell type specific (59). The eradication of cyclin D1 in DT40 lymphoma B cells results in a significant lag in cell cycle progression. The phenotype can be rescued by overexpression of cyclin D1, D2, or D3, but not a mutant cyclin D1 lacking the amino terminal LXCXE sequence corresponding to the pRB binding motif (60). These data suggest that D cyclins are capable of substituting for each other. Substitution *in vivo*, however, would require co-expression in the same cell which does not seem to be the case in all cell types. This is highlighted by the tissue-specific defects of cyclin D1-deficient mice (22, 61). One of the affected tissues is the endochondral skeleton, which shows significant growth retardation, corresponding to a 40% reduction in size of the growth plate proliferation zone evident by three weeks of age (Z. Ali and P. LuValle, unpublished observations).

Cyclin D2 is required for gonadal cell proliferation and is involved in oncogenesis (62). In addition, this cyclin is required for granule cell precursor proliferation and differentiation of stellate interneurons in the cerebellum (63). However, it appears to have no role in skeletal growth. The phenotype of cyclin D3-deficient mice has not been published, but cyclin D3 expression increases dramatically during differentiation of L6 myoblasts (64).

Cyclin E, as mentioned above, is controlled by autonomous mechanisms. Its expression peaks at the G_1/S boundary, although it is grouped together with cyclins D1, D2, and D3 as G1 cyclins. In spite of this grouping, it has more sequence homology with cyclins A, B1, and B2 (S and M phase cyclins) than with the D-type cyclins (58). Cyclin E partners with CDK2, and is immune to inhibition

by the INK4 family of cdk inhibitors (65). Recently, the phenotypes of the cyclin D1-deficient mouse, including the dwarf phenotype (22), were rescued by introduction of cyclin E under the control of the cyclin D1 promoter. Even though cyclin E associated with its normal cdk partner, CDK2, it effectively substituted for the role of cyclin D1, and produced no new phenotypes (66).

Cyclin A1 has been recently described in mice and appears to be expressed only in germ cells (67). Cyclin A2 (referred to here as cyclin A) is ubiquitously expressed and activates CDK2 at the G_1 to S boundary, and CDC2 (CDK1) at the G_2 to M boundary (68). Disruption of the cyclin A gene results in embryonic lethality at around day 5.5, following implantation (69). Therefore, any effect on skeletal growth would be impossible to assess. However, our own data regarding the decrease in cyclin A expression in ATF-2 deficient mice suggests that cyclin A is required at some level for accurate chondrocyte proliferation and endochondral bone growth (70).

5.1.2. INK4 cyclin dependent kinase inhibitors

The INK4 (Inhibitors of CDK) family of cyclin dependent kinase inhibitors act to exclusively interact with and inhibit the activity of CDK4 and CDK6. Like all CKIs, their expression results in cell cycle arrest at G_1 via inhibition of pRB phosphorylation and the subsequent release of E2F transcription factors, required for the transcriptional activation of genes that are essential for DNA synthesis. Deletions found in $p15^{INK4B}$, $p16^{INK4}$ and $p19^{INK4D}$ genes in osteosarcomas suggest that these INK4 family members may play roles as tumor suppressors in bone cells (71, 72). $p18^{INK4C}$-deficient mice display gigantism which may reflect increased skeletal growth, and is similar to the gigantism seen in p27-nullizygous mice (see below) but this aspect of the phenotype has not been analyzed (73). To date, the *in vivo* functions of these CKIs in skeletal growth have not been examined.

5.1.3. CIP/KIP cyclin dependent kinase inhibitors

Unlike the INK4 family of CKIs, the CIP/KIP family (p21, p27, and p57) are capable of inhibiting the activity of all cyclin-CDK complexes that participate in the G_1/S transition (74), including cyclin D-CDK4, cyclin D-CDK6, cyclin E-CDK2, and cyclin A-CDK2 (75)(69). In addition, the CIP/KIP family of CKIs can inhibit cdk activating kinase (CAK) indirectly, resulting in prevention of CDK activation (76, 77).

$p21^{CIP1/Waf1}$ (p21), unlike the other members of this family, can also associate with proliferating nuclear antigen (PCNA), a subunit of DNA polymerase δ, and inhibit DNA proliferation directly (78, 79). P21 expression corresponds to terminal differentiation of many tissues, and has been shown to be up-regulated by the Raf-1 pathway in maturing chondrocytes (18). However, p21-deficient mice develop normally, suggesting that the role of p21 in terminal differentiation is likely to be redundant (80). Nevertheless, fibroblasts from p21 -/- embryos are defective in the ability to arrest in G_1 (80), and p21-/- keratinocytes show increased proliferative potential as well as reduction in the expression of differentiation markers (81). It would be interesting to investigate these characteristics in chondrocytes from p2-/- mice.

