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The Retinoblastoma Protein in Osteogenesis and Osteosarcoma Formation

Pedro G. Santiago-Cardona
Ponce School of Medicine and Health Sciences, Ponce, Puerto Rico

1. Introduction

The retinoblastoma tumor suppressor as a cell cycle regulator, a brief overview

The retinoblastoma tumor suppressor protein (pRb) is a 928 amino acids nuclear phosphoprotein that functions predominantly as a transcriptional regulator (Knudsen and Knudsen, 2006). It possesses a weak, non-specific DNA binding capacity, therefore, its role as a transcriptional regulator requires that it forms part of protein complexes in which its binding partners provide the capacity to interact with *cis* regulatory elements in the promoters of particular target genes. Evidence supporting its predominantly tumor suppressive function rapidly accumulated since its discovery. First, its deletion in humans was found to be an important causative agent in the genesis of malignant tumors of the retina, or retinoblastomas (Cavenee et al., 1983; Friend et al., 1986; Godbout et al., 1983; Lee et al., 1987), hence its name. This was followed by studies with oncogenic viruses such as some strains of the Human Papilloma Virus (HPV), Adenovirus, and the Simian Vacuolating Virus 40 (SV40). These viruses were found to engender an oncogenic programme in their host cells in which virus-encoded oncoproteins inactivate pRb and other important host tumor suppressors (Ludlow et al., 1989). These studies reinforced the conception of pRb as a tumor suppressor by directly showing that abrogation of pRb function is a necessary step in the chain of events resulting in oncogenic transformation. Further research efforts were aimed at elucidating the precise cellular and molecular mechanisms by which pRb exerts its tumor suppressive function. The generation of the first mice in which the gene encoding pRb, *RB1*, was genetically deleted was very informative in regards to pRb function. These studies showed that mice deficient for pRb in a homozygous manner are non-viable and show a host of defects in neurogenesis and hematopoiesis. These homozygous mutants showed an increased pool of immature nucleated erythrocyte progenitors, together with ectopic mitoses in the nervous system. On the other hand, heterozygous mice, while viable, were prone to develop pituitary and thyroid tumors, strictly dependent on the loss of wild type allele of the *RB1* gene (Lee et al., 1992). These early studies suggested that pRb may be essential for the irreversible cell cycle arrest that is now considered to be a precondition of the fully differentiated post-mitotic state. Therefore, absence of pRb loss could result in an enrichment of proliferative cells with a restricted capacity to withdraw from the cell cycle and subsequently engage in a differentiation programme. These studies led to the early suspicion that these pools of undifferentiated

progenitor cells, impaired in their ability to differentiate, could provide a fertile ground for the emergence of tumor forming cells, a suspicion that later studies confirmed. Today, pRb´s tumor suppressive function is widely regarded to depend on a great measure on its capacity to act as a cell cycle repressor, specifically, on its capacity to engender the irreversible cell cycle arrest that is now considered a pre-condition to achieve a fully differentiated state.

pRb´s function as a cell cycle repressor revolves around its capacity to bind and functionally repress the activity of its best characterized binding partners, the E2F transcription factors. These transcription factors, together with their heterodimeric partner DP, trigger the expression of several genes whose products are required for cell cycle progression. Known E2F/DP target genes include proteins involved in DNA synthesis and cell cycle progression such as Thymidine Kinase, Dihydrofolate Reductase (DHFR), DNA Polα, and Types E and A cyclins Cyclins (Knudsen and Knudsen, 2006; Lipinski and Jack, 1999). E2F transcription factors promote cell cycle-related transcription by recruiting pre-initiation complexes consisting of TFIIA and TFIID to E2F-responsive promoters (Nguyen and McCance, 2005; Ross et al., 1999; Zheng and Lee, 2001). As mentioned above, pRb is a phosphoprotein, and it is well established that its function is adversely affected by phosphorylation. In non-dividing cells, pRb is hypophosphorylated and therefore maximally activated, i.e., able to interact with E2F and block its activity (Buchkovich et al., 1989; Cobrinik, 2005; Dyson, 1998; Knudsen and Knudsen, 2006; Knudsen and Wang, 1996). pRb binding to E2F abolishes E2F´s transactivating capacity by recruiting transcriptional repressor complexes to promoters containing E2F binding sites. For example, pRb is known to recruit histone deacetylase (HDAC) enzymes to E2F bound promoters. These HDACs remove acetyl groups from histone proteins, thus strengthening their interactions with DNA thus provoking a local remodelling and condensation of chromatin to make it more compact and therefore less accessible to transcription factors (Lipinski and Jacks, 1999; Steveaux and Dyson, 2002; Zheng and Lee, 2001). pRb also represses transcription directly through direct contact with the basal transcription machinery without the requirement of HDAC activity (Ross and Dynlacht, 1999; Zheng and Lee, 2001).

Under the influence of mitogenic signals acting on a cell, pRb´s capacity to block E2F-dependent transcriptional activity is abolished when it is hyperphosphorylated by heterodimeric complexes containing a Cyclin regulatory component bound to a Cyclin-dependent protein kinase (Cdk). The Cdk component of these complexes gains its catalytic activity only when bound by its cyclin regulatory partner. At least three different Cyclin/Cdk complexes have been shown to phosphorylate pRb during cell cycle progression, each complex phosphorylating pRb in a specific phase of the cell cycle, and each phosphorylation rendering pRb progressively less capable of binding to and inactivating E2F (Harbour and Dean, 2000). Upon cell stimulation by mitogenic growth factors acting via receptor tyrosine kinases and the Ras/MAPK pathway, the mitogen dependent-accumulation of D-type Cyclins drives the formation of complexes between D-type cyclins and Cdk4 and Cdk6 catalytic partners, which phosphorylate pRb in early G1. This relieves the repressive effect of pRb on E2F, the later now being free to command cell cycle-related gene expression. pRb phosphorylation is propagated beyond G1 when E2Fs induce the expression of Cyclins E and A, which in complex with Cdk2 collaborate with CyclinD/Cdk4-6 complexes to sustain phosphorylation during the late G1 and S phases, respectively (Harbour and Dean, 2000; Sheer and Roberts, 1999; Zheng and Lee, 2001). In summary, the concerted actions of these Cyclin/Cdk complexes ensure pRb

hyperphosphorylation and inactivation through the complete cell cycle, allowing the cells to proceed unhampered by pRb function through all phases of the cycle. In this scenario, E2F is free to trigger proliferation-related gene expression thus promoting entry into the S-phase and further progression through of cell cycle (Harbour and Dean, 2000; Zheng and Lee, 2001).

Upon completion of mitosis, and provided that anti-mitogenic signals are enriched in the extracellular milleu, pRb is hypophosphorylated and returned to its active, E2F repressive state (Dyson, 1998). This is engendered due the induction by anti-mitogenic signals of the expression of protein phosphatase 1 (PP1), which de-phosphorylates pRb. Further pRb phosphorylation is prevented when these anti-mitogenic signals induce the activities of Smad proteins, which then relocate to the nucleus upon activation and promote the expression of Cyclin-dependent kinase inhibitors (CKIs) such as p15, p16, p21 and p27. As implied by their name, these CKIs repress the actions of the Cyclin/Cdk complexes responsible for pRb phosphorylation. Thus, the concerted actions of PP1 and CKIs restore pRb to its hypo-phosphorylated, fully functional state (Durfee et al., 1993; Ludlow et al., 1993; Nguyen and McCance, 2005).

It is noteworthy that the paramount biological importance of pRb as a master controller of the cell cycle transcends mammals and is highlighted by the fact that conserved pRb homologues have been identified and shown to play crucial roles in cell cycle control and differentiation in *Drosophila* (Du et al., 1996) and *C. elegans* (Lu and Horvitz, 1998). In both of these organisms pRb performs similar roles in cell cycle regulation and differentiation.

2. pRb inactivation in human cancers: All roads lead to Rome

From the previous description of pRb's mechanism of action, pRb abrogation is expected to lead to a major breakdown in cell cycle control with consequent unrestricted proliferation. A corollary of this statement is that a functional pRb pathway represents a major roadblock to oncogenic transformation. Consistent with this, it is now well established that either pRb itself or proteins that funnel their anti-mitogenic activities through pRb are lost or mutationally inactivated in the vast majority of human tumors (Hanahan and Weinberg, 2011; Nguyen and McCance, 2005). Therefore, it is not an overstatement to say that the pRb pathway is inactivated in most, if not all, human tumors. This observation strongly supports the tumor suppressive nature of pRb, while hinting at the strong selective pressures faced by incipient cancer cells to inactivate pRb.

Given the close relationship between pRb and E2F in cell cycle control, it is not surprising then that some human tumors are comprised by transformed cells bearing mutant *RB1* alleles coding for pRb proteins that are defective in their capacity to block E2F action. This is observed with high frequency in retinoblastomas, osteosarcomas, bladder carcinomas and small-cell lung carcinomas, where the *RB1* gene itself is a usual target of mutational hits (Horowitz et al., 1990). However, given the strong selective pressure for pRb inactivation faced by transformed cells, even tumors comprised of cells with wild type *RB1* alleles usually harbor mutations in genes coding for other pRb pathway components. Excessive expression of Cdk4 or Cyclin D by gene amplication or chromosomal translocation is related to several cancer types. For example, amplification of Cyclin D1 genes have been found in breast, thyroid, head and neck tumors as well as in mantle cell lymphomas, while Cdk4 overexpression or Cdk4 mutations that render it insensitive to CKI inhibition have been

found in melanomas and glioblastomas (Liu et al., 2004; Sherr and McCormick, 2002; Vooijs and Berns, 1999). Other cancer types such as non-small cell lung carcinomas, melanomas, pancreatic carcinomas and T cell lymphomas show mutational inactivation of the CKI p16 (Kaye, 2002). Melanomas are notable for the high frequency with which they bear mutations in the gene coding for the p53 tumor suppressor, a transcription factor that is a potent inducer of the CKI p21, as well as mutations in the p16 gene (Hussussian et al., 1994). Finally, mutations in the *APC* gene, occurring with high frequency in colorectal carcinomas, lead to unrestricted activation of the Wnt signalling pathway, with consequent up-regulation of Cyclin D genes (López-Kostner, 2010). It can be clearly appreciated that all of the mutational scenarios described above result in abrogation of pRb function, even in the ones in which there is a wild type pRb status. In other words, in most human cancers, pRb itself is missing or defective, or it is inactivated due to hyperphosphorylation. Independently of the mode of pRb inactivation, the end result is always unchecked E2F activity. As can be discerned in the examples above, the mechanism of pRb inactivation during tumorigenesis is clearly tissue specific. Nevertheless, independently of the tissue of origin, the acquisition of a fully transformed phenotype is strongly dependent on the acquisition of mechanisms to circumvent pRb activity.

