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Epigenetic Modifications in Osteogenic Differentiation and Transformation

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Abstract Almost all tumors are characterized by both architectural and cellular abnormalities in differentiation. Osteoblast development is relatively well understood, making osteosarcoma a good model for understanding how tumorigenesis perturbs normal differentiation. We argue that there are two key transition points in normal cellular differentiation that are the focus of oncogenic events, in both of which epigenetic processes are critical. The first is the transition from an uncommitted pluripotent precursor (mesenchymal stem cell) to the 'transit-amplifying compartment' of the osteoblast lineage. This transition, normally exquisitely regulated in space and time, is abnormal in cancer. The second involves termination of lineage expansion, equally tightly regulated under normal circumstances. In cancer, the mechanisms that mandate eventual cessation of cell division are almost universally disrupted. This model predicts that key differentiation genes in bone, such as *RUNX2*, act in an oncogenic fashion to initiate entry into a proliferative phase of cell differentiation, and anti-oncogenically into the post-mitotic state, resulting in ambivalent roles in tumorigenesis. Polycomb genes exemplify epigenetic processes in the stem cell compartment and tumorigenesis, and are implicated in skeletal development in vivo. The epigenetic functions of the retinoblastoma protein, which plays a key role in tumorigenesis in bone, is discussed in the context of terminal cell cycle exit. J. Cell. Biochem. 98: 757–769, 2006.

Key words: epignetic; differentiation; cancer; bone; osteoblast

"*A tumor is an actively growing tissue, composed of cells derived from one that has undergone an abnormal type of irreversible differentiation; its growth is progressive, due to a persistent delay in maturation of stem cells*"
—[Berenblum, 1962].

Tumors almost universally show abnormalities in differentiation, known as anaplasia. Cancer is inherently a metazoan problem, and can only occur in the context of tissue-specific differentiation. Anaplasia occurs at both the *cellular* and *architectural* level, and may be due to cell-intrinsic defects, aberrant instructive microenvironmental cues, or both. Distinguishing cellular from architectural derangement is important when integrating experimental data in vitro with in vivo human and animal observations. Perturbations of differentiation programmes in tumor cells can result in abnormal cellular survival, growth, and proliferation, loss of specialised function and acquisition of the ability to invade surrounding tissues [Hanahan and Weinberg, 2000]. Functionally, anaplasia appears to correlate with more aggressive cancer behavior, which suggests that differentiation confers a restraint on tumorigenesis. Animal models showed that loss-of-function of cancer-related genes, whose function is apparently not cell-type specific, gives rise to highly restricted developmental defects and tumor spectra in vivo. For example, inherited heterozygous mutations in the retinoblastoma tumor suppressor gene (*RB*) in humans leads to a highly restricted range of tumors. In the mouse, germline deletion of *Rb* results in embryonic lethality due to developmental defects in specific tissues. This implies that the specific

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molecular events required for tumorigenesis are frequently contingent on commitment to specific cell lineages. The molecular basis for loss of differentiation in carcinogenesis is not well understood, and probably varies between tissue and cancer types. Importantly, differentiation therapy has already been introduced into the clinic, with benefit, giving hope that study of the relationships between differentiation and tumorigenesis will be of use to cancer patients. In this review, we focus on bone development and cancer as model systems for gaining insights into the relationship between differentiation, development, and tumorigenesis.

BONE AND BONE CANCER

Osteosarcoma is the most common bone sarcoma and the third most common malignancy in children and adolescents. Approximately 2,500 cases are diagnosed per year in the United States. Osteosarcoma is defined by the presence of abnormal bone matrix (osteoid). Over 80% of osteosarcomas are graded histopathologically as poorly differentiated [Dahlin, 1957]. Pathologic classification incorporates degree of differentiation in assessing histologic grade, where the absence of differentiation heralds a 10–15% decrease in 5-year survival (AJCC staging manual, 6th edition). Some aspects of the differentiated phenotype are clearly preserved, and the ability of tumor cells to lay down an aberrant matrix is crucial to a diagnosis of osteosarcoma. Alkaline phosphatase (ALP), an early and non-specific marker of the osteoblast lineage, is frequently seen in bone tumors. By contrast, late markers of osteoblast differentiation, such as osteocalcin, are expressed poorly or not at all in most osteosarcomas [Hopyan et al., 1999]. This suggests that *terminal* differentiation, but not differentiation per se, is antithetical to tumorigenesis. Because the osteoblast differentiation program is well understood, osteosarcoma represents an excellent model for interrogating the relationships between differentiation and transformation.