Unlike the p21-null mice, $p27^{Kip1}$ (p27) -deficient mice display a distinct phenotype of enhanced growth in the absence of associated increases in GH and/or IGF-1 levels (82-84), similar to mice that are deficient in $p18^{INK4c}$. G_1 arrest is not affected in embryonic fibroblasts from p27-null mice (82). However, skull size and length of longitudinal bones correspond with the increase in size in the p27 -/- animals (83), suggesting that the growth plate may be directly or indirectly affected by loss of expression of $p27^{Kip1}$.

The $p57^{KIP2}$ Cdk inhibitor (p57) is encoded by a paternally imprinted (maternally expressed) gene in both humans and mice located on human chromosome 11p15.5 (85, 86). It is associated with the heritable Beckwith-Widemann syndrome (BWS), which is characterized by growth abnormalities including gigantism and increased risk of childhood tumors (87). Mice that are mutant for p57 show, among other developmental defects, short limbs as a consequence of delayed cell cycle exit during chondrocyte differentiation, resulting in abnormal endochondral ossification. Specifically, p57, normally expressed in post mitotic growth plate chondrocytes, is absent, and type X collagen expression is reduced in the mutant hypertrophic zone (19, 20). p57, therefore, plays a role in chondrocyte differentiation and growth plate progression. More recently, Caspary et al. (88) generated a mouse model for BWS by combining a null mutation for *p57* and loss of imprinting of *IGF2* . The *IGF2* gene is paternally expressed in mice and humans (44), and its overexpression as a result of loss of imprinting is considered to be causative in the overgrowth phenotype found in BWS (89, 90). Since IGF2 promotes the G_1 to S transition, possibly by increasing cyclin D levels (45), while p57 essentially works in opposition by inhibiting Cdk activity (74), the two genes may act in an antagonistic manner in some tissues (88).

Interestingly, loss of p21 enhances the p57 mutant skeletal phenotype, such that p57 mutant mice that are also null for p21 display additional skeletal abnormalities that included reduced spinal curvature, rib bifurcation, and fusion of the sternum. These phenotypes are likely due to the inability to control cell proliferation and differentiation in the absence of both CKIs (91).

5.1.4. Pocket Proteins

The phosphorylation status of the pocket proteins pRb, P107 and p130, which is regulated by CDK activity, controls G_1 to S progression through interactions with E2F transcription factors (92). Control of levels of pocket proteins appears to be dependent on cell type, status of cell growth, and the specific pocket protein. Recent evidence by Garriga et al. showed that, while pRb and p107 levels were independent of their phosphorylation status, p130 levels were down-regulated in cycling cells (when phosphorylation is high), and up-regulated in quiescent and differentiating cells, when phosphorylation is low (93).

pRB-deficient mice die between E12 and E15 and show defects in lens development, liver erythropoiesis, and neurogenesis (94-96). Overexpression of a pRb minigene in the pRb-minus background partially rescues

these defects; however, the rescued mice appear to have a smaller cartilaginous skeleton than wild type mice (97). Introduction of additional copies of pRb under the control of the human pRb promoter results in dwarfism that is more severe with increased copy number (98). It is interesting that both a deficiency and excess of pRB may result in similar phenotypes. Taken together, these data suggest a direct or indirect role of pRb in the growth of the endochondral skeleton.

Mice in a mixed 129/Sv:C57BL/6J background that are deficient in either p107 or p130 show no obvious defects (99, 100), however, inactivation of both genes in that genetic background results in severe skeletal defects and perinatal lethality (99). Similar to mice that are deficient in p57, these mice demonstrate delayed chondrocyte hypertrophy. Mice from this background that are p107-/- and pRb +/- show a significant reduction in growth compared to double heterozygote littermates, as well as increased mortality during the first three weeks after birth (100).

Remarkably, and in contrast to the phenotypes of the p107 and p130-nullizygous mice described above, mice bred into a 129Sv:BALB/cJ genetic background that are null for p107 show impaired growth, increased levels of Rb, and deregulated cyclin E and A expression such that cyclin D1 expression followed that of cyclins E and A rather than preceding it (101). Mice bred into the same genetic background deficient for p130 die between days 11 and 13 of embryonic development and demonstrate arrested growth (102). Either mutation can be rescued with a single backcross to the C57BL/6 strain, indicating epistatic relationships of modifier genes with both p107 and p130. This may be the case for some other cell cycle genes, as well.