From what was discussed above, it is more than evident that pRb abrogation signifies a major contribution to carcinogenic transformation by removing the primary obstacle to over-proliferation. However, it is widely regarded that oncogenic transformation is rarely, if ever, the end result of mutations in one or just a few genes. On the contrary, it has been established that a minimum of at the very least 6 mutations in critical genes in the same cell are required to drive cells into full malignancy (Hanahan and Weinberg; 2000). It is well known that other aspects of cellular homeostasis, in addition to cell cycle control, must be dysregulated to achieve a fully malignant phenotype. For example, for the development malignat tumors to occur, unrestricted proliferation must be accompanied by other traits such as evasion of apoptosis, increased angiogenic capacity, loss of intercellular contacts, increased proclivity for migratory activity, and production of extracellular matrix degrading enzymes, among others (Hanahan and Weinberg, 2000). Although pRb loss is apparently more relevant for the early stages of hyperplastic proliferation, it is clear that pRb loss at such a stage can enrich the incipient tumor tissue with proliferative cells in which additional mutant alleles are likely to arise due to DNA replication errors during their prolonged and unrestricted proliferation. These mutant alleles can accumulate and propagate in rapidly dividing pRb-deficient cells and they can cooperate with pRb deficiency to drive full oncogenic transformation. It is important to note that pRb has also been assigned a very important role as guardian of the genome (Zheng and Lee, 2001). Therefore, pRb loss has the dual effect of enhancing proliferative capacity while leading to a state of genomic instability. Therefore, pRb null cells are known not only by their capacity to proliferate unrestrictedly, but also by being prone to acquire genetic alterations ranging from point mutations to gross genetic rearrangements. This in turn can result in inactivation of other tumor suppressors and/or in constitutive activation of oncogenes. Thus pRb contributes to early carcinogenesis by allowing the emergence of a pool of rapidly dividing cells that serves as a fertile ground for the acquisition of further genetic changes that will later contribute to the more advanced stages of malignant transformation, and that together with pRb loss confer cells a selective advantage over normal cells.

3. Additional roles for pRb beyond cell cycle control

It was expected that a powerful tumor suppressor such as pRb, whose inactivation has been so intricately linked to the molecular etiology of most human cancers, would become a focus of intense research in cancer biology. Research on pRb has indeed been intensive for over two decades now, and as a result of this, pRb is now appreciated as a complex multifunctional protein with a wider relevance to cellular homeostasis. As a reflection of this, a wide repertoire of pRb-interacting proteins, in addition to E2F transcription factors, has been identified, each of them mediating a particular function, and all of them together reflecting the complex multifunctional nature of this protein. The list of pRb functions has grown over the years and currently includes, among others, roles in stem cell maintenance, senescence, tissue differentiation, morphogenesis and regeneration, modulation of hormone response, genomic integrity, chromosome segregation, cell-to-cell adhesion and global genomic fluidity. In depth-discussion of each of these additional functions is beyond the scope of this chapter and has been reviewed or reported elsewhere (Braig and Schmitt, 2006; Campisi, 2001; Liu et al., 2004; Lundberg et al., 2000; Narita et al., 2003; Sosa-García et al., 2010; Wynford-Thomas, 1999; Xu et al., 1997; Zheng and Lee, 2001). Further underscoring pRb's tremendous biological importance, pRb is now known to be required for the proper formation of the cellular architecture of the placenta. Using a combination of tetraploid aggregation and conditional *RB1* genetic knock-out strategies Wu et al. (2003) were able to identify an important contribution of pRb to extraembryonic cell lineages required for embryonic development and viability. Interestingly, in these studies, most of the neurological and erythoid abnormalities originally described in pRb-null mice were virtually absent in pRb-deficient embryos when these were rescued with a wild type placenta. A defective placenta in the absence of pRb function can significantly contribute to the embryonic lethality of pRb abrogation during development.

3.1 A role for pRb in tissue differentiation

pRb's role as a cell cycle repressor is intricately linked to its role as an inducer of differentiation. This is consistent with the notion that cell differentiation is a post-mitotic state that is achieved only after a cell undergoes an irreversible withdrawal from the cell cycle. Therefore, pRb can be considered as an integrator between permanent cell cycle arrest and the initiation of cellular programmes that culminate in differentiation. pRb's function in this context can be said to consist in ensuring that a cell does not initiate differentiation before arresting its proliferation. As will be discussed below, this is turn predicts that a breakdown of pRb function can result in the accumulation in tissues of proliferating progenitor cells with tumorigenic potential. The phenotype of the pRb knock-out mice described above supports this notion. pRb's contribution to differentiation is complex and at many levels. pRb function confers differentiating cells with the capacity to irreversibly exit the cell cycle while coordinating this exit with the initiation of differentiation. pRb also protects developing tissues from apoptosis, induces and sustains cell type specific-gene expression, and maintains the differentiated post-mitotic state (Lipinski and Jacks, 1999). It is known that in addition to E2F-bound pRb, free unphosphorylated pRb accumulates after cells reach a post-mitotic state and it is this free active pRb that is responsible for driving and sustaining the various aspects of differentiation (Lipinski and Jacks, 1999).

pRb has been intimately linked to the differentiation of several cell types such as cerebellar granule cells, adipocytes, keratinocytes, myoblasts and osteoblasts (Classon et al., 2000;

Landsberg et al., 2003; Liu et al., 2004; Marino et al., 2003). pRb's participation in myogenic, adipogenic and osteogenic differentiation has been particularly well-studied. As will be discussed in details below, pRb's role in differentiation is a dual one, on the one hand promoting terminal cell cycle arrest, an on the other hand, enhancing the activity of tissue-specific transcription factors that in turn trigger the expression of tissue specific differentiation. It is important to note that in both cell cycle repression and in tissue differentiation, pRb functions predominantly as a transcriptional regulator by a mechanism that essentially consists in binding to, and regulating the transactivating capacity of the main transcription factors involved in these processes. However, pRb's effect on transcription is context-dependent, being repressive in cell cycle control while being activating in regards to cellular differentiation. Specifically, while pRb represses the activity of E2Fs transcription factors during cell cycle regulation, it enhances the activity of the transcription factors that drive tissue-specific gene expression during differentiation. Therefore, pRb's capacity to induce terminal cell cycle arrest is tightly coordinated to its capacity to drive cells into differentiation pathways, both roles being evoked in a complementary manner. This is fully consistent with the notion that cell proliferation and differentiation are mutually exclusive processes, and places pRb in the position of an overseer of the mechanisms that prevent the onset of premature differentiation before precursor cells are fully arrested. In terms of protection of tissues undergoing morphogenesis from undue apoptosis, pRb's role seems to be dependent on its capacity to bind and repress E2F1, which is unique among E2F transcription factors for being the only member capable of inducing apoptosis (DeGregori et al., 1997).

As mentioned above, pRb's participation in myogenic, osteogenic and adipogenic differentiation has been particularly well studied. pRb's involvement in myogenic and adipogenic differentiation will be briefly discussed here, while pRb's role in osteogenic differentiation will be the topic of section 5 of this chapter. In regards to myogenic differentiation, it is now well established that it depends on pRb function for the expression of muscle-specific markers (Gu et al., 1993). pRb abrogation severely impairs myogenic differentiation. In addition, pRb-deficient myoblasts cannot maintain a post-mitotic state following differentiation, being susceptible to mitogenic-re-stimulation (Novitch et al., 1999). This again points to a role for pRb in promoting and sustaining the post-mitotic state associated with differentiation. On the other hand, pRb significantly upregulates the expression of MyoD, a myogenic transcription factor, while increasing its transactivating capacity. In this way pRb contributes to the expression of late muscle differentiation markers such as MHC, MCK and MEF2 (Gu et al., 1993; Novitch et al., 1999). A direct pRb-MyoD interaction has been demonstrated *in vitro* (Gu et al., 1993), although there is still controversy as to the possible relevance of this interaction *in vivo* (Nguyen and McCance, 2005). Furthermore, the specific mechanism accounting for the pRb-dependent upregulation of MyoD still awaits clarification. Several scenarios have been proposed to explain pRb's involvement in myogenic differentiation. In addition to directly activating MyoD transcriptional activity, pRb may sequester inhibitors of muscle specific transcription such as HBP-1, leading to a pRb-mediated de-repression of MyoD activity (Nguyen and McCance, 2005; Zheng and Lee, 2001). Therefore, although the details of the mechanisms by which pRb impinges upon myogenic differentiation are still the subject of research, pRb's importance for myogenic differentiation is widely accepted, whether its role consists in directly transcriptionally activating MyoD expression and function, or in removing a block hampering MyoD expression.

In relation to adipogenic differentiation, pRb has also been shown to bind and increase the transactivation capacities of CCAAT/enhancer binding proteins (C/EBPs), which are the central transcription factors driving adipocyte differentiation (Zheng and Lee, 2001). NF-IL6, another transcription factor member of the C/EBP family which is important for leukocyte differentiation, was also shown to be activated by pRb (Chen et al., 1996). pRb is also known to be involved in promoting erythrocyte and neuron differentiation by abrogating the function of differentiation blockers such as Id2 (Zheng and Lee, 2001). Taken together, the findings obtained from studies in differentiation reveal a common mechanism by which pRb regulates differentiation. pRb does so by interacting with transcription factors associated with differentiation, enhancing the activity of those that promote tissue-specific gene expression while blocking the activity of those that hamper such gene expression.
It is noteworthy that the distinct developmental abnormalities observed in the pRb-null mice, i.e., defects in erythropoiesis, lens, skeleton and muscle differentiation, can be explained in light of pRb's functions in differentiation as just described. Defective tissues in these pRb-null mice were predominantly characterized by an enrichment of poorly differentiated progenitor cells, again pointing to an inability to exit the cell cycle in preparation for differentiation. The embryonic lethality can be explained at least in part by the widespread differentiation defects observed in these animals, together with the defects in the placenta described above.

4. Cellular and molecular mechanisms of osteogenic differentiation

The role of pRb in osteogenic differentiation has been well studied and established. Before discussing pRb's participation in this process, an in-depth discussion of the molecular mechanisms associated with osteogenic differentiation is in order.

4.1 Overview of osteogenic differentiation and skeletal morphogenesis

Osteogenic differentiation is a central component of developmental skeletogenesis. Furthermore, it goes beyond embryogensis and continues afterwards as an ongoing process through adult life, intimately linked to bone remodelling. Bone remodelling in post-natal life serves first in the growth phase and later in adult life to replace aging tissue and repair injuries (Day et al., 2005). This necessitates osteoblast proliferation and differentiation through the entire life of an organism in order to continuously supply bone-forming cells and thus maintain bone homeostasis (Mbalaviele et al., 2005). Bone and cartilage are major tissues in the vertebrate skeletal system, which is primarily composed of three cell types: osteoblasts, chondrocytes, and osteoclasts (Day et al., 2005). In bone homeostasis, osteoblasts participate in the synthesis, deposition and mineralization of the matrix that will form the bone, while osteoclasts resorb this mineralized matrix allowing this rigid tissue to remodel (Ducy, 2000).
Bone marrow mesenchymal stem cells are the source of osteoprogenitor cells in adult life. Bone marrow contains a complex and heterogeneous mixture of pluripotent stem cells that can differentiate not only into osteoblasts, but also into fibroblasts, adipocytes, myocytes, hematopoietic cells, and endothelial cells under the induction by systemic or local factors (Marie, 2002). In the developing skeleton, osteoblasts and chondrocytes both differentiate from a common mesenchymal progenitor *in situ*, whereas osteoclasts are of hematopoietic origin and brought in later by invading blood vessels (Day et al., 2005).

