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Fundamental Technological Developments Required for Increased Availability of Tissue Engineering

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1. Introduction

Since the initial excitement surrounding successful clinical studies of skin tissue engineering more than 20 years ago (Gallico *et al.*, 1984), steady progress has been made in enhancing the capabilities of tissue engineering and regenerative medicine. Tissue engineering generally depends upon the use of cultured cells. Since living cells do not fall into any of the existing medical product categories, this has created a great challenge for both regulatory agencies and commercial entities. Although various treatment strategies have been developed, the fundamental technologies and infrastructure to support their widespread adoption are still limited.

In this chapter, attention was focused on fundamental technology development. Three major areas, i.e., introduction of serum-free culture media, development of cell storage technologies and methodological development for quality assurance of the products, are discussed with special reference to future development of tissue engineering.

2. Feasibility of tissue engineering using human bone marrow stromal cells cultivated in serum-free conditions

Tissue engineering is an interdisciplinary approach to regenerate tissue through integration of cell biology and biomaterial/biomedical sciences. The concept of tissue engineering is to regenerate target tissue by mimicking the developmental or regenerative process of that tissue. Thus, it can be considered an ideal therapeutic option for treating various tissue defects. Tissue engineering of skin, cartilage, and bone has already been shown both feasible and effective in several clinical studies, and its efficacy has attracted significant attention from both patients and doctors. However, there are several fundamental technologies which need to be improved before widespread practical use of tissue engineering in hospitals or clinics. In this chapter, the current status of cell culture media used for clinical tissue engineering and the need for the development of safe and reliable serum-free cell culture media will be discussed with special reference to bone tissue engineering.

Patients who lose healthy bone tissue as a result of inflammation or trauma need bone regenerative/reconstructive surgery in order to recover the function of the lost bone. To regenerate the lost bone tissue, autologous bone grafting is the current gold standard, though this technique is a great burden for patients because transplantable autologous bone must be harvested from a healthy site, which causes donor site morbidity and pain. Artificial bone substitutes have been developed as alternatives to autologous bone, though bone regeneration with them is inefficient because they lack osteo-inductive properties. Accordingly, tissue engineering of bone (bone tissue engineering) has attracted significant interest because it is considered less invasive than autologous bone grafting and more efficient than artificial bone substitutes. In fact, cell-based bone tissue engineering which utilizes cells, scaffolds, and bioactive molecules has been shown even more effective than artificial bone substitute in both basic and clinical studies.

For cell-based bone tissue engineering, various tissues derived cells are utilized since osteogenic cells can be harvested from bone marrow, periosteum, and adipose tissue, though recent studies indicate that bone marrow stromal cells (BMSCs, bone marrow derived multipotent mesenchymal stromal cells, or mesenchymal stem cells) are the most reliable cell source because of their superior osteogenic ability (Hayashi *et al.*, 2008). However, it is difficult to obtain adequate numbers of transplantable BMSCs from bone marrow aspirates, as they are rare in the bone marrow (less than 0.01% of marrow cells) (Montzka *et al.*, 2010). Therefore, *ex vivo* expansion of BMSCs is required to obtain a sufficient number of transplantable cells. Since BMSCs require several kinds of supportive factors for their growth, it is standard practice to use fetal bovine serum (FBS), while autologous human serum (HS) and pooled allogeneic HS have also been used. It has been suggested that FBS may not be favorable for clinical applications due to the possible risk of contamination (prions, viruses, zoonosis) or immunological reactions against xenogeneic serum antigens (Agata *et al.*, 2009). Although serious secondary effects of transplanted cells that were cultured in the presence of FBS have not been reported to date, a previous clinical study that utilized BMSCs cultivated in FBS-supplemented media for the treatment of osteogenesis imperfecta showed a 150-fold increase in antibody titer against FBS in the sera of one patient who received BMSCs infusions (Horwitz *et al.*, 2002). Theoretically, use of autologous HS could eliminate the risks of disease transmissions and immune reactions. However, it is not always possible to obtain a sufficient amount of autologous HS for *ex vivo* expansion of BMSCs. In fact, over 400 mL of peripheral blood is usually required to obtain 200 mL of autologous HS, which is only sufficient to support the growth of BMSCs for a few passages. Therefore, collection of a sufficient amount of autologous HS is a considerable burden for anaemic patients as well as for healthy female patients with a low body weight. Use of pooled allogeneic HS cannot overcome this problem because it has been shown that allogeneic HS does not fully support the growth of BMSCs (Kuznetsov *et al.*, 2000). Furthermore, even when a sufficient amount of autologous HS can be obtained from each patient, the constituents of individual HS could vary, which might lead to variations of cell culture outcome. Thus, it is desirable to develop efficient and safe serum-free culture media and eventually serum-independent cell expansion protocols for tissue engineering.