6. PERSPECTIVES

The discussed mechanisms regulating expression of the cyclin D1, cyclin A, and p21 genes represent, to our knowledge, the only data available on the control of cell cycle gene expression in chondrocytes. To obtain a more detailed understanding of chondrocyte proliferation and differentiation, much more work in this field will be necessary. The pathways connecting the mentioned growth factors to cell cycle genes, as well as negative regulation of these genes, have to be analyzed in much more detail. Posttranscriptional control of cell cycle gene expression and protein activity (such as RNA and protein stability, translational control, and cellular localization) will have to be addressed. Most importantly, additional cell cycle genes (such as the cyclin D2 and D3 genes, as well as more CDK inhibitors etc.) will have to be included in these studies, to obtain a more complete picture. As well, signaling from additional growth factors and hormones involved in chondrocyte physiology (such as Indian hedgehog, Growth Hormone, retinoic acid, vitamin D) and from integrin receptors should be of great interest. Finally, due to the complex biology of the skeleton *in vivo*, the results obtained in such studies will have to be confirmed by experiments using transgenic or "knockout" mice to contribute more significantly to our understanding of growth plate function.

Data regarding skeletal growth resulting from genetic manipulation of cell cycle regulators in mice is by no means complete. This is partly due to lack of information about reported skeletal phenotypes in mice that demonstrate growth problems, such as the pRb transgenic mouse containing multiple copies of Rb, and knockouts for cyclin D1, p18INK4c, p21, and p27 (22, 61, 73, 80, 82-84, 98). In addition, many of the aberrant growth phenotypes generated from manipulating growth factors, transcription factors, and other proteins *in vivo* are likely to be caused at least in part by disruption of growth plate function and possibly (directly or indirectly) by altered control of cell cycle regulators (6, 26)(13, 46, 47).

7. ACKNOWLEDGMENTS

Original work form the authors was supported by grants from the Medical Research Council of Canada and the Alberta Cancer Society to P.L. F.B. was supported by postdoctoral fellowships from Deutscher Akademischer Austauschdienst, Deutsche Forschungsgemeinschaft, and the Alberta Heritage Foundation for Medical Research. P.L. is the recipient of an AHFMR Senior Scholar Award. We are grateful to Dr. R.T. Ballock for communication of unpublished results.

8. REFERENCES

1. R. Cancedda, F. D. Cancedda, and P. Castagnola: Chondrocyte differentiation. *Int. Rev. Cytology* 159, 265-358 (1995)
2. B. Lanske and H. Kronenberg: Parathyroid hormone-related peptide (PTHrP) and parathyroid hormone (PTH)/PTHrP receptor. *Crit. Rev. Eukaryot. Gene Expr.* 8, 297-320 (1998)
3. A. C. Karaplis, A. Luz, L. Glowacki, R. T. Bronson, V. L. J. Tybulewicz, H. M. Kronenberg, and R. C. Mulligan: Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev.* 8, 277-289 (1994)
4. N. Amizuka, H. Warshawsky, J. E. Henderson, D. Goltzman, and A. C. Karaplis: Parathyroid hormone-related peptide depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *J. Cell. Biol.* 126, 1611-1623 (1994)
5. A. Vortkamp, K. Lee, B. Lanske, G. V. Segre, H. M. Kronenberg, and C. J. Tabin: Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* 273, 613-622 (1996)
6. B. St-Jacques, M. Mammerschmidt, and A. P. McMahon: Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes and Development* 13, 2072-2086 (1999)
7. H. Zou, R. Wieser, J. Massague, and L. Niswander: Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev.* 11, 2191-2203 (1997)
8. R. Serra, M. Johnson, E. H. Filvaroff, J. LaBorde, D. M. Sheehan, R. Derynck, and H. L. Moses: Expression of a truncated, kinase-defective TGF-β type II receptor in mouse skeletal tissue promotes terminal chondrocyte

