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Bone Stem Cells

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Abstract Osteoblasts are the skeletal cells responsible for synthesis, deposition, and mineralization of the extracellular matrix of bone. By mechanisms that are only beginning to be understood, stem and primitive osteoprogenitors and related mesenchymal precursors arise in the embryo and at least some appear to persist in the adult organism, where they contribute to replacement of osteoblasts in bone turnover and in fracture healing. In this paper, the nature of these cells, whether they constitute a stem cell pool or a committed progenitor pool, and aspects of their apparent plasticity are discussed. Current understanding of differential expression of osteoblast-associated genes during osteoprogenitor proliferation and differentiation to mature matrix synthesizing osteoblasts is summarized. Finally, evidence is discussed that supports the hypothesis that the mature osteoblast phenotype is heterogeneous with subpopulations of osteoblasts expressing only subsets of the known osteoblast markers, raising also the possibility of multiple parallel differentiation pathways and perhaps even different progenitor pools. J. Cell. Biochem. Suppls. 30/31:73–82, 1998.
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Bone formation takes place in the organism during embryonic development, growth, remodeling, fracture repair, and when induced experimentally, e.g., by the implantation of decalcified bone matrix or purified or recombinant members of the bone morphogenetic protein family. This suggests there is a large reservoir of cells in the body capable of osteogenesis throughout life, and the issues addressed in this paper are the nature of these cells, whether they constitute a stem cell pool or a committed progenitor pool, whether there are different stages of committed progenitors recognizable, and identification of transitional steps from stem cell to committed osteoprogenitor to osteoblast.

Osteoblast Ontogeny

Mesenchymal stem cells and multipotential and restricted progenitors. Marrow stroma is clearly highly regenerative, as seen in studies in rodents and humans after high doses of chemotherapy, radiation, or marrow ablation. Friedenstein [1990] first showed that bone marrow stroma contains cells which have the capacity to form bone when transplanted in vivo in diffusion chambers; subsequently, he and others demonstrated that, in addition to bone, cartilage, marrow adipocytes, and fibrous tissue also formed in vivo and that all the tissues could arise from single colonies or CFU-F [summarized in Owen, 1998]. The in vivo analyses of stromal cells has been augmented by functional assays in vitro that show formation of a range of differentiated cell phenotypes. However, whether marrow stroma contains a stem cell—by the definition of self-renewal capacity and ability to repopulate all the appropriate differentiated lineages or even by somewhat less stringent criteria [Morrison et al., 1997]—has still not been rigorously proved, although many in the field have adopted the nomenclature that stromal populations are mesenchymal stem cells [e.g., see Bruder et al., 1998 and references therein], without confirmation of the clonal or single cell origin of the putative stem cell giving rise to the multiple lineages or that the cells exhibit genuine stem cell properties. It is clear that CFU-F are heterogeneous in size, morphology, and potential for differentiation, consistent with the view that they do belong to

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a lineage hierarchy in which only some of the cells are primitive and perhaps multipotential stem or progenitors while others are more restricted [Friedenstein, 1990; Owen, 1998]. This is also consistent with data that shows that only a proportion of CFU-F are CFU-alkaline phosphatase (CFU-AP) and further that only a proportion of these are CFU-osteogenic (CFU-O, clonogenic bone colonies or nodules) [Aubin, 1998] (see below). In many reported experiments, while CFU-F form, the plating densities have been such that colonies clearly merge with and overlap with other CFU-F calling into question their clonality. As recently summarized elsewhere [Owen, 1998], the case for a stromal stem cell rests largely on the data that the cell progeny from a single stromal cell are able to give a full spectrum of stromal cell types in the in vivo open transplant assay. Critical issues of self-renewal, the clonality of progeny, the ratios of stem to other more restricted progenitors in various stromal populations, and the identifiable commitment and restriction points in the stromal cell hierarchy have not been satisfactorily met. This may become increasingly important as work on stromal populations increases in intensity based on the proposed utility of the populations for tissue regeneration and vehicles for gene therapy.

