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Buried Alive: How Osteoblasts Become Osteocytes

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During osteogenesis, osteoblasts lay down osteoid and transform into osteocytes embedded in mineralized bone matrix. Despite the fact that osteocytes are the most abundant cellular component of bone, little is known about the process of osteoblast-to-osteocyte transformation. What is known is that osteoblasts undergo a number of changes during this transformation, yet retain their connections to preosteoblasts and osteocytes. This review explores the osteoblast-to-osteocyte transformation during intramembranous ossification from both morphological and molecular perspectives. We investigate how these data support five schemes that describe how an osteoblast could become entrapped in the bone matrix (in mammals) and suggest one of the five scenarios that best fits as a model. Those osteoblasts on the bone surface that are destined for burial and destined to become osteocytes slow down matrix production compared to neighbouring osteoblasts, which continue to produce bone matrix. That is, cells that continue to produce matrix actively bury cells producing less or no new bone matrix (passive burial). We summarize which morphological and molecular changes could be used as characters (or markers) to follow the transformation process. *Developmental Dynamics 235:176–190, 2006.* © 2005 Wiley-Liss, Inc.

Key words: osteoblast; osteocyte; osteogenesis; intramembranous ossification; preosteoblast; osteoid-osteocyte; preosteocyte

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INTRODUCTION

It has been known, for almost one and a half centuries, that osteocytes are derived from osteoblasts (Gegenbauer, 1864). Osteoblasts (bone forming cells) are of mesenchymal origin, secrete non-mineralized bone matrix (osteoid), and finally become incorporated as osteocytes in mineralized bone matrix. Osteocytes are by far the most abundant cellular component of mammalian bones, making up 95% of all bone cells (20,000 to 80,000 cells per mm^3 bone tissue) that cover 94% of all bone surfaces (Frost, 1960; Marotti, 1996); there are approximately ten times more osteocytes than osteoblasts in an individual bone (Parfitt, 1990). In humans, osteocytes can live long. Frost (1963) estimates the average half-life of a human osteocyte as 25 years. However, when we consider an overall bone-remodelling rate of between 4 to 10% per year (Delling and Vogel, 1992; Manolagas, 2000), the life of many osteocytes may be shorter (Marotti et al., 1990). Furthermore, the lifespan of osteocytes greatly exceeds that of active osteoblasts, which is estimated to be only three months in human bones (Manolagas, 2000) and 10–20 days in mouse alveolar bone (McCulloch and Heersche, 1988). Osteocytes communicate with one another and with cells at the bone surface via a meshwork of cell processes that run through canaliculi in the bone matrix (Palumbo et al., 1990). Thus, bone cells form a functional network within which cells at all stages of bone formation from preosteoblast to mature osteocyte remain connected.

The literature provides us with an astounding number of terms concerning the transition from osteoblast to osteocyte, such as "osteocytes are *encased* in mineralized matrix" (Holtrop,

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1990); "osteoblasts are *buried* within the bone matrix" (Manolagas, 2000); and "osteoblasts *merge progressively* into the bone matrix" (Meunier, 1989). Despite the many different phrases that exist to describe this process, the fundamental question of how osteoblasts are buried remains largely unanswered. This could relate to the fact that most of the research on bone cells to date has been done on osteoblasts and osteoclasts. An electronic database (WebOfScience) search using "osteoblast*," "osteocyte*," and "osteoclast*" as keywords revealed that during the last 30 years, less than 5% of publications mention "osteocyte*," although work on osteocytes is accelerating.

Twenty-five years ago, Knese (1979) proposed two possible mechanisms of osteoblast entrapment: (1) self-entrapment or (2) becoming embedded by neighbouring cells. We explore the transformation by evaluating and synthesizing knowledge regarding the mechanism(s) by which osteoblasts become embedded in bone matrix. We examine different scenarios of how osteoblasts can be turned into osteocytes and discuss morphological and molecular markers that may prove to be important when examining the transformation process. Finally, we suggest a model for the osteoblast-to-osteocyte transformation in mammalian intramembranous bone formation but we also emphasize that there is not only one mechanism for transforming osteoblasts into osteocytes since different mechanisms exist in different bones, different types of bone formation, different positions within a bone, and different vertebrate species.

MODES OF OSSIFICATION

How osteoblasts transform into osteocytes is dependent on the mode of ossification—intramembranous, perichondral, endochondral (see Hall and Witten, 2005, for additional modes of bone formation)—and the type of bone that is being generated (woven or lamellar bone). It may also depend on the location of bone formation, on the species, and on the age and/or gender of the individual.

During intramembranous bone formation, mesenchymal cells differentiate into osteoblasts and bone is formed without replacing a cartilaginous model. Bones that form by this method are called membrane bones. Perichondral ossification is the most common mode of bone formation if a cartilaginous precursor is present. Perichondral ossification usually starts with the transformation of a perichondrium into a periosteum (Scott-Savage and Hall, 1980).

Although found in most vertebrate groups, endochondral ossification is typically studied in mammalian long bones. Endochondral ossification involves a cartilaginous template that is replaced by or remodelled into bone by processes that involve several co-ordinated sequential steps that can include calcification of the cartilage matrix, hypertrophy of chondrocytes followed by apoptosis or transdifferentiation of chondrocytes into osteoblasts (in some long bones), resorption of calcified cartilage, recruitment of osteoblasts, and the deposition of woven bone (and later lamellar bone) on the surface of mineralized cartilage residues (Roach, 1990, 1992; Thesingh et al., 1991; Gerstenfeld and Shapiro, 1996; Buxton et al., 2003; Eames et al., 2003). In advanced bony fish (Acanthomorpha with about 16,000 species), intramembranous, perichondral, and endochondral bone formation result in a bone that contains no osteocytes and so is known as acellular bone (Witten and Huysseune, 2005). The bone of less derived bony fish and tetrapods contains osteocytes and so is cellular bone (Witten et al., 2001; Witten and Hall, 2003). Acellular bone formation includes osteoid and bone matrix deposition but not osteoblast entrapment (Ekanayake and Hall, 1988).

Bone elements can arise via additional and less commonly known modes of ossification. Bone can form by metaplasia of other tissue types, and many tissues have modes of formation that result in structures that are intermediate between dentine and bone or between bone and cartilage (Ørvig, 1951; Huysseune and Verraes, 1990; Hall, 2005; Vickaryous and Olson, 2005; Witten et al., 2005). These intermediate modes of bone tissue formation are not discussed here, but see Beresford (1981, 1993), Hall (1990, 2005), Taylor et al. (1994), Witten and Hall (2002), and Hall and Witten (2005).

OSTEOBLAST FATES AND FUNCTIONS

Osteoblasts are involved in bone matrix mineralization (for a review, see Boskey, 1996). At the end of the bone-forming phase, osteoblasts can have one of four different fates: (1) become embedded in the bone as osteocytes, (2) transform into inactive osteoblasts and become bone-lining cells, (3) undergo programmed cell death (apoptosis), or in some situations (4) transdifferentiate into cells that deposit chondroid or chondroid bone (Manolagas, 2000; Noble et al., 1997; Jilka et al., 1998; Li et al., 2004).