differentiation and osteoarthritis. *J. Cell. Biol.* 139, 541-552 (1997)
9. C. Deng, A. Wynshaw-Boris, F. Zhou, A. Kuo, and P. Leder: Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell* 84, 911-921 (1996)
10. T. M. DeChiara, A. Efstradiatis, and E. J. Robertson: A growth deficiency phenotype in heterozygous mice carrying an insulin-like growth factor 2 gene. *Nature* 345, 78-80 (1990)
11. J. Baker, J.-P. Liu, E. J. Robertson, and A. Efstratiadis: Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 75, 73-82 (1993)
12. M. M. H. Lau, C. E. H. Stewart, Z. Liu, H. Bhatt, P. Rotwein, and C. L. Stewart: Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes Dev.* 8, 2953-2963 (1994)
13. Z.-Q. Wang, M. R. Fung, D. P. Barlow, and E. F. Wagner: Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene. *Nature* 372, 464-467 (1994)
14. M. Amling, L. Neff, S. Tanaka, D. Inoue, K. Kuida, E. Weir, W. M. Philbrick, A. E. Broadus, and R. Baron: Bcl-2 lies downstream of parathyroid hormone-related peptide in a signaling pathway that regulates chondrocyte maturation during skeletal development. *J. Cell. Biol.* 136, 205-213 (1997)
15. R. A. Weinberg: The retinoblastoma protein and cell cycle control. *Cell* 81, 323-330 (1995)
16. C. Sherr and J. M. Roberts: CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes and Development* 13, 1501-1512 (1999)
17. M. C. Stewart, C. E. Farnum, and J. N. MacLeod: Expression of p21CIP1/Waf1 in chondrocytes. *Calcif. Tissue Int.* 61, 199-204 (1997)
18. F. Beier, A. C. Taylor, and P. LuValle: The Raf-1/MEK/ERK pathway regulates the expression of the p21Waf1/Cip1 gene in chondrocytes. *J. Biol. Chem.* 274, 30273-30279 (1999)
19. P. M. Zhang, N. J. Liegeois, C. Wong, M. Finegold, H. Hou, J. C. Thompson, A. Silverman, J. W. Harper, R. A. dePinho, and S. J. Elledge: Altered cell differentiation and proliferation in mice lacking p57(KIP2) indicates a role in Beckwith-Wiedemann syndrome. *Nature* 387, 151-158 (1997)
20. Y. Yan, J. Friesen, M.-H. Lee, J. Massague, and M. Barbacid: Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev.* 11, 973-983 (1997)
21. A. Sunters, J. McCluskey, and A. E. Grigoriadis: Control of cell cycle gene expression in bone development and during c-Fos-induced osteosarcoma formation. *Dev. Genet.* 22, 386-397 (1998)
22. P. Sicinski, J. L. Donaher, S. B. Parker, T. Li, A. Fazeli, H. Gardner, S. Z. Haslam, R. T. Bronson, S. J. Elledge, and R. A. Weinberg: Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* 82, 621-630 (1995)
23. R. Pestell, C. Albanese, A. Reutens, R. Lee, J. Segall, and A. Arnold: The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. *Endocrine Reviews* 20, 501-534 (1999)
24. M. D. Planas-Silva and R. A. Weinberg: The restriction point and control of cell proliferation. *Curr. Opin. Cell Biol.* 9, 768-772 (1997)
