Cell Development, Cell Sources 4

4.1 Cell Development

The skeletal tissue contains various types of mesenchymal cells, such as osteoblasts, osteocytes, chondroblasts, chondrocytes, myoblasts, adipocytes and bone marrow stromal cells. Every cell of the skeletal system, responsible for the regulated synthesis and deposition of extracellular matrix, is necessary to build up a real substitute tissue. The orchestrated work of the different cell types enables the skeleton to perform its unique function. To extracorporally fabricate a bone- or cartilage-like tissue, different cell sources and different cell types are principally suited to tissue-like substitution. The different cell lineages are believed to originate from common progenitors (Taylor and Jones, 1979; Grigoriadis et al., 1988; Owen, 1988; Caplan, 1991; Yamaguchi and Kahn, 1991; Aubin and Liu, 1996; Pittenger et al., 1999) called pluripotent stem cells (Owen, 1988; Caplan, 1991; Pittenger et al., 1999). These progenitor cells acquire specific phenotypes depending on their maturation during differentiation. Stem cells and mesenchymal precursors arise in the embryo and at least some of these appear to persist in the adult organism, where they contribute to the replacement of lost cells in skeletal tissue remodelling and repair. In addition to haematopoietic stem cells, the bone marrow comprises a population of marrow stromal cells or mesenchymal stem cells (MSCs). Mesenchymal stem cells serve as the major reservoir for different classes of stem cells. Stromal cells have multilineage differentiation capacity, thereby generating progenitors with restricted developmental potential, such as fibroblasts, osteoblasts, chondrocytes and adipocyte progenitors (Caplan, 1991; Pittenger et al., 1999) (Fig. 4.1). Maturation of cells happens through stages of proliferation, commitment, progression and differentiation in the organism in order to generate the various tissues (Fig. 4.2). As described in Chap. 2, various hormones and cytokines regulate the fate of the cell during the differentiation process from progenitor cells to mature cells. Among these, members of the transforming growth factor family are the most potent inducers and stimulators of osteogenic and chondrogenic differentiation. For bone development, BMPs as classical examples, not only stimulate osteoprogenitors to differentiate into mature osteoblasts but also induce non-osteogenic cells to differentiate into osteoblast lineage cells (for a review, see Kale and Long, 2000). Other factors (dexamethasone, prostaglandines) also play important roles in the regulation of osteoblast differentiation in an orchestrated way. In this context, it is important to note that molecules of the TGF-β and BMP superfamily are also main regulators of differentiation of stem and precursor cells towards chondrocytes. This is an indication of the complex situation of cell differentiation induction.

