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Embryonic stem cell therapy for osteo-degenerative diseases

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Bone and cartilage normally do not regenerate after injury and current treatment options rely on surgery. Now that directed differentiation of embryonic stem cells into osteoblasts and chondrocytes has been described, these cells might represent a new valuable and unlimited cell source in the field of tissue-engineering.

Bone, the load-bearing tissue inside the body, is fabricated of three characteristic cell types, the chondrocyte, the osteoblast and the osteoclast, a tremendous amount of extracellular matrix and water. The chondrocyte and the osteoblast are the matrix-secreting cells in cartilage and in bone, respectively. Moreover, osteoblasts produce cytokines and activation factors for the haematopoetic osteoclasts, which in turn constantly reshape the deposited matrix in a process called bone resorption. Most of the exclusive molecular markers for cartilage and bone tissue reside in their extracellular matrix [Table 1].

Tissue	Extracellular matrix	TF
Cartilage	Collagen type II	Sox9
	Collagen type X	Scleraxis
	Aggrecan	
	Link Protein	
Bone	Collagen type I	Cbfa1
	Osteocalcin	Osterix
	Bone Sialoprotein	
	Alkaline Phosphatase	

Table 1. Marker genes for cartilage and bone tissue.

Skeletal development *in vivo*

Early embryonic development begins with the fertilisation of the egg by the sperm forming the zygote, followed by blastocyst development [Figure 1]. Bone and cartilage are of mesodermal origin. The majority of bone formation during development occurs via endochondral ossification. Endochondral ossification is initiated by the aggregation and condensation of mesenchymal cells. The condensation phase is followed by differentiation into chondrocytes, which will in turn form a template (anlagen) for subsequent bone development. Chondrocytes go through several steps of maturation, and are organised accordingly. The ends of future bones consist of resting and proliferating chondrocytes, which form a cartilaginous matrix. In the middle of the cartilage, chondrocytes will then differentiate into pre-hypertrophic chondrocytes, then hypertrophic chondrocytes, which stop producing type II collagen, start producing type X collagen, and form a calcified matrix. These cells are located in the region where ossification first occurs, and in fact a bone collar, or periosteum, forms around the areas that contain hypertrophic chondrocytes. Upon completion of the mineralised extracellular matrix the hypertrophic chondrocytes undergo apoptosis. Incoming blood vessels from the surrounding bone collar bring with them osteoblast progenitors, which then differentiate into mature osteoblasts. Vascularisation also brings with it osteoclast progenitors, which reabsorb the mineralised matrix in order to facilitate bone remodelling. Osteoclasts function both during development and during adult life, where they play a role in normal bone remodelling as well as an accelerated role in disease states such as osteoporosis. In contrast to this process, intramembraneous ossification is more straightforward. Here, the mesenchymal tissue aggregates and condenses as it does in endochondral ossification, but the mesenchymal stem cells then differentiate directly into osteoblasts without the formation of a cartilage anlagen.

Genetic markers of bone and cartilage development

Many transcription factors and signaling molecules are involved in skeletal development, however the roles of some are better defined than others. Scleraxis, a basic helix loop helix transcription factor and member of the Sox subfamily, appears to prepare mesenchymal tissue to respond to BMP signalling. Sox9, and later Sox5 and Sox6, are involved in chondrocyte differentiation and formation of the extracellular matrix. The expression of Sox9 appears to be under the control of FGF signalling. Indian hedgehog (Ihh), which is expressed by pre-hypertrophic chondrocytes, maintains the proliferating chondrocyte pool in the growth plate, and controls formation of the bone collar by inducing the expression of parathyroid hormone related protein (PTHrP) in the perichondrium, the area of mesenchymal tissue surrounding the cartilage anlagen. In addition, PTHrP acts on the growth plate to maintain proliferating chondrocytes, and forms a regulatory loop with Ihh. Members of