25. F. Beier, R. J. Lee, A. C. Taylor, R. G. Pestell, and P. LuValle: Identification of the cyclin D1 gene as a target of ATF-2 in chondrocytes. *Proc. Natl. Acad. Sci. USA* 96, 1433-1438 (1999)
26. A. M. Reimold, M. J. Grusby, B. Kosaras, J. W. V. Fries, R. Mori, S. Maniwa, I. M. Clauss, T. Collins, R. L. Sidman, M. J. Glimcher, and L. H. Glimcher: Chondrodysplasia and neurological abnormalities in ATF-2 deficient mice. *Nature* 379, 262-265 (1996)
27. A. Schulze, K. Zerfass, D. Spitkovsky, S. Middendorp, J. Berges, K. Helin, P. Jansen-Durr, and B. Henglein: Cell cycle regulation of the cyclin A gene promoter is mediated by a variant E2F site. *Proc. Natl. Acad. Sci. USA* 92, 11264-11268 (1995)
28. C. Desdouets, G. Matesic, C. A. Molina, N. S. Foulkes, P. Sassone-Corsi, C. Brechot, and J. Sobczak-Thepot: Cell cycle regulation of cyclin A gene expression by the cyclic AMP-responsive transcription factors CREB and CREM. *Mol. Cell. Biol.* 15, 3301-3309 (1995)
29. T. Nakamura, S. Okuyama, S. Okamoto, T. Nakajima, S. Sekiya, and K. Oda: Down-regulation of the cyclin A promoter in differentiating human embryonal carcinoma cells is mediated by depletion of ATF-1 and ATF-2 in the complex at the ATF/CRE site. *Exp. Cell Res.* 216, 422-430 (1995)
30. M. Shimizu, Y. Nomura, H. Suzuki, E. Ichikawa, A. Takeuchi, M. Suzuki, T. Nakamura, T. Nakajima, and K. Oda: Activation of the rat cyclin A promoter by ATF-2 and jun family members and its suppresion by ATF4. *Exp. Cell Res.* 239, 93-103 (1998)
31. M. Sahni, D. Ambrosetti, A. Mansukhani, R. Gertner, D. Levy, and C. Basilico: FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway. *Genes Dev.* 13, 1361-1366 (1999)
32. W. C. Su, M. Kitagaw, N. Xue, B. Xie, S. Garofalo, J. Cho, C. Deng, W. A. Horton, and X. Y. Fu: Activation of Stat1 by mutant fibroblast growth-factor receptor in thanatophoric dysplasia type II dwarfism. *Nature* 386, 288-292 (1997)
33. F. Beier, A. C. Taylor, and P. LuValle: Raf signaling stimulates and represses the human collagen X promoter through distinguishable elements. *J. Cell. Biochem.* 72, 549-557 (1999)
34. L. Wojnowski, L. Stancato, A. Zimmer, H. Hahn, T. Beck, A. Larner, U. Rapp, and A. Zimmer: cRaf-1 protein kinase is essential for mouse development. *Mech. Dev.* 76, 141-149 (1998)
35. R. H. Xu, Z. Dong, M. Maeno, J. Kim, A. Suzuki, N. Ueno, D. Sredni, N. H. Colburn, and H. F. Kung: Involvement of ras/raf/AP-1 in BMP-4 signaling during Xenopus embryonic development. *Proc. Natl. Acad. Sci. USA* 93, 834-838 (1996)
36. L. Stancato, M. Sakatsume, M. David, P. Dent, F. Dong, E. Petricoin, J. Krolewski, O. Silvennoinen, P. Saharinen, J. Pierce, C. Marshall, T. Sturgill, D. Finbloom, and A. Larner: Beta interferon and oncostatin M activate Raf-1 and mitogen-activated protein kinase through a JAK1-dependent pathway. *Mol. Cell. Biol.* 17, 3833-3840 (1997)