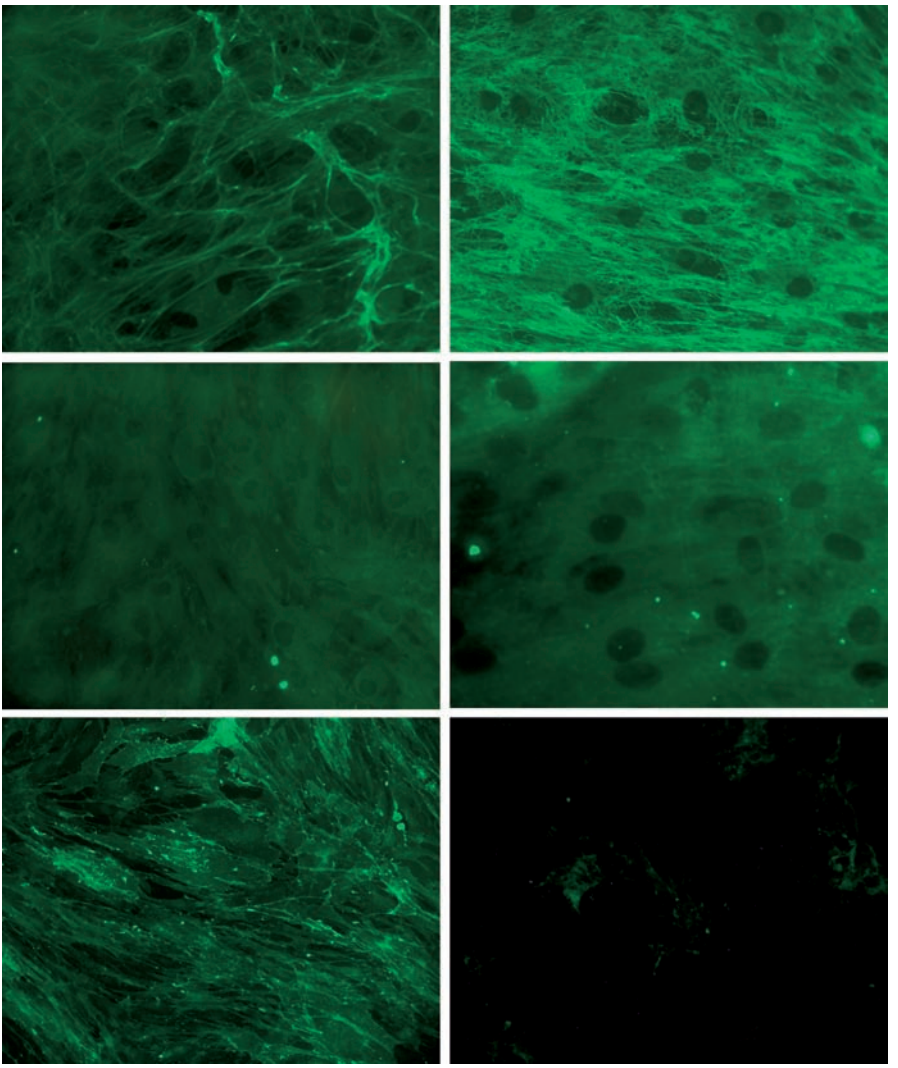

Fig. 4.1. Fluorescent staining of mesenchymal stem cells differentiated to osteoblasts, collagen type I (*top left*), fibronectin (*top right*), osteonectin (*midline left*), bone sialoprotein (*midline right*). For a significant characterisation of the osteoblast differentiation a combination of typical osteoblast and stem cell markers are necessary. For example, stem cells show a positive CD90 signal (*bottom left*) but differentiated osteoblast show no CD90 expression

4.2 Cell Sources

Principal sources of cells for tissue engineering include beneath xenogenic cells, autologous and allogeneic cells (Table 4.1). Each category can be subdivided according to whether the cells are stem cells (embryonic or adult) or whether the cells are in a more differentiated stage (Fig. 4.3). Various mature cell lines as well as multipo-

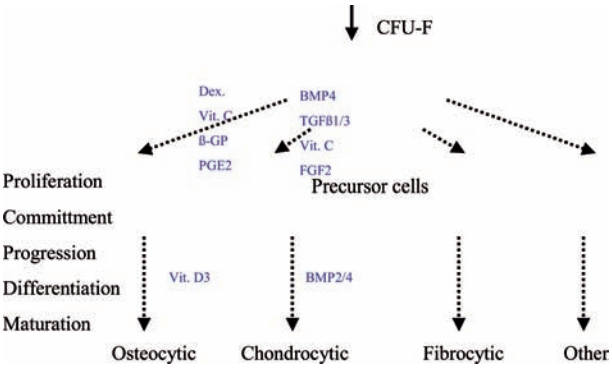

Fig. 4.2. Cell development pathways

Table 4.1. Principal cell sources used in tissue engineering studies

Experimental studies	Preclinical studies	Clinical studies
– Autologous – Allogeneic – Xenogeneic – Immortalised – Non-transformed clonal – Sarcoma	– Autologous – Allogeneic – Xenogeneic	– Autologous

tent mesenchymal progenitors have been successfully established (Yamaguchi et al., 2002) in bone and cartilage tissue engineering approaches. Additionally, other bone and cartilage cell lines like genetically altered cell lines (sarcoma cells, immortalised cells, non-transformed clonal cell lines) have been developed and used to evaluate basic aspects of in vitro cell behaviour in non-human settings.

The stem cell of skeletal tissue is a hypothetical concept with only circumstantial evidence for its existence, and indeed, there seems to be a hierarchy of stem cells each with variable self-renewal potentials (for a review, see Triffitt, 2002). Embryonic and adult stem cells can be distinguished. Embryonic stem cells reside in blastocysts. They were first isolated and grown in culture more than 20 years ago (Martin, 1981). The primitive stem cells renewing skeletal structures have been given a variety of names including connective tissue stem cells, osteogenic cells (Ham, 1969), stromal stem cells (Owen, 1988), stromal fibroblastic cells (Weinberg and Bell, 1986), and mesenchymal stem cells (Caplan, 1991). No nomenclature is entirely accurate based upon the developmental origins or differentiation capacities of these cells, but the latter term, although defective, appears to be in favour at the moment. Stem cells have the capacity for extensive replication without differentiation, and they possess, as mentioned, a multilineage developmental potential allowing them to give rise to not only bone and cartilage, but tendon, muscle, fat, and marrow stroma. This expansion of properties makes stem cells, whether derived from the hematopoetic system (HSCs) or marrow (MSCs), a very interesting source of cells for tissue engineering of bone and cartilage.