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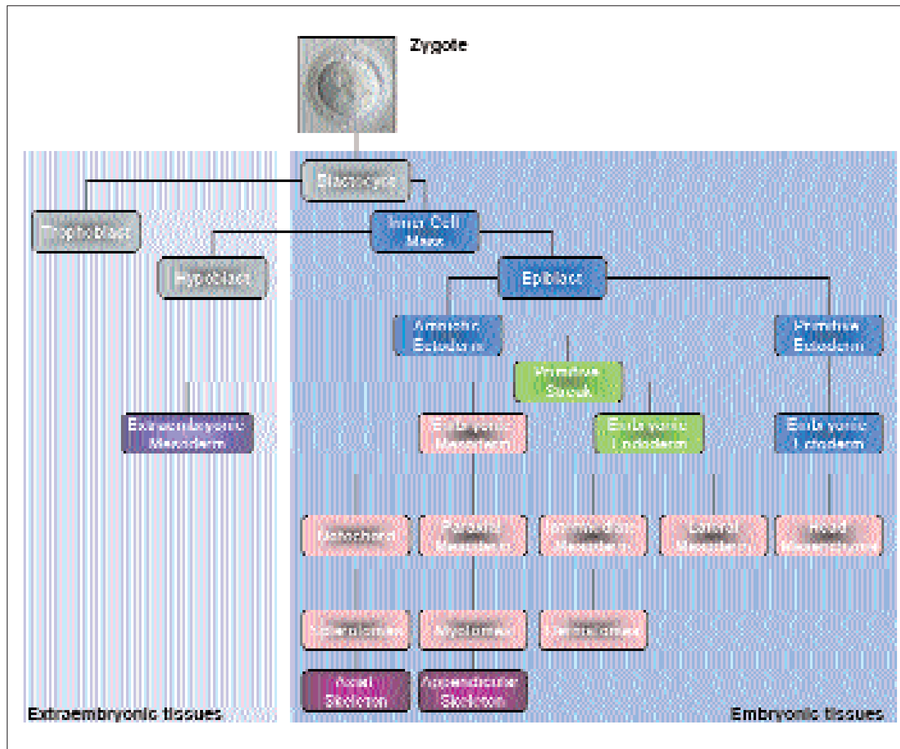

Figure 1. Chart depicting the lineage development in vertebrates through the primary germ layers starting with the zygote. The skeleton, which is composed of chondrocytes, osteoblasts and osteoclasts in the adult, develops from the paraxial mesoderm.

the wnt family control the transition between proliferating and hypertrophic chondrocytes, suggesting that they maintain this control through regulation of cell cycle regulators. Ihh expression is regulated by core binding factor 1 (Cbfa1), a runt family transcription factor that is expressed at low levels in pre-hypertrophic and hypertrophic chondrocytes, and in turn, Ihh induces Cbfa1 expression in the bone collar. Cbfa1 is required for osteoblast differentiation in both endochondral and intramembraneous ossification, since most of the bone-specific genes, such as osteocalcin and collagen type I, contain Cbfa1 binding sites in their promotor regions and are up-regulated upon Cbfa1 induction. Cbfa1 is also responsible for the induction of another recently identified bone-specific transcription factor named osterix.

Bone and cartilage diseases and current treatment options

For decades the treatment of degenerative cartilage and bone diseases defied control by orthopedic surgeons due to the fact that articular cartilage has been reluctant to reveal its secrets as to treatment of its degeneration. Moreover, this challenge is marked by the numerous physiological causes of bone disease. Arthritis and osteoporosis are two of the most common degenerative joint and bone conditions and a major cause of morbidity, disability and claims against health care. Worldwide, an estimated 10 percent of the population aged 15 years and over and 50 percent of people over 60 years reported arthritis as a long-term health problem. There are more than 100 types of arthritic diseases including the non-inflammatory osteoarthritis and inflammatory rheumatoid arthritis, gout, and ankylosing spondylitis. Arthritis is also diagnosed as a secondary symptom in Behcet's and Crohn's disease. Bone diseases such as osteoporosis, Paget's disease and osteogenesis imperfecta decrease quality of life and overall physical health, including loss of the ability to walk, stand up, or dress, and can lead to premature death.

Arthritis and other osteo-/chondrodegenerative diseases can devastate people, but those that suffer from these painful disorders have no effective therapy available to stop the progression of the disease. Patients can only be helped by surgical joint replacement or bone grafting. Common grafts include bone that is donated from another person (allograft), bone that is harvested from the patient (autograft), synthetic constructs made from silicon or a calcium derivative, or bone marrow containing osteoprogenitor cells which are injected into a ceramic scaffold. Operative treatment for hyaline cartilage damage includes microfracture, osteochondral autograft transplantation and autologous chondrocyte implantation. Microfracture which promotes a fibrocartilage healing response is confined to mid-sized injuries and patients with moderate symptoms. Those with significant syndromes must resort to the latter as their only options for relief. Furthermore, patients suffering from bone disease, in particular osteoporosis, are prone to fractures due to weakened bones, which heal slowly. The treatment for these fractures involves the insertion of intermedullary rods into the bone marrow. A major concern, however, is the limited availability of autografts, which significantly reduces the choice of treatable defects, and patients with arthritis or those over the age of 55 are ineligible for any of these treatments.

