Bone is a highly dynamic tissue comprising a mineralized extracellular matrix embedded with bone cells, blood vessels, and nerves. The basic structure and function of bone are reviewed in the handout from Wheaton's Histology Text. Bone contains three main bone-specific cell types: the osteocyte is a mature cell that sits in bone lacunae, communicates with other osteocytes through long cellular processes, senses mechanical stress in bone, and sends signals for bone remodeling as a result of mechanical stress. The responding cells are osteoblasts, cells specialized to secrete the unique collagen-rich extracellular matrix in bone that enables mineralization; and osteoclasts, macrophage-like cells that degrade the bone structure through a combination of localized acidification (removes the minerals) and protease secretion (breaks down matrix). Osteoclasts tunnel through bone and are usually followed close behind by osteoblasts. Bone is in a constant state of remodeling in healthy individuals.

Bone is formed developmentally, and during wound healing, by either endochondral ossification or by intramembranous ossification. In the endochondral ossification process, mesenchymal progenitor cells first form cartilage. The chondrocytes then hypertrophy and the extracellular matrix mineralizes. Blood vessels invade the site, bringing cells that break down the existing matrix. Osteoprogenitor cells the go on to form bone. Long bones are formed by this process during normal development. Intramembranous bone formation is a more direct process, in which osteoprogenitor cells form bone directly. Cranial bones are formed by this process during development. Wound healing in bone may proceed by either process, depending on local environmental factors that include how much the ends of the bone can move relative to each other, with motion favoring the endochondral process.

Osteoprogenitor cells are cells that have to potential to become bone cells, and reside in the periosteum and the marrow. Osteoprogenitor cells are derived from connective tissue progenitor cells that reside also in the surrounding tissue (muscle). A detailed description of the progenitor cells in bone is contained in the Handout by Fleming et al. [2] in the section entitled
"Osteogenic Cells" (pp. 359-362). Please read this section for the details of osteogenic (bone-forming) cells. A summary is as follows:

stem cell → osteoprogenitor cell → preosteoblast → osteoblast
resting → proliferation → matrix deposition → mineralization

2. Clinical problems requiring replacement of bone.

Handouts:
2. Fleming, Cornell and Muschler, Bone Cells and Matrices in Orthopedic Tissue Engineering [2]

An important concept to appreciate is that clinical problems are extremely diverse, and that no one approach will likely suit all potential applications. We focus on those applications where the bone will not heal on its own, and some sort of bone graft is typically used. Some of these diverse applications include:

1. Non-union fractures (fractures that fail to heal)
2. Craniofacial reconstruction
3. Segmental defect due to tumor removal
4. Augmentation of bone around a hip implant revision (i.e., 25% of hip implants are replacements of an existing implant, as the lifespan of a hip implant is only ~10 years)
5. Reconstruction of bone in the jaw for dental purposes -- loss of teeth can lead to resorption of jaw bone as the bone is not being stressed by chewing

In some applications (e.g., dental reconstruction, non-union fracture), there may be enough progenitor cells in the local area that stimulation of these cells will induce local bone formation. In a compromised site, such as a where a tumor was removed and the local tissue irradiated, there may not be many local progenitor cells, and further, it may not be a good idea to release growth factors in a site where a tumor was removed, so alternative approaches must be considered. In addition to the local conditions at the wound site, the patient's age and lifestyle habits (such as smoking) may influence the wound healing. When testing approaches for bone regeneration, animal models that reflect the ultimate clinical application as much as possible should be used.

A review of the current clinical approaches to bone grafting can be found in the handouts by Bauer & Muschler [1] and by Fleming et al. [2]. These approaches include autograft (patients own bone harvested from the iliac crest or other sites), allograft (cadaver bone, washed to remove most cells and often frozen), and alloplast (synthetic materials that serve as a bone-forming scaffold). The handouts describe the attributes and shortcomings of these various clinically-used approaches. In this class, we discuss alternatives that address deficiencies in current clinical practice.
3. Approaches to bone tissue engineering

A. Induction of bone by use of BMPs

Handouts:
1. Groeneveld et al. "Human BMPs..."[3]
   This is a comprehensive review, discussing the basic biologic properties, as well as results of animal and human clinical trials. Read this!
   These are some of the original reviews on the therapeutic potential of BMPs, discussing some of the history and molecular properties of BMPs