37. L. Winston and T. Hunter: JAK2, Ras, and Raf are required for activation of extracellular signal-regulated kinase/mitogen-activated protein kinase by growth hormone. *J. Biol. Chem.* 270, 30837-30840 (1995)
38. J. Ng and D. Cantrell: STAT3 is a serine kinase target in T lymphocytes. interleukin 2 and T cell antigen receptor signals converge upon serine 727. *J. Biol. Chem.* 272, 24542-24549 (1997)
39. R. D. Palmiter, R. L. Brinster, R. E. Hammer, M. E. Trumbauer, M. G. Rosenfeld, N. C. Birnberg, and R. M. Evans: Dramatic growth of mice that develop from eggs microinjected with metallothionin-growth hormone fusion genes. *Nature* 300, 611-615 (1982)
40. L. S. Mathews, R. E. Hammer, R. R. Behringer, A. J. D'Ercole, G. I. Bell, R. L. Brinster, and R. D. Palmiter: Growth enhancement of transgenic mice expressing human insulin-like growth factor I. *Endocrinology* 123, 2827-2833 (1988)
41. O. G. P. Isaksson, C. Ohlsson, A. Nilsson, J. Isgaard, and A. Lindhall: Regulation of cartilage growth by growth hormone and insulin-like growth factor 1. *Pediatric Nephrology* 5, 451-543 (1991)
42. J.-P. Liu, J. Baker, A. S. Perkins, E. J. Robertson, and A. Efstratiadis: Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type I IGF receptor (IgfIr). *Cell* 75, 59-72 (1993)
43. L. Powell-Braxton, P. Hollingshead, C. Warburton, M. Dowd, S. Pitts-Meek, D. Dalton, N. Gillett, and T. A. Stewart: IGF-1 is required for normal embryonic growth in mice. *Genes and Development* , 2609-1617 (1994)
44. T. M. DeChiara, E. J. Robertson, and A. Efstradiatis: Parental imprinting of the mouse insulin-like growth factor 2 gene. *Cell* 64, 849-859 (1991)
45. L. Zhang, M. Kim, Y. H. Choi, B. Goemans, C. Yeung, Z. Hu, S. Zhan, P. Seth, and L. J. Helman: Diminsihed G1 checkpoint after gamma irradiation and altered cell cycle regulation by insulin-like growth factor 2 overexpression. *Journal of Biological Chemistry* 274, 13118-13126 (1999)
46. T. A. Gustafson and W. J. Rutter: The cystein-rich domains of the insulin and insulin-like growth factor 1 receptors are primary determinants of hormone binding specificity. *Journal of Biological Chemistry* 265, 18663-18667. (1990)
47. B. Lanske, A. C. Karaplis, K. Lee, A. Luz, A. Vortkamp, A. Pirro, M. Karperien, L. H. K. Defize, C. Ho, R. C. Mulligan, A. B. Abou-Samra, H. Juppner, G. V. Segre, and H. M. Kronenberg: PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* 273, 663-666 (1996)
48. E. C. Weir, W. M. Philbrick, M. Amling, L. A. Neff, R. Baron, and A. E. Broadus: Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. *Proceedings of the National Academy of Science, USA* 93, 10240-10245 (1996)
49. R. Stanescu, V. Stasnescu, and P. Maroteaux: Achondroplasia: Morphologic and biochemical study of Cartilage. *American Journal of Medical Genetics* 37, 412-421 (1990)
50. F. Rousseau, V. El Ghouzzi, A. I. Delezoide, L. Legeai-Mallet, M. Le Merrer, A. Munnich, and J. Bonaventure: Missense FGFR3 nutations create cysteine residues in thanatophoric dwarfism type I (TD1). *Human Molecular Genetics* 5, 509-512 (1996)
51. J. S. Colvin, B. A. Bohne, G. W. Harding, D. G. McEwen, and D. M. Ornitz: Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nature Genetics* 12, 390-397 (1996)
52. S. Garofalo, M. Kliger-Spatz, J. L. Cooke, O. Wolstin, G. P. Lunstrum, S. M. Moshkovitz, W. A. Horton, and A. Yayon: Skeltal dysplasia and defective chondrocyte differentiation by targeted overexpression of fibroblast growth factor 9 in transgenic mice. *Journal of Bone and Mineral Research* 14, 1909-1915 (1999)
53. C. Li, L. Chen, T. Iwata, M. Kitagawa, X.-Y. Fu, and C.-X. Deng: A Lys644Glu substitution in fibroblast growth factor receptor 3 (FGFR3) causes dwarfism in mice by activation of STATs and INK4 cell cycle inhibitors. *Human Molecular Genetics* 8, 35-44 (1999)
54. M. Pagano, R. Pepperkok, F. Verde, W. Ansorge, and G. Draetta: Cyclin A is required at two points in the human cell cycle. *EMBO J.* 11, 961-971 (1992)
55. M. Ohtsubo, A. M. Theodoras, J. Schumaker, J. M. Roberts, and M. Pagano: Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Molecular and Cellular Biology* 15, 2612-2624 (1995)
56. W. Jiang, S. M. Kahn, P. Zhou, Y.-J. Zhang, A. M. Cacace, A. S. Infante, S. Doi, R. M. Santella, and I. B. Weinstein: Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression and gene expression. *Oncogene* 8, 3447-3457 (1993)
57. D. E. Quelle, R. A. Ashmun, S. A. Shurtleff, J. Kato, D. Bar-Sagi, M. F. Roussel, and C. J. Sherr: Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes and Development* , 1559-1571 (1993)
58. Y. Xiong, J. Menninger, D. Beach, and D. C. Ward: Molecular cloning and chromosomal mapping of CCND genes encoding human D-tye cyclins. *Genomics* 13, 575-584 (1992)
59. H. Matshushime, M. F. Roussel, R. A. Asham, and C. J. Sherr: Colony stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* 65, 701-713 (1991)
60. J. M. Lahti, H. Li, and V. J. Kidd: Elimination of cyclin D1 in vertebrate cells leads to an altered cell cycle phenotype, which is rescued by overexpression of murine cyclins D1, D2, or D3, but not by mutant cyclin D1. *Journal of Biological Chemistry* 272, 10859-10869 (1997)
61. V. Fantl, G. Stamp, A. Andrews, I. Rosewell, and C. Dicksen: Mice lacking cyclin D1 arew smaller and show defects in eye and mammary gland development. *Genes and Development* 9, 2364-2372 (1995)
62. P. Sicinski, J. L. Donaher, Y. Geng, S. B. Parker, H. Gardner, M. Y. Park, R. L. Robker, J. S. Richards, L. K. McGinnis, J. D. Biggers, J. J. Eppig, R. T. Bronson, S. J. Elledge, and R. A. Weinberg: Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* 384, 470-474 (1996)
63. J. M. T. Huard, C. C. Forster, M. L. Carter, P. Sicinski, and M. E. Ross: Cerebellar histogenesis is disturbed in mice lacking cyclin D2. *Development* 126, 1927-1935 (1999)