The proportion of osteoblasts following each fate is not the same in all mammals and is not conserved among all taxa or all types of bone. Parfitt (1990) report that in human cancellous bone, 65% of the osteoblasts undergo apoptosis and only about 30% transform into osteocytes. Aubin and Liu (1996) give a figure of 10–20% for the number of osteoblasts transforming into osteocytes. Banks (1974) estimated that 10% of osteoblasts transform to osteocytes in the antlers of the white-tailed deer (*Odocoileus virginianus*). In advanced bony fishes with acellular bone, the number of osteoblasts that turn into osteocytes is, of course, zero (Ekanayake and Hall, 1987; Witten et al., 2004).

The age of the animal may also influence the number of osteoblasts that transform into osteocytes. In trabecular bone of ageing beagles, the number of bone-lining cells decreases (Miller et al., 1980) suggesting, either that fewer osteoprogenitors differentiate into osteoblasts (i.e., there is a smaller pool of osteoblasts) or that the proportion of cells following each developmental fate is altered.

Once embedded into the bone matrix, the former osteoblasts, now osteocytes, cease their activity. An important role of osteocytes and their network of cell processes is to function as strain and stress sensors, signals that are very important for maintaining bone structure (see Burger et al., 2003; Knothe Tate et al., 2004, among others). Osteocytes communicate with neighbouring osteocytes and with

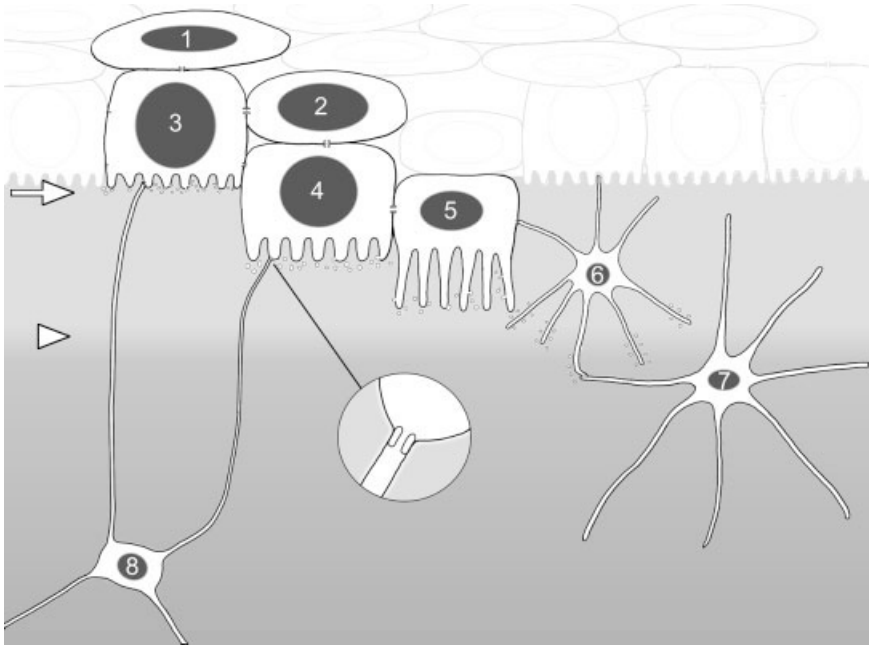

Fig. 1. Diagram showing the transitional cell types between preosteoblasts and osteocytes during osteoblast transformation and their relationships to one another during the second phase of intramembranous ossification (i.e., when transformation occurs). The diagram is not to scale. The preosteoblast (1) layer consists of proliferating cells. Gap junctions are present between all cells for direct communication. Enlargement shows gap junction between the cell process of an osteocyte and an embedding osteoblast. Arrow indicates osteoid deposition front; arrowhead indicates mineralization front. During the transformation process, cellular organelles decrease and the total cell body volume decreases substantially. 1. preosteoblast, 2. preosteoblastic osteoblast, 3. osteoblast, 4. osteoblastic osteocyte (Type I preosteocyte), 5. osteoid-osteocyte (Type II preosteocyte), 6. Type III preosteocyte, 7. young osteocyte, 8. old osteocyte. Diagram drawn by Tim Fedak (www.figs.ca).

cells on the bone surface via a meshwork of cell processes, which are located inside canaliculi within the bone matrix (Palumbo et al., 1990) (Fig. 1). While cross-talk between osteoblasts (bone deposition) and osteoclasts (bone resorption) (Lacey et al., 1998; Yasuda et al., 1998) has been established, there is also the likelihood of cross-talk between osteocytes and osteoclasts (Burger et al., 2003). Thus, a functional network of bone cells that extends from preosteoblast to mature osteocyte is important for maintaining the integrity of bone as a tissue.

Another function of embedded osteoblasts (osteocytes) within the bone cell network is the ability of some osteocytes to deposit and resorb bone around the osteocyte lacuna in which they are housed, thus changing the shape of the lacuna. This process, called osteocytic osteolysis, is often not regarded as characteristic of human osteocytes, but evidence for its occurrence has been observed in many vertebrate species, such as bats (Doty and Nunez, 1985; Kwiecinski, 1985; Kwiecinski et al., 1987), hamsters (Steinberg et al., 1981), squirrels (Haller and Zimny, 1978), rats (Bélanger, 1977a; Tazawa et al., 2004); rabbits (Zhang et al., 2000), snakes (Alcobendas et al., 1991), eels (Lopez et al., 1980), salmon (Hughes et al., 1994a), carp (Witten et al., 2000) and an unidentifiable Cretaceous reptile (Bélanger, 1977b). Osteocytic osteolysis may be limited to situations such as lactation, hibernation, or pregnancy that require increased mobilization of minerals from the skeleton (see Haller and Zimny, 1978; Steinberg et al., 1981; Kwiecinski, 1985; Hall, 2005).

THE CELLS INVOLVED

Many researchers consider the transformation process to involve three cell types: preosteoblasts differentiate into osteoblasts, which become trapped as osteocytes. All agree that the transformation involves a range of morphological changes such as decrease in cell body size, increase in cell processes, and changes in intracellular organelles (Marotti, 1977; Palumbo, 1986; and see Knothe Tate et al., 2004 for a recent review). Palumbo (1986) estimates a total reduction in cell body volume of ~70% between the osteoblast and the osteocyte stage. We first describe these more familiar cell types and then discuss various intermediate cell types that have been proposed.

Three types of osteoblasts are usually identified at the bone surface, based on function and morphology: preosteoblasts, active osteoblasts, and inactive (or resting) osteoblasts (Holtrop, 1990). Mammalian preosteoblasts have been described as less cuboidal in shape than active osteoblasts. They are located at a distance from the bone surface, do not deposit bone matrix, and can still divide (Fig 1). Preosteoblasts already produce collagen type I precursor molecules,

References from Table 1.