Embryonic skeletogenesis *in situ* starts with the condensation of undiffentiated mesenchymal cells. These condensations, also called anlagen, occur in structures and locations that prefigure each future skeletal element (Ducy, 2000; Hall and Miyake, 2002). Depending on the anatomic location, skeletogenesis can occur by two distinct mechanisms: intramembranous and endochondral ossification (Day et al., 2005). Intramembranous ossification occurs by the direct transformation of mesenchymal cell within condensates into osteoblasts, and is limited to bones of the cranial vault, some facial bones, and part of the mandible and clavicle (Day et al., 2005). On the other hand, the axial and appendicular skeletal elements, i.e., bones that participate in joints and bear weight such as long bones, the spine and ribs, form by endochondral ossification. In this mechanism, the condensed embryonic mesenchyme first transforms into a cartilage template of the future bone while osteoblasts differentiate and mature in the periphery of the cartilage (perichondrium) to form bone collars. The whole template is later remodelled and ossified to produce the mature bone when a collagen type I-rich extracellular matrix (ECM) becomes mineralized by the action of mature osteoblasts (Day et al., 2005; Ducy, 2000).

Osteogenic differentiation is a major driving force of skeletogenesis by providing a constant pool of differentiated osteoblasts, which will form the bone structure by synthesizing, depositing and mineralizing the bone matrix. *In vitro* studies of osteogenic differentiation have supplemented *in vivo* studies and have contributed significantly to the elucidation of the details of this process. Osteogenic differentiation is a complex multi-step process that can be roughly divided into two major stages. The first stage involves the commitment of bone marrow stem cells to the osteogenic lineage, a commitment that imposes a restriction to their pluripotency. This leads to the production of a pool of osteoprogenitor cells that eventually convert to pre-osteoblasts or immature osteoblasts, that will then differentiate into fully mature osteoblasts upon receiving the appropriate stimuli. While osteoprogenitor cells still retain some level of plasticity in their differentiation potential, pre-osteoblast are irreversibly committed to differentiate into osteoblasts. In the second stage, irreversibly committed pre-osteoblasts fully differentiate into mature osteoblasts with bone-producing capabilities. This second stage entails a series of intermediate steps along the osteoblast lineage, each characterized by the expression of specific differentiation-stage-specific markers. Based on the expression patterns of differentiation markers studied in osteoblasts cultured in differentiation inducing medium, most of the steps along the differentiation pathway have been elucidated. Based on the outcomes of in vitro studies (Aubin, 1999; Marie, 2002; Marom, 2004), the second stage can be further subdivided into four main periods as follows. First, pre-confluent proliferation supports expansion of the pre-osteoblasts, which are also active in the biosynthesis of the type I collagen that predominates in bone ECM. This period results in the formation of a confluent monolayer of pre-osteoblasts anchored to a collagen type I ECM. At this time, and in addition to type I collagen, genes related to proliferation (e.g., c-myc, c-fos and c-jun) and cell cycle progression (e.g., histones and cyclins) are expressed together with genes encoding cell adhesion-related molecules (e.g., fibronectin, cadherins, integrins). This period culminates with the establishment of confluent monolayers of post-mitotic pre-osteoblasts that have undergone the contact-dependent growth arrest normally experienced at high cellular densities. In the second period, a second wave of post-confluent proliferation ensues, but only in a very limited population of pre-osteoblasts that become irreversibly committed to enter the full differentiation programme. This post-confluent proliferation allows clonal

expansion to increase the mass of future bone forming cells. It occurs at multiple foci scattered through the monolayer of growth-arrested pre-osteoblasts and supports their multilayering to develop bone-forming nodules. Eventually cells within each nodule become growth arrested and start expressing markers of osteoblast differentiation. This step is characterized by the expression of genes that support organization, maturation, and mineralization of the bone ECM (Aubin, 1999; Marie, 2002). This is a post-proliferative gene expression pattern restricted to subgroup of cells within the nodule and serves predominantly to render the ECM competent for mineralization, a process that is essential for the complete expression of the mature osteoblast phenotype. The genes predominantly expressed at this time include genes coding for Alkaline Phosphatase (AP), a cell surface glycoprotein early marker involved in ECM mineralization and in the synthesis and deposition of type I collagen and other non-collagenous bone matrix proteins (Marom et al., 2004). The third period is completely post-mitotic and involves gene expression related to subsequent and more advanced stages of differentiation, specifically related to the ordered deposition of hydroxyapatite, which is the predominant bone mineral. The main characteristic of this period is the appearance of a mineralized bone ECM with which fully mature osteoblasts interact. Osteopontin, Bone Sialoprotein (BSP) and Osteocalcin, which are late markers of full osteoblast differentiation, exhibit maximal expression at this time when maturation of osteoblasts and mineralization of bone tissue reach their peaks (Aubin, 1999; Marie, 2002; Marom, 2004). Osteoponting and BSP are secreted proteins, they bind cell surface integrin receptors, and regulate mineralization (Marom, 2004). Osteocalcin is a matrix protein that regulates osteoclast activity (Marom, 2004), which must be later balanced with osteoblast function to sustain proper bone remodelling and homeostasis. *In vivo*, mature osteoblasts that are actively forming the bone matrix have cuboidal shapes, they line the forming bone, and form extensive cell-to-cell contacts. Once the bone matrix has been deposited, most of these cuboidal osteoblasts become flattened, and a fraction of them loose cell-to-cell adhesion and become embedded within the matrix to become osteocytes (Marie, 2002). Finally, the fourth period is not directly involved in the initial osteogenic differentiation related to skeletogenesis, but is more related to editing and remodelling of the bone ECM. Consistent with editing and remodelling, this period is characterized by increased expression of not only collagen type I, but also of collagenase enzyme. This period is also characterized by apoptosis of osteoblasts and a compensatory proliferative activity that replenishes the osteoblasts lost to apoptosis. As discussed above, there is a reciprocal and functionally coupled relationship between proliferation and differentiation, since full differentiation and maturation of osteoblasts in the third period needs to be preceded by their terminal withdrawal from the cell cycle. The first three periods described above are visually represented in Figure 1.

4.2 Runx2 as a master switch of osteogenic differentiation and bone formation

The events described above are notable for the progressive expression of markers associated with each differentiation step and culminating with the expression of genes that are typical of the fully differentiated osteoblast such as Osteocalcin, Bione Sialoprotein and Osteopontin. The main players in this sequential pattern of differentiation specific gene expression are usually tissue-specific transcription factors that temporally regulate the expression of these markers. In the process of osteogenic differentiation, the predominant, and so far considered the most important, tissue-specific transcription factor identified is the

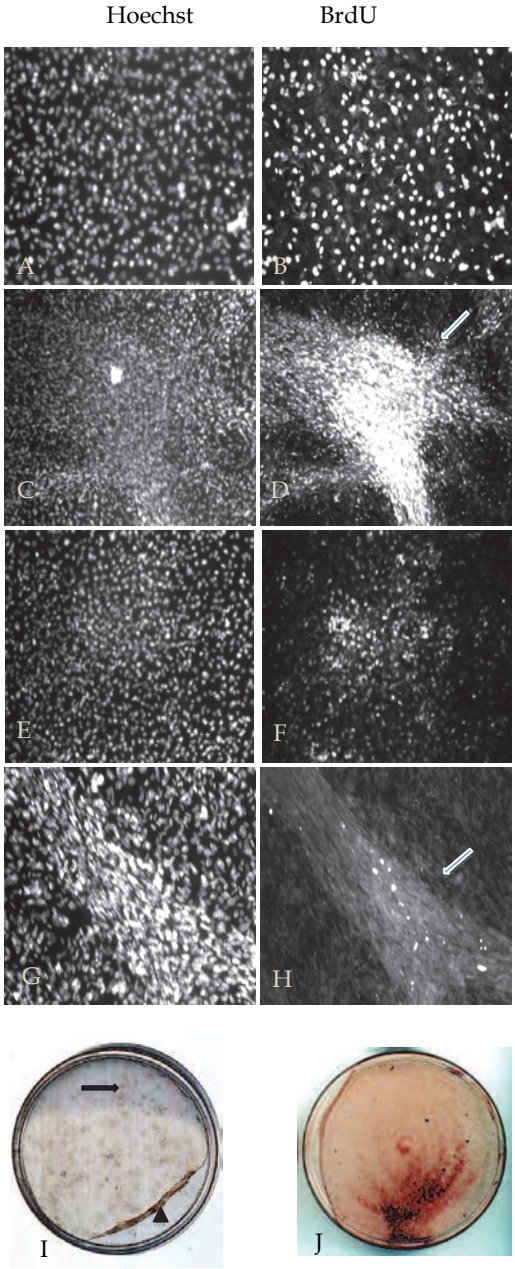

Fig. 1. Main events associated with osteogenic differentiation. The calvaria osteoblast cell line MC3T3-E1 can be induced to differentiate *in vitro* in the presence of ascorbic acid and β-glycerophospahate. This system has been very useful to study the main events and the

transcriptional changes associated to osteogenic differentiation. Panels A, C, E, G and I show total nuclei stained with Hoechst nuclear stain, while panels B, D, F, H and J show nuclei of proliferating cells immunostained for BrdU. Pre-confluent pre-osteoblasts show robust proliferation until high-density cultures are reached (A, B), in which eventually cells undergo contact-dependent growth arrest to produce a confluent cell monolayer. In this stage cells predominantly express collagen type I and genes involved in cell proliferation. At 7 days after confluence (dac), a second wave of proliferation ensues to allow clonal expansion of pre-osteoblast that are irreversibly committed to produce mature osteoblasts (C, D). This clonal expansion results in the formation of multi-layered bone-forming nodules (arrow in D) analogous to the anlagen formed *in vivo*. Notice that cell proliferation is restricted to the bone-forming nodule while cells at the periphery of the nodule are growth arrested, as determined by their lack of incorporated BrdU. At this stage collagen type I continues to be produced robustly, while Alkaline Phosphatase expression is initiated in preparation for the mineralization of the collagen I matrix. At 14 dac, cell proliferation starts to decrease within the bone-forming nodules (E, F), in preparation for the mineralization of the matrix. At 21 dac (G, H), proliferation has completely stopped within the bone-forming nodules (arrow in H). At this time point the bone-forming nodules are apparent to the unaided eye due to their refractive properties and can be seen as mineral- and matrix-dense areas (arrow in I) interspersed through the cell monolayer, which in turn is embedded in a dense collagen type I matrix, seen peeling-off the culture plate in I and J (arrowheads). At this time point markers of late differentiation such as Osteocalcin, Osteopontin and Bone Sialiprotein are being expressed, while mineralization of the bone matrix can bee seen by staining the cultures with alizarin red, which stains bone mineral.

