NWO | Huygens Lecture 2003

Antonios G. Mikos

Tissue Engineering

Christine L. Mummery

Stem cells and tissue engineering – spare parts medicine?

The Hague, November 2003
Netherlands Organisation for Scientific Research

Colophon

Print

Netherlands Organisation for Scientific Research (NWO)
P.O. Box 93138
2509 AC The Hague
The Netherlands
Tel.: +31 70 344 07 13
news@nwo.nl
www.nwo.nl/huygens

In association with

NRC Handelsblad, Rotterdam
City council of The Hague

Editor-in-chief

David Redeker, NWO

Editor

Dave Thomas, Native Speaker Translations

Design

Christiane Renard, NWO

November 2003

ISBN 90-70608-91-X

NWO | Huygens Lectures

The Netherlands Organisation for Scientific Research (NWO) and the newspaper *NRC Handelsblad* have organized NWO | Huygens Lectures since 1992. The lectures providing the publice at large a unique opportunity to listen to and meet famous scientists. The city council of The Hague acts as a host.

For the year 2003 lecture, NWO invited the American bioengineer Antionos G. Mikos. He is J.W. Cox Professor of Bioengineering and Chemical Engineering at Rice University (Texas). Mikos is famous for his research on tissue engineering. Second speaker is the Dutch biologist Christine L. Mummery. She works at the Hubrecht Laboratory of the Netherlands Institute for Developmental Biology (NIOB-KNAW). She is an highly appreciated spokesperson on human embryonic stem cell technology.

The successful NWO | Huygens Lectures are being organized for the twelfth time. Predecessors of Professor Mikos were:

2002	Frans de Waal
2001	Wilfred van Gunsteren
2000	Carl Wieman
1999	Antonio Damasio
1998	Tony Hoare
1997	Paul Colinvaux
1996	Stanley Prusiner
1995	Jonathan Spence
1994	Pierre-Gilles de Gennes
1993	Bert Bolin
1992	Nathan Glazer

Antonios G. Mikos is the J.W. Cox Professor of Bioengineering and Chemical Engineering at Rice University (Texas, United States of America). He received his Dipl.Eng. (1983) from the Aristotle University of Thessaloniki, Greece, and his Ph.D. (1988) from Purdue University. He was a postdoctoral researcher at the Massachusetts Institute of Technology and the Harvard Medical School before joining the Rice Faculty in 1992 as an assistant professor.

His research focuses on the synthesis, processing, and evaluation of new biomaterials for tissue engineering, carriers for controlled drug delivery, and non-viral vectors for gene therapy. Further information about these research interests can be found on Mikos' website (www.ruf.rice.edu/~mikosgrp/).

He is the author of over 240 publications and 17 patents, and the editor of 6 books. He has been elected a Fellow of the International Union of Societies for Biomaterials Science and Engineering and the American Institute for Medical and Biological Engineering.

His work has been further recognised with the *Clemson Award for Contributions to the Literature* of the Society for Biomaterials, the *Phoenix Pharmazie-Wissenschaftspreis*, the *Young Investigator Research Achievement Award* of the Controlled Release Society, the *Outstanding Young Investigator Award* of the Materials Research Society, the *Whitaker Young Investigator Award* of the Biomedical Engineering Society, and the *Victor K. LaMer Award* of the American Chemical Society. He is a founding editor of the journal *Tissue Engineering*.

Christine L. Mummery obtained her Ph.D. in biophysics from the University of London in 1978. She then worked as a postdoctoral researcher at the Hubrecht Laboratory of the Netherlands Institute for Developmental Biology where she became interested in the mechanisms of differentiation during early embryonic development. Mummery became a group leader at the Hubrecht Laboratory in 1993 and she has recently been appointed Professor of Cardiovascular Development at the Faculty of Medicine, Utrecht University.

Her current research focuses on the functional analysis of TGFβ signalling during early differentiation in mammalian development. The following model systems are used for this, transgenic and knock-out mouse embryos, cultures of human and mouse embryonic stem cells, and embryonal carcinoma cells. More recently, her research has also addressed the possible use of human stem cells as a source of cardiomyocytes for transplantation to infarcted or ischaemic hearts. Further information about these research interests, including a selection of published articles, can be found on the Hubrecht Laboratory's website (www.niob.knaw.nl/researchpages/mummery/mummery1.htm).

Mummery is also the Netherlands Institute of Developmental Biology's spokesperson on human embryonic stem cell technology (see a recent interview in the *NRC Handelsblad* newspaper (in Dutch): www.nrc.nl/nieuws/binnenland/1007015542165.html).

Antonios G. Mikos

Tissue Engineering

Abstract

Utilising biologically active factors, cells, and novel biomaterials, tissue engineers have started to repair damaged tissues successfully. Since its infancy only 15 years ago, the field of tissue engineering has already produced a number of commercially available products for the clinical treatment of extensive burn wounds and skin ulcers. Current research at Rice University focuses on the development of new polymeric materials for cell and drug delivery and the design of sophisticated bioreactors for dynamic cell culture. This research has made significant progress towards the treatment of a number of orthopaedic disorders, such as large bone defects resulting from cancer resections and cartilage lesions of osteoarthritic tissue. Further research in this field will result in improved biomaterials and a greater understanding of cellular processes which in turn will lead to an increased range of commercially engineered tissue products to treat a host of degenerative conditions, such as heart, liver, and kidney failure.

Introduction

Age-related diseases are becoming increasingly important in Western societies as the average life expectancy increases due to better health care and advances in medicine. Most of the health problems affecting the elderly occur when vital organs and tissues become less efficient and fail to function adequately. Age-related degenerative conditions include arthritis and failure of the heart, liver and kidneys. Such tissue and organ damage may be the result of tissue overuse or misuse. Furthermore, traumatic injuries, immune diseases, cancer, and other conditions leading to irreparable tissue loss afflict numerous individuals, irrespective of age.

For all of these cases of tissue degeneration, therapy is limited due to the complex structure and functioning of the body's tissues. Current treatments focus on reducing the negative side effects of these conditions by means of medication or surgical procedures. For instance, pain and blood pressure are often controlled by medication. Surgical procedures include catheter insertion or vessel dilation procedures to improve the function of heart muscles and the implantation of artificial prostheses to restore joint function. However, the transplantation of whole organs or the use of tissues from one's own body is limited to a small number of patients due to a shortage of donor organs and the possibility of complications at the donor site.

An exciting new strategy is poised to revolutionise the treatment of these patients with degenerative tissue conditions: the creation of man-made tissues or organs, known as neo-organs. This strategy, which has emerged from the field of tissue engineering, will overcome the problems associated with tissue transplantation.[1] Scientific research has led to an increased knowledge of the molecular processes guiding cell proliferation and differentiation and of the biological processes mediating wound healing.[2] Tissue engineering approaches combine three main factors for the creation of neo-organs and tissues: reparative cells, signalling molecules, and scaffold carriers.

Which organs or tissues can be "engineered" using these reconstructive techniques? Current tissue engineering strategies are targeting the recreation of numerous tissues within the human body.[3] However, the most promising advances have been demonstrated with tissue-engineered skin. Several commercial products are already available and are being used to engineer skin to treat the many patients who suffer from severe burn wounds or from skin necroses resulting from diabetic ulcers or drug abuse. In the United States, approximately two million people per year suffer from burns, and 13,000 patients require skin grafts. In some cases (approximately 1500 patients per year), these burns cover over 20 percent of the patient's body, and skin grafting is not a feasible treatment option. Luckily, due to advances in tissue engineering, commercially available skin substitutes can be employed. These products, approved by the United States Food and Drug Administration, are essentially living cell sheets that can be used for skin replacement.

Intensive orthopaedic research has also led to the development of commercially available tissue-engineered products for bone and cartilage repair. While advances have been made in the area of cartilage tissue engineering, further research is necessary for the treatment of severe degenerative conditions such as osteoarthritis (degradation of the cartilage which cushions joints) a disease estimated to affect some 20.7 million people in the United States.[4] Yet, it is difficult to predict how many of these 20 million or so patients would therapeutically benefit from tissue-engineered cartilage, since the progressive state of this disease varies from patient to patient. However, orthopaedic tissue engineering research can also help with the treatment of bone and joint cancer. In 1999, approximately 2000 people suffered from these diseases and they were generally treated with artificial joints or inert metal prostheses.[4] These replacement parts often promote infections and may wear and loosen with time. For instance, the average life expectancy of an artificial hip joint is only about 15 years.[4] Furthermore, younger patients may outgrow implanted prostheses and thus need to undergo several replacements.