1. Aubin and Liu (1996); 2. Candeliere et al. (2001); 3. Nakashima and de Crombrugghe (2003); 4. Liu et al. (1997); 5. Roach (1994); 6. Nampei et al. (2004); 7. Wetterwald et al. (1996); 8. Hadjiargyrou et al. (2001); 9. Schulze et al. (1999); 10. Litvin et al. (2004); 11. Fox and Chow (1998); 12. Burger et al. (2003); 13. Kalajzic et al. (2004); 14. Bianco et al. (1993); 15. Chen et al. (1993); 16. Sandberg et al. (1988); 17. Bianco et al. (1990); 18. Horiuchi et al. (1999); 19. Grzesik and Gehron Robey (1994); 20. Heersche et al. (1992); 21. Becker et al. (1986); 22. Hughes et al. (1994b); 23. Clauss et al. (1993); 24. Middleton et al. (1995); 25. Martineau-Doize et al. (1988); 26. Wang et al. (1995); 27. Dodds et al. (1994); 28. Jamal and Aubin (1996); 29. Machwate et al. (1995); 30. Rouleau et al. (1988); 31. Ishidou et al. (1995); 32. Mark et al. (1988); 33. Turksen and Aubin (1991); 34. Lee et al. (1993); 35. Kobayashi and Kronenberg (2005); 36. Kamiya et al. (2001); 37. Lazowski et al. (1994); 38. Mizoguchi et al. (1997); 39. Sasano et al. (2000); 40. Väkevä et al. (1990); 41. Romanowski et al. (1990); 42. Bianco et al. (1988); 43. Ikeda et al. (1992); Hall and Miyake (1995); 45. Inada et al. (1999); 46. Ducy et al. (1997); 47. Ducy et al. (2000); 48. Nah et al. (2000); 49. Wong et al. (1992).

TABLE 1. Molecular Markers (mRNA, protein) Involved During Endochondral (EC) and Intramembranous (IM) Ossification[a]

	Preosteoblast				Osteoblast				Transitional cells				Osteocyte				References
	mRNA		Protein		mRNA		Protein		mRNA		Protein		mRNA		Protein		
	EC	IM	EC	IM	EC	IM	EC	IM	EC	IM	EC	IM	EC	IM	EC	IM	
Extracellular Matrix Proteins																	
Bone sialoprotein (BSP)	-	+/-	-	+/-	++	+/-	++	+/-			+		+	+/-	+	+/-	1, 2, 5, 14, 15, 29, 35, 36
Osteocalcin (BGP)	+/-	-	+/-	-	++	+/-	++*	+/-					+	-	+/-	+/-	1, 2; 5, 29, 32, 35, 36, 38, 43
Osteopontin (SPP1)	-	+/-	++	-	+/-	+/-	++	+/-			+		++	+/-	++	+/-	1, 2, 5, 8, 15, 32, 35, 43
Collagen I	+	+/-	+/-	++	++	++	++	++					+/-	+/-	+/-	+	1, 16, 20, 21, 29, 35, 38, 39
Collagen II			++	++													48, 49
Collagen III			++			-		-									1, 16, 35
Osteonectin (SPARC)			++	++			++	++			++	++			++	++	9, 41, 42
Fibronectin			+/-	++			++	++			++	++			++	++	1, 8, 19, 39
Tenascin C			++	++				++								-	39, 40
Decorin	+	+			++	+	++						+	+			1, 17, 36
Biglycan	+	+			+	+	+						+	+			1, 17, 36
vitronectin			-				++				++				++		1, 19
Membrane Proteins																	
ALP		++	++	++	++	++	++	++							-	-	1,2, 4, 14, 33, 34, 40
IGF-RI	++				++								-				1, 24, 26
IGF-RII	++				++												1, 26
EGF-R				++				-									1, 25
PTH/PTHrP-R	+	++	++		++	++	+						-	+			1,2, 30, 34
E11 antigen					++		++	-	++		++		++		++	++	1, 7, 8, 9
BMP-RI			+				++										1, 31
Vitamin D3 receptor								++				++				++	1
$Alpha_4$ Integrin			+/-				++				+				+/-		1, 19
$Alpha_v$ Integrin			+				++				++				+		1, 19
$Alpha_5ß$ integrin			+				++				+				+/-		1, 19
$ß_3/ß_5$ integrin			++				++				++				++		1, 19
Secreted Proteins																	
CD44			+				+/-								++		1, 22, 28
IGF-I	+/-				++								-				1, 24, 26, 37
IGF-II	++				++								-				1, 24, 26
IL-1ß					+/-												1, 27
IL-6	++				++												1, 27
Thrombospondin			+				++				++				++		1, 19
Osf 2 or periostin	++	++			++	++							-	-			10, 18
Nitric oxide															++		1, 11, 12
DMP-1							-								++		1, 13
BMP2							++								+		37, 44
BMP4							++								+		37, 44
PTHrP		+/-				+/-											2
TIMP			++	++			++	++							-	-	1, 23
MEPE							-								++		6
Key Transcription Factors																	
Runx2/cbfa1	++	++			++	++											3, 45, 46
Osterix	-	+/-			++	++					++		++				2, 3, 45, 46
Msx2	++	+/-			-	+/-							-	-			2, 3, 47
hXBP-I			++	++			++	++							-	-	1, 23
c-fos	+/-	+/-			-	+/-							-	-			1, 2, 16, 29

[a]Only 10–20% of osteoblasts follow this fate. No data from cell lines have been included below. Much of the molecular expression data presently available do not include the transitional stages between osteoblast and osteocyte. Some of the expression data for transitional stages can be inferred from the expression of cell stages before and after the transformation period (gray shaded fields). The expression data of some key regulatory factors that act upstream of these markers are included. hXBP-1, human X box binding protein 1; IGF, insulin-like growth factor; EGF, epidermal growth factor; ALP, Alkaline phosphatase; TIMP, tissue inhibitor of metalloproteinase; MEPE, matrix extracellular phosphoglycoprotein; Osf2, osteoblast specific factor 2; DMP1, dentin matrix acidic phosphoprotein 1; *Negative in human bone, ++ present, + weak expression, +/− variable expression (positive or negative depending on study), − absent or present at levels below detection

which, after post-translational modification, assemble into collagen fibrils (Manolagas, 2000). A number of bone-specific markers are reliably expressed by preosteoblasts, namely osteonectin, alkaline phosphatase, several IGFs, hXBP-1, TIMP, tenascin C, EGF-R, IL-6, PTH/PTHrP receptor, several integrins, and periostin (osf2) (Table 1). Preosteoblasts differentiate into active bone matrix-secreting osteoblasts, which are typically cuboidal in shape (in mammals) and ultimately responsible for depositing organic bone matrix.

Osteoblasts have a large eccentric nucleus with one to three nucleoli, and prominent RER (rough endoplasmic reticulum) and Golgi areas. They extend cellular protrusions or pseudopodia towards the osteoid seam (Palumbo, 1986) (Fig.1), do not divide, and can be distinguished from preosteoblasts by the upregulation of a suite of bone markers, the main ones being bone sialoprotein, osteocalcin, E11, BMP-R1, vitamin D3 receptor, vitronectin, thrombospondin, decorin, several BMPs, and, of course, collagen type I (Table 1).