Runt-related transcription factor 2 (Runx2), formerly known as Core Binding Factor α1, or Cbfa1. Runx2 is the earliest molecular marker of the osteoblastic lineage, its expression is both necessary and sufficient to induce osteoblast differentiation (Bialek et al., 2004; Ducy, 2000; Komori, 2002). Evidence pointing to the pivotal role of Runx2 in the regulation of osteogenesis has accumulated to the extent that Runx2 is now considered the main intrinsic regulator of osteogenic differentiation. Runx2 was first identified as the nuclear protein binding to an osteoblast-specific cis-acting element activating the expression of Osteocalcin, the most osteoblast-specific gene. Sequences analyses followed by DNA binding assays located putative Runx2 binding sites in the promoters of other major osteoblast specific genes such as Bone Sialoprotein, Osteopontin and the α1 type I collagen gene (Ducy, 2000). Further confirming the regulatory effect of Runx2 over these genes, *in vitro* studies showed that addition of Runx2 anti-sense oligonucleotides to cultured osteoblasts specifically decreased their expressions (Banerjee, et al., 1997; Ducy, 2000). More importantly, forced expression of Runx2 in non-osteoblastic cells such as primary fibroblasts can activate the expression of Osteocalcin and Bone Sialoprotein in them (Ducy, 2000). The accumulated evidence suggests that Runx2 expression is a key event in the commitment of multipotent mesenchymal stem cells to the osteogenic lineage (Komori, 2002). In addition to participating in the early commitment stage, Runx2 function is also apparently necessary at the later stages of osteogenic differentiation since it is also required for the induction of alkaline phosphatase activity, expression of bone matrix protein genes, and mineralization of that matrix to form bone structures (Banerjee et al., 1997; Ducy, 2000; Komori, 2002; Otto et al., 1997).

The importance of Runx2 for osteogenic differentiation was also established *in vivo*. In mouse embryogenesis, by 12.5 days post-coitum (dpc) every anlage expresses high levels of Runx2. Runx2 is expressed in every future osteoblast, independently of its embryonic origin and regardless of the future mode of ossification, whether intramembranous or perichondrial (Ducy, 2000). Two groups independently deleted the Runx2 gene in mice using homologous recombination (Komori et al., 1997; Otto et al., 1997). Consistent with an important role in osteogenesis, the Runx2 homozygously deficient mice, although normally patterned and of nearly normal size, have skeletons that are entirely cartilaginous. Histologically, these animals lack osteoblasts and their skeletons show a complete lack of bone tissue. Those mice were able to construct a nearly complete cartilage model of the skeleton, but having lost all bone matrix production, failed to mineralize the cartilage scaffold. Further analysis of Runx2 deficient mice revealed that osteogenic differentiation was arrested in the absence of Runx2, demonstrating both that Runx2 is important to that process and that there is no other parallel pathway that can replace its absence (Ducy, 2000). At the molecular level, *in situ* hybridization studies established that there is no expression of differentiation markers expressed exclusively in osteoblasts such as Osteopontin Bone Sialoprotein and Osteocalcin (Komori et al., 1997; Otto et al., 1997), indicative of the absence of mature osteoblasts in these mice. Further supporting a role for Runx2 in bone formation, the heterozygous mice showed a phenotype strongly suggestive of the syndrome known as cleidocranial dysplasia, which arises in humans as a consequence of Runx2 haploid insufficiency and is characterized by generalized bone defects including a ridged skull and lack of clavicles (Otto et al., 1997). Additional evidence has consistently documented a role for Runx2 in the maintenance of the osteoblast differentiated state. For example, Runx2 was shown to regulate the rate of bone mineral deposition by differentiated osteoblasts. Consistent with this, Runx2 expression is sustained post-natally in mice and in fully differentiated osteoblasts (Ducy, 2000). As stated above, Runx2 is an inducer of the expression of Osteocalcin, a gene that is an exclusive trait of fully differentiated osteoblasts (Aubin, 1999; Ducy, 2000). Taken together, the data summarized above suggest that Runx2 modulates commitment of pluripotent stem cells to an osteogenic lineage, while being also a major force driving cells into the osteogenic differentiation. Once osteoblasts reach their maturity, Runx2 regulates their functions and sustains their differentiated state. Clearly then, Runx2 acts as a master regulator of osteoblast differentiation and bone synthesis acting in several stages of the process.

It is important to note that Runx2, although considered a master regulator of the osteogenic differentiation, it is by no means the only osteoblast specific transcription factor related to osteogenic differentiation. In fact, Runx2 is known to act in close concert with another osteoblast specific transcription factor known as Osterix (Osx). In fact, Runx2´s function in the initial commitment to the osteoblast lineage strongly depends on having Osx acting downstream of Runx2. Inactivation of Osx, even in the presence of a fully functional Runx2, results in the formation of ectopic chondrocytes at the expense of osteoblasts (Day et al., 2005). These studies indicate that the initial Runx2-induced commitment to the osteoblastic lineage needs Osx activity to be sustained, and that in the absence of Osx this commitment is fragile, with cells retaining a certain degree of plasticity (Day et al., 2005). These results further indicate that the concerted action of Runx2 and Osx is required not only for determination of one cell type, but also for suppressing the genetic and molecular programs

leading to another cell type. Consistent with this, expression of Runx2 in an osteochondral progenitor cell line inhibited chondrocyte differentiation (Lengner et al., 2005).
The mechanisms related to the regulation of Runx2 function have been the subject of intensive research, and there are data suggesting that the regulation of Runx2 function itself may be complex. In light of Runx2´s powerful osteogenic effect, it is puzzling that in mice development, Runx2 expression precedes the appearance of osteoblasts by at least 4 days. Runx2 expression is detected in lateral plate mesoderm as early as E10 during mouse development (Bialek et al., 2004), yet expression of molecular markers of differentiated osteoblasts cannot be detected before E13 at the earliest, and in most skeletal elements, replacement of the cartilaginous template by bone does not occur before E15 (Bianco, et al., 1991). It is puzzling then how such a powerful inducer of osteogenesis can be present in the embryo for a time window of approximately 4 days without exerting its powerful osteogenic effect. Some observations have suggested answers to this puzzle. It has been observed that Twist 1 and 2, which are basic helix-loop-helix (bHLH) containing transcription factors, are expressed in Runx2-expressing cells throughout the skeleton during early development, and osteoblast-specific gene expression ensues only after their expression decreases. Therefore, an inverse correlation has been found between Twist expression and expression of osteoblast differentiation markers (Bialek et al., 2004). This has led investigators to propose that Runx2 action may be blocked in the very early stages of skeletogenesis and that Twist proteins may have a leading role in this Runx2 repression. Supporting this, it has been shown that Twist-1 and -2 deficiency unleashes premature osteoblast differentiation. Conversely, Twist-1 overexpression inhibits osteoblast differentiation. It was later discovered that twist proteins inhibit osteoblast differentiation by interacting with Runx2's DNA binding domain, thus abrogating Runx2´s DNA binding capacity and transactivating activity without affecting its expression levels (Bialek et al., 2004). This study reveals that osteoblast differentiation is a negatively regulated process early during skeletogenesis, despite the normal expression of Runx2, and that relief of inhibition by Twist proteins is a mandatory event preceding osteoblast differentiation. It is tempting to speculate that the action of Twist proteins permits the building up of enough cellular mass in the mesenchymal condensates by blocking premature onset of osteogenic differentiation. This will allow enough building-up of cellular mass that later will ensure that the appropriate bone density is attained by the forming skeleton. Therefore, Twist proteins block the premature onset of the osteogenic differentiation programme.

4.3 Regulation of osteogenic differentiation

While Runx2 and Osx provide a determinant major force in driving commitment to the osteoblastic cell lineage and together keep the cell differentiating along that pathway, its is clear that these transcription factors trigger differentiation-specific gene expression as a response to external osteogenic stimuli acting on pluripotent stem cells. Several well-studied external ligands with powerful osteogenic influence include Bone Morphogenetic Proteins (BMPs), Transforming Growth Factor-β (TGF-β), Glucocorticoids, Parathyroid Hormone (PH), Estrogen, Insulin-like growth factors (IGFs), Fibroblast Growth Factors (FGFs), Indian Hedgehog, Retinoic Acid (RA) and 1,25-dihydroxyvitamin D_3 (Canalis et al., 1993). While the osteogenic effect of these ligands have been established, the mechanisms that sensitize subpopulations of cells in the bone marrow to be responsive to some stimuli and not others are still under intense investigation. These osteogenic ligands exert their effects by acting

through specific signaling pathways that most likely impinge upon Runx2 and/or Osx if they are to elicit an osteogenic response. Consistent with this, several intracellular signalling pathways have been identified in relation to osteogenic differentiation that serve as bridges linking the actions of these osteogenic ligands with Runx2 and Osterix regulation. Two of these are the Wnt/β-catenin signalling pathway and the Akt pathway. Each of these will be discussed in the following section and used to illustrate the mechanisms that integrate the action of osteogenic external stimuli to the regulation of Runx2 and Osx.

4.3.1 The Wnt pathway in osteogenic differentiation

The Wnt/β-catenin pathway has proved to be a very important signalling pathway controlling the embryonic patterning and morphogenesis of various tissues, including bone. Briefly described, the Wnt pathway is activated by several Wnt ligands that interact with Frizzled receptors in the surface of Wnt-responsive target cells. Activation of the Wnt pathway blocks the degradation of β-catenin, an adherens junction component that is normally targeted for proteasome-mediated degradation after detaching from the membrane. Blocking of β-catenin degradation by Wnt activity leads to its accumulation in the nucleus and subsequent binding to TCF/Lef Transcription factors. This β-catenin/TCF/Lef complex acts as a transcription factor that induces transcription of various target genes depending on the biological context (Logan and Nusse, 2004). As previously explained, chondrocytes and osteoblasts share a common bi-potential precursor within a subpopulation of mesenchymal stem cells, and therefore there must be mechanisms in place to ensure a balanced discrimination between osteogenesis and chondrogenesis during vertebrate skeletogenesis. Bi-potential progenitor cells within early mesenchymal condensations can differentiate into both osteoblasts and chondrocytes as they co-express Sox9 and Runx2. While the importance of Runx2 for osteogenic differentiation was described above, Sox9 is a transcription factor required for chondrocyte cell fate determination and marks early chondrogenic differentiation of mesenchymal progenitors (Akiyama et al., 2002). Inactivation of Sox9 blocks chondrocyte differentiation and leads to ectopic expression of osteoblast-specific genes in targeted progenitor cells (Akiyama et al., 2002). Conversely, cultured Runx2-/- calvarial cells differentiate into chondrocytes *in vitro* when treated with BMP-2 (Kobayashi et al., 2000). Therefore, there appears to be a competition or mutual suppression between the genetic pathways leading to osteoblastic and chondroblastic diferentiation in the common mesenchymal progenitors during both endochondral and intramembranous ossification. Interestingly, Wnt/β-catenin signalling has been implicated in the mutual exclusivity between these pathways, and appears to play a very important role in controlling the balance between the chondrogenic and osteogenic differentiation. The expression patterns of many Wnt ligands during skeletal development suggest the hypothesis that they may be actively signalling the mesenchymal condensations and affecting the balance between osteoblasts and chondrocyte differentiation within the condensations (Parr et al., 1993). Consistent with a role for Wnt/β-catenin in early specification, Wnt/β-catenin pathway activity was found to be upregulated in osteogenic mesenchymal condensations and in the differentiating osteoblasts (Day, et al., 2005). Furthermore, Wnt/β-catenin signalling prevented osteoblasts from differentiating into chondrocytes (Hill et al., 2005). Osteoblast precursors lacking β-catenin are blocked from differentiating into osteoblasts and develop into chondrocytes instead (Hill et al., 2005). *In vivo* ectopic Wnt/β-catenin signalling leads to the enhanced ossification and suppression of

chondrocyte formation. Conversely, genetic inactivation of β-catenin causes ectopic formation of chondrocytes at the expense of osteoblast differentiation during both intramembranous and endochondral ossification, leading to disrupted normal skeletal development (Day et al., 2005). Moreover, inactivation of β-catenin in mesenchymal progenitor cells *in vitro* causes chondrocyte differentiation under conditions allowing only osteoblasts to form (Day et al., 2005). Taken together, these data show that Wnt/β-catenin is essential in determining whether mesenchymal progenitors will become osteoblasts or chondrocytes regardless of regional localization or ossification mechanisms (Day et al., 2005). Specifically, it inhibits chondrocyte differentiation, likely by suppressing Sox9 activity, and promotes osteogenic differentiation and bone formation, by enhancing Runx2 activity.