In the past, engineering bone or cartilage usually required handling of autologous cells. Tissue samples from patients have to be isolated by enzymes such as collagenase and hyaluronidase to remove extracellular matrix components. An underlying problem with this technique is the small number of available progenitors, as adult stem cells usually are very scarcely supplied within the tissue. In fact, the existence of tissue-specific stem cells for some organs is overall questionable. In addition, the ability of harvested cells to undergo proliferation is limited with increasing age of the donor. During *in vitro* expansion of the precious harvested material, cell culture has to be

carefully executed to avoid contamination. The limited therapeutic opportunities have led investigators to focus on more appropriate bioregenerative tissue engineering approaches, which could be specifically tailored for a patient's need.

EMBRYONIC STEM CELLS

Embryonic stem (ES) cells represent a valuable source for cell transplantation since their characteristic features include an unlimited self-renewing capacity and a multilineage differentiation potential. The emergence of the ability to manipulate ES cells is among the most significant advances in the biological sciences. For decades, genetically modified murine ES cells have been used to create transgenic mouse models for the study of mammalian development and gene function [Figure 2]. Furthermore, their pluripotency has been exploited to investigate the cellular factors that are necessary to direct their differentiation into a variety of different tissue types, including those that do not normally regenerate, such as neurons, cardiomyocytes, pancreatic islets, and chondrocytes. The recent discovery of human ES cells has opened up a myriad of therapeutic uses for these cells. In fact, murine ES-derived glial precursors and cardiomyocytes have been successfully transplanted, integrated and shown to be functionally active at the transplantation site [1, 2]. Moreover, the *in vitro* differentiation of ES cells is useful in drug discovery to assess the teratogenic potential of pharmaceutical compounds [3, 4].

Murine ES cells are typically expanded *in vitro* in static monolayer cultures, either on a feeder layer of irradiated mouse fibroblasts, or in medium containing serum and leukaemia inhibitory factor (LIF). LIF stimulates the self-renewal of undifferentiated ES cells while suppressing differentiation by activating transcription of the pluripotency gene Oct4. When ES cells are placed in a medium without LIF, they stop proliferating and differentiate. Clusters of contracting cardiomyocytes form spontaneously during *in vitro* development of ES cells via embryoid bodies and provide a dramatic example of ES cell differentiation potential. The yield of differentiation of ES cells into an intended lineage can be greatly enhanced by the addition of growth factors or induction substances. Whereas protocols for the differentiation of cardiomyocytes, neuronal cell types, insulin-producing cells or adipocytes from ES cells have been available for more than two decades, it is only within the last five years that their differentiation into elements of the skeleton has been reported.

OSTEOGENESIS AND CHONDROGENESIS IN ES CELLS

A new model system for evaluating bone development has recently evolved using mouse ES cells. Uniform embryoid bodies are created from ES cells in 'hanging drops' [Figure 3] in the presence of defined growth factors and supplements. An alkaline environment is necessary to force the cells to secrete a mineralised matrix, which is provided by addition of a phosphate source. Vitamin D_3, retinoic acid, BMP-2 and dexamethasone can act as additional triggers to activate the alkaline phosphatase enzyme and to express a variety of bone marker genes, including Cbfa1, collagen type I, osteocalcin and bone sialoprotein, in a temporally-coordinated fashion [5-7]. BMP-2 is also used to stimulate chondrogenic differentiation. Current research relates to the identification of factors that can improve the output of osteoblasts and chondrocytes. Upon exposing differentiated EBs to various cocktails, improved yield can be detected both by fluorescence activated cell sorting or quantitative PCR. Using these approaches, for example, we have recently demonstrated that the yield of chondrocytes within differentiations can be increased from 7 to 57% simply through the continuous supplementation of culture with BMP-2. This improved efficiency reflects the important role that BMP-2 plays both early in mesenchymal cell development as well as in cartilage formation. Additionally, using appropriate supplements, bone can be derived from cartilage differentiations. Consequently, we and others have suggested that *in vitro* both types of bone formation endochondral (indirect via cartilage) and intramembraneous (direct) can be mimicked [8, 9]. It has also become clear that in the tissue engineering field a critical aspect for successful generation of functional tissue is not only the supplementation with diverse growth factor 'cocktails', but also 3D queues are necessary for the cell to adopt a certain fate. These 3D signals can be provided in culture through growth on scaffolds or in so-called 'pellet cultures', in which chondrocytes are grown in non-attaching aggregates [10].