NORMAL BONE DEVELOPMENT AND DIFFERENTIATION

Bone development is relatively well understood at the cellular and molecular level (reviewed by [Aubin, 1998]). A key feature of bone development is that differentiation is accompanied by a progressive loss of proliferative capacity, as illustrated in Figure 1. There are two key steps in cellular differentiation that appear critical to tumorigenesis. The first key transition point is the step between a mesenchymal stem cell and a lineage-restricted progenitor cell. The osteoblast lineage begins with multi-potent mesenchymal stem cells, located in the periosteal surfaces and within bone marrow stroma. Bone has a vast potential for regeneration from pluripotent mesenchymal stem cells [Aubin, 1998]. It is important to note that the majority of these progenitor cells are thought to be quiescent in the adult skeleton, and quiescence or low proliferative index is a general property used in other systems to identify stem cells (label retention; [Watt, 2001]). Stem cells are activated by tightly regulated and complex signals, whose interactions are beginning to be understood. Following initial lineage commitment, a phase of lineage expansion ensues which culminates normally in permanent cell cycle withdrawal. The initial cell division is asymmetric, giving rise to another stem cell (self-renewal) and a committed osteoprogenitor. Following commitment, the stem cell gives rise to the transit-amplifying compartment [Watt, 2001]. This phase is associated with intensive proliferative activity. The pre-osteoblast is an intermediate stage, which expresses both STRO1, ALP, parathyroid hormone receptor, and type I collagen, and is committed to the osteoblast lineage with extensive replicative capacity, but no self-renewal capacity [Gronthos et al., 1999]. In vitro, the use of agents such as retinoic acid can induce further differentiation in the pre-osteoblast. The mature osteoblast expresses ALP, osteopontin, bone sialoprotein, and osteocalcin, and lies adjacent to newly synthesized osteoid. This stage, which is responsible for the laying down of bone, has limited replicative potential [Stein et al., 1996]. The cumulative effect of the recruitment of stem cells and their expansion, and the functional capacity of mature osteoblasts, is measured by rates of bone formation in vivo. The second key step initiates terminal differentiation and permanent cell cycle withdrawal. The terminal stage of the bone lineage is the post-mitotic osteocyte, often found isolated within bone, presumably embedded within advancing osteoid. As an alternate fate, a proportion of cells in the transient amplifying compartment may also terminate in apoptosis.

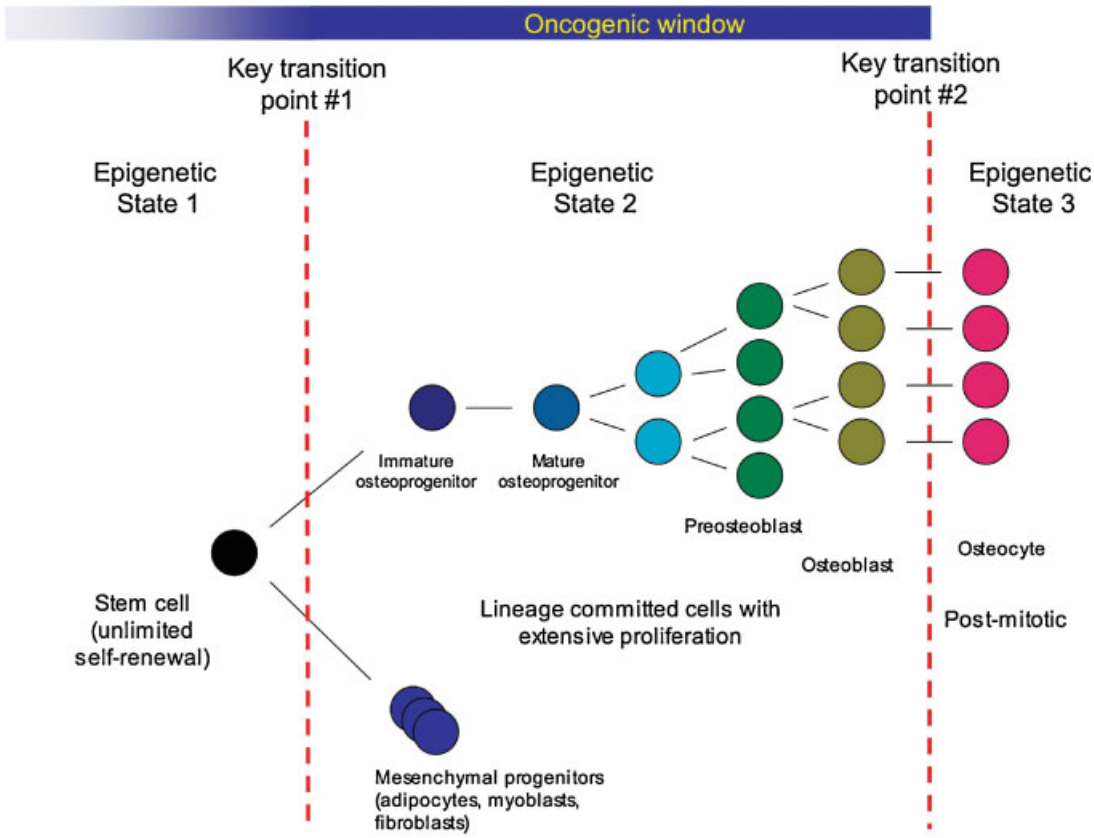

Fig. 1. Model of osteoblast differentiation. There are three compartments (stem cell, transit-amplifying compartment, and terminal differentiation) and two key transitions (see text for details). The oncogenic window refers to those stages of differentiation that are permissive for tumorigenic events. The transition between the stem cell and transit-amplifying compartment is notionally reversible, at least under tumorigenic circumstances, whereas the transition between the transit-amplifying compartment and the terminally differentiated compartment is not. Each stage is characterized by epigenetic templates that are established at the transitions. Evidence for this model is presented in the text.