64. M. Kiess, R. M. Gill, and P. A. Hamel: Expression of the positive regulator of cell cycle progression, cyclin D3, is induced during differentiation of myoblasts into quiescent myotubes. *Oncogene* 10, 159-166 (1995)
65. C. J. Sherr and J. M. Roberts: Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.* 9, 1149-1163 (1995)
66. Y. Geng, W. Whoriskey, M. Y. Park, R. T. Bronson, R. H. Medema, T. Li, R. A. Weinberg, and P. Sicinski: Rescue of cyclin D1 deficiency by knockin cyclin E. *Cell* 97, 767-777 (1999)
67. C. Sweeney: Cyclin A is expressed in germ cells in the mouse. *Development* 122, 53-64 (1996)
68. E. A. Nigg: Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *BioEssays* 17, 471-480 (1995)
69. M. Murphy, M.-G. Stinnakre, C. Senamaud-Beaufort, N. J. Winston, C. Sweeney, M. Kubelka, M. Carrington, C. Brechot, and J. Sobczak-Thepot: Delayed early embryonic lethality following disruption of the murine cyclin A2 gene. *Nature Genetics* 15, 83-86 (1997)
70. F. Beier, A. C. Taylor, and P. LuValle: ATF-2 is necessary for maximal activity and serum induction of the cyclin A promoter in chondrocytes. *J. Biol. Chem.* in press (2000)
71. C. W. Miller, A. Aslo, M. J. Campbell, N. Kawamata, B. C. Lampkin, and H. P. Koeffler: Alterations of the p15, p16, and p18 genes in osteosarcoma. *Cancer Genetica and Cytogenetics* 86, 136-142 (1996)
72. C. W. Miller, C. Yeon, A. Aslo, S. Mendoza, U. Aytac, and H. P. Koefler: The p19INK4D cyclin dependent kinase inhibitor gene is altered in osteosarcoma. *Oncogene* 15, 231-235 (1997)
73. D. S. Franklin, V. L. Godfrey, H. Lee, G. I. Kovalev, R. Schoonhoven, S. Chen-Kiang, L. Su, and Y. Xiong: CDK inhibitors p18INK4c and P27KIP1 mediate two separate pathways to collaboratively supress pituitary tumorigenesis. *Genes and Development* 12, 2899-2911 (1998)
74. M. Roussel: The INK4 family of cell cycle inhibitors in cancer. *Oncogene* 18, 5311-5317 (1999)
75. I. Reynisdottir, K. Polyak, A. Iavarone, and J. Massague: Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes and Development* 9, 1831-1845 (1995)
76. J.-Y. Kato, M. Matsuoka, D. K. Strom, and C. J. Sherr: Regulation of cyclin D-dependent kinase 4 (cdk4) by cdk4-activating kinase. *Molecular and Cellular Biology* 14, 2713-2721 (1994)
77. K. Polyak, M.-H. Lee, H. Erdjument-Bromage, A. Koff, P. Tempst, J.-M. Roberts, and J. Massague: Cloning of p27Kip1, a cyclin-cdk inhibitor and potential mediator of extracellular mitotic signals. *Cell* 78, 59-66 (1994)
78. R. Li, S. Waga, G. J. Hannon, D. Beach, and B. Stillman: Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature* 371, 534-537 (1994)
79. S. Waga, G. J. Hanon, D. Beach, and B. Stillman: The p21 inhbitor od cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* 369, 574-578 (1994)
80. C. Deng, P. Zang, J. W. Harper, S. J. Elledge, and P. Leder: Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* 82, 675-684 (1995)
81. C. Missero, F. D. Cunto, H. Kiyokawa, A. Koff, and G. P. Dotto: The absence of p21Cip1/Waf1 alters keratinocyte growth and differentiation and promotes ras-tumor progression. *Genes Dev.* 10, 3065-3075 (1996)
82. K. Nakayama, N. Ishida, M. Shirane, A. Inomata, T. Inoue, N. Shishido, I. Horii, D. Y. Loh, and K. Nakayama: Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 85, 707-720 (1996)
83. H. Kiyokawa, R. D. Kineman, K. O. Manova-Todorova, V. C. Soares, E. S. Hoffman, M. Ono, D. Khanam, A. C. Hayday, L. A. Frohman, and A. Koff: Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell* 85, 721-732 (1996)
84. M. L. Fero, M. Rivkin, M. Tasch, P. Porter, C. E. Carow, E. Firpo, K. Polyak, L. H. Tsai, V. Broudy, R. M. Perlmutter, K. Kaushansky, and J. M. Roberts: A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* 85, 733-744 (1996)
85. S. Matsuoka, J. S. Thompson, M. C. Edwards, J. M. Barletta, P. Grundy, L. M. Kalikrin, J. W. Harper, S. J. Elledge, and A. Feinberg: Imprinting of the gene encoding a human cyclin-dependent kinase inhibitor, p57Kip2, on chromosome 11p15. *Proc. Natl. Acad. Sci.* 93, 3026-3030 (1996)
86. I. Hatada and T. Mukai: Genomic impriniting of p57Kip2, a cyclin dependent kinase inhibitor, in mouse. *Nature Genetics* 11, 204-206 (1995)
87. I. Hatada, H. Ohashi, Y. Fukushima, Y. Kaneko, M. Inoue, Y. Komoto, A. Okada, S. Ohishi, A.Nabetani, H. Morisaki, M. Nakayama, N. Niikiwa, and T. Mukai: An imprinted gene p57Kip2 is mutated in Beckwith-Weidemann syndrome. *Nature Genetics* 14, 171-173 (1996)
88. T. Caspary, M. A. Cleary, E. J. Perlman, P. Zhang, S. Elledge, and S. M. Tilghman: Oppositely impreinted genes p57Kip2 and IGF2 interact in a mouse model for Beckwith-Wiedemann syndrome. *genes and Development* 13, 3115-3124 (1999)
89. R. Weksberg, D. R. Shen, Y. L. Fei, Q. L. Song, and J. Squire: Disruption of insulin-like growth factor 2 imprinting in Beckwith-Wiedemann syndrome. *Nature Genetics* 5, 143-150 (1993)
90. W.Reik, K. W. Brown, H. Schneid, Y. LeBouc, W. Bickmore, and E. R. Maher: Imprinting mutations in the Beckwith Wiedemann syndrome suggested by an alteered imprinting pattern of the IGF2-H19 domain. *Human Molecular Genetics* 4, 2379-2385 (1995)
91. P. Zhang, C. Wong, D. Liu, M. Finegold, J. W. Harper, and S. J. Elledge: p21Cip1 and p57Kip2 control muscle differentiation at the myogenin step. *Genes and Development* 13, 213-224 (1999)
92. X. Grana, J. Garriga, and X. Mayol: Role of the retinoblastoma protein family,pRB, p107, and p130 in the negative control of cell growth. *Oncogene* 17, 3395-3382 (1998)