Interestingly, the Wnt/β-catenin pathway can also shed some light into the molecular mechanisms distinguishing endochondral from intramembranous ossification. It has been shown that β-catenin expression is transiently kept low in cells within the mesenchymal condensations that prefigure the future bones that arise by the endochondral mechanism, which requires the formation of a cartilage template that is later ossified (Day et al., 2005). This decreased β-catenin expression proportionally diminishes Wnt/β-catenin activity inside the mesenchymal condensations during endochondral bone formation in such a manner that at first only chondrocytes can form and osteoblast differentiation is repressed in the core of the mesenchymal condensations. Importantly, in this mechanism, osteoblast differentiation is later initiated at the periphery of the cartilaginous structure, where Wnt/β-catenin signalling is up-regulated. This agrees with the observation that several Wnt-activating ligands are expressed only at the periphery of the newly formed cartilage in the limb (Parr et al., 1993), while some Wnt antagonists, including Sfrp2 and Sfrp3, are expressed within the chondrogenic mesenchymal condensation (Day et al., 2005). In addition, it has been shown that Sox9 promotes the degradation of β-catenin (Akiyama et al., 2002), explaining the lack of Wnt/β-catenin activity in cartilage structures. The difference in Wnt/β-catenin signalling activity in the mesenchymal condensations during intramembranous and endochondral ossifications may be controlled by more upstream events. Further studies have shown that β-catenin and BMP-2 synergize to promote osteoblast differentiation and new bone formation (Mbalaviele et al., 2005). Thus, Wnt/β-catenin signalling may drive osteogenic lineage allocation by enhancing mesenchymal cell responsiveness to osteogenic factors such as BMP-2. Therefore, the function of Wnt/β-catenin during osteogenic differentiation is a dual one, consisting in the repression of chondrogenic differentiation in a subgroup of cells within the mesenchymal condensates, while making them more sensitive to the strong osteogenic influence of BMP-2.

4.3.2 The Akt-PI3K pathway in osteogenic differentiation

As explained above, some of the potent osteogenic ligands that act on mesenchymal cells include, among others, PDGF, IGF and VEGF. These external ligands impinge upon osteogenesis acting through different signalling mechanisms involving the Akt-PI3K pathways. Akt is a serine-threonine kinase activated by various ligands including IGFs through the phosphatidylinositol 3-kinase (PI3K) pathway (Scheid and Woodget, 2001). The Akt kinase is a key component of the signaling events elicited by potent bone anabolic factors. Akt enhances transcription factor-dependent osteoblast differentiation, acting specifically on Osx. As explained above, Runx2 exerts its effect on osteogenesis by requiring

the downstream action of Osx. The interplay between external osteogenic stimuli and the activity of osteoblast-specific transcription factors is further illustrated by the observation that BMP-2, a potent osteogenic ligand, increases the levels of Osx in a manner that requires Akt activity. Akt phosphorylates and increases the osteogenic activity of Osx. It has been found that Akt phosphorylates Osx, increasing its stability, osteogenic activity and transactivation capacity. These results suggest that Akt activity enhances the osteogenic function of Osx, at least in part, through protein stabilization and that BMP-2 regulates the osteogenic function of Osx, at least in part, by activating Akt (Choi et al., 2011).

Akt activity is also required for Runx2 function. Interestingly, the interplay between Akt and Runx2 may be at the core of molecular mechanisms involved in the migration of mesenchymal stem cells that makes them to coalesce into mesenchymal condensations. As explained above, mesenchymal condensation of osteoblast precursors to form the anlagen that prefigure future bones is an essential pre-requisite for bone formation. Therefore, migration, segregation and arrangement of osteoblast precursors in relation to other pluripotent mesenchymal stem cells are important pre-requisites for skeletogenesis. Studies about the interaction between Runx2 and Akt may shed light into the question of how progenitor cells migrate and condensate to form the anlagen. These studies also point to chemiotaxis as a mechanism to direct migration of mesenchymal towards a common location. PDGF, IGF, and VEGF work as chemotactic factors through PI3K, and PI3K-Akt signalling is a major pathway for chemotaxis through tyrosine kinase receptors in fibroblasts. Akt likely mediates cell migration at least partly by activating Rho GTPases such as Rac1 and their effectors such as the p21-activated protein kinase, or Pak1 (Fujita et al., 2004; Ridley et al., 2003). It is tempting to speculate that this pathway is involved in migration and chemioattraction of mesenchymal stem cells, but further experimentations will have to be done to empirically establish this. Studies on Akt have shed some light in the mechanisms by which Runx2 may contribute to early commitment to an osteogenic lineage (Fujita et al., 2004). These studies have uncovered that at least some aspects of Runx2 function need Akt activity. For example, they showed that Runx 2 induces osteoblast migration by coupling with Akt-PI3K signalling. As expected, overexpression of Runx2 enhanced osteoblastic differentiation of C3H10T1/2 and MC3T3-E1 cell lines, but a novel finding was that Runx2 osteogenic effect was blocked by treatments that blocked Akt-mediated signalling, such as anti-IGF-1 antibodies, the drug LY294002 (a PI3K inhibitor) or adenoviral introduction of a dominant-negative Akt. In these studies, PI3K-Akt signalling enhanced Runx2´s DNA binding capacity and Runx2-dependent transcription. These results implicated Akt into the Runx2-mediated effect in inducing osteogenesis. Runx2 activation also induced cell migration, whereas the migratory enhancement produced by Runx2 was decreased by the Akt-abolishing treatments mentioned above. Furthermore, Runx2 up-regulated PI3K subunits (p85 and p110beta) and Akt (Fujita, et al., 20004).

In addition to a possible role for Runx2 in migration and condensation of mesenchymal cells to form the anlagens, Runx2-mediated migration may also play a role in bone remodelling by inducing the displacement of osteoblasts to the surface of bone that has undergone osteolysis by osteoclasts. This is supported by the fact that Runx2 expression is strongly induced in osteoblasts after bone fracture (Kawahata et al., 2003). This suggests that Runx2-mediated chemotaxis may be important for the migration of osteoblastic cells to the healing area. However, further investigation is required to confirm this, as well as to establish the involvement of Akt in osteoblast migration associated to bone healing.

4.3.3 Regulation of osteogenic differentiation by cell-to-cell adhesion

In addition to the external osteogenic ligands described in the previous section, cell-to-cell and cell-to-substrate adhesion is recognized as a major driving force of the osteogenic differentiation programme. That cell-to-cell and cell-to-substrate adhesion are integral components of bone formation and maintenance is widely accepted, and no discussion of osteogenic differentiation would be complete without this topic. Cell-to-cell adhesion plays an important role in the early mesenchymal condensation of osteoblast precursors that precedes ossification (Hall and Miyake, 2000). Embryonic bone development occurs by migration, aggregation, and condensation of immature mesenchymal progenitor cells to form the cartilaginous anlage. During these processes, pre-osteoblasts must be sorted from other mesenchymal cells, migrate and then align with neighboring osteoblasts (Kawaguchi et al., 2004). Cell-to-cell interactions permit cells to synchronize activity, equalize hormonal responses, and diffuse locally generated signals (Stains and Civitelli, 2005a, 2005b). These intercellular interactions also enable the establishment of concerted gene expression patterns among the cells that comprise the mesenchymal condensates.

Several lines of evidence highlight the importance of cell adhesion, whether it be to other cells or to the ECM, as an additional source of osteogenic cues that are at least as important for osteogenic differentiation as the ones provided by the soluble osteogenic factors mentioned above. For example, the maturation and organization of the ECM contributes to the shut down of proliferation, suggesting that attachment of osteoblasts to a well-organized matrix constitutes by itself a signal to stop proliferation. Culture conditions that enable the differentiation of primary calvaria osteoblasts necessarily involve high cell density with its consequent contact-dependent growth arrest. Therefore, induction of osteogenic differentiation *in vitro* only occurs at high cell density and is mediated by the establishment of cell-to-cell contacts. This is further supported by the observation that continuous passage of sub-confluent cultures, which avoids the attainment of high cellular densities, prevents osteogenic differentation. Formation of bone-nodules *in vitro* is also only achieved after osteoblast cultures have attained sufficient cellular density, suggesting that cooperativity among cells is required to form these structures (Aubin, 1999). This has been termed as a "community effect" in which the establishment of a group of differentiated osteoblasts may be dependent on cell-to-cell interactions that occur only when a critical number of cells is reached (Aubin, 1999). In summary, it can be truly said that osteogenic differentiation is a cell density dependent process.

Functional studies using neutralizing antibodies or anti sense oligonucleotides to disrupt the function of cadherins, which are one of the major protein components of the adherens junction that mediate cell-to-cell adhesion, show that cadherin-mediated cell-to-cell adhesion is involved in the control of osteoblast gene marker expression and differentiation (Marie, 2002). Hormonal and local soluble factors known to regulate osteoblast function also regulate N-cadherin expression and subsequent N-cadherin-mediated cell-to-cell adhesion associated with osteoblast differentiation and survival. Alterations of N-cadherin expression are associated with abnormal osteoblast differentiation and osteogenesis in pathological conditions (Marie, 2002).