More definitive evidence for the existence of multipotential mesenchymal progenitor or stem cells has been obtained by analysis of the in vitro differentiation outcomes of clonally derived immortalized (e.g., via large T antigen expression) or spontaneously immortalized cell lines derived from stroma, bone, or other mesenchymal/mesodermal tissues, such as the mouse embryonic fibroblast line C3H10T1/2, the rat calvaria-derived cell lines RCJ3.1 and ROB-C26, and the mesodermally-derived C1 line. While none of these lines behaves exactly alike or necessarily responds comparably to various inducers or regulators, and the self-renewal aspect of the "stemness" of the cells cannot be rigorously assessed, they do have some common features, including the explicit demonstration that a clonal population is capable of giving rise to multiple differentiated cell phenotypes including osteoblasts, chondroblasts, myoblasts, and adipocytes. Further analysis of subclones of RCJ 3.1 and C3H10T1/2 cells suggested the existence of a lineage hierarchy in which the multipotential cell gives rise to more restricted bi- or tripotential cells, and these ultimately give rise to monopotential progenitors. However, both a stochastic process with an expanding hierarchy of increasingly restricted progeny (e.g., RCJ3.1; Figs. 1, 2) and a nonrandom, single step process in which multipotential (i.e., tripotential) progenitors become exclusively restricted to a single lineage by particular culture conditions (environment) and inducers (e.g., C1) have been proposed [see discussion in Aubin et al., 1993]. We have discussed in detail the multiple caveats affecting interpretation of the results of clonal lines, including the limitations of analysis of end-stage phenotypes under only given sets of conditions and the concomitant limitations in being able to accurately discern commitment or restriction points. Nevertheless, it is interesting to consider that the different models needn't be considered entirely exclusive since the imposition of culture condition restraints may shift markedly the frequency of apparently random or stochastic commitment/restriction events to favor particular outcomes, as might also be achieved in vivo under particular environmental conditions or at particular developmental times.

Committed osteoprogenitors, i.e., progenitor cells restricted to osteoblast development and bone formation, can be identified by functional assays of their differentiation capacity in vitro or, as so-called above, the CFU-O assay, in not only stromal cell populations but also populations derived from calvaria and other bones (see below). However, in addition to committed osteoprogenitors, several investigators have documented mixed colony types and/or manipulated cultures such that apparently committed cells expressed alternate differentiation patterns. This has been of particular interest for the relationships between adipocytes and osteoblasts, an issue of significant clinical interest in osteoporosis and the aging or immobilized skeleton [summarized in Aubin and Heersche, 1997]. A number of studies on human bone-derived cells, both populations derived from human trabecular bone and clonally-derived lines of human bone marrow stromal cells, have supported the observations on rodent marrow stromal populations that a cell exists that appears to be at least bipotential for adipocytes and osteoblasts and that an inverse relationship between the osteoblast and adipocytic phenotypes in marrow stroma may reflect the ability of single or combinations of agents to alter