The function of persisting terminally differentiated cells is not well understood.

In many systems, such as muscle and neural tissue, cellular differentiation correlates closely with cellular contribution to organ development, such that terminally differentiated cells are those that contribute to tissue function. But this may not be the case in bone. Two lines of evidence support the idea that bone formation may be a function of cells within the transit-amplifying compartment rather than the post-mitotic compartment. First, increased proliferation of transit-amplifying cells and decreased egress from the transit-amplifying compartment lead to increased bone mass. For example, inactivation of the Wnt co-receptor, LRP5, leads to loss of bone mass in vivo and decreased proliferation of the transient amplifying compartment in bone [Westendorf et al., 2004]. More importantly, in vivo loss of $p27^{KIP1}$, leading to defective terminal cell cycle egress in osteoblasts, also is associated with an increase in bone mass and expansion of osteoid in vivo [Thomas et al., 2004] and in vitro [Drissi et al., 1999]. Second, the canonical late marker of terminal differentiation, osteocalcin, appears to negatively regulate skeletal mass [Ducy et al., 1996], suggesting that the terminal phase of osteoblast differentiation may function to set boundaries on the anabolic phase of bone formation, rather than directly contribute to bone formation. Thus, inactivation of mechanisms that are critical to terminal cell cycle exit may paradoxically increase bone mass in vivo.

The process of bone formation is therefore critically dependent on coordination of proliferation and differentiation (for review see [Stein et al., 1996]). Conceptually, tumorigenesis probably involves disturbances of mechanisms that appropriately constrain the initiation of proliferation by tumor stem cells, or that allow persistent expression of stem cell-like

features in apparently partially committed cells. The similarities between stem cell properties and those of transformed cells are striking. Both cell types possess unlimited self-renewal, express telomerase, and are undifferentiated as defined by the absence of lineage-restricted markers (reviewed in [Sharpless and DePinho, 2004]). These features raise the possibility that mechanisms that maintain 'stemness' are important to tumorigenesis (disruption of the first transition point in Fig. 1). Accumulating evidence suggests that similar signaling pathways, including those relevant to bone, contribute to the regulation of self-renewal in progenitor and tumor cells [Reya and Clevers, 2005]. There is also good evidence (summarized below) that cancers are characterized by disruption of mechanisms that enforce terminal cell cycle exit (the second key transition point in Fig. 1).

The notion that both lineage commitment and terminal differentiation are both key stages in tumorigenesis has several significant implications. First, it suggests that transcriptional regulators of differentiation may play ambivalent roles in tumorigenesis, since they preside initially over lineage commitment and expansion, but later over terminal aspects of differentiation. Second, there are probably both cell intrinsic and extrinsic mechanisms involved in regulating both key transition points, which may be perturbed in cancers. In particular, the growing recognition that non-cell autonomous mechanisms are critical to tumor formation has led to speculation about the key role of epigenetic (mutation-independent) processes in establishing heritable, stem cell-like states in tumorigenesis. There is also evidence that epigenetic mechanisms are important at the second transition point to the terminally differentiated state. These aspects are discussed in detail in this review, with emphasis on bone development and transformation where data is available.

TRANSCRIPTIONAL REGULATION OF OSTEOBLAST DIFFERENTIATION AND TRANSFORMATION

While the signaling pathways that initiate recruitment of stem cells towards a specific lineage are still to be fully characterized, the transcriptional mediators regulating osteogenic differentiation have recently been mapped in some detail [Ducy, 2000]. Critical among these is runx2 (CBFA1/Osf2/PEBP2a), a key transcriptional regulator of osteoblast differentiation, belonging to the runt family of transcription factors [Ogawa, 1993]. Mice nullizygous for *runx2* exhibit a complete lack of ossification [Komori et al., 1997], while runx2 postnatally regulates expression of bone-specific genes such as osteopontin and osteocalcin, and controls bone matrix deposition [Ducy et al., 1999]. Notably, runx2 activity is required for expression of genes, such as osteocalcin, associated with terminal differentiation. Runx2 function is modified dramatically by the co-operating and antagonistic actions of other proteins. This is a rapidly expanding field of study. CBFβ, a heterodimeric partner, is required for full activity, but other co-regulators include TLE2, a groucho family member; MAPK; TGFβ through direct interactions with SMAD3; and LEF1 (reviewed by [Lian et al., 2004]). Transcriptional splicing events give rise to functionally distinct isoforms [Harada et al., 1999]. Downstream effectors of runx2 function, such as osterix, are critical to bone development [Nakashima et al., 2002]. Taken together, these data suggest that the transcriptional activity of runx2 depends critically on transcriptional splice patterns, co-activators and -repressors, post-translational modifications, and the integrity of downstream effectors.