Many researchers have reported extensive heterogeneity in osteoblast gene and protein expression patterns (Aubin et al., 1992, 1993; Heersche et al., 1992; Chen et al., 1993; Ikeda et al., 1995; Liu et al., 1997; Candaliere et al., 2001) (Table 1). For example, in adult rat bone marrow stromal cell cultures, adjacent osteoblasts that appear identical morphologically express very different levels of osteoblast-associated markers such as osteocalcin, osteonectin (SPARC), and galectin-3 (Malaval et al., 1994). This heterogeneity is not regarded as the result of the cell cycle stage and is still present when analyses are restricted to post-proliferative osteoblasts (Liu et al., 1997). A recent study of the osteoblasts of 21-day-old fetal rat calvaria found that despite all cells appearing histologically similar, the only markers expressed by all osteoblasts, irrespective of their position in the calvaria, were alkaline phosphatase and the pth/pthrp receptor (Candeliere et al., 2001) (Table 1). Both markers are also expressed by preosteoblasts, although the levels are lower in preosteoblasts than in osteoblasts (for pth/pthrp receptor), and are not detectable above background staining in osteocytes. Other osteoblast markers (osteocalcin, *msx-2, c-fos,* parathyroid hormone-related protein) are differentially expressed at both mRNA and protein levels in subsets of osteoblasts depending on their location or environment within calvaria (Candeliere et al., 2001). All markers tested in this study are present in post-proliferactive cells so cell cycling cannot account for the differential expression patterns. Anatomical site, developmental age, species, and mode of ossification can all influence the gene expression profile of osteoblasts (Heersche and Aubin, 1990; Aubin et al., 1993; Gehron Robey et al., 1993; Liu et al., 1997). Candeliere et al. (2001) also found that preosteoblasts and osteocytes differentially express a repertoire of genes.

As bone matrix deposition continues, osteoblasts become embedded in the cells secretory product, the osteoid. Cells at this early stage of osteoblast to osteocyte differentiation have been called "large osteocytes," "young osteocytes," or osteoid-osteocytes (Baud, 1968, Semba et al., 1966). These cells are larger than mature ("older") osteocytes and have a well-developed Golgi apparatus for collagen storage. At the transmission electron microscope level, collagen fibrils can be seen surrounding these matrix-producing cells (Hancox and Boothroyd, 1965).

On mineralization of the osteoid, osteocyte ultrastructure undergoes further changes, a reduction in the ER and Golgi apparatus corresponding to a decrease in protein synthesis and secretion (Dudley and Spiro, 1961). Now, many of the previously expressed bone markers are down regulated or switched off in the osteocyte (e.g., osteocalcin, bone sialoprotein, collagen type I, alkaline phosphatase, IGFs, integrins, periostin, and others) (Table 1). Some studies have shown that depending on the type of bone formed and the activity and size of the committed osteoblast, the newly embedded osteocyte may be variable in size and shape in comparison with older, more mature, osteocytes already embedded in bone matrix (Boyde, 1980; Marotti et al., 1990). Furthermore, the shape of embedded osteocytes may depend on the bone type. For example, in woven bone, which is laid down rapidly with randomly oriented collagen fibres, the osteocytes are isodiametric (Currey, 2003). In lamellar bone, however, which is laid down more slowly, osteocytes are flattened and oblate with their short axis parallel to the thickness of the lamella (Currey, 2003). Mature osteocytes are finally situated within lacunae in the bone matrix, are stellate-shaped, and have long cell processes (Fig.1).

The Transitional Cell Stages

Since osteocytes derive from osteoblasts, transitional cell stages between differentiated osteoblasts and osteocytes should be identifiable based on both, morphological and molecular characters (Fig. 1, Table 1).

Some authors distinguish intermediate or transitional stages between osteoblasts and osteocytes, namely osteoid-osteocytes (Palumbo, 1986), osteocytic osteoblasts (Nijweide et al., 1981), or preosteocytes (Holtrop, 1990) for the bone cell completely surrounded by osteoid. More recently, Nefussi et al. (1991) distinguish between osteoblastic osteocytes and osteoid osteocytes. The osteoblastic osteocyte is younger and will become an osteoid osteocyte. These authors also distinguished an intermediate pool of cells between preosteoblasts and osteoblasts that they term preosteoblastic osteoblasts. In summary, in the transition from preosteoblast to osteocyte, they identify six cell types (Fig. 1). The preosteoblastic osteoblast will not necessarily transform into an embedded osteocyte and merely replaces the "lost" osteoblast from the osteoblast layer.

Palumbo et al. (1990) distinguish three cell types from osteoblast to mature osteocyte. The type I preosteocyte is also known as the osteoblastic osteocyte. Type II preosteocytes are osteoid-osteocytes, while type III preosteocytes are cells that are partly surrounded by mineralized matrix. Despite the fact that many workers refer to any bone cell surrounded by osteoid or matrix as an osteocyte, it is important to remember that the initial enclosure of an osteoblast by osteoid is not the end of the process of osteoblast-to-osteocyte transformation. This stage, as all other stages, is part of a continuum of differentiation, which is why different authors can

identify distinct features at each stage. We summarize the stages from preosteoblast to osteocyte in Figure 1.

It is not only the presence or absence of bone markers that alters during the transformation process but also their levels of expression. Naturally, quantitative characters, such as mRNA and protein expression levels, are more difficult to describe than qualitative characters. A molecular marker that has been proposed to characterize the intermediate cell types in the osteoblast-to-osteocyte transformation is an epitope that binds an antibody called E11 (Table 1). Wetterwald et al. (1996) originally identified the E11 antibody, which recognizes osteoblasts, preosteocytes, and osteocytes. Liu et al. (1997) then used this marker to distinguish between mature osteoblasts on osteoid (i.e., osteoblastic osteocytes) and those embedded in mineralizing osteoid (preosteocytes). The expression pattern of this marker was, however, contradicted by Schulze et al. (1999). They detected E11 expression in osteocytes and their processes but not in osteoblasts. More recently, Hadjiargyrou et al. (2001) confirmed E11 expression in osteoblasts and osteocytes during fracture healing in rat femora. The variable expression pattern of this marker may be the result of working with different populations of osteoblasts and osteocytes by these researchers, as this is the situation for many of the other osteoblast-associated markers (Aubin, 1998).

In addition to the great repetoire of genes expressed at each of the above stages, there are also differences between bones of different ages. Differential expression of non-collagenous matrix proteins was observed in rat femora and calvaria by Ikeda et al. (1992). For example, osteocalcin is weakly expressed in newborn rat bone (2 day) compared to older rat bones (8 weeks).

In summary, although a number of molecular markers for preosteoblasts, osteoblasts, and osteocytes are known, their level of expression is variable at different stages during osteogenesis, and because of this there is great heterogeneity in the expression profiles with some lineage markers (Aubin, 1998) (Table 1). Consequently, the identification of intermediate cell types still depends largely on morphological characteristics (Fig. 1, Table 1). A combined effort using a number (or all) of these markers and looking specifically at the osteoblast-osteocyte transformation could prove to be extremely useful in our understanding of this dynamic process. From our analysis, those that may prove to be helpful are alkaline phosphatase, the insulin-like growth factors and their receptors, TIMP, hxBP-1, osf2, nitric oxide, and DMP1 (Table 1). To this end, Bonewald and colleagues have established several osteocyte-like mouse cell lines from long bones (called MLO cell lines) (Aarden et al., 1996; Caplan et al., 1983; Kato et al., 2001). The advantage of cell culture methods is that one can manipulate cell culture factors to influence proliferation, differentiation, matrix synthesis, and function (Tenenbaum and Heersche, 1982; Aubin et al. 1992; Stein and Lian, 1993). One of the difficulties with culturing bone cells, however, is that osteocytes do not divide in situ and require the presence of their extracellular mineralized matrix to maintain their differentiated state and normal function (Kalajzic et al., 2004). In addition, differences in maturation of bone cells in vivo compared to in vitro have been observed (Litvin et al., 2004). For this reason we have not included expression data from cell culture work in Table 1. The lack of molecular data for transitional cell stages is highlighted in Table 1, although some data can be inferred from the expression pattern before and after the intermediate stages (grey shading in Table 1).