Recently, several companies have launched complete serum-free culture media that can support the growth of human mesenchymal stem cells without the addition of sera (Table 1). Although the number of studies that have investigated the potential of these serum-free media is still limited, it has been suggested that these serum-free media can support the

growth of human somatic (postnatal/tissue) stem cells even more efficiently than conventional serum-based media (Lindroos *et al.*, 2009, Ishikawa *et al.*, 2009, Hartmann *et al.*, 2010). In support of this conclusion, our previous study of human BMSCs showed that the efficacy of cell growth was greater in StemPro® SFM (Invitrogen, Carlsbad, California, U.S.A.) than FBS-containing medium (Agata *et al.*, 2009). Similar findings have been reported with MesenCult®-XF (STEMCELL TECHNOLOGIES, Vancouver, BC, Canada), STK2® (DS Pharma Biomedical Co.,Ltd., Osaka, Japan), and the xenogenic-free (xeno-free) version of StemPro® SFM, all of which have been developed for xeno-free as well as serum-free cultivation of human somatic stem cells (Lindroos *et al.*, 2009, Ishikawa *et al.*, 2009, Hartmann *et al.*, 2010). These data indicate that currently available xeno-free, serum-free media may have the potential to replace conventional serum-based media in clinical tissue engineering, though further basic studies are required to ensure its safety and efficacy. To develop a protocol for bone tissue engineering with serum-free media, we now discuss current findings regarding the character of serum-free expanded cells.

Name of the medium	Company	Of note	*In vitro* osteogenic potential	*In vivo* osteogenic potential	Reference
StemPro SFM	Invitrogen (Carlsbad, California, U.S.A.)		Quantitative alkaline phosphatase (ALP) assay	Ectopic bone formation	Agata *et al.*, 2009,
StemPro SFM XenoFree	Invitrogen (Carlsbad, California, U.S.A.)	Xenofree	Quantitative ALP assay, ALP staining	?	Lindroos *et al*, 2009
MesenCult-XF	STEMCELL TECHNOLOGIES (Vancouver, BC, Canada)	Xenofree	Alizalin red staining	?	Hartmann *et al*, 2010
STK2	DS Pharma Biomedical Co.,Ltd. (Osaka, Japan)	Xenofree	Alizalin red staining	?	Ishikawa *et al.*, 2009

Table 1. List of currently available commercial serum-free media and the osteogenic ability of postnatal stem cells cultivated in each product

Since the type of expansion medium used in primary culture may affect the viability and type of cell population generated, it is important to compare the cell populations grown in serum-free and serum-containing medium. For this purpose, Lindroos *et al*. investigated cell surface marker expression by cells cultured in FBS- or HS-containing media and those cultured in serum-free media using human adipose stem cells. They reported that the expression profiles of examined cell surface antigens were not statistically different (Lindroos *et al.*, 2009). Our previous study investigated cell surface marker expression by human BMSCs cultured in serum-free medium. It also showed that the expression profiles of most of the examined antigens were comparable in both serum-free and serum-containing groups, though there were some differences in the expression of CD105 and CD146 (Agata *et al.*, 2009). Since the mean fluorescence intensity of the CD105 antigen was stronger in serum-free expanded BMSCs, it is possible that a larger population of CD105-positive cells was obtained by growth in serum-free medium. In contrast, the CD146-positive fraction was more evident in cells cultured in serum-based medium and only a limited number of cells were positive for CD146 in the serum-free group (Agata *et al.*, 2009). It is not clear whether serum-free conditions alter the expression of both of these surface markers or whether the conditions selectively support the growth of the $CD105^{positive}$