There is good evidence in vivo to suggest that the runt family appears to function as a tumor suppressor in hematologic cancers [Lund and Van Lohuizen, 2002]. *RUNX1* (*AML1*) is mutated in human leukemia, and mice expressing loss-of-function runx1 mutants are prone to leukemia [Perry et al., 2002]. CBFβ, the heterodimeric partner of runx proteins, is frequently the subject of translocation events in leukemias (reviewed by [Ito, 2004]). *RUNX3* has been reported to be subject to genomic deletion or promoter hypermethylation in gastric cancers [Li et al., 2004]. The evidence for runx2 is more circumstantial, and is based on in vitro data, suggesting that runx2 expression varies with cell cycle status, inhibits osteoblast proliferation, and promotes terminal differentiation [Pratap et al., 2003; Thomas et al., 2004; Galindo et al., 2005]. Further evidence comes from data demonstrating that the retinoblastoma tumor suppressor protein (pRb) co-operates with runx2 to promote differentiation.

The cell cycle regulatory pathway centered around pRb is inactivated in almost all human cancers, but individual tumor types seem to target specific components to achieve this effect (reviewed by [Korenjak and Brehm, 2005]). pRb itself is frequently somatically inactivated in osteosarcomas [Horowitz et al., 1990], while inherited heterozygous loss of the *RB* gene confers a 500-fold greater incidence of osteosarcoma than the general population [Abramson et al., 1984]. The Rb pocket proteins family play roles in mesenchymal differentiation [Korenjak and Brehm, 2005], and several lines of evidence implicate pRb in osteogenesis (reviewed by [Thomas et al., 2003]). pRb co-activates runx2, through direct physical interactions at sites of active transcription, and loss of function of pRb attenuates terminal osteoblast differentiation in vitro [Thomas et al., 2001]. Conversely, runx2 co-ordinates terminal cell cycle exit through induction of the CDK2 inhibitor, $p27^{KIP1}$, which in turn is required for normal bone development in vitro and in vivo, and is lost in de-differentiated human osteosarcomas [Thomas et al., 2004]. More recently, Benevolenskaya et al. [2005] showed that Rbp2 may function as a repressor of runx2-dependent transcription, and that pRb acts to displace Rbp2 from osteoblast-specific promoters thereby activating differentiation. Interestingly, pRb is likely to influence osteoblast differentiation through additional mechanisms involving chromatin structure (see below). These data suggest that runx2 may function in pathways that hinder tumorigenesis.

However, there is considerable debate regarding the role of runx2 in cancer [Blyth et al., 2005]. Under some circumstances, runx2 appears to act as an oncogene. Runx2 co-operates with Myc to cause lymphomas in mice based on proviral insertion sites studies (e.g., [Vaillant et al., 1999]). The same group reported that ectopic expression of runx2 resulted in transformation of p53-null fibroblasts [Wotton et al., 2004], and more intriguingly, that runx1 acts as a dominant oncogene in T-cell lymphoma [Wotton et al., 2002]. Amongst other possible interpretations, it is intriguing to postulate that stage-specific signals may cause runx proteins to act under different circumstances in both an oncogenic and tumor suppressor role. This is consistent with an oncogenic role for runx2 in the expansion, and a tumor suppressor role in terminal differentiation phases of osteoblast ontogeny, which might account for the absence of mutations in *RUNX2* itself. Thus, the effect of *RUNX2* expression may depend on the differentiation stage in which it functions.

THE ONCOGENIC WINDOW

Several studies have observed that oncogenic effects depend on the differentiation stage in which the oncogene is activated (reviewed by [Weinstein, 2002]). Of particular relevance to this review, this point was confirmed in an elegant conditional transgenic model of *MYC*-induced osteosarcomas [Jain et al., 2002]. Brief inactivation of *MYC* resulted in sustained regression of the tumors, with differentiation of tumor cells into mature osteocytes. This observation alone is, perhaps, unsurprising. However, Jain et al. [2002] went on to show that re-activation of *MYC* did not restore tumorigenic properties, but rather induced apoptosis. These observations were interpreted to suggest that brief inactivation of *MYC* 'appears to cause epigenetic changes in tumor cells that render them insensitive to *MYC*-induced tumorigenesis'. These studies support the notion that terminally differentiated cells lie outside an 'oncogenic window,' and thus are not permissive for tumorigenesis. The role of epigenetic processes is discussed in more detail below.