Further research is also necessary to address blood vessel, retina, and nerve regeneration, as well as liver, bladder, kidney, intestine, and heart valve repair. Tissue engineering approaches for these conditions offer an enormous potential for improving and extending patients' lives, as there are often no alternative therapies. For example, the treatment options for patients suffering from liver failure, as a result of viral infections, chronic hepatitis, or alcohol abuse, are currently limited to transplantation. However due to donor shortage, only 32 percent of the 17,000 patients in the United States suffering from liver failure will receive a transplant within one year. Therefore, a huge patient population could potentially benefit from tissue engineering strategies focused on liver repair and regeneration.[5]

The failure or disruption of neural tissue is another potential application for engineered tissues. In the United States alone, between 7600 and 10,000 spinal cord injuries occur every year, with motor vehicle collisions being the main cause of these.[6] These accidents often lead to lifelong disability and at present there are no options for healing or partially restoring neural function. Such treatment options could improve the quality of life for individuals with neural damage as well as reducing the health expenses for nursing these patients.

Despite humans having two kidneys, renal failure is also a significant cause of mortality in the United States. Only about 20 percent of the 58,000 patients on the waiting list for a kidney transplant can expect to receive a replacement organ within the next year. In fact, in 2002, kidney transplantations were limited to 11,000 patients.[5] For patients awaiting transplants, long-term, renal replacement therapy by means of haemodialysis or other types of dialysis provides only an intermittent filtration function and continues to be associated with unacceptably high rates of mortality. Therefore, a tissue engineered renal replacement device would be a welcome alternative to haemodialysis or transplantation.

The long-term outlook for patients suffering from heart disease is not much better. Only 2200 of the 3600 patients who currently need a new heart will receive one within the next year.[5] However,

this statistic only includes patients in need of a complete heart replacement. Other potential therapies for tissue-engineered constructs include the replacement of damaged blood vessels or heart muscles following a heart attack and the replacement of damaged or non-functioning heart valves. All of these potential applications of tissue-engineering could lead to a tremendous improvement in patient lifespan as well as a significant reduction in health costs.

These numerous medical disorders with insufficient treatment options, illustrate that advances in modern medicine are both urgent and necessary. Tissue engineering strategies provide a means of reducing patients' pain and suffering as well as long-term health costs. These benefits mean that the creation of engineered tissues is an ideal alternative to difficult and costly organ transplantations and prosthetic implantations.

Principles of Tissue Engineering

Although specific techniques and technologies must be deployed to engineer each type of target tissue, all of the approaches are based on common key tissue engineering principles. In fact, physicians have been using some of these principles for years. In the simplest case of a relatively small tissue defect, a therapeutic molecule, such as a drug, can be administered or applied locally at the defect site by injection. These molecules are used to "engineer" or influence the behaviour of nearby cells. Chemotactic factors may be used to encourage cells near the defect site to migrate into the wound. Additional protein molecules, known as growth factors, can be used to guide cell development towards a particular cell lineage or type. However, this simple strategy for delivering therapeutic molecules is only successful in stimulating tissue repair when the surrounding tissue possesses sufficient cellularity to support self-healing. Therefore, large defects require more complicated tissue engineering techniques.

Moreover, a method of retaining growth factors or other therapeutic agents at the defect site may be necessary. In this case, tissue engineers use degradable implants to deliver these

bioactive molecules to the injured tissue in a controlled manner. Thus, the implant serves as a means of releasing the drug at the defect site whilst also serving as temporary, three-dimensional scaffolding to which cells from the surrounding tissue may attach and proliferate. However, as new tissue infiltrates the scaffold, enzymatic and cellular processes will bring about its degradation so that the defect is ultimately bridged by new tissue. Scaffolds may be fabricated from a wide variety of biodegradable materials, including polymers, such as those used in producing soluble sutures for surgical applications.

An alternative and perhaps more ambitious procedure involves first of all seeding or filling degradable, porous scaffolds with cells. In many cases, a small number of the patient's own cells may be harvested prior to scaffold seeding and expanded *in vitro* using standard cell culture techniques. After seeding, the cell-scaffold composite is then transplanted into the wound site, where the cells replicate and reorganise to form the new tissue or neo-organ. Bioactive molecules may also be incorporated into the scaffold, prior to seeding, to guide the co-transplanted cells.

Scaffolds for either drug or cell delivery must be designed to address a number of challenges. For instance, scaffolds must be fabricated from materials which release the selected growth factors or drugs within a therapeutic time-frame and dosage. Additionally, scaffolds should provide sufficient mechanical support or flexibility for the desired tissue application. At present *in vitro* experiments and *in vivo* implantations are being carried out to improve the biological response to the scaffolds and the ultimate utility of these scaffolds for tissue engineering.

Synthetic, biodegradable polymers with specific mechanical properties, porosities, and degradation rates can be easily and reproducibly fashioned into implants for drug and cell delivery. As these key material parameters can be tailored in the laboratory, research in the field of tissue engineering has largely focused on the use of polymeric materials in scaffold fabrication. Unlike scaffolds derived from natural materials, such as collagen, gelatine, or fibrin, the properties of polymeric scaffolds are independent

of the batch or source, since polymers are synthesised according to systematic protocols. Therefore, polymeric scaffolds can also be used as standardised experimental tools in cell cultures to study the effect of various stimuli on cell attachment, growth, and differentiation. Such studies provide critical information on cell-biomaterial interactions and reduce the number of experimental animal studies needed. The use of synthetic polymeric scaffolds also circumvents the concerns about immune rejection or possible disease transmission from natural materials derived from tissue from a cadaver or tissue from an animal.

The research in the field of tissue engineering at Rice University focuses on designing new biomaterials for orthopaedic and dental applications, such as bone and cartilage repair. Research within Prof. Mikos' Laboratory has led to the development and characterisation of several novel polymers useful in drug, gene, and cell delivery for both soft and hard tissue applications.

The success of a tissue engineering strategy very much depends upon the choice of scaffolding material. Therefore, these new polymers must be biocompatible and easy to sterilise and should degrade into products that can be metabolised or excreted from the body. The polymer must also be easily and reproducibly processed to form a scaffold (**figure 1**) with a shape and structure defined by the defect volume. The mechanical properties of the scaffold are of particular importance in load-bearing tissues such as bone. Thus, the rate of scaffold degradation should match the rate of tissue regeneration so as to maintain mechanical integrity and to avoid collapse or damage to surrounding tissue. The appropriate degradation rate also allows co-transplanted cells sufficient time and space to proliferate and secrete extracellular matrix molecules as the tissue regenerates. Polymer biodegradability can be tailored by altering a number of chemical and physical parameters, such as polymer composition, molecular weight, and crystallinity. However, scaffold processing and environmental conditions, such as temperature, pH, and mechanical loading, will also affect the degradation process.

The use of a scaffold is imperative since most mammalian cell types are anchorage-dependent and require a suitable substrate to survive, function, and proliferate. Thus, scaffolds are necessary to support co-transplanted cells, as well as those cells recruited from the surrounding tissue by bioactive molecules. The morphology of these cells is directly related to their function. Therefore, by engineering specific surface patterns on a polymeric scaffold, the cell shape can be controlled and the expression of a differentiated cell phenotype can be induced. For example, rounded cell morphology is often maintained by confining cells within microstructures. However, these topological constraints may also affect cell proliferation and migration.