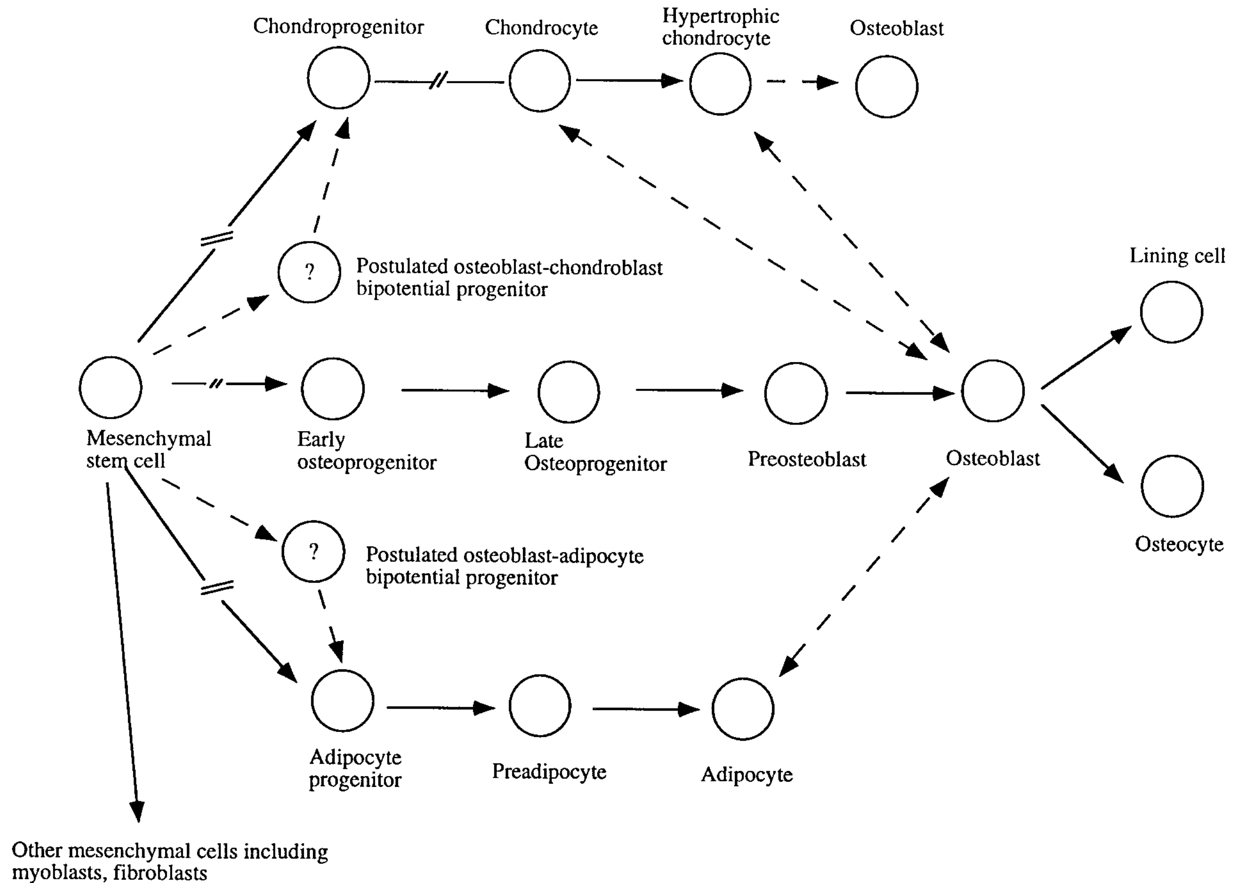
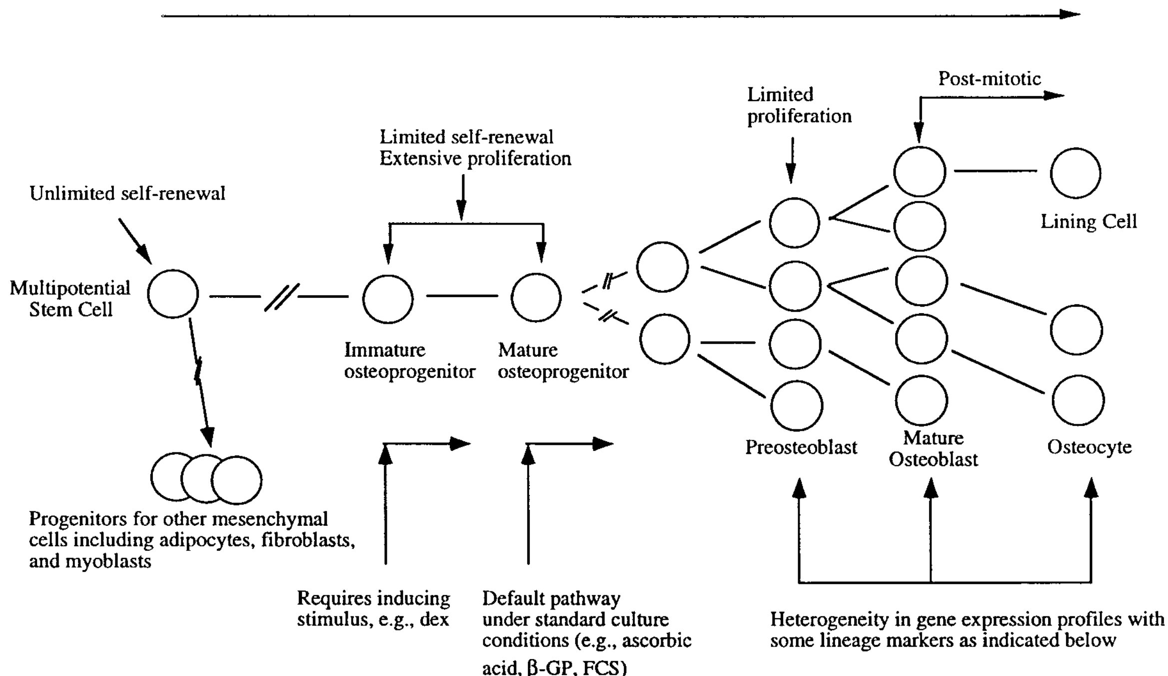