THE TRANSFORMATION PROCESS

The transformation from osteoblast to osteocyte occurs during several of the ossification processes described. We concentrate on intramembranous ossification, which although a continuous process, can be subdivided into two broad phases: (1) condensation of cells and initial synthesis of collagen fibrils, and (2) polarized secretion of bone matrix (Fig. 2).

Initially, a condensation of mesenchymal cells (committed to an osteoblast fate) develops, within which osteoblasts differentiate and begin depositing collagen type I fibres with some type II and III. Collagen type III may form an initial framework on which bone deposition takes place (Carter et al., 1991). During fracture healing, collagen type III forms a scaffold for the migration of osteoprogenitors and capillary in-growth, only later being replaced by collagen type I (Bierbaum et al., 2003). A view outlined in most textbooks (e.g., Windle, 1976, figs. 6–16), present in the minds of most researchers, and based on studies in mammals, is that the osteoblasts within the condensation deposit this collagen in a polarized manner (i.e., matrix secretion occurs from one cell surface only) (Fig. 2B–D). What is not clear is whether these polarized osteoblasts are organized (Romer, 1970; Windle, 1976) or not (Bloom and Fawcett, 1969, fig. 10.20, also suggested by Ferretti et al., 2002) (compare Fig. 2B,C). It is also unknown whether the *very first* cells to deposit collagen fibrils at the centre of an osteogenic condensation (before they line up along the bone surface) are also polarized; they may secrete fibres from all cell surfaces (Fig. 2A). Nevertheless, during this (first) phase of intramembranous ossification, osteoblasts appear to undergo self-burial (Parfitt, 1990).

Recently, bone acidic glycoprotein-75 was found to be expressed during the very early stages of intramembranous osteogenesis and may play a role in defining condensed mesenchyme regions (Gorski et al., 2004), together with tenascin C and other extracellular matrix molecules (Hall and Miyake, 2000).

Once this initial matrix (osteoid) is laid down, osteoblasts line up along the edge of the bone spicule to deposit more bone matrix, thus increasing the size of the bone. Once the osteoid has reached a particular thickness, additional osteoblasts become trapped. This second phase of intramembranous ossification is discussed in the schemes below.

Possible Schemes of the Transformation Process

We consider four schemes for how an osteoblast could become trapped within bone matrix (Fig. 3A–D). A fifth scheme (Fig. 3E) represents the

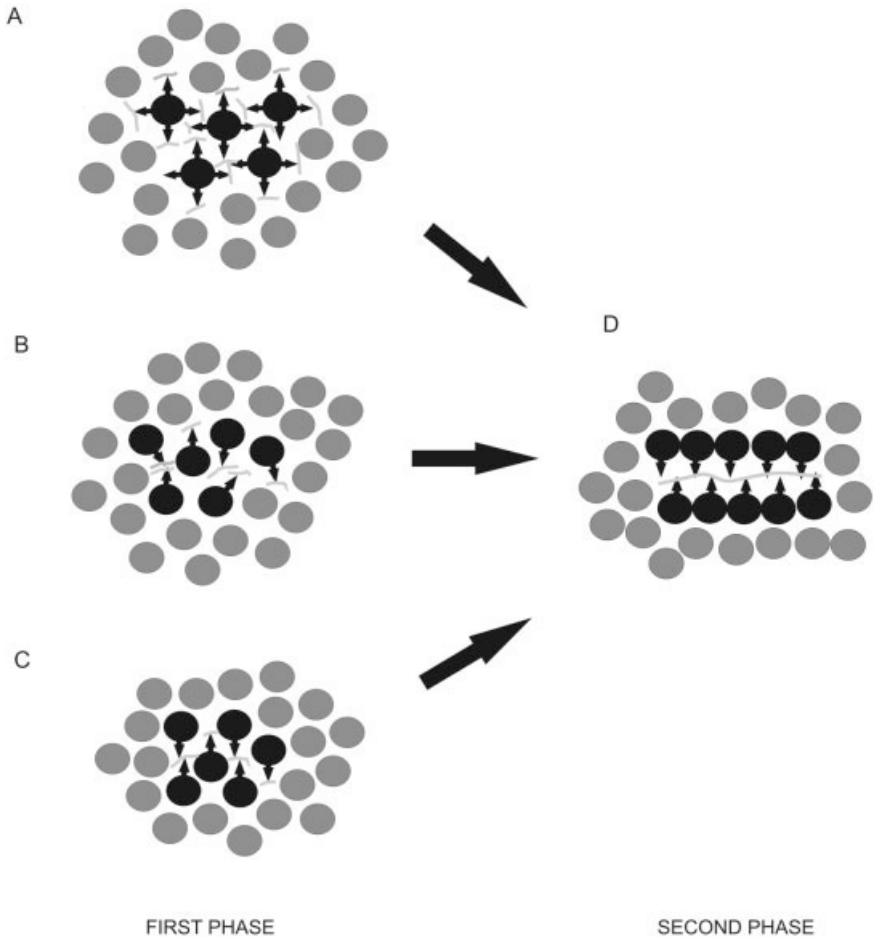

Fig. 2. A diagram of the possible ways in which the first collagen fibres are laid down by cells within a condensation during the first phase of intramembranous ossification prior to lining up on a bone surface. Secreted collagen fibrils are shown in grey shading and the arrows indicate the direction of secretion. **A:** The cells secrete collagen in all directions and are not polarized. **B:** The individual cells are polarized but secrete collagen in an apparently random fashion. **C:** The individual cells are polarized and secrete collagen in an organized fashion towards the centre of the condensation. **D:** The situation is similar to C except that the cells are lined up when they secrete collagen fibres. See text for literature cited to support these possibilities.

likely way in which osteoblasts avoid becoming trapped in bone matrix in the osteocyte-deprived (acellular) bone of higher teleosts (Ekanayake and Hall, 1987, 1988; Witten et al., 2004). These schemes are outlined below.

1. Osteoblasts are unpolarized and lay down bone in all directions. That is, the cells become trapped by their own secretions (Fig. 3A).
2. Individual osteoblasts are polarized (i.e., lay down bone in one direction only), but those within the same generation (layer) are polarized differently to those within adjacent layers. Consequently, bone is deposited in all directions and osteoblasts become trapped (Fig. 3B).
3. Osteoblasts of each generation are polarized in the same direction: one generation "buries" the preceding one in bone matrix (Fig. 3C). We are unaware of any evidence having been provided for this mechanism.
4. Within one generation, some osteoblasts slow down their rate of bone deposition or stop laying down bone so that they become trapped by the secretion of their neighbouring cells (Fig. 3D). This scheme was proposed by Palumbo et al. (1990) and Nefussi et al. (1991).
5. Osteoblasts are highly polarized and function as a unit to lay down bone synchronously. All cells move away from the osteogenic front as bone matrix is deposited, ultimately resulting in acellular bone (Fig. 3E) (Ekanayake and Hall, 1987, 1988; Witten, 1997).