SCALABLE ENGINEERING OF STEM CELL CULTURE SYSTEMS

One of the major obstacles in the path to successful clinical implementation of this technology is the generation of sufficient numbers of transplantable cells. Bioreactors provide a scalable, robust, and reproducible process to expand cells for clinical purposes. Critical environmental conditions for the proliferation of mouse neural stem cells (NSCs) in suspension culture bioreactors include oxygen level, pH, agitation rate, and osmolarity [11]. The development of the suspension culture protocols for NSCs has laid the groundwork for experiments related to the large-scale expansion of other stem cells, including ES cells. Murine NSC suspension culture protocols are currently adapted to the scaled-up culture of ES cells. ES cells aggregate to form 'embryospheres' in suspension bioreactors, which are molecularly different from embryoid bodies. Over 97% of cells within these embryospheres are expressing the pluripotency markers nanog and Oct4, indicating that the majority of the ES cells in the embryospheres are undifferentiated [unpublished observation]. This is possible through controlled manipulation of the shear field on the embryospheres and maintenance of the average aggregate diameter below sizes that would cause necrotic centres to develop. Not only can this approach be applied to the expansion of large numbers of undifferentiated stem cells, but can also be used for controlled generation of their progeny.

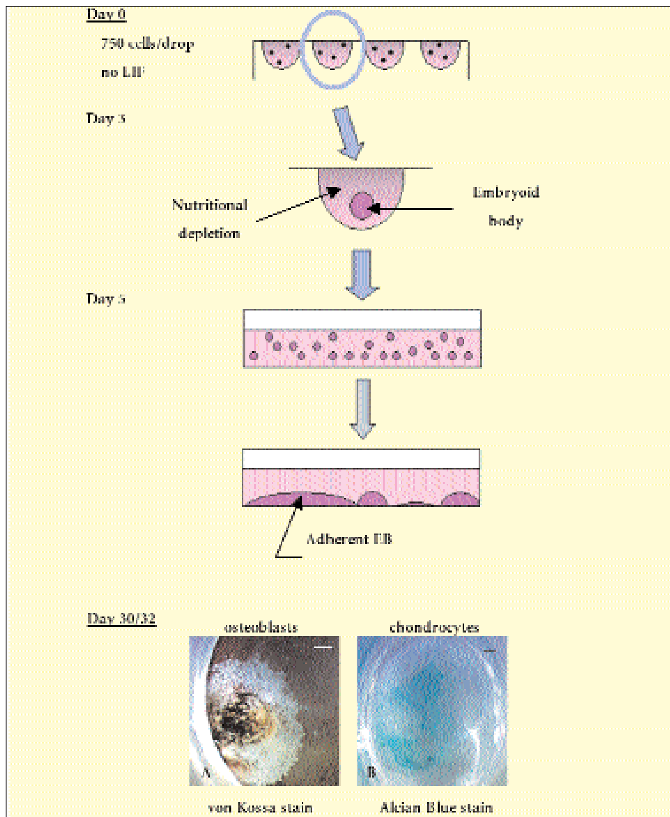

Figure 2. Hanging drop protocol to induce differentiation in ES cells. Drops of single cell suspension are seeded onto the lid of a petri dish. After three days, so-called embryoid bodies (EBs) have formed through aggregation. Due to depletion of nutrients in the drops, EBs are transferred to suspension culture for an extra two days. Appropriate supplementation with growth factors will lead to formation of osteoblasts and chondrocytes after 4-5 weeks, which stain positive for mineralized calcium (A) and proteoglycans (B), respectively. Bar 160 μm (A) and 423 μm (B).