Fig. 1. Postulated steps in the osteoblast lineage outlining some of the potential commitment and restriction points and plasticity discussed in the paper. Updated from Aubin et al. [1993].

the commitment or at least the differentiation pathway these bipotential cells will transit [Gimble et al., 1996]. In many cases, combinations of glucocorticoids and $1{,}25(OH)_2D_3$ are used, and often individual colonies are seen in which both osteoblast and adipocyte markers are present simultaneously. Dedifferentiation has been opined to account for observations in some cultures of stroma in which highly differentiated adipocytes are thought to revert to a less differentiated, more proliferative fibroblastic precursor phenotype and then to osteogenic phenotype [summarized in Owen, 1998]. On the other hand, Nuttall et al. [1998] found that osteoblasts, differentiated to the point of already expressing osteocalcin, were able to undergo rapid differentiation events that led to essentially 100% of the formerly osteoblastic cells expressing adipogenesis. Thus, although osteocalcin is a very late marker of osteoblast maturation, the data are consistent with the cells being able to transdifferentiate to an adipogenic phenotype. Taken together, the data support the notion that there may be plasticity amongst at least some cells of the mesenchymal lineages.

A variety of observations have suggested that a restricted bipotential progenitor for bone- and cartilage-forming cells (osteo-chondroprogenitor) may exist [for discussion of earlier literature, see Aubin et al., 1993]. However, as raised above for the adipocyte-osteoblast phenotypes, plasticity may also characterize the osteoblast-chondroblast lineages. For example, further differentiation from a hypertrophic chondrocyte to osteoblast has been proposed [Cancedda et al., 1995] in a chick model system. Transdifferentiation from hypertrophic chondrocyte to osteoblast involving asymmetric divisions has been documented by Roach et al. [1995] also in a chick model. Further, hypertrophic chondrocytes seem also to be able to reverse their phenotype by stopping type X collagen expression and starting again expression of markers such



Collagen type I	?	-	+	++	++	++	-
Alkaline phosphatase	?	-	-	+	++	+++	-
Osteocalcin	-	-	-	-	-	- → +++	-
Bone sialoprotein	-	- ↔ ++		-	- → +++	- → +++	- → +++
Osteopontin	?	-/+	-/+	-/+	- → +	- → +++	- → +++
PTH/PTHrP-R	?	-	-	-/+	+++	+++	++
PTHrP	?	-/+	-/+	+	++	++	-
Cbfa-1	++	?	?	?	?	++	?
Msx-2	?	-	-	-	++	+ → -	-
CD44	?	-/+	-/+	-/+	+	++	++
E11	?	-	-	-	- → +	- → +++	+ → -

Fig. 2. Postulated steps in the osteoblast lineage implying recognizable stages of differentiation as detectable from in vitro and in vivo experiments. Superimposed on this scheme are several well-established markers of the osteoblast and current thinking as to how their expression changes through differentiation stages. -, no detectable expression; -/+ - +++, expression ranging from detectable to very high, -→+++, heterogeneous expression in individual cells.

as aggrecan [Chen et al., 1995]. It is also striking that normal hypertrophic chondrocytes express a variety of markers also expressed by osteoblasts, including bone sialoprotein (BSP), osteopontin, osteonectin, PTH receptor, and alkaline phosphatase (ALP). Therefore, further analyses with appropriate new markers including e.g., the alternatively spliced variants of type II collagen, type IIA, and type IIB [Lui et al., 1995], are required to address whether a hypertrophic chondrocyte transdifferentiates to an osteoblast, goes through a further maturational progression to an osteoblast, or other kinds of plasticity of expression between the two lineages exist (Fig. 1). These observations, together with discrepancies between results in calvaria versus stromal and other populations, underscore the need for experiments to distinguish the molecular mechanisms underlying the ability of cells to express multipotentiality, commit to a restricted phenotype, and/or display plasticity. However, the possible presence

of undifferentiated/uncommitted stem cells and multi- and bipotential progenitors in cultures that may also contain monopotential progenitors at higher frequencies will complicate the ability to unambiguously discriminate the nature of the cells being affected.