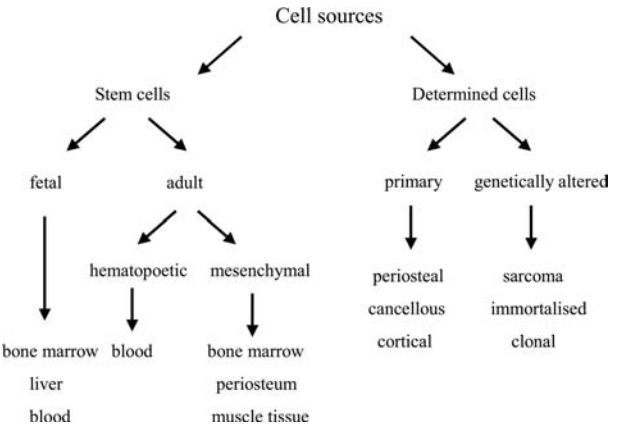

Fig. 4.3. Cell development pathways

Differentiated Phenotypes Derived from MSCs

- Fibroblast
- Osteoblast
- Chondrocyte
- Adipocyte
- Skeletal myocyte
- Cardiac myocyte
- Myofibroblast
- Endothelial cell
- Pericyte
- Tenocyte
- Thymic stromacell

MSCs are present in fetal tissue, post-natal bone marrow and also in the bone marrow of adults (Caplan, 1991). A number of markers are expressed on MSCs. Some of these have been used not only to characterise these cells but also to enrich MSCs from populations of adherent bone marrow stromal cells. However, none of these markers seem to be specific for MSCs. Although the bone marrow serves as the primary reservoir for MSCs, their presence has also been reported in a variety of other tissues (periosteum, muscle connective tissue (Nathanson, 1985; Nakahara et al., 1991), fetal bone marrow, liver, and blood (Campagnoli et al., 2001)). Transfer of these tissues (e.g. coverage of cartilage defects by periosteum) may act also through stem cell-induced repair mechanisms. Whereas MSCs have been identified in fetal blood (Campagnoli et al., 2001) and infrequently observed in umbilical cord blood (Erices et al., 2000; Gutierrez-Rodriguez et al., 2000; Mareschi et al., 2001), it is not definitively solved whether MSCs are present in steady state peripheral blood of adults or not, but the number of stem cells in peripheral blood is probably extremely low (Zvaifler et al., 2000).

A main problem in using adult stem cells in a given clinical situation is the difficulty in obtaining a significant number of stem cells to generate cell constructs of large size. Limitations of adult stem cell harvesting have therefore given rise to the use of fetal stem cells, particularly on fetal bone marrow cells (FBM). This cell source

seems to be a promising source for tissue engineering approaches, since they have a significantly reduced immunogenity, compared to adult stem cells. In contrast to adult tissues, fetal tissues produce more abundant trophic substances and growth factors, which promote to a greater extent cell growth and differentiation. Research on fetal tissue transfer in animal models and in human experimental treatments have confirmed distinct advantages over adult tissues, including lowered immunogenicity and a higher percentage of primitive cells. Due to the lowered immunogenity, fetus-derived stem cells appear to remove, to some extent, the problems of tissue typing. As such cells also have a high capacity to differentiate into the complete repertoire of mesenchymal cell lineages, combined with the ability for rapid cellular growth, differentiation and re-assembly, fetal cells may become a more important subject in tissue engineering strategies. (Fig 4.4) Fetal stem cells have, beneath their enormous potential for biomedical and tissue engineering applications, some serious limitations. There is a lack of methods that enable tissue engineers to direct the differentiation of embryonic stem cells and to induce specific functions of the embryonic cell-generated tissue after transplantation (Wobus, 2001). Two other issues are of main concern (Wobus, 2001). (1) It must be assured that the cultivation and transplantation of such stem cells are not accompanied by a tumorigenic differentiation. As it was shown that undifferentiated ES cells give rise to teratomas and teratocarcinomas after implantation in animals, this potential misdevelopment constitutes a major problem for the clinical use of such cells. (2) It must be assured that the in vitro generation of cells does not lead to an immunological incompatibility towards the host tissue. Immunological incompatibilities can be avoided by using the somatic cell nuclear transfer methodology (SCNT) (Alison et al., 2002). However, nuclear cloning methods are often criticised for this potential risk. The main problem in employing fetal cells for use in tissue engineering is not only obtaining such cells, but perhaps more important, the clinical, legal and ethical issues involved in such treatment strategies, which are probably the most difficult barrier to overcome (see Chap. 12).