There is some debate as to whether the oncogenic window includes the stem cell compartment, the transit-amplifying compartment, or both [Huntly and Gilliland, 2005]. Cancer cells share the proliferative characteristics of the transit-amplifying compartment, and the capacity for indefinite self-renewal of stem cells. Whether cancers arise as a consequence of mutational or epigenetic events in transit-amplifying cells that confer immortality; or whether such events disrupt the precise constraints on stem cell proliferation prior to true lineage commitment, is open to question. It has been argued that cancer-initiating cells arise in the stem cell compartment, perhaps by epigenetic mechanisms (reviewed by [Feinberg et al., 2006]). The cancer stem cell theory posits that a sub-population of cells within each cancer possess 'cancer initiating properties,' analogous to the physiologic ability of stem cells to fully reconstitute organ development (reviewed by [Huntly and Gilliland, 2005]). This property, along with infinite self-renewal and multi-potentiality, is the defining characteristic

of a true stem cell. Stem cells would appear to lie within the oncogenic window, and the identification of the *BCR-ABL* fusion gene in all hemopoietic lineages in chronic myeloid leukemia supports this notion [Huntly and Gilliland, 2005].

In osteosarcomas, it has been suggested that a sub-population of multi-potent cancer stem cells without lineage commitment gives rise to aberrantly differentiated osteoblastic progeny [Gibbs et al., 2005]. In this study, a population of multi-potent osteosarcoma-derived cells were identified which express stem cell markers, as well as markers of ectoderm and endoderm, as well as mesodermal gene expression. It is not clear whether the same cells express non-mesodermal genes and mesodermal genes, nor which population possesses cancer-initiating properties [Gibbs et al., 2005]. Clinically, there are uncommon primary sarcomas of bone that lack lineage-restricted features, and are designated malignant fibrous histiocytoma of bone [WHO, 2002], while other tumors commonly contain regions with chondroblastic, osteoblastic and fibroblastic differentiation, suggesting that the cell of origin retains some degree of multi-potentiality. These observations suggest that the cancer-initiating cell has stem cell-like characteristics, including multi-potentiality.

However, we favor the notion that osteosarcomas can arise in a transit-amplifying compartment, perhaps in addition to the stem cell compartment. Clonal cell lines derived from primary osteosarcomas, which presumably fulfill the criteria for cancer stem cells, express osteoblastic markers such as ALP in every cell [Thomas and Kansara, unpublished data]. This suggests that, at least for a subset of osteosarcomas, the cancer-initiating cell shares features of a committed osteoprogenitor. Additionally, it has been argued that a relationship exists between proliferative rate and the acquisition of mutagenic events [Cohen and Ellwein, 1991]. This may favor the accumulation of oncogenic events in the transit-amplifying compartment, while stem cells are typically quiescent. In support of this concept, osteosarcomas are most frequently observed in adolescence, a stage of intensive skeletal growth entailing increased osteoblast activity. Furthermore, Paget's disease of bone, a benign condition characterized by dramatically increased bone formation and resorption, is also associated with an increased risk of osteosarcoma. These data suggest that increased osteoblastic activity is associated with tumorigenesis. Third, stem cells are up to 100-fold more resistant to mutagenic events than somatic cells, consistent with the necessity for conserving the genetic code in cells that give rise to multiple tissues [Cervantes et al., 2002]. This resistance appears in part due to enhanced apoptotic responses to genotoxic stress and DNA damage [Hong and Stambrook, 2004; Saretzki et al., 2004]. The efficiency of such processes appears inversely related to degree of differentiation. Thus, it may be that genes that initiate lineage commitment and expansion, under the influence of genes such as *RUNX2*, create conditions favoring the acquisition of tumorigenic events.

NON-CELL AUTONOMOUS REGULATION OF OSTEOBLAST DIFFERENTIATION AND TRANSFORMATION

Carcinogenesis may be a process akin to development gone awry (reviewed by [Weaver and Gilbert, 2004]), and non-cell autonomous (microenvironmental) temporospatial cues are critical to normal development. There is growing recognition of the central importance of microenvironmental context in driving tumor formation, illustrated strikingly by a recent study in which nitrosomethylurea (NMU)-treatment of cleared mammary fat pads in vivo was sufficient for the development of epithelial neoplasia, while NMU treatment of epithelial cells alone did not result in tumors [Maffini et al., 2004]. This experiment implies a dominant role for microenvironmental processes in regulating tumor formation, at least in a murine mammary tumor model. In many cases, oncogenic effects of signaling molecules may be related to physiologic functions in maintenance or expansion of stem cell compartments. Examples of secreted signaling molecules implicated in bone development and cancer include the Wnts and related proteins, hedgehogs, notch, transforming growth factor-β, parathyroid hormone-related protein, and receptor activator of NFkB ligand. The Wnt pathway illustrates the potential role of environmental cues in determining the balance between differentiation and proliferation. Wnt signaling is critical to development, stem cell biology and tumorigenesis [Reya and Clevers, 2005], and is also to bone development [Westendorf et al., 2004]. Wnts stimulate expansion of the transit-amplifying