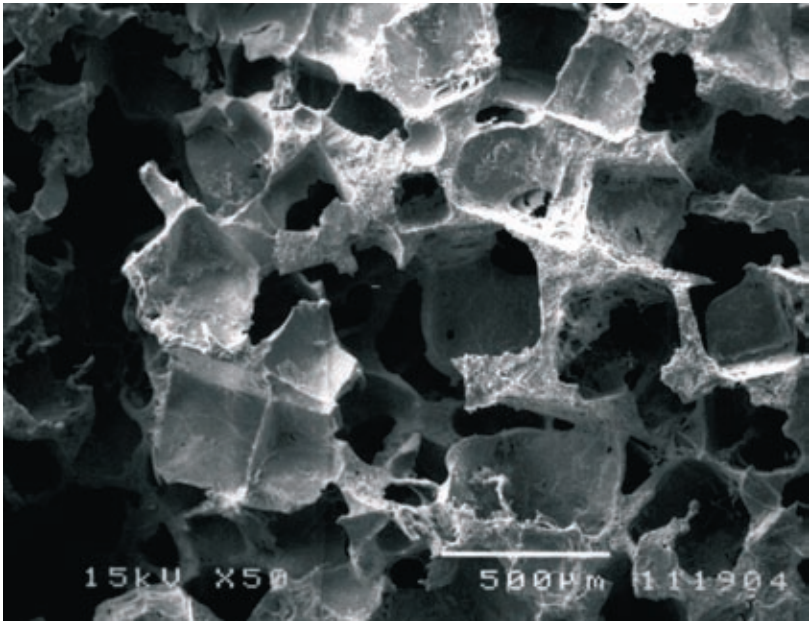


Figure 1: Porous, biodegradable scaffolds obtained by photo-cross-linking a polymer around a pore-forming chemical (sodium chloride).

Yet, scientists can also use other scaffold parameters to further modify cell behaviour. For instance, the chemical composition of the polymer often determines cell adhesion to scaffolds. In general, polymers are classified as hydrophobic or hydrophilic, depending on their composition and water solubility. Hydrophobic polymers

are generally water-insoluble polymers, which tend to adsorb proteins and other fatty molecules from the surrounding culture media or body fluid. In contrast, hydrophilic polymers have a high affinity for water, leading to protein-repellent surfaces, and therefore, non-adhesive surfaces for cells.

Hydrophilic polymers are often preferred as they provide tissue engineers with a precise means of influencing cell attachment. A hydrophilic surface reduces the attachment ability of all cells. Therefore by modifying either the bulk polymer or the scaffold surface with selective adhesion ligands, the number and type of cells recruited can be controlled. For example, researchers in Prof. Mikos' laboratory have coupled a sequence of three amino acids (arginine-lysine-aspartic acid) with a novel, hydrophilic polymer.[7] Although this peptide sequence interacts with the receptors of many cell types, this modification provides an efficient means of modulating cell density and morphology on scaffold surfaces. As shown in **figures 2a, 2c and 2d**, inert polymeric surfaces modified with this peptide facilitate the attachment and spread of cells to form networks with deposited extracellular matrix fibres. The quantity of attached peptide influences the extent to which cells spread and differentiate (**figures 2c and 2d**). However, unmodified, hydrophilic surfaces result in low attachment of cells, which generally retain a spherical morphology and are unable to spread (**figure 2b**). Longer peptide sequences facilitate a greater degree of control over the specific cell type recruited to scaffolds, as well as the modulation of its function.

As well as the response to signals from peptides within the extracellular matrix or scaffold surface, cells also respond to soluble bioactive molecules such as hormones, growth factors, and drugs to promote blood vessel formation. Such molecules are therefore frequently incorporated into a biodegradable scaffold for controlled release to the surrounding tissue, so as to further influence cell behaviour. These so-called inductive factors can be directly incorporated into the cell carrier during scaffold processing or first encapsulated in a release vehicle before mixing with the polymer. Protein encapsulation within carriers such as biodegradable microparticles (**figure 3**) or nanoparticles, protects

these molecules from the scaffold-processing environment and also provides an additional means of controlling release rates. The *in vivo* release of bioactive molecules is governed by diffusion through the carrier and scaffold, as well as by the rate of polymer degradation. Retaining the biological activity of these drugs has been a major concern in delivering large proteins, such as growth factors, but in general, carrier systems considerably extend the half-life of proteins compared to a simple injection of these molecules into a defect.

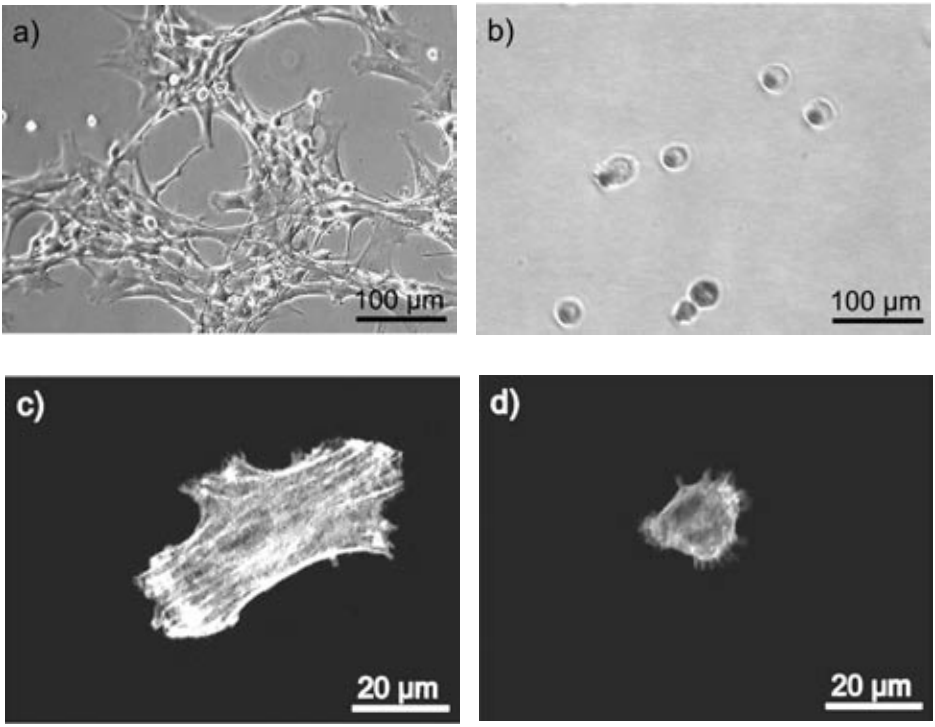


Figure 2: Cell adhesion to a peptide-modified surface (a, c, d) and an unmodified surface (b). On peptide-modified scaffolds, individual cells easily attach and spread in accordance with the peptide concentration, but on unmodified surfaces, cells remain non-adherent and rounded.

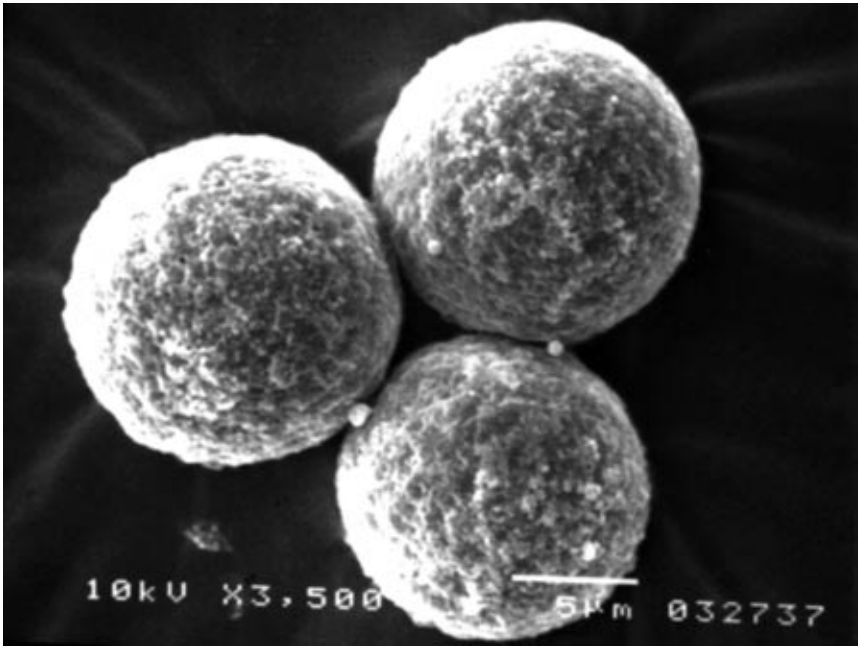


Figure 3: Biodegradable polymer microparticles, commonly used in drug delivery strategies, display rough surface characteristics after 14 days of degradation.