The data emphasize the need for more markers and for more rigorous assessment of the functional capacity of individual progenitors from various sites in bone and in the skeleton, have important consequences for determining formation of fully functional differentiated cells, and raise serious questions about defining a mesenchymal stem cell, the nature of commitment and restriction points, and the role plasticity plays in the lineage. In the last several years, significant strides have been made to identify the molecular mechanisms underlying lineage restriction, commitment, and/or differentiation within some of the mesenchymal lineages. The master genes, exemplified by the MyoD, myogenin, and Myf-5 helix-loop-helix transcription factors in muscle lineages, is one paradigm in which one transcription factor is induced and starts a cascade that leads to sequential expression of other transcription factors and of phenotype specific genes [reviewed in Rawls and Olson, 1997]. A factor of a totally different family, the nuclear receptor family member peroxisome proliferator activated receptor γ2 or PPARγ2, plays a key role in adipocyte differentiation [Tontonoz et al., 1994]. Only recently, Cbfa-1, a member of yet another family of transcription factors, the runt homology domain family, has been found to play a crucial role in osteoblast development in embryogenesis and in the relationships between chondrocytic and osteoblastic lineages [e.g., Banerjee et al., 1997; Ducy et al., 1997; Mundlos et al., 1997]. Many questions remain as to the precise role of Cbfa-1 in commitment versus differentiation events and osteoblast phenotypic expression, however, it has offered important new and evolving insights.

Osteoprogenitor Cells and the Osteoblast Differentiation Sequence

Cellular features. Committed osteoprogenitors, i.e., progenitor cells restricted to osteoblast development and bone formation, can be identified by functional assays of their differentiation capacity to form bone nodules in vitro. Based on morphological and histological studies, osteoblastic cells in vivo are categorized in a presumed linear sequence progressing from osteoprogenitor to preosteoblasts, osteoblasts, and then lining cells or osteocytes (Fig. 2). These earlier morphological definitions are now being supplemented by elucidation of cell and tissue specific macromolecules, including e.g., the bone matrix molecules (type I collagen, osteocalcin, osteopontin, bone sialoprotein (BSP), amongst others) and transcription factors that regulate them and commitment/differentiation events (e.g., AP-1 family members, Msx-2, Cbfa-1). Cells virtually identical to the same morphological stages described in vivo can be identified and the matrix contains the major bone matrix proteins when bone nodules form in vitro. The nodules represent the end product of the proliferation and differentiation of relatively rare osteoprogenitor cells present in the starting cell population. Estimates by limiting dilution have indicated that these osteoprogenitor cells are present at a measurable but low frequency of rat calvaria populations (i.e., $<1\%$), and rat and mouse bone marrow stroma (i.e., $1/2–1 \times 10^5$ of the nucleated cells of unfractionated marrow or $<1\%$ of the stromal layer; references in [Aubin, 1998]) under standard isolation and culture conditions. The number of nodules or colonies forming bone can be counted for an assessment of osteoprogenitor numbers recoverable from calvaria or other bones and bone marrow stroma under particular assay conditions. However, evidence from rat calvaria cell bone nodule assays suggest the existence of at least two distinct populations of osteoprogenitors. One population appears capable of constitutive differentiation in vitro, i.e., in standard culture conditions (ascorbic acid, β-glcyerophosphate, fetal calf serum) differentiation leading to the mature osteoblast phenotype appears to be a default pathway, while the other, apparently less differentiated, population undergoes osteoblastic differentiation only following the addition of specific inductive stimuli [Turksen and Aubin, 1991]. Thus the addition of dexamethasone or other steroids (e.g., progesterone) [Ishida and Heersche, 1997] or other factors such as bone morphogenetic proteins (BMPs) [Hughes et al., 1995] increases the number of bone nodules or bone colonies in calvaria-derived and bone marrow stromal cell cultures, suggesting the presence of "inducible" osteoprogenitor cell populations as well.