Many attemps have been undertaken to refine procedures for the propagation and differentiation of stem cells. Despite the various advantages of using tissue-de-

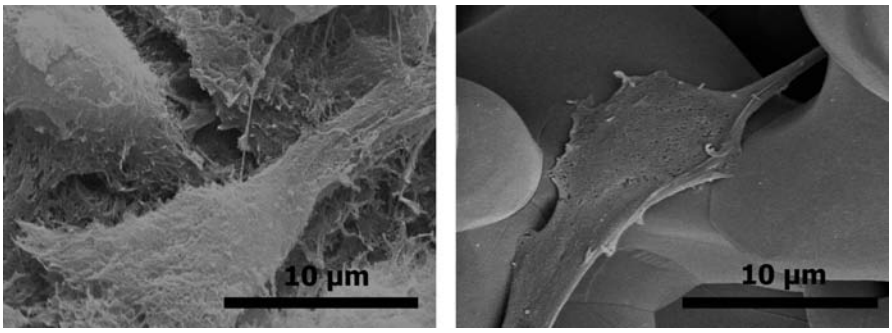

Fig. 4.4. Fetal stem cell-based tissue engineering. Fetal stem cell cultured under defined conditions on an artificial material display the phenotypic appearance of mature bone cells. a Scanning electron microscopical picture of osteoblasts at the surface of a bone trabecula. b SEM view of a murine fetal stem cell cultured on a PLA/PGA scaffold

rived adult stem cells over other sources of cells, there is some debate as to whether large enough populations of differentiated cells can be grown in vitro rapidly enough when needed clinically. At present, stem cells are, for example, not able to differentiate definitively into osteocyte-like cells, which are competent to mineralise the pericellular region in a bone-like manner under in vitro conditions (Jaiswal et al., 1997; Plate et al., 1998). This must be considered as one limitiation for the use of stem cells in extracorporal skeletal tissue engineering. Much more basic research is therefore necessary to assess the full potential of stem cell therapy to reconstitute skeletal mass. It is expected that many future studies will be directed towards the development of gene therapy protocols employing gene insertion strategies (Evans and Robbins, 1995). The concept that members of the bone morphogenetic proteins (BMP) and the transforming growth factor-beta (TGF-beta) superfamily will be particularly useful in this regard has already been tested by many investigators (Lieberman et al., 1998; Oakes and Lieberman, 2000). Genetic engineering to shape gene expression profiles may be, therefore, a future route for the use of stem cells in human tissue engineering, but this approach is at the moment a long way from clinical applications (see Chap. 12).

Differentiated osteoblast- or chondrocyte-like cells serve as common cell lines in evaluating preclinical and clinical aspects of bone and cartilage tissue engineering. Osteoblasts, derived from multiple sites of the skeleton, can be harvested in the form of precursor cells, lining cells, mature osteoblasts or osteocytes. Cell separation is then performed by various techniques in order to multiply distinct cell lines in culture (see Chap. 5). Chondrocytes can also be obtained from the various cartilage-containing tissue sites. They are easy to harvest and are, in most situations, in a mature differentiation stage.

A complementary approach in experimental settings can be distinguished from the use of primary cells: specifically modified cells. Whereas primary cells are commonly used in clinical cell-based engineering strategies, some of the genetic alterations of cells for use in bone or cartilage reconstruction have, up to now, been restricted to laboratory studies. Experiments using i*n vitro* assay systems have yielded not only much basic information concerning the cultivation of these cell lines, but can also be used for tissue engineering studies in basic and preclinical investigations. Each cell source has its inherent advantages and limitations.