$CD146^{dim}$ population. Nonetheless, cells grown in serum-free media do appear to be different from those grown in serum-containing media, and the information regarding BMSCs grown in serum-containing media may not be used as a reference. Therefore, the feasibility of bone tissue engineering with serum-free expanded BMSCs should be independently investigated from the beginning, though there have already been several clinical trials to show the safety and efficacy of bone tissue engineering with BMSCs grown in serum-containing media.

One of the most important things that should be assured for use in a clinical setting is that transplanted BMSCs do not form tumors in the recipient following transplantation. Since our previous study showed that transplanted BMSCs grown in serum-free medium did not form tumors in nude mice (Agata *et al.*, 2009), it might be possible that BMSCs expanded in serum-free medium are as safe as those expanded in serum-containing medium. However, further studies are required to confirm their safety because few studies have transplanted serum-free expanded somatic stem cells. Together with cell transplantation analyses, genomic and chromosomal stabilities must be analyzed, because these data can support the safety of serum-free expanded BMSCs.

In addition to confirming the safety of such transplants, assurance of the osteogenic differentiation ability of transplanted BMSCs is important in clinical bone tissue engineering. BMSCs grown in serum-containing media are known to differentiate into the osteogenic lineage when they are cultured in osteogenic induction medium (serum-containing media supplemented with dexamethasone, ascorbic acid, and β-glycerophosphate). However, it was still necessary to determine whether somatic stem cells grown in serum-free media would behave similarly in the presence of the same osteogenic components. To date, adipose stem cells, umbilical cord tissue-derived mesenchymal stem cells, and BMSCs those grown in serum-free media have been shown to differentiate into osteogenic cells in the conventional induction medium (Lindroos *et al.*, 2009, Ishikawa *et al.*, 2009, Hartmann *et al.*, 2010). However, it remains unknown whether conventional osteogenic induction medium is optimal for their differentiation, because some of the manufacturers recommend a specially formulated kit for osteogenic induction of serum-free expanded cells. Therefore, we explored osteogenic induction of BMSCs expanded in serum-free medium, using both a conventional osteogenic induction medium and the commercially supplied osteogenesis kit (Agata *et al.*, 2009). Results of alkaline phosphatase (ALP) assays showed that both treatments were able to induce osteogenic differentiation of serum-free expanded BMSCs, though the increase of ALP activity was more rapid with the osteogenesis kit (Fig. 1A). We also performed *in vivo* transplantation experiments to investigate possible differences in bone forming abilities between cells grown in the two media. As shown in Figure 1B - 1E, cells treated with both osteogenic medium and the osteogenesis kit were able to form bone *in vivo*, and there was no significant difference in the efficacy of bone formation (Fig. 1B, 1C: osteogenic medium; Fig. 1D, E: osteogenesis kit). These data indicate that bone tissue engineering with serum-free expanded BMSCs can be achieved with either the conventional osteogenic induction medium or the osteogenesis kit. However, these treatments may not be ideal for induction of osteogenic differentiation of serum-free expanded BMSCs, because both media (even the commercially supplied kit) contain some serum-derived components. Therefore, to enhance the safety of clinical bone tissue engineering, a completely serum-free osteogenic induction media should be developed.