compartment. Mice lacking the Wnt co-receptor Lrp5 have decreased bone mass and decreased osteoblast proliferation [Kato et al., 2002]. In humans, gain-of-function mutations in *LRP5* lead to increased bone mass [Boyden et al., 2002], while loss-of-function mutations have been the cause of osteoporosis-pseudoglioma syndrome [Gong et al., 2001]. During osteoblast differentiation, Wnt signaling contributes to expansion of the transit-amplifying compartment, but is followed by expression of negative regulators of Wnt signaling. For example, Wnt inhibitory factor 1 and SFRP2 are both expressed at high levels in association with osteoblast differentiation in vitro, concomitant with expression of late markers of the osteoblast lineage such as osteocalcin [Vaes et al., 2005]. Thus, Wnt signaling may act as one example of a molecular switch integrating proliferation and differentiation in the osteoblast lineage, and could therefore determine the oncogenic or tumor suppressor activity of runx2.

An interesting implication of the importance of microenvironmental signals in tumor progression is that it predicts a key role for epigenetic processes [Feinberg et al., 2006]. Discussed in greater detail below, epigenetic mechanisms are those which result in heritable changes in gene expression without changes in gene sequence, in contrast to genetic mechanisms, which are based on sequence alterations. It is notable that, unlike genetic events, epigenetic events are under some circumstances reversible. The clonogenic effect of microenvironmental signals requires a mechanism for 'fixing' the fate choices induced by those signals, such that all progeny of the recipient progenitor cell express the tumorigenic programme. It is unlikely that aberrant microenvironmental signals act by inducing genetic changes in the recipient tumor progenitor. Epigenetic patterning, however, provides an excellent mechanism for transmission of accumulated oncogenic signals in a clonal fashion to all daughter cells. In effect, the tumorigenic consequences of microenvironmental signals are likely to be the abnormal application of epigenetic templates that maintain cellular states favoring tumor formation. This interpretation is supported by the NMU-mutagenesis studies described earlier, in which microenvironmental signals are critical to the emergence of the epithelial tumor clone.

There is additional evidence to suggest that epigenetic events are dominant over mutational events, based on the reversibility of both loss of differentiation and tumor phenotype. This evidence comes from studies of the contribution of cancer-derived genomes to embryonic development following nuclear transfer [Blelloch et al., 2004; Hochedlinger et al., 2004] and blastocyst injection [Mintz and Illmensee, 1975]. Nuclei derived from a wide range of cancer cells were able to support normal pre-implantation development to the blastocyst stage at frequencies between 0% and 12% [Hochedlinger et al., 2004]. Nuclei derived from doxycycline-inducible *ras+/ink4a−/−* melanoma, and fibroblasts were used to generate chimeric mice. While these mice developed cancers with higher penetrance, shorter latency, and an expanded spectrum compared to the donor mice, the ES cells supported differentiation into multiple lineages, including melanocytes, lymphocytes, and fibroblasts. The tumors derived from ES-cell derived chimeras on activated alleles of *RAS* carried identical genomic profiles compared to the donor tumor. Embryonal carcinoma cell nuclei were also used to reconstitute a broad range of mature neuroepithelium, epithelium, and mesothelial tissues [Blelloch et al., 2004]. It appears that, just as microenvironmental signals can establish pro-oncogenic epigenetic templates, under different circumstances microenvironmental signals can revert tumorigenic epigenetic templates. It is difficult to account for such observations if mutations are the basis for tumorigenicity and loss of developmental potential in tumor cells.

EPIGENETIC PROCESSES

The eukaryotic genome is vastly more complex than that of prokaryotes. In addition to sequence-dependent determinants of functional specificity, which include regulatory sequences and their complementary *trans* factors, non-sequence dependent (epigenetic) mechanisms have evolved to effectively restrict the available genome that is accessible to transactivation. This is logical and elegant in metazoan structures, given that lineage commitment during development means that although stem cells must carry the entire human genome, cells within individual tissues are required to express only a fraction of all genes. Epigenetic processes contribute to development, differentiation, aging, carcinogenesis, and autoimmunity (reviewed in [Strathdee et al., 2004]). Epigenetic

processes may take several forms. One form of epigenetic silencing involves methylation of the C5 position of cytosine bases, usually in the context of cytosine-phospho-guanine dinucleotide pairs, which are often found in clusters called CpG islands located at the promoter regions of about 50% of human genes. Methylation of CpG islands causes stable heritable transcriptional silencing through binding of methyl-DNA-specific proteins to affected CpG islands attracts histone-modifying enzymes, which focally establish a silenced chromatin state. A second form of epigenetic regulation of gene expression affects chromatin structure through covalent modification of histone proteins. This field of biology is rapidly evolving, with the recognition that acetylation and methylation of nucleotides and histones results in the establishment of chromatin structures that constrain transcriptional competence. Recent data suggest that processes involved in histone modification may also control DNA methylation, linking diverse mechanisms of epigenetic regulation [Vire et al., 2005].