Current Research Projects

One challenge which tissue engineers currently face centres on harvesting or recruiting the correct quantity and type of cells necessary to achieve the repair of large tissue defects. To address this concern, one of our current projects is investigating the expansion and differentiation of a small number of precursor cells found in adult humans. These cells, known as mesenchymal stem cells, can be obtained from a small biopsy of a patient's own bone marrow. It has been shown that these progenitor cells have the "potential" to differentiate into various cell types, including the cells of bone, cartilage, muscle, tendon, and adipose tissue. Since the aspiration of human bone marrow is a well-established clinical procedure, this strategy offers a promising cell source for the repair of a number of tissues.

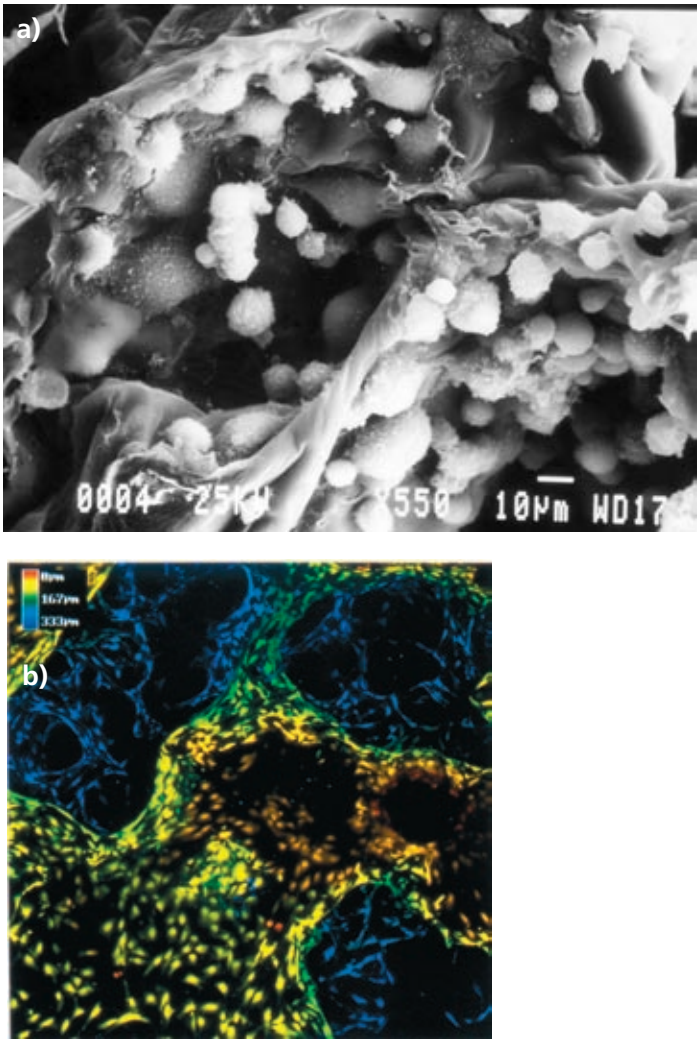


Figure 4: Evaluation of cell-scaffold constructs.
a) SEM image of rat mesenchymal stem cells cultivated in polymeric scaffolds.
b) CSLM image of the three-dimensional arrangement of cells in the newly formed tissue.

After the initial bone marrow harvest, a two-dimensional culture period is used to separate mesenchymal stem cells from the cells responsible for haematopoiesis (maintenance of the body's blood and immune systems). This procedure allows non-adherent, haematopoietic cells to be removed from the anchorage-dependent, mesenchymal stem cells. After this separation, the stem cells are further cultured to increase the number of cells available. Finally, supplements added to the culture media allow tissue engineers to guide or differentiate these cells towards a particular tissue type. For instance, for bone repair, mesenchymal stems cells are differentiated to osteoblasts (bone-forming cells).

To evaluate the quality of tissue obtained during culture, several indicators of bone formation are assessed during the culture period. In particular, biochemical assays are performed to quantify the cells and proteins within cell-scaffold constructs. Histological techniques are also employed to evaluate cell morphology and calcium deposition in the newly formed tissue. Sophisticated techniques, such as scanning electron microscopy (SEM) and confocal scanning laser microscopy (CSLM), allow researchers to analyse the three-dimensional arrangement of cells in the resulting tissue constructs (**figures 4a and 4b**). Finally, micro-computed tomography provides a means of analysing and quantifying the polymer remaining in large tissue blocks excised from animal studies.

In static *in vitro* cultures, diffusion limitations often restrict cell survival in the interior of large scaffolds. However, by providing a constant fluid flow, bioreactor culturing ensures a proper nutrient and oxygen supply to all of the cells in the scaffolds. Using an innovative, flow-perfusion bioreactor designed in our laboratory in collaboration with Prof. John A. Jansen (University of Nijmegen, the Netherlands), cells seeded in meshes (**figure 5a**) are distributed throughout the exterior and interior of these scaffolds. In response to the constant flow of the culture medium, these seeded cells secrete a dense extracellular matrix and form new pores in well-structured sheets, as shown in **figures 5b and 5c**.

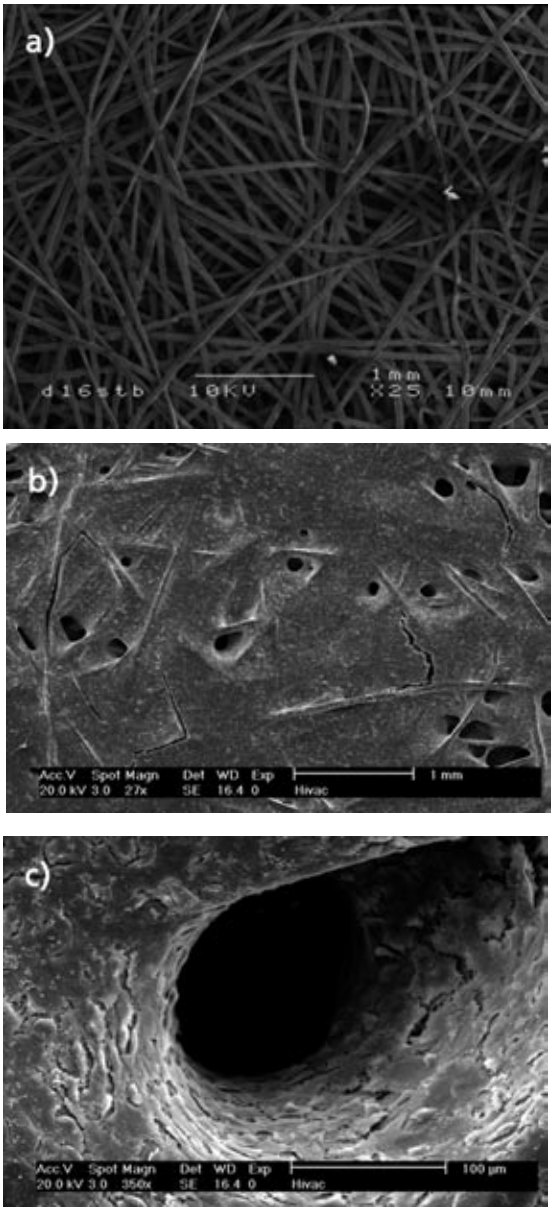


Figure 5:
a) A fibre mesh scaffold prior to cell seeding and bioreactor culture.
b) The dense extracellular matrix secreted by cells cultured in perfusion flow.
c) New pore formation within meshes cultured in the perfusion flow bioreactor.

The use of culture medium flow significantly enhances matrix formation, calcium deposition, and cellularity within these meshes compared to static culture.[8] This dynamic culture system also provides a means of assessing the effects of various stimuli on cell behaviour within scaffolds. Since osteoblasts within the body's intrinsic bone environment constantly experience dynamic mechanical forces, bioreactor culturing may help to mimic these effects *in vitro.*

The cultivation of these cell-seeded scaffolds and their subsequent *in vivo* implantation offers a promising treatment for large tissue defects. However, for irregular shaped defects, the implantation of prefabricated scaffolds may not be feasible. Moreover, in the case of small areas of tissue degeneration, such as cartilage lesions in a knee joint, invasive implantation surgery may lead to further tissue damage. Therefore, injectable polymeric materials offer an alternative means of delivering cells or bioactive molecules to these defects. However, as with prefabricated scaffolds, the processing conditions of injectable materials should not adversely affect co-delivered cells or growth factors.