The Decision to Transform Into an Osteocyte: Morphological Perspective

Candaliere et al. (2001) raised the point that differences in the developmental age of osteoblasts may account for the expression pattern of gene heterogeneity between osteoblasts, such that, at least in calvaria, osteoblasts express different sets of genes as they progress along bone surfaces away from the bone suture. This hypothesis implies that osteoblasts of different developmental stages are present along bone surfaces; we know that only some osteoblasts make the transition to osteocytes. The decision to follow the osteocyte fate could very well depend on or be controlled by the gene expression profile of the surface osteoblast (which is in contact with underlying osteocytes). The tools to track individual osteoblasts as they transform into osteocytes are available but need to be applied to the transitional cell stages as we are only beginning to understand osteoblast gene heterogeneity.

It has also been proposed that osteoclast signals may modulate osteoblasts given the cross-talk between these two cell types (Candaliere et al., 2001). Thus, a combination of communication between embedded osteoblasts, osteocytes, and osteoclasts, together with unique gene profiles, could decide the fate of an osteoblast. Throughout the whole process of transformation, the differentiating cell remains in contact with cells in both the osteoblast layer and with embedded osteocytes, even as morphological characters change (Palumbo et al., 1990).

According to Palumbo et al. (1990), the decision to transform into an osteoblast is as follows. During bone formation, processes on the vascular surface of the osteocytes continue to grow to enable osteocytes to remain in contact with the active osteoblast layer and to modulate their activity. When these vascular-facing processes stop growing, they produce a signal that induces the recruitment of those osteoblasts with which they are losing

contact. The committed osteoblasts are then transformed into osteoblastic osteocytes. The signal to stop growing a vascular process may by issued by the osteoblasts with which they have contact or it may be due to the gradual reduction in the vascular supply to the osteocytes as new layers of bone are laid down on the osteogenic front. In either event, the active lifespan of the osteoblast (prior to entrapment) is independent of the amount of bone matrix it produced (as shown in the mouse periodontium by McCulloch and Heersche, 1988).

The Decision to Transform Into an Osteocyte: Molecular Perspective

Several factors have been reported to modulate osteoblast function and/or are involved in controlling the decision to transform into an osteocyte. Here we discuss some of the key players.

The transcription factors, *Runx2* and *Osterix*, are crucial for osteoblast differentiation during both intramembranous and endochondral ossification (Nakashima and de Crombrugghe, 2003; Inohaya and Kudo, 2000; Kobayashi and Kronenberg, 2005). *Runx2/Cbfa1* directly activates a number of osteoblast/osteocyte markers such as type I collagen, osteopontin, bone sialoprotein, and osteocalcin (Aubin, 1998; Inohaya and Kudo, 2000; for further discussions, see Eames et al., 2003). A homozygous mutation of this gene in mice causes a complete lack of bone formation with arrested osteoblast differentiation (Komori and Kishimoto, 1998; Otto et al., 1997). This transcription factor is, however, not expressed by preosteoblasts, nor is it expressed in osteo-chondroprogenitor cells (Nakashima and de Crombrugghe, 2003), which are common precursor cells that have the potential to differentiate along either the osteoblast/osteocyte or chondroblast/chondrocyte lineages (for more information on this cell type, see Hall, 2005). *Osx* is probably downstream of *Runx2* since no *Osx* transcripts are detectable in *Runx2* null mice (Nakashima and de Crombrugghe, 2003). Several other transcription factors are also involved in osteoblast proliferation and differentiation—*Msx1, Msx2, Dlx5, Dlx6, Twist, Runx2, Osx, Sp3*—but have no bearing on our discussion on the transformation from osteoblast to osteocyte.