Also relevant:
Lauffenburger & Linderman, material from the textbook "Receptors: Models for Binding, Trafficking, and Signaling"[7]

The dry weight of bone comprises 70% inorganic materials and 30% organics; 90% of the organic material is Type I collagen, while the other 10% comprises proteins that induce mineralization and signal for regeneration. In the 1960's, Urist demonstrated that demineralized bone (i.e., bone exposed to acid to dissolve the inorganic component, leaving the organic matrix = Demineralized Bone Matrix or DBM) could induce ectopic bone formation via the endochondral process. [note ectopic = any site that is not the normal physiological site] It was hypothesized that a diffusible factor was present in DBM. Characterization of the properties of DBM led to identification and cloning of a molecule (now called BMP-2) that could induce ectopic bone formation on its own. The BMP family has grown substantially (see handouts for specifics of the different family members) and it is now recognized that these molecules also play important and essential roles in development.

Several commercial efforts are directed at developing BMPs for applications in bone repair and regeneration. BMPs induce cell migration, proliferation, and differentiation, and it is not entirely clear yet which of these processes dominate in vivo. In order for BMPs to be effective, progenitor cells that can be induced to form bone must be present in the local area. This means that BMPs will not likely be effective in very large defects, or defects which have been compromised by irradiation or infection.

An essential problem in making BMPs a clinically useful therapy is finding an appropriate delivery vehicle (see comments at the end of the Groeneveld article). We discussed the fundamental aspects of receptor-ligand binding in class, and analyzed the requirements for inducing a physiologically relevant gradient that could be sensed by cells. A key observation was that for ligand concentrations greater than about 5K_d, most receptors would be occupied and thus the cell could not sense a gradient from front to back. We also noted that for ligand
concentrations less than ~ 0.1 K_d, the absolute number of receptors occupied was low, and thus
the cell might not be able to tell the difference between front and back. In applying these
observations to the specific problem of BMP release, we noted that it would be difficult to
achieve a physiologically-relevant gradient of BMP to induce migration.

The homework problem due 11/8 focuses on analysis of data on BMP release from
different carrier vehicles in subcutaneous implants. The data are presented as % TCA-
precipitable \(^{125}\text{I}\)-labelled material retained in the implant (this means measurement of protein
instead of protein + free label). The homework problem focuses on analyzing the data to
estimate the relative fluxes from each release device, and the approximate concentration of free
BMP within each type of graft. The results of the analysis allow you to compare concentrations
of BMP in the vicinity of the device with the physiological properties of the cells (K_d and
receptor number; K_d \~ 0.01-0.1 nM and R_T \~ 1000-10,000). This problem is not neat and tidy --
and that fact that it is not should illustrate the need for such quantitative analysis in tissue
engineering!

B. Use of Bone Progenitor Cells in Bone Tissue Engineering

Handouts:
1. Bruder and Caplan. "Bone regeneration through cellular engineering"[8]
   This is a review of using purified mesenchymal stem cells
2. Fleming, Cornell and Muschler, Bone Cells and Matrices in Orthopedic Tissue Engineering
   [2]
   This is a general review of bone tissue engineering, putting use of progenitor cells in
   context of several therapies
3. Majors, et al., "Characterization of human bone marrow stromal cells with respect to
   osteoblastic differentiation," [9]
   This is a research paper describing how to characterize bone progenitor cells from human
   marrow.

A promising approach in bone tissue engineering is the use of progenitor cells added to bone
grafts, instead of trying to recruit them using BMPs. Very generally, there are two broad
approaches to using such cells. First, cells may be isolated from donor tissue (such as marrow)
and expanded in vitro. This approach would be particularly useful if immunological barriers to
transplantation could be overcome, enabling a single cell source to provide transplants to many
patients. It may also be necessary in patients that have a severe deficit of bone progenitor cells
available in marrow (e.g., elderly women) or in patients who have very large defects. This
approach is described in the handout by Caplan and Bruder [8].