Whether all such progenitors belong to the same unidirectional lineage pathway (i.e., immature progenitors induced by a variety of

agents to undergo differentiation to mature osteoblasts) or constitute recruitment from other parallel lineages remains to be explicitly established. It is also worth considering whether the osteoprogenitors in calvaria and stroma are the same. As discussed in more detail below, they do appear to reach similar endpoints with respect to osteoblast marker expression and mineralization. Recent data have indicated that, in rat stromal populations, as in rat calvaria-derived populations, there are two pools of osteoprogenitors: ones that differentiate in the absence of added glucocorticoids and ones that do so only in its presence, although the number of the former type is relatively low and so detectable only at relatively high plating cell densities and the latter comprise the majority [Aubin, 1998]. Whether the two sorts or stages of progenitors are identical in other features to the progenitors in calvaria remains to be assessed rigorously, but in rat stroma, unlike in rat calvaria, limiting dilution analysis indicates that more than one cell type is limiting for nodule formation until high cell densities are reached suggesting a cell nonautonomous aspect to differentiation of the stromal progenitors and a role for heterotypic cell-cell interactions [Aubin, 1998]. The relationship of these to the osteoprogenitors that apparently reside in the nonadherent fraction of bone marrow and are assayable under particular culture conditions, e.g., in the presence of PGE_2 (rat) [Scutt and Bertram, 1995] or as colonies in soft agar or methylcellulose (human) [Long et al., 1995] also remains to be determined. Direct and unambiguous comparisons are not yet possible but should be advanced as more markers become available. In this regard, isolation and fractionation of cells with tools such as the STRO-1 antibody against an unknown human epitope on all relatively undifferentiated stromal cells and that is lost as the cells differentiate to osteoblasts and other stromal lineages [Gronthos et al., 1994], the SB-10 antibody recognizing ALCAM [Bruder et al., 1998] and HOB-26 [Joyner et al., 1997] also both identified in human cell populations, may be helpful. The latter two in particular appear to label both bone marrow stromal cell populations and the relatively immature fibrous periosteal cells in bones such as calvaria.

Given its prominence in assays of osteoprogenitors, it is also worth considering whether the activity of dex is direct or is mediated through other cytokines and growth factors. Clearly, dex modulates production of cytokines that regulate the differentiation pathway, e.g., downregulating endogenous production of LIF which appears to be inhibitory at a late progenitor/preosteoblast stage [Malaval et al., 1998] and upregulating the stimulatory BMP-6 [Boden et al., 1997], in autocrine regulatory feedback loops. A growing list of both systemic and locally active hormones and cytokines has been shown to regulate osteoblast activity and/or differentiation. One of the most interesting and recurrent features of regulation of osteoblasts by exogenous agents is the biphasic pattern of response, inhibition, or stimulation depending on such parameters as the duration of exposure to the agent, its concentration, and the presence or absence of other agents. In addition, there is growing evidence provided from the bone nodule assay systems that at least some of the actions of growth and differentiation factors are dependent on the relative stage of differentiation (either more or less mature) of the target osteogenic cells, with both stimulatory/inhibitory responses in proliferative progenitors and stimulatory/inhibitory responses of sensitive differentiation stage-specific precursors. The molecular mechanisms underlying these complex effects are poorly understood, however localization and determination of levels of expression of cytokine receptors and ligands within specific subgroups of osteogenic cells as they progress from a less to a more differentiated state may help to dissect both the developmental and regulatory pathways (e.g., PTH/PTHrP-R, Fig. 2) [see also Discussion in Aubin and Liu, 1996].

The osteoprogenitors measurable in functional bone nodule assays appear to have a limited capacity for self-renewal in both calvaria and stromal populations, consistent with their being true committed progenitors with a finite lifespan. Morphologically recognizable osteoblasts associated with bone nodules appear in long-term bone cell cultures at predictable and reproducible periods after plating. Recent time lapse cinematography of individual progenitors forming colonies in low density rat calvaria cultures indicated that the immature progenitors requiring glucocorticoids divide approximately eight times prior to overt differentiation, i.e., to achieving cuboidal morphology and matrix deposition [Aubin and Liu, 1996]. Interestingly, however, measurement of the size distribution of large numbers of individual bone

colonies in low density cultures shows a normal distribution of sizes, suggesting a stochastic component to the differentiation program.