Roadblocks to therapeutic application

Transplantation of undifferentiated ES cells can result in the formation of teratomas and teratocarcinomas, and when implanted into joints, ES cells destroy the joint due to teratoma formation [12]. Even when they are differentiated down specific lineages, grafts can be contaminated with residual pluripotent cell types leading to the formation of teratomas. Here, teratoma formation appears to correlate with the degree to which cells are differentiated and enriched in culture before grafting. Success in the field of tissue engineering and transplantation is thus predicated on driving differentiation of the cell source along a certain lineage, expansion and purification of the cell type. Currently, there is limited knowledge about the factors that direct specific ES cell differentiation in most lineages. With the limited differentiation paradigms that exist, it is not possible to achieve 100% percent efficiency in differentiation. Accordingly, there is concern that poorly defined differentiations could lead to tumour formation upon transplantation. This risk can be mitigated by purging strategies, which eliminate undifferentiated precursor cells, or cell purification strategies based on the capture of cell surface markers, or engineered antibiotic resistance under cell type-specific promoter control.

Furthermore, the cell to be transplanted has to display crucial functional properties on top of its molecular identity in order to restore pathologically altered function. Moreover, appropriate carrier systems have to be defined that deliver the cells to the injury site and provide structural integrity while the cell source integrates into the host tissue. On the pathway to the clinic, a major step will be the critical investigation of the combinatory effect of growth factor cocktails and well-timed application on the implant, which was found to be crucial in the ES system. As we gain more and more insight into the identity of the morphogens for osteoblast and chondrocyte, it might be possible to orchestrate massive regeneration by the clever combination of 3D scaffolds and morphogenic factors, thereby not obviating traditional drug treatment.

Perspective

It is important to note that this technology will lead to eventual application in the clinic. Already researchers have begun to transfer their knowledge of mouse ES cell osteo- and chondrogenesis over to the study of

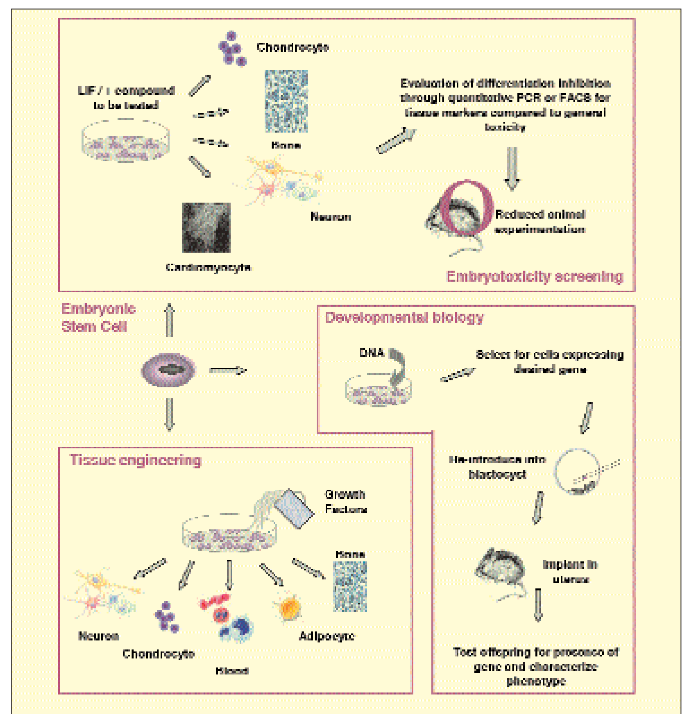

Figure 3. Application of embryonic stem cells. ES cells are used in embryotoxicity assays in the pharmaceutical industry, where the inhibition of their differentiation capacity is consulted to evaluate new drug compounds. Genetically modified ES cells are widely used to generate transgenic mouse models, which aid in understanding functions of genes. Recent efforts in the stem cell field centre around the in vitro engineering of tissues through directed differentiation of ES cells.

human ES cells. Although human ES cell research has been encumbered by regulatory oversight in many countries, a few laboratories will lead the world towards understanding the developmental pathways by which ES cells differentiate, which will enable the creation of a source of cells that can be manipulated to correct for a particular defect. With somatic nuclear transfer, the transfer of a post-mitotic somatic cell nucleus into an enucleated oocyte, scientists now have the tool to generate 'personal' ES cell lines (ntES cells), which may provide an infinite source of autologous cells. Here, another challenge will be the availability of a sufficient number of oocytes. Recently, derivation of gametes from ES cells has been described where oocytes can be differentiated *in vitro* from ntES cells, providing a first step in overcoming this hurdle [13].

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