Osteoblasts express various cadherins, the predominant and most closely associated with the osteoblast phenotype being OB- (also known as cadherin-11) and N- cadherin, although epithelial cadherin (E-cadherin) has been also found to be expressed in human bone marrow stromal cells (Turel and Rao, 1998), murine calvaria cells, rat osteosarcoma cells (Babich and

Foti, 1994; Tsutsumimoto et al., 1999), and human calvaria cells (Marie, 2002). During differentiation, progenitor cells express a changing repertoire of cadherins, which serves as a molecular fingerprint for identifying the differentiation stage and commitment of the progenitor (Stain and Civitelli, 2005a and 2005b). Thus, as cell-to-cell interactions are essential for cell aggregation and cell specification during embryogenesis, it can be said that cadherin mediated cell-to-cell interactions define if not direct, cell fate decisions in adult bone marrow mesenchymal stem cells (Stain and Civitelli, 2005a and 2005b). Each mesenchymal lineage has a characteristic cadherin expression profile, OB-cadherin being expressed constitutively in the osteoblast lineage while N-cadherin is expressed widely in mesenchymal lineage cells (Stain and Civitelli, 2005a and 2005b). The expression of N-cadherin in mesenchymal stem cells varies with cell differentiation towards the osteogenic, myogenic or adipogenic pathway. In mesenchyal stem cells, N-cadherin mRNA levels increase during osteogenic and myogenic differentiation while decreasing during adipogenic differentiation (Shin et al., 2000). OB-cadherin follows the same pattern (Kawaguchi et al., 2001; Shin et al., 2000). As adipogenesis proceeds, N-cad and OB-cadherin are further down regulated to the point that mature adypocytes do not express any of these cadherins (Kawaguchi et al., 2001; Shin et al., 2000). On the other hand, as mesenchymal cells progress towards myoblastic differentiation OB-cadherin decreases and M-cadherin becomes dominant (Shin et al., 2000). Therefore, OB-cadherin appears to be the only cadherin expressed exclusively in fully differentiated osteoblasts. Regarding the osteogenic lineage, N-cadherin is expressed at all stages of bone formation, although at various levels of expression, while OB-cadherin, although present in most stages of differentiation, seems to be significantly up-regulated in more mature osteoblasts. Osteoblasts also express R-cadherin/cadherin-4, but it is rapidly down regulated as differentiation advances (Stain and Civitelli, 2005a and 2005b), therefore its levels are negligible in mature osteoblasts. This suggests that R-cadherin may have an early role in lineage commitment, while being unnecessary during more advanced stages of differentiation, and fully dispensable in the mature osteoblast.

In osteogenic differentiation *in vitro*, N-cadherin mRNA levels increase at the stage of nodule formation and mineralization (Lin et al., 1999) and is further enhanced to accompany the later expression of Alkaline Phosphatase and Osteocalcin (Ferrari et al., 2000). The importance of N-cadherin for the expression of these markers of bone differentiation has been established *in vitro* by approaches such as culturing osteoblasts in the presence of N-cadherin inhibitory peptides (Cheng et al., 2000; Ferrari et al., 2000), neutralizing antibodies (Oberlander and Tuan, 1994), anti-sense oligonucleotides (Hay et al., 2000), and transfection with gene constructs encoding for mutant N-cadherins with dominant negative effects (Cheng et al., 2000; Ferrari et al., 2000). All of these treatments were shown to perturb cell-to-cell adhesion while adversely affecting osteogenic differentiation. As a consequence of these treatments, Alkaline Phosphatase expression in osteoblast cultures was down-regulated (Ferrari et al., 2000), expression of bone matrix proteins such as Bone Sialoprotein, Osteocalcin, and type I collagen, was reduced, and matrix mineralization was impaired (Cheng et al., 2000; Ferrari et al., 2000). Importantly, inhibition of N-cadherin function with one of the strategies mentioned above also lead to an impairment of BMP-2's osteogenic effect (Hay et al., 2000), again suggesting that BMP-2's osteogenic effect is cell density dependent and strongly linked to the establishment of intercellular contacts. According to these studies, treatment of cells with rhBMP-2 induced a rapid and transient increase in N- and E- cadherin mRNA and protein levels. It also induced cadherin-mediated adhesion

which was blocked by anti E- and N- cadherin neutralizing antibodies. In addition, these antibodies decreased basal Alkaline Phosphatase activity as well as the rhBMP-2 induced activity. Treatment with cadherin neutralizing antibodies had the same detrimental effect on Runx2 function and Osteocalcin expression. As mentioned above, other local regulators of osteogenic differentiation are the FGFs. FGFs also appear to act at least in part by influencing cell-to-cell adhesion. FGF-2 transcriptionally increases N-cadherin mRNA levels in human calvaria osteoblasts. Specific anti-N-cadherin antibodies abolished FGF-2's capacity to promote cell aggregation (Debiais et al., 1998). N-cadherin expression has been shown to be regulated in osteoblasts by both BMPs and FGF. Regulation of the transcription of the N-cadherin gene is very complex and involves the PIP3 pathway downstream of FGF (Marie, 2002). Taken together, the studies summarized above show beyond doubt the strong interdependence that exists between external osteogenic signals and the establishment of intercellular adhesion. However, the details of the mechanisms explaining their mutual interdependence still await clarification.

There is evidence suggesting that elevated N-cadherin levels are necessary to maintain the osteoblastic differentiated state. For example, loss of N-cadherin expression with concomitant disruption of cell-to-cell adhesion allows osteoblasts to escape from their interactions with other osteoblasts and become embedded in the bone matrix thus becoming osteocytes (Ferrari et al., 2000; Kawaguchi et al., 2004). Therefore, loss of cellular adhesion due to downregulated N-cadherin levels may be related to the transformation of osteoblasts into osteocyte.

The exact roles of both OB- and N-cadherin in osteoblast differentiation and function, however, still remain elusive. It is possible, however, that they may have complementary or overlapping functions during osteogenic differentiation. Studies to distinguish their functions are made difficult by the fact that homozygous genetic deletion of N-cadherin in mice results in a lethal phenotype (Stains and Civitelli, 2005a and 2005b). This argues against the possibility that OB-cadherin can fully or partially compensate for N-cadherin deficiency. In contrast, genetic ablation of OB-cadherin in mice results in viable animals that appear normal at birth, despite slight reductions in calcification of the cranial sutures and femoral metaphysis (Stains and Civitelly, 2005a and 2005b; Kawaguchi et al., 2004). There is a modest osteopenia in OB-cadherin null mice by three months of age, characterized by diminished mineralizing surface and trabecular bone volume. This defect is cell autonomous since osteoblast function is impaired *in vitro.*

The difficulty of the embryonic lethality of N-cadherin deficiency has been overcome by using a conditional genetic knock-out approach (Castro et al., 2004). Mice expressing a conditional dominant negative N-cadherin mutant showed a delay in reaching peak bone mass as a result of impaired osteogenic differentiation. Bone formation rate in these mice is reduced 74% compared to wild type litter-mates controls. Consistent with an early role for cell adhesion molecules in lineage commitment, mice expressing the mutant N-cadherin also displayed an osteogenic to adipogenic shift, with 27% increase in the percent of body fat relative to controls. Bone marrow mesenchymal cells from these animals were skewed towards adipogenic commitment rather than osteogenic (Castro et al., 2004). Osteoblast differentiation was delayed in calvaria isolated from these transgenic mice (Castro et al., 2004). Nevertheless, further investigations need to be conducted in order to dissect the specific contribution of each cadherin to osteogenic differentiation and osteoblast function, as well as to clarify the specific mechanism and signalling pathways by which they act during these processes.

4.3.4 Regulation of osteogenic differentiation by epigenetic mechanisms and micro RNAs

The studies discussed above represent the foundation of our knowledge about osteogenic differentiation. Research on that area is still intense to this day, and recent findings add more levels of complexity to the regulation of osteogenic differentiation. Recent advances on bone research have uncovered the participation of epigenetic events in the regulation of osteogenic differentiation. Epigenetics encompasses all mechanisms that affect gene transcription without altering nucleotide sequence. These mechanisms invariably involve modifying either histones or the DNA itself by the addition of functional groups such as methyl and acetyl groups that will affect the interaction between DNA and chromatin proteins. The effect on gene expression can be either repressive or activating depending on the nature of the modification. Epigenetics these days is widely recognized as a major influence in the regulation of gene expression during development. On the other hand, several pathologies such as cancer, have been associated to abnormal epigenetic modifications (Cui et al., 2011). Recent investigations have focused on the role of epigenetic regulation in lineage-specific differentiation of mesenchymal stem cells, showing that unique patterns of DNA methylation and histone modifications play an important role in the induction of mesenchymal stem cell commitment and differentiation toward specific lineages (Teven et al., 2011). Epigenetic mechanisms may contribute to the up-regulation of osteoblast-specific genes during osteogenic differentiation. For example, it has been shown that CpG regions in promoters of Runx2 and Osterix, which are master transcription factors during osteogenic differentiation, are demethylated during the increase in gene expression associated with osteogenic differentiation. Conversely, enforced hypermethylation of these promoters by inactivation of Gdd45 suppressed the expression of osteoblast-specific genes with concomitant interruption of osteogenic differentiation (Zhang et al., 2011). These studies showed the important influence that epigenetic controls can exert over osteogenic differentiation, while pointing to Gadd45 as a possible player in the mechanisms involved in stem cell differentiation. Other studies have shown that acetylation of histones H3 and H4, as well as a decreased level of DNA methylation, increase accessibility of the Osteocalcin promoter to osteoinductive transcription factors (Teven et al., 2011). Furthermore, *in vitro* induced osteogenic differentiation of mesenchymal stem cells correlates with a decrease in Osteopontin promoter methylation together with increased Osteopontin expression (Teven et al., 2011).

While epigenetic mechanisms control gene expression by influencing chromatin condensation and thus the access of transcriptional complexes to gene promoters, micro RNAs, or miRNAs, silence gene expression by promoting the highly specific degradation of particular mRNAs via mechanisms that involve the actions of Risc and Dicer protein complexes. miRNAs are a diverse class of small non-coding RNA molecules that function as negative gene regulators (Ambros, 2004; Bartel, 2004), and they are now well-established silencers of gene expression during embryonic development. As in the case of epigenetic modifications, abnormal expression of miRNAs has been detected in several diseases (Maire et al., 2011; Mirabello et al., 2011). Recent studies have revealed the contribution of miRNAs to osteogenic differentiation (Zhang et al., 2011). It was recently discovered that a series of miRNAs controls osteogenic lineage progression by targeting Runx2. During both osteogenic and chondrogenic differentiation, these miRNAs were found to be inversely expressed relative to Runx2 in a lineage-related pattern in mesenchymal cell types (Zhang et al., 2011). Based on 3-UTR luciferase reporter, immunoblot, and mRNA stability assays, it

was found that each miRNA directly attenuates Runx2 protein accumulation. miRNAs have also been implicated in the regulation of lineage commitment; it has been shown that a particular miRNA, designated as MiR-637, maintains the balance between adypocites and osteoblasts by directly targeting Osterix (Zhang et al., 2011). This miRNA suppressed the growth of mesenchymal stem cells and induced S-phase arrest. Expression of miR-637 was increased during adipocyte differentiation whereas it was decreased during osteoblast differentiation, which suggests that miR-637 could act as a mediator of adipo-osteogenic differentiation. Osterix was shown to be a direct target of miR-637 which significantly enhanced adipocyte formation and suppressed osteoblast differentiation in mesenchymal stem cells by directly suppressing Osx expression. Furthermore, miR-637 also significantly enhanced de-novo adipogenesis in nude mice (Zhang et al., 2011).