A great deal of evidence supports the idea that DNA methylation patterns are essential for normal development, cellular differentiation, and tumorigenesis (reviewed by [Jones and Laird, 1999; Arney and Fisher, 2004]). In bone, reduced CpG methylation has been shown to be associated with transcriptional activation of the bone-specific rat osteocalcin gene in osteoblasts [Villagra et al., 2002]. While overall methylation is decreased in cancer, ~1% of genes are newly silenced by promoter methylation [Costello et al., 2000]. Aberrant de novo methylation of CpG islands is found early during carcinogenesis. Interestingly, the number of cancer related genes affected by epigenetic inactivation may exceed the number inactivated by mutation (reviewed by [Jones and Baylin, 2002]). Some examples of silencing by DNA promoter methylation include $p16^{INK4A}$, *p73*, *MLH1*, *BRCA1*, *E-cadherin*, *APC*, and *VHL*. Interestingly, methylation changes in cancer cells are not limited to hypermethylation. A small group of genes, including *C-JUN*, *C-MYC*, and *TCLI*, may become hypomethylated and reactivated in the course of tumor progression [Yuille et al., 2001]. Of relevance to osteosarcoma, the *RB* gene has been shown to be silenced by promoter methylation [Sakai et al., 1991]. Interestingly, many secreted inhibitors of Wnt signaling, including SFRPs and WIF1, have been shown to be silenced by promoter methylation in human cancer [Suzuki et al., 2002].

EPIGENETICS AND THE TRANSITION BETWEEN STEM CELL AND TRANSIT-AMPLIFYING COMPARTMENT

As noted earlier, epigenetic mechanisms have been proposed to be critical to disruption of the first transition point from stem cell to transit-amplifying compartment [Feinberg et al., 2006]. Evidence of the importance of epigenetic processes in tumorigenesis and development comes from recent studies of the polycomb group (PcG) of proteins (reviewed by [Valk-Lingbeek et al., 2004]). PcG proteins play critical roles in assignment of epigenetic states, through histone modification. Three PcG complexes have been characterized to date. The first, polycomb repressive group 2 (PRC2) initiates silencing through methylation of lysine residues on histones H3 and H1. The second, PRC1, is involved in maintenance of stable states of gene repression, in part by mechanisms that recognize methylated lysines on histone H3. The third complex, PRC3, was identified recently and targets specific lysine residues (K27 on H3 and K26 on H1) via Eed proteins [Kuzmichev et al., 2005]. The developmental importance of these complexes is indicated by early embryonic lethality in mice with loss of function of components of polycomb complexes.

The polycomb family of proteins appear to specifically regulate the transition from the stem cell compartment to the transit-amplifying compartment following lineage commitment. This is exemplified by BMI-1, a key component of PRC2. *Bmi-1*-deficient mice manifest defects in hemopoietic and neuronal development, consistent with a requirement of Bmi-1 in maintaining the activity of stem cells in these compartments [van der Lugt et al., 1994]. Moreover, Bmi-1 is in turn regulated by Sonic hedgehog, a key morphogen in skeletal development. Enhancer of zeste (Ezh2), a component of PRC2, is required for blastocyt development and the generation of embryonic stem cell lines [O'Carroll et al., 2001], implying a role in early embryonic stem cell function. For example, Ezh2, the PRC1 component Rnf2, and Mph1/Rae28 are highly expressed early embryonic development [Valk-Lingbeek et al., 2004]. These data collectively suggest that polycomb

genes affect epigenetic processes important to maintenance of the stem cell phenotype.

There is considerable evidence that polycomb genes play key roles in the biology of cancer stem cells. Bmi-1 acts as an oncogene in vivo causing B- or T-cell leukemia [van Lohuizen et al., 1991], and is overexpressed in a variety of human cancers [Vonlanthen et al., 2001]. Bmi-1 may act in part by stimulating proliferation by repression of the Ink4a/Arf locus [Jacobs et al., 1999], although the developmental defects in Bmi-1 deficient mice are only partially rescued by loss of the Ink4a/Arf locus. Among other PcG proteins, Ezh2 is overexpressed in prostate cancer, where it plays a role in proliferation [Varambally et al., 2002], although probably not through Arf [Bracken et al., 2003]. Cbx7, a novel PcG protein, was identified in a screen to identify genes that bypass replicative senescence [Gil et al., 2004]. Downregulation of Cbx7 resulted in increased expression of Ink4a and Arf, and is highly expressed in prostate cancers.