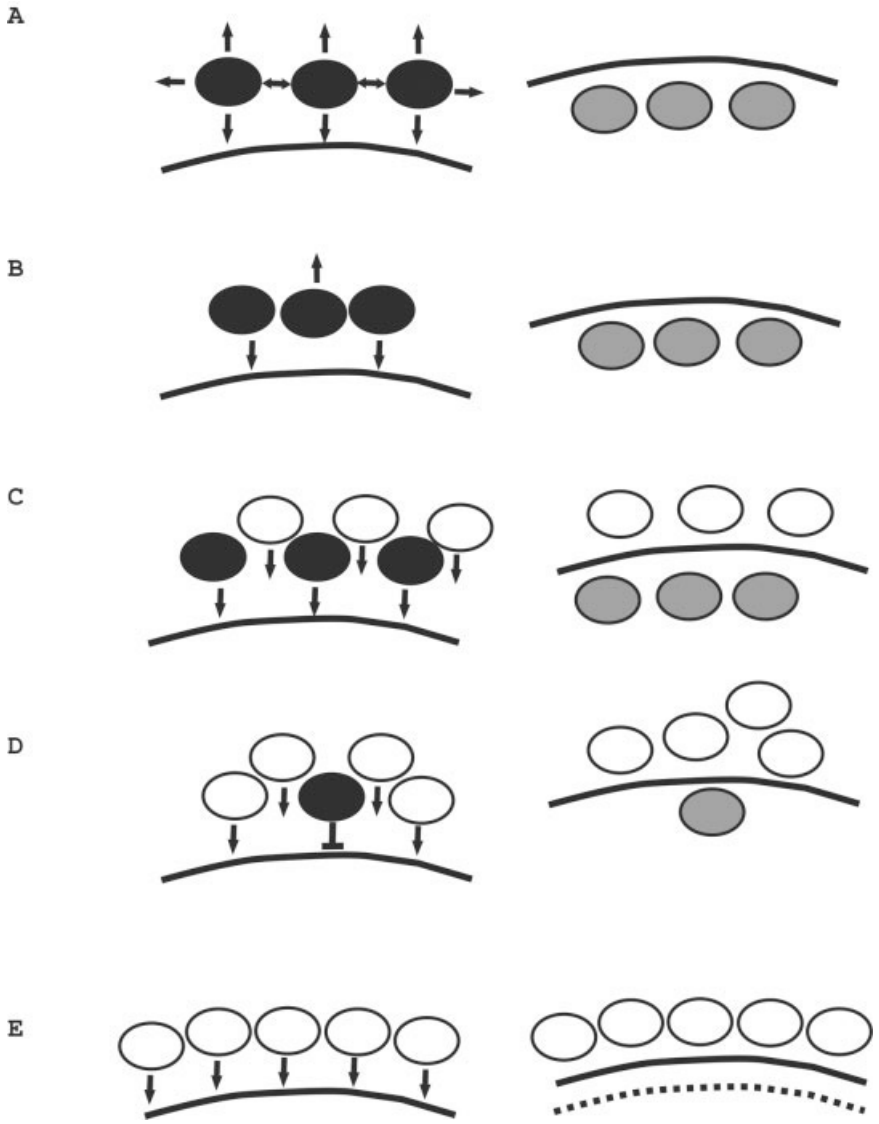

Fig. 3. A schematic of the four different schemes (**A–D**) by which osteoblasts could become entrapped in bone matrix as osteocytes and one scheme (**E**) that leads to bone without osteocytes (acellular bone). The column on the left is the situation before osteoblasts become embedded in osteoid and the column on the right depicts their positions in the osteoid during the next deposition phase. Arrows indicate the direction of matrix deposition by osteoblasts towards the osteogenic front. Solid line indicates the bone surface. Black shaded cells indicate those osteoblasts that will become trapped in the next phase of matrix deposition as osteocytes (grey shading). The cell in D (column 1) stops or slows down matrix secretion and becomes "buried" by its neighbours (passive). The dotted line in E indicates the previous bone surface, which was displaced by the deposition of acellular bone. In all five schemes osteoblasts line up along the bone surface. A: Osteoblasts secrete matrix in all directions. B: Each osteoblast is polarized in a different direction but still secretes matrix in one direction only. C: One generation of osteoblasts buries the next generation. D: The osteoblast to be embedded slows down its matrix production compared to neighbouring osteoblasts. E: Matrix secretion does not embed cells. See text for detailed discussion.

Some important insights can be gained from clinical studies on osteoporosis and craniosynostoses. Leptin is a gene product synthesized by adipocytes but that may serve an important signal to modulate osteoblast function (Gordeladze et al., 2002) and could explain how obesity has a protective effect on osteoporosis. Mice homozygous for the *obese* gene have abnormal glucose and fat metabolism, reduced stature, and do not produce

leptin. Recently, it has been shown that leptin protects cells (including osteoblasts) from apoptosis and facilitates the transformation from osteoblast to preosteocyte. A widely held view amongst researchers who study osteoporosis is that a decrease in bone formation is the result of a reduction in the lifespan of the osteoblast population. This reduced lifespan could be the result of an early incorporation into bone matrix (hence increase in transformation rate to becoming osteocytes [Lips et al., 1978; Darby and Meunier, 1981; Dempster et al., 1983]).

A recent investigation by Borton et al. (2001) suggested that attenuating TGFβ-related signaling mechanisms can increase the propensity of an osteoblast to mature into an osteocyte, and decrease the duration of its productive functioning by shortening its lifespan, ultimately resulting in osteopenia. TGFβ is also produced by osteoblasts and is incorporated into bone matrix (Seyedin et al., 1986; Robey et al., 1987). The model that has been put forward is that osteoblast matrix production rate and osteoblast incorporation into matrix as osteocytes is regulated in part by TGFβ-related signaling mechanisms (Jordan et al., 2003).

Researchers studying craniosynostoses (premature fusion of skull sutures) have identified genes involved in blocking osteoblast differentiation, namely FGFs and Wnt genes and Sox2 (Masukhani et al., 2005). It is thought that FGFs may act via Wnt genes to inhibit osteoblast differentiation and that Sox2 is FGF-inducible.

Genetic models of bone-specific markers (such as knockouts, gain- and loss-of-function mutants, transgenic mice, etc.) can make important contributions to our understanding of the transformation process. Recent studies have identified some factors (e.g., LRP5, sclerostin, *Fgfr2c*, matrix metalloproteases, and glucocorticoids) that have an impact on osteoblast apoptosis and/or activity (Babij et al 2003; Winkler et al., 2003; Eswarakumar et al., 2004; Karsdal et al., 2004; O'Brien et al., 2004) and thus indirectly on the decision process to become an osteocyte (or not). Kalajzic et al. (2004) generated a transgenic mouse with a dentin matrix protein 1 cis-regulatory system that drives green fluorescent protein as a marker for living osteocytes. These studies (and others) provide us with potential future models in which to explore the transformation process in more detail. However, at present none of these studies shed light on the specific mechanism of how the transformation from osteoblast to osteocyte takes place.

How Long Does It Take?

The transformation from osteoblast to osteocyte takes about three days in the femoral metaphysis of 2-week-old rabbits (Owen, 1963). Young (1962) gives an estimate of 2 days for newborn rat tibiae and ribs, while Kember (1960) estimates 5 days for the same animals. In newborn mice, labelled with tritiated thymidine, labelled osteocytes are only seen from day 10 in the periodontal surfaces of the molar root, but in the periodontal alveolar bone osteoblast-osteocyte tranformation is much longer, about 19 days. It is clear from this study that the time to transform is not consistent in all bones/sites of the same animal (McCulloch and Heersche, 1988). At both surfaces, bone apposition rates were similar. That is, transformation rate is independent of bone apposition rate. Interestingly, Ten Cate and Mills (1972) have shown that the source of the progenitor cells for the periodontal surface of the alveolar bone and of the osteoblasts lining the endosteal and periosteal surfaces in alveolar processes is not the same. That is, the origin of the osteoblasts may influence the transformation time to osteocytes. More recently, different rates of bone formation have been reported along the human ilium of pre-menopausal women (Parfitt, 1990) and during calvarial development in rats (Candaliere et al., 2001).

It is clear from the above studies and others that even within one bone element in a restricted population, different rates of bone formation occur. It has also been shown that bone deposition rates are significantly affected by environmental/experimental conditions, skeletal element, and age (Stark and Chinsamy, 2002). In summary, many recent studies investigate bone deposition rates but few focus on the osteoblast-osteocyte transformation other than the early studies mentioned above, which indicate that this process is site-specific depending on the origin of the osteoblasts, the bone, and possibly the age and sex of the animal but independent of the bone deposition rate.

How Does It Happen?

It is thought that osteoblasts control the initiation of mineralization of bone matrix by leaving behind matrix vesicles in the osteoid. As noted above, E11 is one marker expressed during the transition from osteoblast to osteocyte. Recently it has been suggested that E11 is necessary for the formation of fully mineralized vesicles on the developing cellular processes of osteoid-osteocytes (Barragan et al., 2004). Lengthening and narrowing of cell processes results in the mineralized vesicles becoming associated with collagen-mediated mineralization.

It has also been suggested that osteoid-osteocytes may participate in matrix secretion and mineralization and may be involved in the orientation of collagen fibres (as are osteoblasts) (Palumbo, 1986). Palumbo (1986) also suggested that these functions of the osteoid-osteocyte are performed from the mineral-facing side; the vascular side does not have any cell processes at this stage of the osteoblast-to-osteocyte transformation (Fig. 1). This observation implies that mineralization of any osteoid matrix situated between the osteoblast layer and the osteoid-osteocyte is dependent on the activity of osteoblasts (or osteoblastic-osteocytes) and not on osteoid-osteocytes (Palumbo, 1986).

Perhaps one of the most detailed studies on how osteoblasts become osteocytes stems from the examination of intramembranous bone formation in rat calvaria by transmission electron microscopy. Based on their observations, Nefussi et al. (1991) hypothesized the following mechanism for osteoblast entrapment: one of the aligned polarized osteoblasts decreases its activity and loses its alignment with neighbouring active osteoblasts (now called osteoblastic osteocytes) (Fig. 1). These cells slowly become embedded in the matrix formed by the aligned osteoblasts but

maintain lateral cell contacts with these cells. Concurrently, the preosteoblast above the cell that is being transformed into an osteocyte (called the preosteoblastic osteoblast), moves in to occupy the space in the active osteoblast layer so that the aligned layer of active osteoblasts is not disrupted (Fig. 1). In other words, according to Nefussi et al. (1991), embedding of an osteoblast is a passive process.