4.3 Genetically Modified Cell Lines

Cell lines that are in use for evaluating aspects of extracorporal bone tissue engineering include, besides primary cells, the following cell types: (1) osteosarcoma cell lines; (2) intentionally immortalised cell lines; and (3) non-transformed clonal cell lines. (Jones et al., 1991). In the following we will describe these different cell types.

Osteosarcoma cell lines are known to display patterns of gene expression, modes of adhesion, and signal transduction pathways that in certain aspects resemble those of normal, non-transformed bone cells. Most of the osteosarcoma cell lines used, however, do not display a complete pattern of in vitro differentiation. The development of established clonal osteoblast-like cells from rat osteosarcomas (MG-63, UMR and ROS series) provided cell lines that were homogeneous, phenotypically stable, and easy to propagate and maintain in culture (Wada et al., 1998). They share

many of the properties of non-transformed osteoblasts. But, as with cancer cells, these cells are transformed and display an aberrant genotype, have an uncoupled proliferation/differentiation relationship, and exhibit phenotypic instability in long-term culture. Therefore, these osteoblast-like cells do not reflect the normal phenotype of primary osteoblast-like cells. Therefore, as substrate-dependent cell reactions are difficult to assess, these cells seem not to be suitable to evaluate biomaterial-related aspects in tissue engineering and cannot be introduced into clinical engineering techniques.

Other approaches have been done by using clonally derived immortalised or spontaneously immortalised cell lines (neonatal mouse MC3T3E1 and fetal rat RCJ cell lines; Elgendy et al., 1993). Although none of these cell lines behave exactly alike and their behaviour in cell culture differs considerably (Aubin, 1998b), they do have some common features (alkaline phosphatase activity, collagen type I production, bone-like nodule formation). Despite these common features cells can be in different stages of growth and development under cell culture conditions. They have therefore various phenotypic features, depending on the cell culture situation. Conditionally transformed immortalised human osteoblast cell lines were developed by various researchers aimed to investigate the behaviour of osteoblasts towards external stimuli. Xiaoxue and co-workers (2004) for example assessed the generation of an immortilised human stromal cell line, which contains cells able to differentiate into the osteoblastic cells. Concerning the use of immortalise cells in ex vivo tissue engineering approaches, it is important to recognise that all cell lines impose the disadvantage of having unique phenotypes, so that the morphological sensitivity towards a changing environment (material surface, external stimuli) is impaired.

Considering the features of genetically altered cells, it becomes obvious that non-transformed and primary cultured osteoblasts are advantageous in extracorporal tissue engineering, since these cells display a well-defined inverse relationship of proliferation and differentiation (Owen et al., 1990). Measures of osteoblast-specific matrix protein expression define valuable reference points for the study of regulated osteoblast physiology, especially when a substratum-dependent reaction is under investigation. Oreffo and Triffitt (1999) demonstrated that primary cells are able to react sensitively to minor structural alterations in their surroundings, a key feature which is advantageous in tissue engineering concepts. To assess cellular reactions towards scaffolds the use of primary and non-transformed cells is advisable. It should be noticed that the reaction of cells towards the material is also dependent on the cellular maturation stage (Boyan et al., 1996). Additionally, it should be emphasised that the behaviour of osteoblasts on artificial surfaces is dependent on the experimental cell culture conditions (for a review, see Coehlo and Fernandes, 2000).

Clonal sarcoma cell lines with cartilage phenotypes were established from various sources (for a review, see Kudawara et al., 2004). The human chondrosarcoma cell line HCS-2/8 exhibits a polygonal to spherical morphology as the cells become confluent. After reaching confluence, the cells continue to proliferate slowly and form nodules, which show metachromatic features (for a review see Vautier et al., 2003). Electron microscopically, the cells resemble features of chondrocytes and produce an extracellular matrix consisting of thin collagen-like fibrils. Immortalised chondrocytes have been generated that serve as reproducible models for studying chondrocyte function. Immortalisation of chondrocytes increases the life span and proliferative capacity but does not necessarily stabilise the differentiated phenotype.

Immortalisation of primary human chondrocytes has been done with SV40-TAg, HPV-16 E6/E7, and telomerase by retrovirally mediated transduction, and selection for neomycin resistance are described. It was observed that stable integration of an immortalising gene stabilises proliferative capacity, but not the differentiated chondrocyte phenotype.

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