Molecular and biochemical features. How do cells progress from an early progenitor to a fully functional matrix synthesizing osteoblast—through quantal leaps or gradual transitions? The process has been subdivided into three developmental time stages: 1) proliferation, 2i) extracellular matrix development and maturation, and 3) mineralization, with characteristic changes in gene expression [Stein et al., 1996]. Genes associated with proliferative stages, e.g., histones, proto-oncogenes such as c-fos and c-myc, characterize the first phase, while certain cyclins, e.g., cyclins B and E, are upregulated postproliferatively [Stein et al., 1996]. Expression of the osteoblast-associated genes is asynchronously acquired and/or lost as the progenitor cells differentiate. For example, alkaline phosphatase increases then decreases when mineralization is well progressed; osteopontin peaks twice during proliferation and then again later but prior to certain other matrix proteins including BSP and osteocalcin; BSP is first detected in differentiated osteoblasts forming bone; and osteocalcin appears with mineralization [Aubin and Liu, 1996; Stein et al., 1996]. However, some discrepancies have been noted in the results from different labs, and at least some of the variations being observed may reflect inherent differences in the populations being analyzed, i.e., as reflecting different mixtures of more or less mature progenitors and more mature cells and perhaps some species differences.

To further analyze the differentiation sequence and explore the possibility that more transitional stages characterize osteoblast development than those detailed above, we combined global amplification poly(A)PCR, which allows entire gene repertoires to be amplified in sample sizes as small as single cells and colonies of rat calvaria cells, with immunolabelling, which allows visual or positional cues to be used in tandem. Since osteoprogenitors giving rise to bone nodules in vitro undergo a series of amplifying divisions, i.e., in the order of eight to ten population doublings prior to overt morphological differentiation [Aubin and Liu, 1996], replica plating was used to capture calvaria osteoprogenitors as early as only a few cell divisions from their in vivo lifetime and poly(A)PCR to amplify their gene repertoires. Gene expression profiles were compared in these replicated colonies with bone colonies identified morphologically as being early or young bone colonies versus more mature or terminally differentiated/heavily mineralized colonies. In addition to the standard repertoire of probes to osteoblast-associated genes (type I collagen, alkaline phosphatase, osteopontin, BSP, osteocalcin, PTH/PTHrP-R), probes for potential regulatory molecules including PTHrP, and growth factor receptors, e.g., FGFR-1 and PDGFRα, were used. All these genes were found to be modulated during the progression from committed osteoprogenitor to preosteoblast and then to mature osteoblast stage. All osteoblast-associated markers analyzed were upregulated prior to cessation of proliferation in precursors except osteocalcin, which was upregulated only in post-proliferative osteoblasts. Based on the simultaneous expression patterns of these multiple markers, we have now categorized osteoblast differentiation into a *minimum* of seven transitional stages [Aubin and Liu, 1996; Liu and Aubin, 1994, submitted], not the three stages discussed earlier. Several other striking features were noted from the analysis, one of the most interesting being striking intercellular heterogeneity in expressed gene repertoires at every stage of the differentiation process, consistent with similar observations in the osteoblasts formed in vitro in marrow stromal populations [Malaval et al., 1994] (Fig. 2). A further analysis of the most mature cells in mineralizing bone colonies confirmed that the heterogeneity of expression in cells classed as mature osteoblasts is extensive, appears not to be related to cell cycle differences and extends to virtually all osteoblast-associated markers analyzed to date in vitro [Liu et al., 1997]. The question becomes compelling as to whether this extensive diversity in vitro is a consequence of the in vitro environment or has comparable in vivo correlates. A few examples have been noted of differential expression of osteopontin, osteocalcin and BSP in different groups of osteoblasts [references in Liu et al., 1997]. These observations support the notion that osteoblast heterogeneity may reflect specialized subpopulations with functional heterogeneity in vivo [see also Rodan et al., 1988], but none of these earlier studies addressed whether whole repertoires of osteoblast-associated genes underwent simultaneous changes in vivo.