Our laboratory has developed a novel, degradable polymer capable of forming a gel-like material for the injectable delivery of cells or drugs to soft tissues. This water-soluble polymer can be mixed with a cell or drug solution and then cured into a gel state by temperature-initiated or photo-initiated cross-linking reactions. The resulting gel, known as a hydrogel since it easily absorbs water, allows for living cells to be embedded within the polymer matrix.[9] The gelation of this novel polymer occurs within approximately ten minutes at physiological temperature (37°C), which means that this system can be used as an injectable cell carrier. Mesenchymal stem cells have been successfully encapsulated using this technique. These embedded cells remain viable and produce a calcified matrix after 21 days in culture, as can be seen in **figure 6**. These results indicate that cells not only survive the cross-linking procedure, but are also able to differentiate into functional osteoblasts.

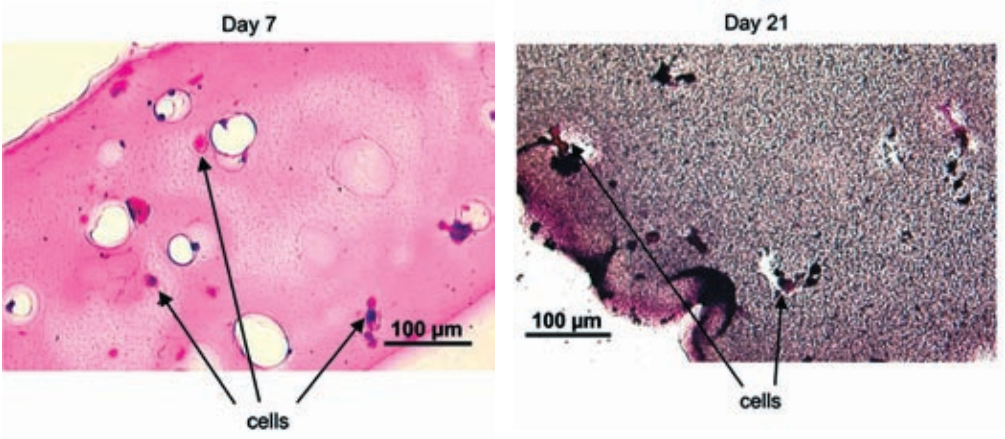


Figure 6: Mesenchymal stem cells encapsulated in a synthetic hydrogel. After 21 days, a significant calcium matrix, stained in black, indicates the differentiation of these cells along an osteoblastic lineage.

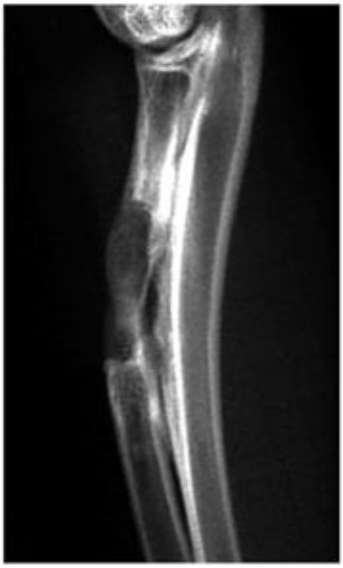
As stated earlier, the incorporation of growth factors or other bioactive molecules within either injectable hydrogels or prefabricated scaffolds provides an additional means of improving the quality of engineered tissue. Our laboratory has developed a number of exciting polymeric delivery systems for the controlled delivery of therapeutic agents. For instance, growth-factor-loaded microparticles have been encapsulated into hydrogels and incorporated into porous, polymeric scaffolds. More sophisticated, multi-layered systems, which allow the release of multiple factors in a time-dependent fashion are currently under development. By altering key material parameters, such as microparticle loading or polymer molecular weight, various rates of release can be obtained.

In all cases, it is essential to optimise the release rate needed for tissue regeneration using a given delivery strategy. For instance, our laboratory has developed porous, polymeric scaffolds for the controlled delivery of a recently discovered therapeutic peptide. This small peptide derived from a natural protein, has been shown to induce both bone and cartilage formation. Degradable scaffolds, with unloaded and peptide-loaded microparticles embedded within the scaffold pores, were fabricated to investigate the effect of the controlled release of this peptide *in vivo*.[10]

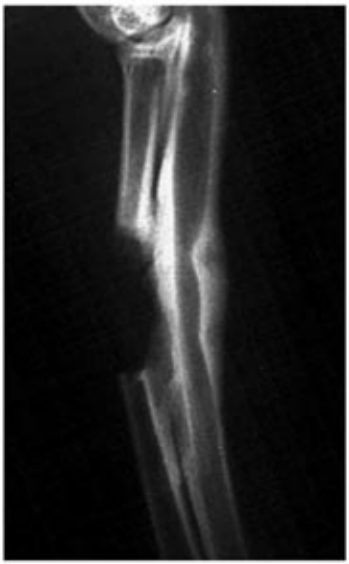
These scaffolds were then implanted into bone defects in rabbits. In particular, a significant portion of the rabbit radius was removed to create a void known as a critical size defect, which cannot heal if left untreated. X-ray photos taken after eight weeks and images generated by micro computed tomography (**figure 7**) demonstrated a greater amount of bone formation when this peptide was released.

Many researchers are also pursuing gene therapy as an alternative means of delivering growth factors over a prolonged period of time. These strategies are seeking to genetically modify cells so that they continually produce a desired therapeutic protein. The modification of cells can be performed *in vitro* prior to the cell seeding of a prefabricated scaffold, or by directly implanting the polymeric gene delivery systems *in vivo* to modify the cells surrounding a defect site.[11] As the protein encoded by the desired gene will ultimately be produced and secreted from modified cells, this method of drug delivery might help to preserve protein activity. If the gene concerned is not incorporated into the cell genome, the resulting genetic modification will not be transferred to daughter cells. In such cases the protein encoded by this gene will only be produced for the lifetime of the modified cells and this will help to prevent excessive or abnormal tissue growth.

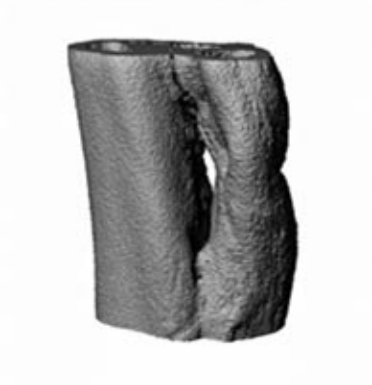
In our laboratory, cells have been genetically modified to produce bone morphogenetic protein-2 (BMP-2), a bone growth factor. **Figure 8** compares the bone formation in critical size, cranial defects of rats following the implantation of meshes (stained black) seeded with either unmodified or BMP-2 producing cells. Successful bone bridging, as indicated by the red stain, can be seen in the case of genetically modified cells, but meshes seeded with unmodified cells result in inadequate bone fill. Therefore, these results demonstrate that a growth factor released from genetically modified cells is capable of inducing bone formation.



Peptide release



No peptide release



Peptide release

No peptide release

Figure 7: X-rays and micro CT scans taken after 8 weeks demonstrate enhanced bone repair in defects treated with peptide-releasing scaffolds compared to defects treated with unloaded scaffolds.

Figure 8: Genetically modified, BMP-2-releasing cells successfully bridge critical size cranial defects in rats, whereas unmodified cells result in limited bone formation.

Challenges

One of the greatest challenges facing the field of tissue engineering is how to overcome limitations in nutrient diffusion in large size tissue defects. Today's engineered tissues are generally realised in smaller animal models, and significantly larger tissues are needed for many clinical applications. However, larger cell-scaffold constructs often lack sufficient oxygen and nutrients to support tissue growth due to the limited blood vessel system present. If the tissue concerned cannot obtain enough oxygen, necrosis might result. Researchers hope to overcome this problem by studying how new blood vessels spontaneously form in tumours, thus allowing these unwanted cell growths to expand and spread. By reproducing the molecular and cellular events which enable blood vessel formation, researchers hope to produce engineered tissues with a good vessel system.

A greater understanding of the cellular processes involved in healing and tissue regeneration is also necessary to improve tissue engineering strategies for both cell and drug delivery. The isolation and culture conditions for cell-based therapies must also be optimised to reduce the time needed to culture cells as well as the number of cells required.

Moreover, recent work on nano-structured and nano-reinforced materials has demonstrated that substantial improvements can be made to the physico-chemical properties of scaffolds.