5. A role for pRb in osteogenic differentiation and osteosarcoma formation

There is strong evidence supporting a role for pRb in osteogenic differentiation and bone formation. A corollary of a role for pRb in osteogenic differentiation is that loss of pRb function will deviate osteoblast function away from the production of normal bone and redirect it towards the production of bone tumors or osteoasrcomas. Supporting this notion is the observation that pRb deletion seems to be a strong causative agent in the formation of osteosarcomas or bone tumors, and osteosarcomas are second only to retinoblastomas in people with inherited mutations in the *RB1* gene (Lueder et al., 1986).

Several observations strongly link pRb to osteogenic differentiation and osteosarcoma. First, the pocket family of proteins, to which pRb belongs together with p107 and p130, are already established regulators of the differentiation of mesenchymal lineages, specifically in chondrogenesis, myogenesis and adipogenesis (Chen et al., 1996; Gu et al., 1993; Novitch et al., 1999). Second, viral oncoproteins that target pRb prevent osteogenesis (Thomas et al., 2001). Third, and perhaps the strongest evidence linking pRb to osteogenic differentiation, is the observation that re-expression of pRb in the pRb-null Saos-2 human osteosarcoma cell line induces a senescence phenotype together with the expression of markers suggestive of bone differentiation (Sellers et al., 1998). These observations prompted several groups of researchers to investigate in more depth the exact mechanisms relating pRb to osteogenic differentiation. Several discoveries resulted from these investigations that further established a connection between pRb and bone. For example, it was shown that expression of HPV16-E7, a Human Papilloma Virus-encoded oncoprotein that binds and inactivates pRb, disrupted osteogenic differentiation (Thomas et al., 2001). In these studies, HPV16-E7 expression abolished most landmarks of osteogenic differentiation. Furthermore, pRb loss was able to abolish most aspects of the BMP-2-induced osteogenic differentiation, suggesting that BMP-2´s osteogenic action requires a functional pRb. Especially impaired by pRb deficiency were matrix mineralization and Osteocalcin expression, suggesting that pRb's intervention occurs in late differentiation inducing the expression of markers of the fully differentiated state and maintaining gene expression patterns associated with terminal differentiation. Interestingly, BMP-2 was still able to induce early markers of differentiation such as Runx2 and Alkaline Phosphatase expression even in the absence of pRb, suggesting a minimal impact of pRb loss the expression of early markers of differentiation. This is consistent with data obtained from human osteosarcoma tumors where Alkaline Phosphatase is expressed even in pRb-null tumor samples, while Osteocalcin is usually reduced or absent (Thomas et al., 2003). When the relevance of pRb for osteogenic

differentiation was further probed, it was discovered that pRb forms a strong association with Runx2 and that pRb/Runx2 complexes bind to osteoblast specific promoters and induce their transcription. Furthermore, pRb was shown to significantly increase Runx2 transactivating capacity (Thomas et al., 2001). Interestingly, Runx2 is still being produced in the absence of pRb, but apparently lacks transactivation capacity. It is important to note, that naturally occurring pRb mutants, some presumed to confer sensitivity to osteosarcoma, are impaired in their capacity to bind and activate Runx2 (Thomas et al., 2003). Taken together, these studies have shown that pRb positively regulates Runx2's activity as a transcription factor. Given pRb's well-established role as a cell cycle repressor, these findings begged the question as to whether pRb's capacity to induce Runx2 activity and osteogenic differentiation is related to its capacity to arrest the cell cycle, or if these two functions are mechanistically distinct. Along these lines, all three pocket proteins were able to induce growth arrest in Saos-2, but only pRb increased activity of Runx2, suggesting that growth arrest per se does not increase Runx2 activity (Thomas et al., 2001). Furthermore, pRb mutants that fail to bind E2F and induce cell cycle arrest were nevertheless able to induce expression of osteoblast markers in Saos-2 cells (Sellers et al., 1998). From these studies it was concluded that although pRb engenders two tumor suppressive functions, one as a cell cycle repressor and the other as an inducer of differentiation, these two can nevertheless be mechanistically dissociated.

It is interesting to note that BMP-2 has been reported to increase the levels of the CKI p21 (Thomas et al., 2003; Yamato et al., 2000). Furthermore, in an *in vitro* model, p27, another CKI, has been observed to increase with osteogenic differentiation, specifically during matrix formation and mineralization stages (Drissi et al., 1999). The mechanisms by which BMP-2 increases p21 await further clarification. However, it is tempting to propose a model in which BMP-2 activates Runx2 function by increasing CKI levels with consequent inactivation of the Cyclin/Cdk complexes that phosphorylate pRb. In such a scenario, active, hypophosphorylated pRb will be able to, first, bind and inactivate E2F thus promoting cell cycle exit, and second, bind to Runx2 and enhance its capacity to initiate osteoblast-specific gene expression. Such a mechanism not only would explain BMP-2's strong osteogenic effect, but would also explain pRb/Runx2 activation in response to BMP-2.

The experiments discussed above in which pRb deletion abrogates predominantly markers of late osteogenic differentiation such as Osteocalcin and matrix mineralization (Thomas et al., 2001) suggest a role for pRb in the latest stages of osteogenic differentiation and in the maintenance of the osteoblast differentiated phenotype. However, the possibility that BMP-2 may activate pRb, and consequently Runx2, by increasing CKI expression, opens the door for an intervention of pRb earlier in osteogenic differentiation, particularly in the earlier stages involving proliferative arrest and commitment to an osteoblastic lineage. Therefore, pRb's strongest influence on osteogenic differentiation could be first during early commitment, and then later during the attainment of the fully mature osteoblastic phenotype, and subsequently for the maintenance of such a state. In fact, recent evidence supports a role for pRb in the earlier commitment stages of osteogenic differentiation. Conditional deletion of pRb in osteoprogenitor cells in mice resulted in an increased pool of mesenchymal progenitor cells in calvaria of pRb-deficient mice. These pRb-deficient progenitors showed clear adipogenic ability with increased multipotency (Calo et al., 2010; Gutierrez et al., 2008), suggesting that pRb loss resulted in an inability to irreversibly enter the osteogenic differentiation pathway. Interestingly, the ossification defects observed in

pRb deficient mice can be suppressed by deletion of E2F1 (Berman et al., 2008), suggesting that the impaired osteogenesis observed upon pRb loss could be a consequence of over-proliferating osteoprogenitors that are unable to undergo irreversible cell cycle withdrawal in order to commit to a specific cell lineage. Therefore, pRb's role in the early commitment stages of osteogenic differentiation clearly depends on pRb's capacity to induce terminal cell cycle withdrawal. On the other hand, pRb's involvement in the induction of the expression of late markers of differentiation such as Osteocalcin, appears to be independent of pRb's capacity to repress cell cycle progression by inactivating E2F, since, as discussed above, pRb mutants that are unable to bind E2F and induce a proliferative arrest are nevertheless capable of inducing the expression of late markers of osteogenic differentiation (Sellers et al., 1998).

Work done in our laboratory has also contributed to the elucidation of pRb's role in osteoblast differentiation and function. We have uncovered a role for pRb as a regulator of osteoblast cell adhesion, and our data suggest that promoting the proper cell-to-cell contacts is another mechanism by which pRb regulates osteoblast differentiation and function. This function could synergize with the previously reported pRb function of enhancing Runx2 mediated transcription of osteoblast-specific genes. Our data show that pRb regulates the expression of a wide repertoire of cell adhesion genes in osteoblasts and also regulates the assembly of adherens junctions, which are membrane-associated complexes involved in cell adhesion (Sosa-García et al., 2010). We generated pRb knock-out mice in which the *RB*1 gene was excised specifically in osteoblasts using the cre-lox P system and found that osteoblasts from pRb knock out mice did not assemble adherens junction at their membranes. pRb depletion in wild type osteoblasts using RNAi also disrupted adherens junctions. Microarrays comparing pRb-expressing and pRb-deficient osteoblasts showed that pRb controls the expression of a number of genes coding for cell adhesion proteins, including cadherins. Furthermore, pRb knock-out mice showed bone abnormalities consistent with osteoblast adhesion defects. Importantly, we found that deleting pRb led to a decrease in the expression of OB-cadherin, which is a cadherin type expressed exclusively in osteoblasts. This decrease in OB-cadherin was accompanied by an increase of comparable magnitude in the expression of N-cadherin, probably compensatory in nature (Sosa-García et al., 2010). Therefore, pRb loss in osteoblasts can lead to a dramatic disarray in the expression of cell adhesion molecules which in turn may negatively affect osteogenic differentiation. Taken together, our data suggest that pRb is required to temporally regulate changes in the expressions of cadherins during osteogenic differentiation, such that expression of specific cadherins is triggered with the right timing during differentiation. pRb loss, by promoting unregulated cadherin expression, could hamper the proper homotypical intercellular contacts, resulting in defective osteoblast differentiation and function with consequent disruption of bone integrity or formation of bone tumors.

5.1 pRb loss in osteosarcomas

Osteosarcomas are relatively rare forms of pediatric cancers, with approximately 1000 new cases diagnosed yearly in the USA (Sandberg and Bridge, 2003). They are, however, a particularly common non-hematologic malignancy in children (Sandberg and Bridge, 2003). Osteosarcomas typically arise in the metaphyseal regions of long bones, within the medullary cavity, and penetrate the cortex of the bone to involve the surrounding soft tissues (Sandberg and Bridge, 2003). The distal femur, proximal tibia, and proximal

humerus represent the three most common sites of tumor formation. It is noteworthy that almost all osteoarcomas are of high grade, are poorly differentiated, and have a poor prognosis, with 10-20% of diagnosed cases having detectable metastases at diagnosis (Dahlin, 1975). Pulmonary metatases are the most common cause of death (Broadhead et al., 2011). Further indicating the aggressive and malignant nature of this tumor type, only approximately 10% of patients with osteosarcomas achieve long-term disease free intervals (Sandberg and Bridge, 2003). Given the important role of pRb for osteogenic differentiation, it is not surprising that there is abundant evidence pointing to pRb loss as a strong causative factor for osteosarcomas. The incidence of osteosarcoma is increased 1000-fold in patients who inherit mutations in the *RB1* gene, relative to the general population (Lueder et al., 1986). Also, pRb loss occurs in about 70% of sporadic osteosarcomas (Araki et al., 1991). Loss of heterozygocity at the *RB1* gene is present in 60-70% of tumors and it has been proposed as a poor prognostic factor in osteosarcomas (Araki et al., 1991). Patients with hereditary retinoblastomas, which as discussed previously arise after homozygous loss of the *RB1* gene, have a high risk of second cancers, 50% of which are osteosarcomas (Lueder et al., 1996). Therefore, the strong association between pRb loss and osteosarcoma formation has been well established. From these observations, and consistent with pRb's role in osteogenic differentiation, it is apparent that bone tissue is particularly sensitive to the loss of pRb's tumor suppressive function. It is important to note that existing data indicate that osteosarcoma tumors display a broad range of genetic and molecular alterations, including the gains, losses, or arrangements of chromosomal regions, which in turn could result in the inactivation of tumor suppressor genes and in the deregulation of major signaling pathways. However, except for p53 and pRb mutations, no consensus changes have been identified in all osteosarcoma tumors (Tang et al., 2008). To determine if pRb and p53 losses are sufficient for osteosarcoma formation, attempts to generate a mouse model of osteosarcomas were done using conditional and transgenic mouse strains to inactivate pRb and p53 specifically in osteoblast precursors (Berman et al., 2008). Consistent with the available tumor data, and suggesting that abrogation of p53 and pRb function suffices to trigger the events associated with osteosarcoma formation, the resulting pRb; p53 double mutant animals, although viable, developed early onset osteosarcomas with complete penetrance. These mice tumors displayed many of the characteristics of their human counterparts, including being highly metastatic (Berman et al., 2008).