Where does all of this fit into bone and bone cancers? Mice deficient in Bmi-1, Mel-18, Rae28, Ring1A, and M33 polycomb genes manifest disturbances involving the antero-posterior aspect of the skeleton [Valk-Lingbeek et al., 2004]. Mice lacking both Bmi-1 and M33 demonstrated abnormal Hox gene expression and complex abnormalities of skeletal patterning [Bel et al., 1998]. Array data suggest that the expression levels of Ezh2 mRNA in bone and bone marrow are amongst the highest in the adult mouse [Su et al., 2004], but there is little data available specifically interrogating expression of polycomb genes in bone, especially in man. Given the impact of polycomb genes on bone development, there is scant information regarding the role of polycomb genes in osteosarcoma. A recent study suggested that ectopic expression of Bmi-1 in mesenchymal stem cells resulted in immortalization, interestingly without loss of differentiation [Takeda et al., 2004]. A recent study suggested that epigenetic processes assign stem cell-like or differentiated properties in primary cultures of osteosarcoma cells [Gibbs et al., 2005].

EPIGENETICS OF THE TRANSITION BETWEEN THE TRANSIT-AMPLIFYING COMPARTMENT AND TERMINAL DIFFERENTIATION

At the other end of the differentiation program, epigenetic processes are also at work. Evidence for this comes from recent data indicating that pRb has a key role in establishment of chromatin structures in senescence, in a manner reminiscent of polycomb functions. At the second key transition point, terminal differentiation is characterized by irreversible cell cycle withdrawal, a feature shared by senescence. Senescence was originally described as a phenomenon observed in long-term culture of primary cells, in which the cells entered an irreversible, non-proliferating state after a variable number of population doublings ex vivo (the Hayflick limit) [Hayflick and Moorhead, 1961]. Senescence may also function as a tumor suppressor response, analogous to apoptosis. The introduction of oncogenic alleles of ras provokes a senescent state that depends on intact functioning of the retinoblastoma and p53 pathways [Serrano et al., 1997]. The re-expression of pRb in osteosarcoma cell lines provoked both aspects of terminal differentiation, but also senescence (reviewed by [Thomas et al., 2003]). In addition to evidence of a role in histone modification at specific promoters mediated by involvement in multi-protein histone deacetylating complexes, recent evidence suggests that pRb may play a role in establishing epigenetic patterning of the genome. Senescence-like states are associated with the establishment of 'senescence-associated heterochromatic foci,' which coincides with the recruitment of heterochromatin proteins and pRb to E2F-responsive promoters [Narita et al., 2003]. Interestingly, these foci depend upon intact functioning of the pRb pathway, and are not seen in reversibly arrested cells in human fibroblasts. More recently, pRb was shown to control histone methyltransferase activity by direct interactions with SUV4-20H1 and SUV4-20H2, which methylate lysine 20 on Histone H4 [Gonzalo et al., 2005]. The trimethylation of Histone H4 is associated with pericentric and telomeric heterochromatin. In cells lacking pRb (alone or in combination with other pocket proteins), there is a decrease in global cytosine methylation, an increase in Histone H1 and 3 acetylation, and a decrease in Histone H4 methylation. Gonzalo et al. [2005] postulate that pRb is involved in maintaining overall chromatin structure and in particular constitutive heterochromatin, which leads to genomic instability and aneuploidy. It is notable that osteosarcomas are characteristically aneuploid tumors. More controversially, some evidence

has been presented that pRb may interact with the polycomb pathway, either directly [Dahiya et al., 2001], or by regulation of polycomb proteins such as EZH2 [Bracken et al., 2003].

Unlike stem cell recruitment, it is not clear whether the epigenetic patterning associated with senescence/terminal differentiation is regulated by extrinsic signals. Two possibilities exist. First, it may be that commitment to any cell lineage involves the automatic acquisition of 'mortality.' One mechanism may be the loss of expression of stem cell functions that maintain 'immortality,' such as the expression of telomerase. The telomere 'clock' starts counting from the moment of lineage commitment, resulting after a variable number of passages in mandatory terminal cell cycle exit in terminal differentiation [Reddel, 1998]. The second possibility is that extrinsic signals are either required for, or contribute to, terminal cell cycle exit. If true, contextual signals may be sufficient to initiate terminal differentiation/senescence, an interesting subject currently little studied. The 'culture shock' theory of in vitro senescence is consistent with this model [Sherr and DePinho, 2000]. Induction of senescence in primary cell cultures is, in part, dependent on culture conditions, changes in which can result in replicative senescence at between 15 and 88 passages [Gospodarowicz et al., 1981]. In vitro, these conditions include the presence of serum, extracellular matrix, and growth factors, but it is not understood whether or how these factors might operate in vivo to regulate either development or tumorigenesis.

CONCLUSIONS

The crucial role of epigenetic events in both development and tumorigenesis is clear. In bone, there is remarkably little data on the role of epigenetic processes in development or tumorigenesis, despite detailed knowledge of transcriptional regulation of osteoblast differentiation. The ability of tumor nuclei to recapitulate normal development suggests that epigenetic programming may in some cases be sufficient for tumorigenesis, and more importantly, be reparable in response to external signals. It may be possible to manipulate such signals therapeutically. The use of all-trans retinoic acid in acute promyelocytic leukemia represents proof of principle that exploiting non-cell autonomous signals to effect changes in differentiation state is a reasonable strategy.

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