Nevertheless significant developments in the field of tissue engineering have provided a solid framework from which to develop additional clinical therapies for a host of degenerative conditions. In particular, engineers have shown that degradable, polymeric scaffolds can be used to support and guide developing tissue as well as to provide a means of delivering reparative cells or bioactive agents. Furthermore, injectable polymeric materials offer less invasive methods of treating some degenerative conditions. Tissue engineering has made tremendous advances within its relatively short history and the strategies developed will undoubtedly revolutionise the future of medicine.

Acknowledgments

Prof. Mikos acknowledges the significant contributions of his undergraduate, graduate, and postgraduate associates to the work presented here and is indebted to his fellow researchers for their inspiration. Most of the tissue engineering research in Prof. Mikos' laboratory has been funded by the National Institutes of Health in the United States.

References

1 Langer, R. & Vacanti, J.P. (1993). Tissue engineering. Science 260, 920-926.

2 Mooney, D.J. & Mikos, A.G. (1999). Growing new organs. Scientific American 280, 60-65.

3 Bonassar, L.J. & Vacanti, C.A. (1998). Tissue engineering: the first decade and beyond. Journal of Cellular Biochemistry 30-31, 297-303.

4 Center for Disease Control and Prevention. www.cdc.gov/health/ (2003).

5 United Network for Organ Sharing. www.unos.org/ (2003).

6 Understanding Spinal Cord Injury. www.neurolaw.com/ (2003).

7 Shin, H., Jo, S. & Mikos, A.G. (2002). Modulation of marrow stromal osteoblast adhesion on biomimetic oligo [poly(ethylene glycol) fumarate] hydrogels modified with Arg-Gly-Asp peptides and a poly(ethylene glycol) spacer. Journal of Biomedical Materials Research. 61, 169-179.

8 Bancroft, G.N., Sikavitsas, V.I., Van den Dolder, J., Sheffield, T.L., Ambrose, C.G., Jansen, J.A. & Mikos, A.G. (2002). Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner. Proceedings of the National Academy of Sciences of the United States of America 99, 12600-12605.

9 Shin, H., Temenoff, J.S. & Mikos, A.G. (2003). In vitro cytotoxicity of unsaturated oligo [poly(ethylene glycol) fumarate] macromers and their cross-Linked hydrogels. Biomacromolecules 4, 552-560.

10 Hedberg, E.L., Tang, A., Crowther, R.S., Carney, D.H. & Mikos, A.G. (2002). Controlled release of an osteogenic peptide from injectable biodegradable polymeric composites. Journal of Controlled Release 84, 137-150.

11 Godbey, W.T., Wu, K.K. & Mikos, A.G. (1999). Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. Proceedings of the National Academy of Sciences of the United States of America 96, 5177-5181.

Christine L. Mummery

Stem cells and tissue engineering – spare parts medicine?

Introduction

Imagine. A year ago you were lying in bed. Tired. Too tired to get dressed. Heart failure following a heart attack. Just like 700,000 other people in the Netherlands. Yet next week you will take part in the Rotterdam marathon! Impossible? If we believe reports in the media, stem cells and tissue engineering will make this dream a reality. Just as it will heal Christopher Reeve's spinal cord lesion and allow him to walk again or will cure Michael J. Fox of his Parkinson's disease.

Such scenarios are not yet a reality but neither are they complete science fiction. For many years, scientists have known that tissues and organs in the adult body with the capacity to repair themselves, such as the skin, intestine or blood, contain unspecialised stem cells. These *adult stem cells* can divide; some of their progeny "differentiate" and undergo changes so that they mature into cells of specific tissue types, whilst others remain undifferentiated and form a resident population of stem cells ready to respond to the body's demands for replacement whenever damage occurs. Until recently it was thought that tissues incapable of repairing themselves, such as the heart, brain and central nervous system, did not contain a resident stem cell population. However, there is now some evidence to suggest that stem cells may exist in these tissues but in insufficient numbers for tissue repair.

However, the current interest in stem cells was not fuelled by the studies on adult stem cells. Rather the excitement was caused by the discovery of embryonic stem cells in humans. Embryonic stem cells are unusual, as unlike adult stem cells these can develop into *all* of the somatic tissues of the adult body. They are therefore the most important potential source of cells for organ repair and restoring organ function.

However, they are obtained from very early human embryos, embryos which are left over from the frequently successful *in vitro* fertilisation (IVF) treatment of childless couples. Even though these embryos are destined to be destroyed, using them to obtain embryonic stem cells is controversial, and some regard it as ethically unacceptable because of their potential to become new human beings.[1] The prediction that stem cells would be obtained from human embryos was made more than twenty years ago and despite the development of *in vitro* fertilisation in many countries, very few have legislation that covers the use of embryos for this purpose. The Netherlands is one of the few countries that has such legislation in place. Since 1 September 2002, the use of these discarded embryos for stem cell isolation has been permitted, as long as permission has been obtained from the Central Committee for Research Involving Human Subjects (Dutch acronym: CCMO).

The history of embryonic stem cells

How could scientists and even politicians have predicted that embryonic stem cells would become a reality in humans? They certainly had no glass ball, but in fact they did not need one. Simply following the research of a group of developmental biologists over the past twenty years would have been sufficient to make this prediction. These developmental biologists studied a type of tumour, a so-called teratocarcinoma, which can occur in the testes of both mice and men. These tumours can be quite remarkable in the mixture of tissues they contain, with hair, muscle, bone and even complete sets of teeth being found in the very largest. Two examples are shown in **figure 1**; one is very small and only visible on a histological slide, whilst the other weighs more than one kilogram.

One of the earliest references to these tumours is on a clay tablet dating from 600-900 BC. The tablet is devoted to methods of predicting the future and says:
"When a woman gives birth to an infant that has three feet, two in their normal position (attached to the body), and the third between them, there will be great prosperity in the land".

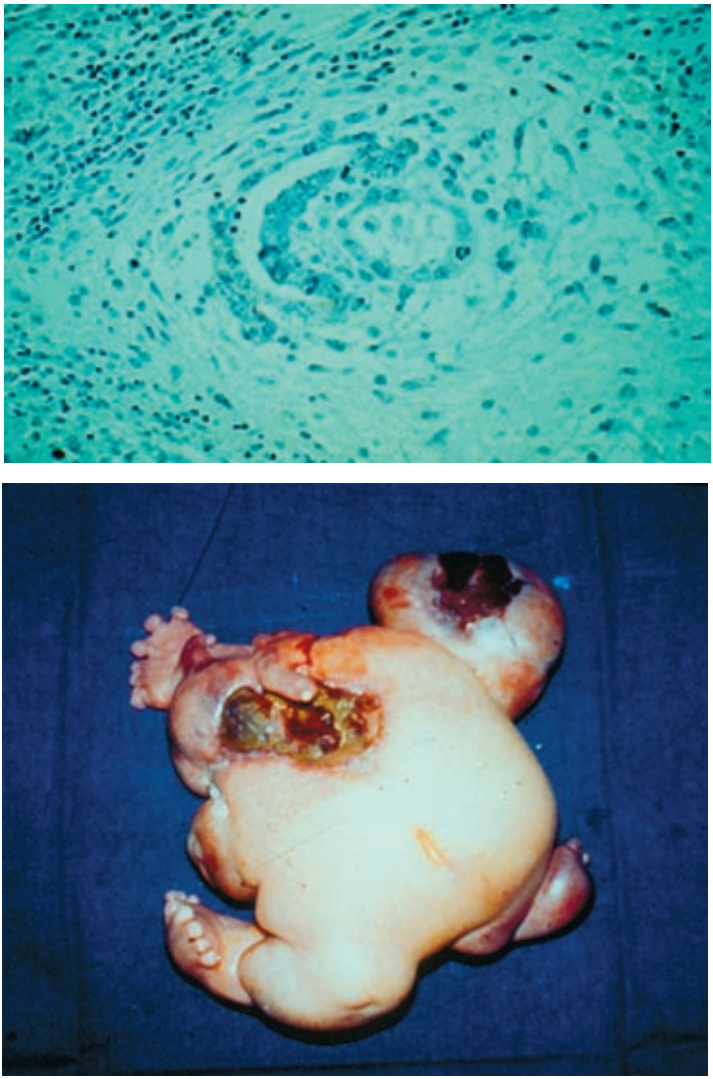


Figure 1: "Teratocarcinoma": tumour of the testis in mouse and man.
(Courtesy of Prof. Dr. J.W. Oosterhuis, JNI, EUR/RUG)

Perhaps such an optimistic forecast foretells the valuable insights modern biology would gain from studying such teratocarcinomas. As you can see, these tumours resemble a disorganised foetus and the one shown here does indeed seem to have a third foot. In the mid 1970s, developmental biologists discovered that teratocarcinomas could be induced in mice by removing early embryos from the uterus and placing them under the skin. They also discovered that these tumours contained undifferentiated stem cells. These embryonal carcinoma stem cells could be isolated and grown in culture without losing the capacity to differentiate into many different cell types. They could behave as a tumour cell or an embryonic cell depending on their environment. This was most strikingly demonstrated when they were introduced into embryos; if these embryonal carcinoma cells from a brown mouse were placed into a blastocyst from an albino mouse, the pups delivered by the foster mother were brown and white. The stem cells had formed so-called chimeric embryos and had contributed to all of the adult animal's somatic tissues, most obviously in the skin.