To test the latter possibility, we recently analyzed osteoblast phenotype in 21day fetal rat calvaria, the same bone used for isolation of the cell populations for studies summarized above, and a bone characterized by well-demarcated maturational zones comprising nascent bone, cortical bone, and remodeling or spongy bone. Of the markers studied, only two, alkaline phosphatase and PTH/PTHrP receptor, appeared to be “global” or “ubiquitous” markers expressed by all osteoblasts in vivo. Strikingly, all other markers analyzed, including osteopontin, BSP, osteocalcin, PTHrP, c-fos, msx-2, and E11, were differentially expressed at both mRNA and protein levels in only subsets of osteoblasts, depending on the maturational state of the bone and the age of the osteoblast, and on the environment (endocranium, ectocranium) and the microenvironment (adjacent cells in particular zones) in which the osteoblasts reside [Candeliere et al., 1997, submitted] (Fig. 2). The observations imply that histologically identical, fully differentiated, cuboidal matrix-synthesizing osteoblasts are molecularly heterogeneous. The biological or physiological consequences of the observed differences is not known, but they support the notion that not all mature osteoblasts are functionally identical and predict that the make-up of different parts of bones may be significantly different, as previously suggested by the observations that the presence of and amounts of extractable noncollagenous bone proteins are different in trabecular versus cortical bone and in different parts of the human skeleton [Ninomiya et al., 1990]. They also suggest also that the global or ubiquitously expressed molecules, type I collagen, PTH/PTHrP-R and alkaline phosphatase, serve common and nonredundant functions in all osteoblasts, and that only small variations in expression of these molecules may be tolerable [Rodan et al., 1988]. Differentially expressed lineage markers, on the other hand , e.g., BSP, osteocalcin, osteopontin, and E11, vary much more, both between osteoblasts in different zones and between adjacent cells in the same zone—also consistent with the five-fold differences observed with these molecules between individual cells in vitro [Liu et al., 1997]. These markers may have specific functions associated with only some positionally or maturationally defined osteoblasts. In this regard, it is striking that all of the noncollagenous matrix molecules analyzed are extremely heterogeneously expressed by osteoblasts.

The nature of the signals leading to diversity of osteoblast gene expression profiles is not known. Cross-talk between cells of various lineages, e.g., the dura matter and osteoclasts which reside on the endocranial but not ectocranial surfaces, may comprise one signal. Differential expression could also develop as a consequence of variations, perhaps even relatively small variations, in the cellular microenvironment, including the degree of and nature of the crystal structure of the deposited mineral. Clearly, the presence of particular transcription factors, e.g., Msx-2, at some sites and not others provides clues to expression of some of the molecules. However, the fact that the heterogeneity is apparently controlled both transcriptionally and post-transcriptionally implies that the regulation may be complex. The molecular mechanisms and transcription factors responsible for the coordinate expression and regulation of specific genes during osteoblast differentiation and development are only beginning to be deciphered [Aubin and Liu, 1996; Stein et al., 1996]. The observations suggest that it will be important to analyze their expression not only globally but at the individual osteoblast level. Another unanswered question remains whether the striking diversity of marker expression in different osteoblasts is non-reversible or reversible in either a stochastic manner or governed by changes in a microenvironmental signal or receipt of hormonal or growth factor cues or both. Since the heterogeneity observed extends to expression of potential regulatory molecules such as cytokines and their receptors, it suggests that autocrine and paracrine effects may be elicited on or by only a subset of cells at any one time and the responses to such stimuli could themselves be varied. We also cannot rule out the possibility that the heterogeneity can be subdivided further, a scenario that seems likely as new markers for the lineage are identified.

Concluding Remarks

The developmental history of cohorts of cells, including osteoblasts and their precursors and the specific bones in which they reside, undoubtedly influences both their ultimate phenotype and their ability to be regulated by particular molecules at particular times of their developmental, differentiation, and maturational lifetimes. The fact that not only osteoblasts, but

also preosteoblasts and osteocytes, differ in phenotypic expression in the different zones of the calvaria also supports the possibility that lineage progression from progenitor to terminally differentiated osteoblast and osteocyte may follow different pathways depending on where in a bone a particular cell is residing. One limitation in the field remains an inability to enrich for and isolate the earliest progenitors in the lineage, especially normal—nontransformed, nonestablished—progenitors. It is also clear that more markers—specifically ones that may demarcate various important transitional stages—would advance understanding in the field. As raised above, a few monoclonal antibodies are becoming available that may help in these areas. Many investigators have also embarked on searches for novel genes that may be expressed preferentially at particular transitional stages in the lineage, all of which approaches should aid in resolution of many of the issues raised here.

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I apologize to all those investigators whose work could not be referred to directly due to space limitations, but many important references can be found in the reviews quoted.

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