93. J. Garriga, A. Limon, X. Mayol, S. G. Rane, A. J. H, E. P. Reddy, V. Andres, and X. Grana: Differential regulation of the retinoblastoma family of proteins during cell proliferation and differentiation. *Biochemical Journal* 333, 645-654 (1998)
94. T. Jacks, A. Fazeli, E. M. Schmitt, R. T. Bronson, M. A. Goodell, and R. A. Weinberg: Effects of an Rb mutation in the mouse. *Nature* 359, 295-300 (1992)
95. E. Y.-H. P. Lee, C.-Y. Chang, N. Hu, Y.-C. Wang, C.-C. Lai, K. Herrup, W.-H. Lee, and A. Bradley: Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* 359, 288-294 (1992)
96. A. R. Clarke, E. Robanus-Maandag, M. v. Roon, N. M. v. d. Lugt, M. v. d. Valk, M. L. Hooper, A. Berns, and H. t. Reile: Requirement for a functional Rb-1 gene in murine development. *Nature* 359, 328-330 (1992)
97. E. Zacksenhaus, Z. Jiang, D. Chung, J. D. Marth, R. A. Phillips, and B. L. Gallie: pRb controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. *Genes Dev.* 10, 3051-3064 (1996)
98. Y.-J. Bignon, Y. Chen, C.-Y. Chang, D. J. Riley, J. J. Windle, P. L. Mellon, and W.-H. Lee: Expression of a retinoblastoma transgene results in dwarf mice. *Genes Dev.* 7, 1654-1662 (1993)
99. D. Cobrinik, M.-H. Lee, G. Hannon, G. Mulligan, R. T. Bronson, N. Dyson, E. Harlow, D. Beach, R. A. Weinberg, and T. Jacks: Shared role of the pRb-related p130 and p107 proteins in limb development. *Genes Dev.* 10, 1633-1644 (1996)
100. M.-H. Lee, B. O. Williams, G. Mulligan, S. Mukai, R. T. Bronson, N. Dyson, E. Harlow, and T. Jacks: Targeted disruption of p107: functional overlap between p107 and Rb. *Genes Dev.* 10, 1621-1632 (1996)
101. J. E. LeCouter, B. Kablar, W. R. Hardy, C. Ying, L. A. Megeney, L. L. May, and M. A. Rudnicki: Strain-dependent myeloid metaplasia, growth-deficiency, and accelerated cell cycle in mice lacking the Rb-related p107 gene. *Mol. Cell. Biol.* 18, 7455-7465 (1998)
102. J. E. LeCouter, B. Kablar, P. F. M. White, C. Ying, and M. A. Rudnicki: Strain-dependent embryonic lethality in mice lacking the retinoblastoma-related p130 gene. *Development* 125, 4669-4679 (1998)

Key words: Cell Cycle, Cyclin, Cyclin Dependent Kinases, CDK Inhibitors, Pocket Proteins, Chondrocytes, Growth Plate. Review

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