After differentiating, embryonal carcinoma cells are no longer malignant; thus they became not only a useful model for studying development, but were also of interest to oncologists testing differentiation-induction as therapy for teratocarcinoma in humans. Although this ultimately failed, pathologists gathered several diagnostic markers for the undifferentiated cells which they could use to establish if a teratocarcinoma contained these stem cells. This is very useful in determining the therapy and prognosis. Teratocarcinoma used to be the second highest cause of death in young men but chemotherapy now cures 99 percent of cases. It is one of cancer research's success stories and its most well-known victim, the cyclist and multiple Tour de France winner Lance Armstrong, is proof of a real cure.

In the meantime developmental biologists were addressing the question of whether it would be possible to isolate stem cells directly from mouse embryos, without an intermediate teratocarcinoma stage. In 1981, two groups succeeded in establishing mouse embryonic stem cell lines in culture.

Due to the similarities between mice and human teratocarcinomas, it was then predicted that embryonic stem cells could be isolated from human embryos. Initially the motivation for this was the study of early human embryonic development, but later the possibilities for cell transplant therapies became evident. The first attempts to obtain human embryonic stem cells were made in the mid 1980s, when embryos could not be frozen successfully; left-over embryos were discarded immediately after IVF. The attempts were unsuccessful, possibly because the quality of IVF embryos was not up to present-day standards, and such attempts were mostly discontinued when the freezing of embryos became common practice. Yet had someone succeeded at that time, we might have been twenty years further in this research. Nevertheless, a few groups continued with this line of research. In 1994, Bongso, an IVF specialist in Singapore, succeeded in growing cell lines from human blastocysts but was not able to validate them rigorously. Thomson in the United States also continued with his research. He first of all reported successful embryonic stem cell derivation in two species of primates in 1995 and 1996. In 1998 the group made their breakthrough when they published the same success for humans.

How he did this is shown in **figure 2**. Four to five days after fertilisation, mouse and human embryos have formed a blastocyst. The outer layer, the trophectoderm, gives rise to the extraembryonic tissues, such as the placenta and the membranes surrounding the foetus. Some of the inner cells give rise to all of the cells of the new individual, including the germ cells which go on to become the eggs and sperm of the next generation.

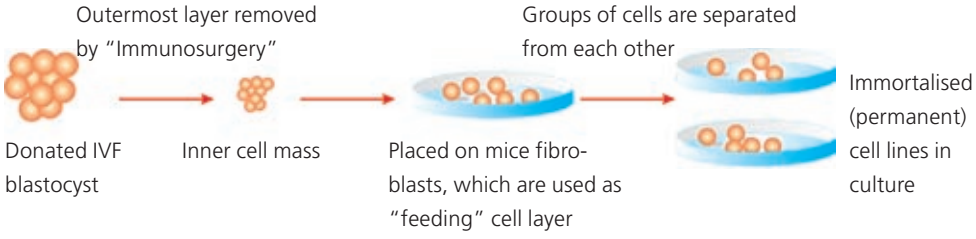


Figure 2: Derivation of embryonic stem cells from human IVF embryos. Science vol. 286, 6 november 1998.

If the inner cells are carefully separated from the trophectoderm and cultured in a special fluid, stem cells will grow that retain their potency (potential) to form all cells of the human body. An embryonic stem cell line is thus established.

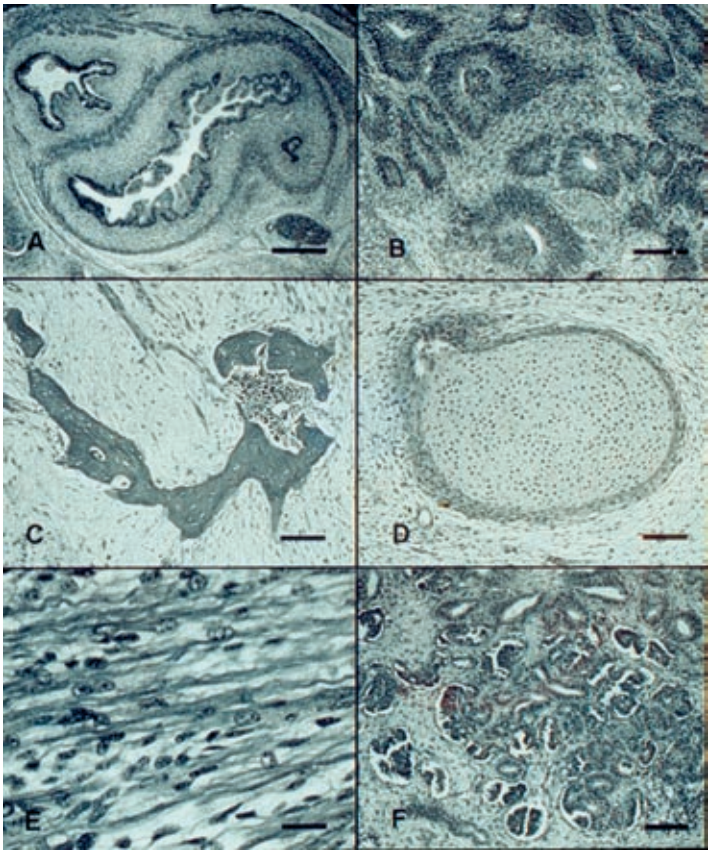


Figure 3: Teratocarcinoma in mice derived from human embryonic stem cells. (From: Thomson et al, 1998, Science)

In humans it is not possible to verify an embryonic stem cell phenotype by generating chimeric embryos, as is the case in mice. Therefore, the markers developed by pathologists to diagnose embryonal carcinoma stem cells in tumours proved their worth and provided the first evidence of these cells' undifferentiated phenotype; their capacity to form teratocarcinomas containing many tissue types in immunodeficient mice confirmed their

pluripotency. **Figure 3** shows what one of these human embryonic stem cell-derived tumours looked like and even the untrained eye can see something that looks like a cross-section through the intestine.[2] There is also tissue that looks like brain, cartilage, bone and skeletal muscle as well as kidney tubules that were later shown to produce urine!

Controlled stem cell differentiation

Being able to obtain stem cells from human embryos and demonstrate their ability to differentiate into many cell types in a teratocarcinoma, is of course still a far cry from producing a transplantable cell that can cure a disease. We need to be able to control the differentiation process and obtain many millions of cells of the same type that will survive transfer into a patient. This could be into diseased tissue, as in heart failure, or into healthy tissue where a cell can nevertheless function, such as the transplantation of pancreas cells for the treatment of diabetes. The β-islet cells of the pancreas can produce insulin in response to glucose even when they are not in the pancreas and it might, for example, be easier to put them under the kidney capsule than into the pancreas itself. Of course transplanted cells should also be able to communicate with any remaining healthy tissue at the transplant site. Cardiomyocytes transferred into an infarcted heart must not start beating independently of the host heart cells. Similarly nerve cells transplanted into a site of spinal cord injury must be able to communicate with cells in their environment.

How are we going to control the differentiation of stem cells? This is perhaps one of the biggest challenges that needs to be overcome before we can apply stem cell therapy. The transfer of undifferentiated embryonic stem cells to damaged tissue and tissue undergoing repair is likely to be a dangerous option; when used in combination with immunosuppressive drugs the cells could potentially form teratocarcinomas at the transplant site, as has already been observed in immunodeficient mice. The principal option with embryonic stem cells is to use partially or fully differentiated cells for transplantation.