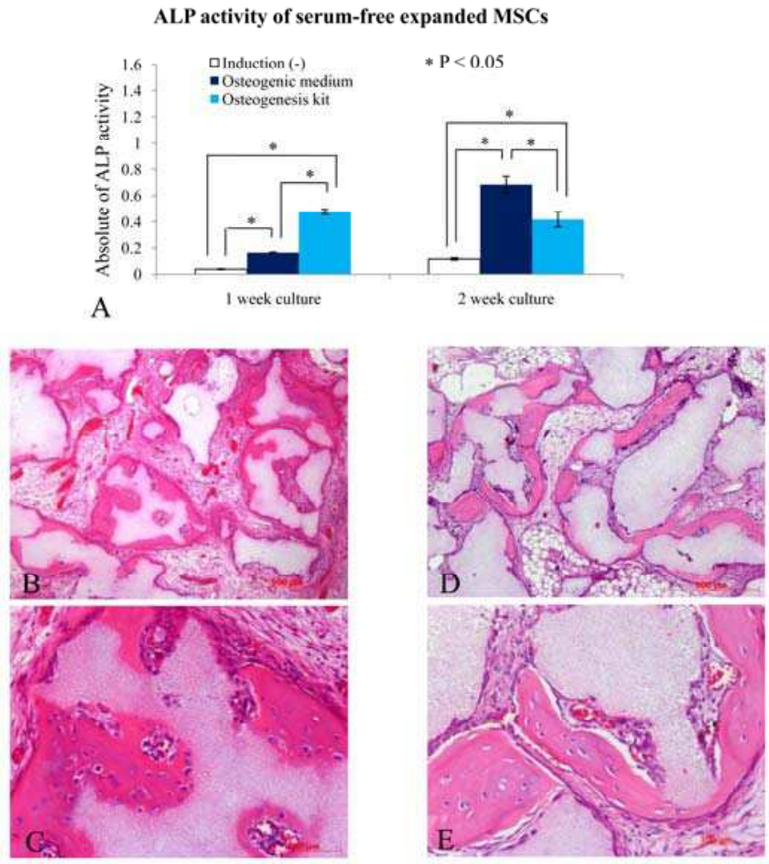

Fig. 1. Osteogenic abilities of serum-free expanded BMSCs after osteogenic induction with either osteogenic medium or osteogenesis kit (From Agata *et al.*, 2009 with permission)

Collectively, these data indicate that bone tissue engineering with BMSCs expanded in currently available commercial serum-free media is feasible, though further studies regarding the characteristics of the cells and the safety of serum-free expanded cells are required. In addition, further improvements in serum-free media are desirable because currently available xeno-free, serum-free media contain allogeneic human proteins, which may cause unknown disease-transmissions and immune reactions. Furthermore, related products for serum-free media such as cell culture dish coating materials, which are required for the efficient adhesion and proliferation of primary culture cells in serum-free culture system, should also be improved because no allogeneic-free materials are currently available.

3. Cell storage technologies

Cell storage technologies are essential for efficient, safe, and widespread use of tissue engineering. Storage technologies for cells and tissue-engineered products are required for their timely and efficient distribution. Furthermore, storage of stem cells (stem cell banking) is expected as a reservoir of stem cells for future use and also for public cell banking. Currently, cryopreservation is the most reliable and established technology to store tissues and cells. However, some novel technologies such as freeze dry technology have been

investigated. In this chapter, we focus on the characteristic features of tissue-engineered products for cryopreservation and recent developments in storage technologies. Furthermore, potential future applications of stem cell banking are discussed.

3.1 Storage of tissue-engineered products

The storage of tissue-engineered products is an attractive target for technological development. Since tissue-engineered products usually consist of living cells, technical issues include the limited shelf life of the cells and the use of specialized conditions for transportation. Without storage, treatment with cells requires timely harvesting from the donor, which significantly affects the availability of tissue-engineered products. If the tissue-engineered products can be used as off-the-shelf products such as bioartificial bone substitutes, it would significantly enhance the adoption of this alternative.

3.1.1 Cryopreservation of tissue-engineered products

Currently, cryopreservation is the only available strategy for the storage of tissue-engineered products. However, tissue-engineered products usually consist of multiple layers of cells and, in most cases, the cells are seeded on scaffold made of biomaterials, which complicates the development of efficient freezing storage protocols (Pancrazio *et al*., 2007). Furthermore, the scale-up of cryopreservation procedures from the cellular level to a macroscopic tissue scale introduces new problems related to heat and mass transfer phenomena in larger systems (Karlsson and Toner, 1996). Although it has been shown that frozen storage is feasible for some of the tissue-engineered products such as bone (Kofron *et al*., 2003), it is more difficult than that for isolated cells and requires special considerations.