Emerging evidence suggests osteosarcoma should be regarded as a differentiation disease, thus establishing a potential link between defective osteogenic differentiation and bone tumorigenesis. Pathologic and molecular features of most osteosarcoma tumors strongly suggest that they may be caused by genetic and epigenetic disruptions of osteoblast differentiation pathways (Haydon et al., 2007). Potential cancer stem cells responsible for osteosarcoma development have yet to be identified, lending further credence to the notion that osteosarcomas may arise from progenitors with impaired differentiation capacity. This view is further supported by the observation that osteosarcoma tumors are comprised of cells that exhibit characteristics of undifferentiated osteoblasts (Haydon et al., 2007; Thomas et al., 2004; Zenmyo et al., 2001). In one study, 81% of osteosarcomas were either poorly differentiated or undifferentiated (Thomas et al., 2004), and the late marker of osteogenic differentiation, Osteocalcin, was undetectable in >75% of osteosarcomas (Hopyan et al., 1999). *In vitro* studies further support this view by showing that terminal osteoblast differentiation, mediated by Runx2 and p27, is disrupted in osteosarcoma (Thomas et al., 2004). The appreciation of osteosarcomas as arising due to

differentiation defects comes as no surprise given that it is now widely recognized that pRb loss is one of its causative factors. By being incapable of irreversibly withdrawing from the cell cycle, osteoprogenitor cells that have undergone pRb loss will continue their proliferation while being unable to initiate their differentiation pathways. In the absence of pRb, Runx2 transactivating capacity will be severely diminished, as it has indeed been shown to occur (Thomas et al., 2001), with the consequent loss of expression of bone markers. This may explain the commonly observed absence of Osteocalcin expression in osteosarcoma tumors, as well as their poorly differentiated state. The increased pool of poorly differentiated and rapidly dividing osteoprogenitors may also be susceptible to additional transforming events that could cooperate with pRb loss to further advance the genesis of osteosarcomas (Gutierrez et al., 2008). In addition, as described above, pRb expression has been shown to be important for the expression of OB-cadherin, which is the cadherin type that is unique to the fully mature and differentiated osteoblast, as well as for the establishment of the cell-to-cell adhesion that is so important for osteoblast differentiation and function (Sosa-García et al., 2010). pRb loss could cause major disruption of cell-to-cell adhesion, and this could in turn promote later stages of tumorigenesis in which osteosarcoma cells spread, invade and colonize other tissues. Therefore, pRb loss facilitates various stages of osteosarcoma formation, from the early disruption of proper lineage commitment with a consequent disruption of osteogenic differentiation, to facilitating later stages of metastasis by disrupting intercellular adhesion.

6. Summary

Over two decades of research on pRb have demonstrated this protein to be a truly potent tumor suppressor. Its potency as a tumor suppressor stems from the fact that it has been implicated in a wide range of cellular process that go way beyond cell cycle repression, and that range from tissue differentiation to intercellular adhesion. Due to the involvement of pRb in such a diverse variety of cellular processes it is only natural that its absence or inactivation, such as is observed in most human cancers, leads to a major disarray in cellular homeostasis. Cells whose physiology is disrupted at many levels by pRb loss are fertile ground for the accumulation of additional genetic alterations, which in turn could cooperate with pRb loss to drive tumorigenesis. pRb's role as a cell cycle repressor is a complement to its role as an inducer of differentiation. By blocking the former and inducing the later, pRb's function may be at the center of the mechanisms that ensure that proliferation and differentiation remain mutually exclusive cellular behaviors. In terms of osteogenic differentiation, pRb function is now recognized as being essential for this process. pRb's roles in osteogenic differentiation are summarized in Figure 2. BMP-2, a potent osteogenic inductor, may exert its osteogenic influence, at least in part, by acting through pRb, specifically by increasing the p21 levels that will in turn block phosphorylation and inactivation of pRb. As described above, increased levels of p27 have also been demonstrated during osteogenic differentiation (Drissi et al., 1999), and this may also contribute to pRb activation by collaborating with p21 in the repression of Cyclin/Cdk complexes. Once active, pRb represses the E2F-mediated expression of proliferation genes, thus leading to the cell cycle arrest necessary for commitment and initiation of differentiation. Concomitantly, excess hypophosphorylated pRb binds and enhances the activity of Runx2, the main transcription factor driving osteoblast-specific gene expression.

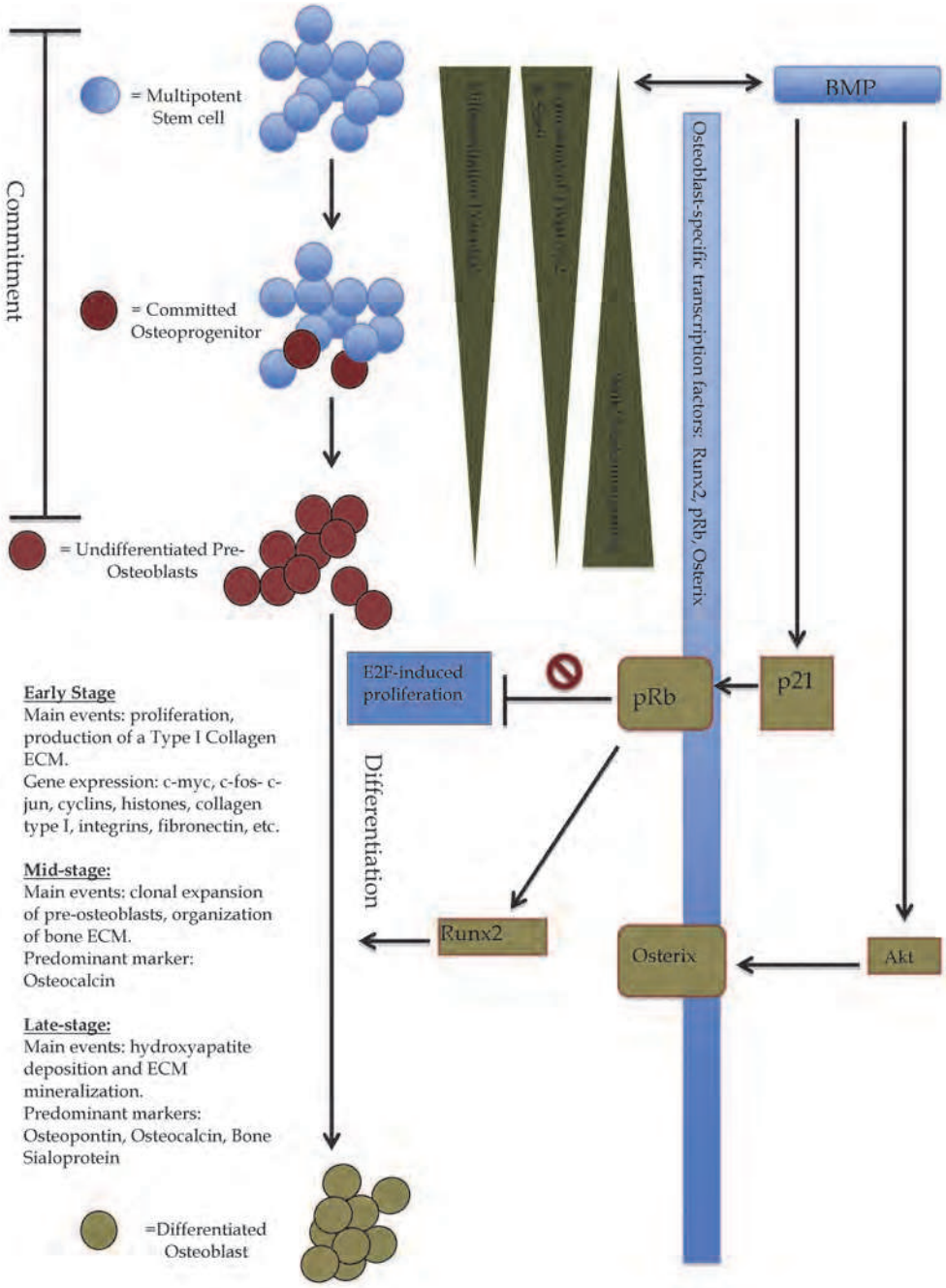

Fig. 2. The process of osteogenic differentiation starting from the commitment of multipotent stem to the osteoblastic lineage and culminating with the production of fully

mature osteoblasts (shown in the figure from top to bottom). Commitment to the osteoblastic lineage results in the progressive restriction of differentiation potential, possibly due to decreased expression of inducers of chondroblastic differentiation (Sox9) together with decrease in repressors of the osteoblastic lineage (Twist-1 and -2). This in turn allows the activation of the Wnt/beta-catenin pathway, which furthers strengthens osteogenic commitment possibly by sensitizing cells to the effect of BMP-2. In addition to synergizing with the Wnt/beta-catenin pathway, BMP-2 leads to increased p21 levels, which by inactivating Cyclin/Cdk complexes, promotes pRb activation. Increased levels of p27 have also been demonstrated (not shown in the figure), and may also contribute to pRb activation by collaborating with p21 in the repression of Cyclin/Cdk complexes. Once active, pRb represses the E2F-mediated expression of proliferation genes, thus leading to the cell cycle arrest necessary for the initiation of differentiation. Concomitantly, hypophosphorylated pRb binds and enhances the activity of Runx2, the main transcription factor driving osteoblast-specific gene expression. Commitment and differentiation are further strengthened by Osterix, which acts downstream of Runx2 and is also activated by BMP-2 acting through Akt. Once cells are irreversibly committed and become growth arrested, differentiation proceeds through the three main stages shown in the figure, each of them characterized by a predominant event and a specific gene expression pattern.

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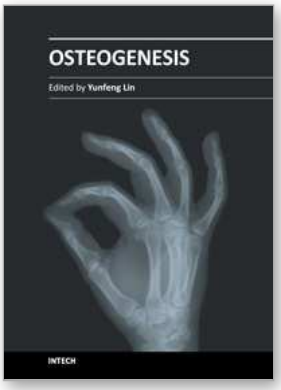

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This book provides an in-depth overview of current knowledge about Osteogenesis, including molecular mechanisms, transcriptional regulators, scaffolds, cell biology, mechanical stimuli, vascularization and osteogenesis related diseases. Hopefully, the publication of this book will help researchers in this field to decide where to focus their future efforts, and provide an overview for surgeons and clinicians who wish to be directed in the developments related to this fascinating subject.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821