An alternative approach is to isolate cells from marrow in the operating room and add
them to the graft. This approach is described in the handout from Fleming, Cornell, and
Muschler[2]. It is preferable to perform only a single procedure on a patient, and further,
manipulation of cells outside the operating room (OR) increases risk. However, since connective
tissue progenitors (CTPs), or cells that go on to form bone, are present at very low concentrations
in marrow (see handout by Majors et al.[9]), strategies for effective use of these cells must be
developed. In particular, it is desirable to enrich the number of CTPs that are placed in a device
above the concentration which is obtained in a standard marrow aspirate. The reason for this is two-fold. First, it is desirable to have as many CTPs present as possible to speed the bone healing process. Second, in a large defect, the overall cell concentration may be limited by the availability of oxygen (see homework #7). Thus, enriching progenitors and purging non-essential cells may be necessary to keep the overall cell concentration in the implant low enough that all cells remain viable. Note that the cell concentration that is acceptable diminishes as the size of the graft increases (see homework for the functional dependence). This may explain in part why experiments that succeed in rats are more problematic in larger animals, although biological factors may play a role as well.

In class, we discussed how CTPs are assayed in vitro -- cells from anti-coagulated marrow aspirates are spun down to remove the red blood cells, and the cells are plated in serum-containing medium onto polystyrene petri dishes. After a 24-hour attachment period, petri dishes are washed. Only 1/20000 cells on average stays attached, and 99+% of these cells form colonies (i.e, consist of more than 8 cells after 6 days). CFUs (colony-forming units) progress toward differentiation down the osteoblastic pathway, as described in the paper by Majors et al. [9]. Why do cells attach to the polystyrene? Serendipity! but we can speculate….we discussed in class the phenomenon of protein adsorption, and how adsorbed proteins mediate cell attachment through cell surface receptors.

In a nutshell, proteins in aqueous solution are surrounded by a well-ordered shell of water. Likewise, hydrophobic surfaces such as polystyrene petri dishes have a well-ordered layer of water at the fluid-solid interface. Remembering that $\Delta G = \Delta H - T\Delta S$, we note that if a protein adsorbs to a surface, all that well-ordered (low-entropy) water escapes to the chaos of the liquid phase…thus the entropy increase is huge. The enthalpic term is usually small compared to the entropic term, making adsorption an overall favorable process. Upon adsorption the conformation of the protein may change, as it stretches out to maximize interactions with the surface.

We also discussed the way that ECM proteins such as fibronectin interact with cells (see handout by Ruoslahti [10]). Most matrix proteins are large multimeric molecules, with sites for binding other matrix proteins, growth factors, and cell adhesion receptors. The protein acts in part as a structural scaffold to present all these binding sites. As detailed in the handout by Ruoslahti, the regions within molecules such as fibronectin that interact with cell surface receptors such as integrins are actually quite small peptide sequences. The prototypical adhesion peptide sequence is the RGD (arginine - glycine - aspartate) peptide, which enables adhesion mediated by two specific integrins. Hundreds of other comparable sequences have been identified in fibronectin and other matrix molecules.

The consequences of this are two-fold. First, when a protein adsorbs to a surface, there is no way to predict its conformation and thus no way to predict how it will interact with cells -- the peptides responsible for integrin binding may end up adsorbed to the surface and unavailable; or only some of the many sites within the protein may be available. But second, it may be possible to use small synthetic peptides in a rational way to induce cell adhesion.

Thus, we discussed that one approach to building a bone scaffold would be to create a surface that would select progenitor cells out of marrow using a rational approach based on peptide recognition. Although specific peptides that recognize bone progenitors have not yet been definitively identified, a candidate set of peptides is available through mapping of the laminin molecule. It is reasonable to suppose that a suitable selective peptide could be identified by screening a small set of peptides.
Finally, we discussed some of the properties of scaffolds for CTP enrichment and bone repair. The scaffold will likely need a defined shape (e.g., to fit the defect) and a complex architecture. We noted that it is desirable to have a large surface area within a porous architecture to enable cell attachment, but that it is also desirable to have large channels (~200 - 600 microns) for rapid tissue and blood vessel ingrowth. Thus, the optimal architecture may comprise a porous scaffold with defined channels.

4 Rosen, V. and Thies, R. S. (1992) Trends Gen. 8