Water transport processes may cause difficulties for tissue-scale freezing. While cells at the surface layer would respond to freeze-induced osmotic changes much like cells in suspension, interior cells would dehydrate as a response to the increased intracellular tonicity in the dehydrate surface layers. Accordingly, interior cells dehydrate more slowly than surface cells, which may affect their survival (Karlsson and Toner, 1996). Heat transport limitation in larger tissue may also affect survival. Due to the macroscopic size of tissue-engineered products and its finite thermal conductivity, there may be large thermal gradients from the surface to the interior of the samples. The presence of a thermal gradient during cooling and warming phases makes it difficult to choose optimal temperature change protocols for both surface and interior cells. Moreover, osmotic effects (water movement from inside-unfrozen cells to outside-frozen cells) during cooling, reduces cell survival. Accordingly, it may not be possible to recover full viability throughout the tissue (Karlsson and Toner, 1996).

One of the key decisions in achieving successful freezing of tissue-engineered products is the choice of cryoprotectant. Cryoprotectants minimize damage caused by ice crystal formation and should induce an amorphous state, rather than ice crystals during the cooling and warming phases. Although the use of cryoprotectants is mandatory, currently available reagents are cytotoxic to some extent. Since tissue-engineered products are larger than isolated cells, longer incubation times with cryoprotectant are necessary which may result in a lower survival rate. On the other hand, short incubation times may not allow enough cryoprotectant to penetrate relatively thick tissue-engineered products and cause ice crystallization and cell death in internal layers. The pre-incubation time used for penetration of cryoprotectant should balance damage caused by toxicity and freezing/warming.

3.1.2 Technology development for the storage of tissue-engineered products

Two major approaches to cryopreservation are known, i.e., conventional freeze-thaw procedures and vitrification, which is defined as a glass-like solidification (Karlsson & Toner, 1996). While freeze-thaw procedures minimize the probability of intracellular ice formation, vitrification attempts to prevent ice formation throughout the entire sample during the cooling and warming process (Kuleshova *et al.*, 2007). Recently, the potential of vitrification has been tested for tissue-engineered constructs. Since tissue-engineered products consist of multicellular layers and often include biomaterials with varying coefficients of expansion compared with cells, cryopreservation using conventional freeze-thaw procedures with slow cooling rates has achieved limited success. Accordingly, vitrification could be an attractive alternative technology.

Vitrification has been investigated for tissue-engineered bone and blood vessels. Liu & McGrath (2003) explored the potential of vitrification for the cryopreservation of tissue-engineered bone constructs consisting of a hydroxyapatite scaffold-cell complex. Cell survival was 92.0% for suspended cells and 43.0% for attached cells. In terms of tissue-engineered blood vessel constructs, the effects of vitrification and conventional cryopreservation were compared (Elder *et al.*, 2005). Collagen-based vascular constructs were used as models in this study. Morphological changes associated with ice formation were visible within tissues preserved using traditional cryopreservation but not in tissue preserved using vitrification. The metabolic assay results indicated that vitrified tissue had viability similar to fresh controls. More recent study with tissue-engineered blood vessels using polyglycolic acid scaffold showed that ice formation in tissue-engineered blood vessels was negligible in the vitrified specimens but extensive ($68.3 \pm 4.5\%$ of vessel area) in the extracellular matrix of frozen specimens. The vitrified tissue had a viability similar to fresh controls and the contractility results for vitrified samples were >82.7% of fresh controls but markedly reduced in the frozen samples (10.7% for fresh controls) (Dahl *et al.*, 2006). Vitrification is a feasible storage method for tissue-engineered blood vessel constructs, and their successful storage brings these constructs one step closer to clinical utility. Although it is a promising technique, higher concentration of cryoprotectant should be used, which could potentially damage the cells. Accordingly, it is still technically difficult and its utility for tissue-engineered products remains to be elucidated.