The osteoblastic osteocyte continues its passive embedding procedure so that by the end of this stage, this cell is completely out of the osteoblast layer. This cell is now morphologically distinct—reduced cell size, decreased organelles—and is called an osteoid-osteocyte (Fig. 1). These authors could not deduce whether the osteoid between this cell type and the osteoblast layer was solely synthesized by the active osteoblast layer or by cells in the osteoid layer. Palumbo et al. (1990) concluded that the osteoblastic osteocyte (type I preosteocyte) and the osteoid osteocyte (type II preosteocyte) are still polarized towards the mineral surface and produce matrix. Matrix production by type III preosteocytes is minimal since mineralizing matrix vesicles are never found on the vascular surface.

Palumbo et al. (1990), examining the parallel-fibred bone of newborn rabbit tibiae, also concluded that entrapment was a passive process. Cellular changes previously reported during osteoblast-to-osteocyte transformation, such as decrease in cell size and organelle number (Palumbo, 1986; Aarden et al., 1994), are indicative of a cell whose activity has declined and appear to support this hypothesis.

More recently, osteoblast entrapment was discussed by Ferretti et al. (2002) in the context of a TEM study of osteoblasts in the calvariae and long bones of newborn rabbits and embryonic chickens. They consider intramembranous bone formation as static osteogenesis because it is performed by stationary osteoblasts that transform into osteocytes at the site where they differentiated. They describe cells irregularly arranged in cords of two to three cell layers at the site of new bone trabeculae formation. Each of these cells is polarized in a different direction with respect to adjacent ones (as revealed by the position of cell organelles with respect to the nucleus and the presence of newly secreted type 1 collagen fibrils). Movable osteoblasts, polarized in the same direction, then differentiate along the trabeculae previously laid down by the "stationary" osteoblasts. So, according to this study, intramembranous ossification is biphasic, consisting of a stationary and a dynamic phase. Surprisingly, they do not describe a condensation stage prior to bone formation so it is difficult to interpret their descriptions. Ferretti et al. (2002) found no significant structural or ultrastructural differences in stationary compared with dynamic osteoblasts. In addition they conclude that two different mechanisms of osteoblast-to-osteocyte transformation takes place: "stationary" osteoblasts transform into osteocytes by self-burial, whereas "dynamic" osteoblasts are selected to transform into osteocytes by the secretory activity of neighbouring osteoblasts.

CHOOSING A MODEL

During the first phase of intramembranous bone formation (when preosteoblasts are within condensations) (Dunlop and Hall, 1995; Hall, 2005), osteoblasts may deposit collagen in all directions (Fig. 2A) (Ham and Cormack, 1979), may be polarized but orientated differently and thus deposit collagen in various directions (Fig. 2B) (Bloom and Fawcett, 1969, also suggested by Ferretti et al., 2002), may be polarized and directed towards the centre of the condensation (Fig. 2C), or may be polarized and aligned, thus depositing collagen in one direction only (Fig. 2D) (Romer, 1970; Windle, 1976). The remaining question that we cannot as yet answer is whether cells committed to become osteoblasts must also become polarized and aligned. To answer this question, more detailed histological studies on early osteogenic condensations are required. Molecular markers for early condensations such as *Osterix*, osteopontin, osteonectin, and alkaline phosphatase (Liu et al., 1997; Aubin, 1998), although also expressed during later stages, can certainly help to identify cells within a condensation that develop into osteoblasts. Dunlop and Hall (1995) showed that alkaline phosphatase is even activated before the condensation of osteoblast precursors during osteogenesis in the first mandibular arch in chicks. At present, alkaline phosphatase remains the earliest marker for osteogenic condensations and demonstration of the enzyme accompanied by structural and ultrastructural studies should reveal if osteoblast precursors and early osteoblasts are polarized and into which direction the first matrix is secreted (Fig. 2).

The second phase of intramembranous ossification is better understood and appears to us far more coordinated in time and space. Osteoblasts are definitely polarized at this stage; the polarized activity of osteoblasts undergoing transformation into osteocytes has been shown based on the position of three main cell organelles: nucleus, endoplasmic reticulum, and Golgi apparatus (Dudley and Spiro, 1961; Pritchard, 1972; Palumbo, 1986). But the question remains, are osteoblasts simply buried by the next generation of osteoblasts (Fig. 3C) or do they contribute to their own burial by stopping and/or slowing down matrix deposition before becoming embedded in the bone matrix (Fig. 3D). Nefussi et al. (1991) suggest that, indeed, a combination of both processes may occur.

Once a certain amount of osteoid has been deposited, and we do not know whether the amount is critical, but a minimum of osteoid is perhaps required to ensure the spacing of osteocytes (osteocyte generations) in the bone matrix, osteoblasts become trapped, probably by the mechanism first proposed by Nefussi et al. (1991) and outlined in scheme D (Fig. 3D). After all, it is evident that osteoblasts can slow down their matrix production, for example, when they transform into bone-lining cells or before undergoing apoptosis. Thus, it appears reasonable to assume an osteoblast destined to become an osteocyte slows down matrix production in comparison to neighbouring osteoblasts and in this way would become passively entrapped in the bone matrix. Even if a polarized osteoblast in the process of becoming trapped does not

stop matrix production completely but continues to secrete matrix at a slower rate, the entrapment would still be a passive process, in the sense that neighbouring cells are responsible for the entrapment. As we know from matrix production by odontoblasts and osteoblasts, which form dentine and acellular bone, respectively, in advanced bony fish (Acanthomorpha), a polarized and synchronized production of matrix by aligned cells does not lead to the embedding of cells into the matrix. If we accept scheme four (Fig. 3D), then the developmental heterogeneity of osteoblasts provides the embedding of some osteoblasts into the bone matrix.

PERSPECTIVE

Future research that focuses on the mechanisms that underlie osteoblast heterogeneity might not only provide a better understanding of how osteoblasts become embedded in bone matrix but also contribute to our understanding of synchronized processes, such as dentine and acellular bone formation, when cells do not become embedded in the matrix. There is so far no evidence to suggest that some osteoblasts are predetermined to become embedded (and hence slow down matrix production) within the newly secreted osteoid, although Lanyon (1993) suggested that the osteoblasts that secrete less matrix and, ultimately, become osteocytes may be those with prior connections to underlying osteocytes. The key to understanding the transformation of osteoblasts to osteocytes and how the latter are buried alive may reside in viewing all bone cells (preosteoblasts, osteoblasts, bone-lining cells, osteocytes) as a continuum, as a tissue, with cells in different developmental stages. To characterize these different developmental stages and to understand their function in time and space, it will certainly be helpful to close the gaps, regarding the expression of molecular markers during the transformation process.

Currently, bone is often still viewed as a hard substance (although we call it hard tissue), with the focus on how the mineralized material is deposited by osteoblasts. However, bone is a dynamic tissue composed of living cells, 95% of which are osteocytes. In this review, we raised the question "how the tissue called bone develops" in order to increase attention to osteocytes.

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