We can try empirical strategies to induce differentiation to the various cell types required and this is largely what is taking place now. For example, vitamin A in combination with fibroblast growth factor has been shown to be highly effective in inducing mouse embryonic stem cells to form nerve cells. This recently gave spectacular results when these nerve cells were used to "cure" mice with Parkinson's disease. Yet these of course mouse nerve cells transplanted into sick mice. Although fibroblast growth factor and vitamin A also induce human embryonic stem cells to form nerve cells, and just a year ago it was found that these nerve cells could be injected into the brains of young mice and survive, we are still a long way off from curing a patient. This research is still in its infancy and at present it is impossible to predict when things might happen.

Although an empirical approach such as that used for these nerve cells will undoubtedly be useful, we have chosen to invest in fundamental research on the mouse embryo with the intention of finding clues as to how we can best control differentiation. For example, an embryo knows how to make a heart; can we learn the embryo's tricks and do the same? And can analysis of the connective tissue at the sites in the embryo where differentiation takes place provide clues as to the best extracellular scaffold we can engineer for each cell type?

We believe that we can learn from embryos and to illustrate this point, one of our first experiments on human embryonic stem cells is shown in **figure 4**. The picture on the top of figure 4 shows colonies of several thousand, undifferentiated human embryonic stem cells growing on a carpet of mouse "feeder cells", which are necessary to keep them in this undifferentiated state. In this aspect human embryonic stem cells differ from those in mice. Mouse cells also needed to grow on a carpet of these feeder cells until the growth factor these secreted was identified and could just be added as a culture medium supplement. Unfortunately this same growth factor does not work for human embryonic stem cells and researchers are in hot pursuit of the human embryonic stem cell factor, in the hope of being the first bring it onto the market.

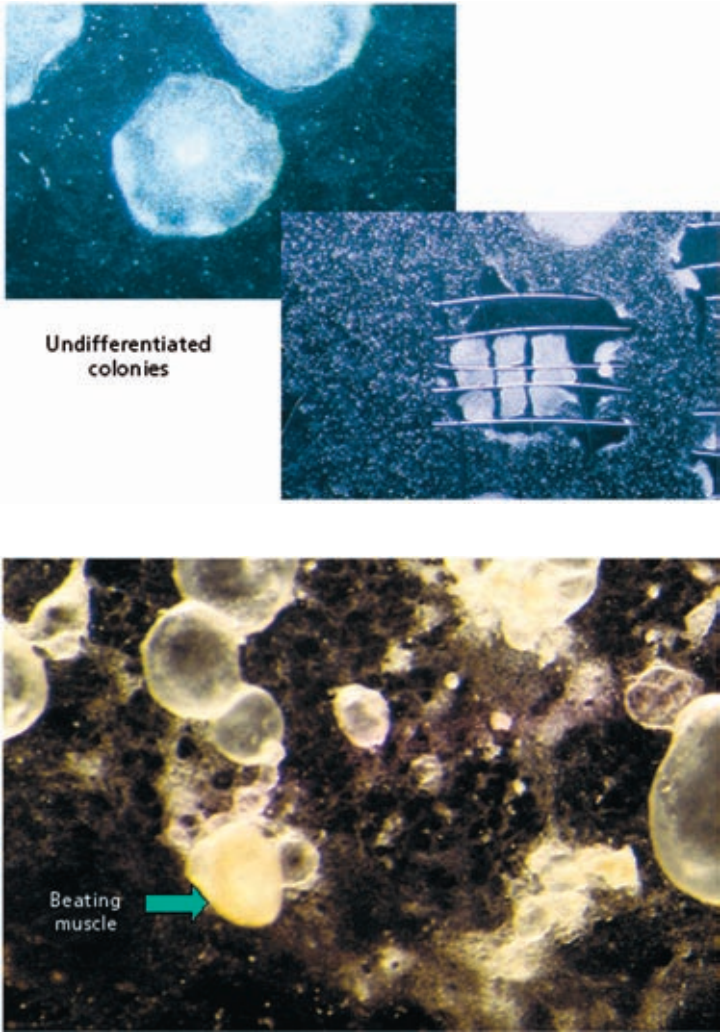


Figure 4: Human embryonic stem cells in culture. (J.W. Leeuwis; D. Ward)

If it were to become available, the isolated factor could certainly simplify and standardise the cell culture procedure and reduce the labour necessary just to maintain the cells. More importantly, it would allow the large-scale generation of cells for transplantation to scaffolds and to human patients, who are of course many times larger than mice. The colonies of cells shown in this figure are similar to the cells we brought to the Netherlands from Australia in 2000. Evidence in both chickens and frogs had shown that endoderm, the cell layer adjacent to the region where the heart forms in an embryo, is essential for the differentiation of cardiomyocytes from the mesoderm. We had also shown that endoderm-like cells in culture, had a similar effect on mouse embryonal carcinoma cells; if the two cell types are grown together, the undifferentiated embryonal carcinoma cells formed heart cells and started to beat.[3] Therefore our first experiment was very simple; to grow human embryonic stem cells on top of these endoderm-like cells and see what happened. On 14 February 2001, Valentine's Day, we obtained beating muscle in the human embryonic stem cell cultures. The first heart cells continued to beat for more than six weeks. Later we discovered that we could freeze and thaw these cells and they would still resume beating. We also discovered that they were electrically coupled as heart cells should be and could form gap junctions with primary human cardiomyocytes, at least in culture. They might therefore be able to communicate correctly when transplanted to a real heart.

Adult stem cells

Adult stem cells are an alternative to embryonic stem cells. Mesenchymal stem cells from bone marrow and adipose tissue are already finding applications in tissue engineering for bone and cartilage, whilst stem cells from pancreatic ducts of deceased donors can now be expanded in culture and used to treat "brittle" or unstable diabetes in patients. At present two donors are needed for the treatment of each patient but it is hoped that in the future, one donor will be able to help five patients. However, what should we make of recent reports about unexpected "plasticity" in adult stem cells? This has been evidenced mostly in mice but also to a

certain extent in certain human adult stem cells, where cells have appeared to show a broader capacity to differentiate than was previously thought.[4] Bone marrow stem cells have been reported to give rise to heart cells, for example, and neural stem cells to bone. If adult stem cells are as good as embryonic stem cells then why would we want to continue research on embryonic stem cells? Adult stem cells would be immunologically and ethically preferable, but are they indeed plastic? At present the evidence is inconclusive. There is evidence of transdifferentiation of marked cells *in vivo* but the numbers are low; not enough to cure and possibly the result of fusion between somatic cells and stem cells. The answer has yet to be found.

Conclusion

It is clear that some adult stem cells will grow and function on appropriate scaffolds that can be successfully transplanted to animals and patients. The age of tissue engineering has dawned. It is also clear that the hard evidence on pluripotent differentiation of stem cells is restricted to embryonic stem cells. However, with these cells it is still essential to test the safety and increase in numbers before either the cells or the cells on scaffolds are transplanted. The steps now being taken in the Netherlands to initiate tissue engineering and stem cell research programmes are commendable, and will hopefully complement similar initiatives taken by the National Institute of Health (NIH) in the United States and the Medical Research Council (MRC) in the United Kingdom. We believe that although the Netherlands is a small country, it nevertheless possesses the skills required to help combat debilitating chronic diseases in an aging population.

References

1 De Wert, G. & Mummery, C.L. (2003). Human embryonic stem cells: research ethics and policy. Human Reproduction 18, 1-11.

2 Thomson, J., Itskovitch-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. & Jones, J.M. (1998). Embryonic stem cells derived from human blastocysts. Science 282, 1145-1147.

3 Mummery, C.L., Ward-van Oostwaard, D., Doevendans, P.A., Spijker R., Brink S. van den, Hassink, R., Heyden, M. van der, Opthof, T., Pera M., Brutel de la Riviere, A., Passier, R. & Tertoolen, L. (2003). Differentiation of human embryonic stem cells to cardiomyocytes: the role of co-culture with visceral endoderm-like cells. Circulation 107, 2733-2740.

4 Passier, R & Mummery, C.L. (2003). The origin and use of embryonic and adult stem cells in differentiation and tissue repair. Cardiovascular Research, in press.