One of the most awaited technologies for the preservation of tissue-engineered product is long-term unfrozen storage (more specifically, dry storage) at ambient temperature. This approach allows storage without dependence on expensive freezers or liquid nitrogen, which require daily maintenance. This "off-the-shelf" availability and low cost should facilitate the usage of tissue-engineered products. Unfortunately, this is not yet a reality. However, many organisms can undergo a phenomenon called anhydrobiosis to survive in a completely dehydrated state for an extended time and resume activity upon rehydration (Crowe *et al.*, 2002). The sugar trehalose is found at high concentrations in many anhydrobiotic organisms. Thus, the addition of trehalose is considered a key factor in achieving freeze-dried storage. In mammalian cells, freeze-drying of platelets was reported using trehalose (Crowe *et al.*, 2005). Although recovered platelets were strongly attenuated, the survival rate exceeded 90%. Drying of nucleated cells is apparently more challenging. However, the addition of p29, a small a-crystallin stress protein, together with trehalose showed increased resistance to dryness in 293 cells (Ma *et al.*, 2005). Although "off-the-shelf"

tissue-engineered products stored at ambient temperature are not available, the steady progress of research in this area may provide a functional protocol at some point in the future.

3.2 Cryopreservation of putative stem/progenitor cells

Storage of putative stem/progenitor cells is also an attractive research target in tissue engineering. Collection of cells for tissue engineering is less invasive than conventional tissue or organ transplantation. However, repetitive collection of cells can stress patients. If the stem/progenitor cells could be stored, it is possible that they could be repeatedly used for future therapy, reducing the burden and the cost to patients. The concept of banking stem/progenitor cells in storage is not new. Stem/progenitor cell populations decrease in size with age (D'Ippolito *et al*., 1999; Zhou *et al*., 2008). If the stem/progenitor cells were harvested at an early age and the cells could be stored, it is possible that those cells could be used later in the life of the donor for autologous transplantation. In this chapter, we also focus on cell banking.

3.2.1 Banking of somatic stem/progenitor cells

Currently, one of the most established and widely accepted stem cell bank systems is umbilical cord blood banking. However, the establishement of somatic stem cell bank other than cord blood (non-hematopoietic stem cells) is still underway. The nature of somatic stem/progenitor cells is much different from that of embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. Somatic stem/progenitor cells possess limited ability to differentiate compared with ES cells and iPS cells. Furthermore, accumulating evidence suggests that somatic stem cells may lose their plasticity soon after cultivation is initiated (Sugiura *et al*., 2004). Thus, expansion and storage of autologous somatic stem cells for personal future use could be impractical, particularly if the cells carry a genetic predisposition for the disease that is being treated. Improved understanding of somatic stem/progenitor cells is required for more varied applications.

3.2.2 Banking of ES cells and iPS cells

Recently, the establishment of "stem cell banks" has been reported in several countries. Those stem cell banks, mostly government-supported, aim to provide a resource for storing, characterising and supplying ethically collected, quality controlled stem cell lines for research and ultimately for treatment (London *et al*., 2004; Nakamura, 2010). There is no doubt that those stem cell banks can facilitate research in this area and contribute to the availability of those rare resources such as human embryonic stem (ES) cells and somatic stem cells to researchers who do not have access. Recently, production of induced pluripotent stem (iPS) cell lines from human somatic cells has been reported (Nakagawa *et al*., 2008). Theoretically, it is possible to generate personalized iPS cells for therapeutic use without ethical problems and also immunological rejection, though it may not be practical due to the time and cost required for the production and quality assurance. To overcome this problem, the establishment of HLA-haplotype banking for human iPS cells has been argued (Nakatsuji *et al*., 2008). A similar idea was originally reported for human ES cell lines (Taylor *et al*., 2005). HLA-haplotype banking may provide a more efficient and safe alternative. They estimated that only 50 iPS cell lines would be necessary to find a three-locus match for 90.7% of the Japanese population. At present, the safety of iPS cells for

clinical use is the major concern. The development of safe and effective clinical applications with iPS cells would enhance their appeal.

4. Non-invasive image-based cell quality evaluation technology for cell therapy

In clinical tissue engineering, both safety and efficacy requirements must be satisfied. In the production of conventional chemical-based pharmaceutical drugs, the quality and efficacy of the product is guaranteed through adherence to strict regulations. However, cell-based materials, including tissue-engineered products, require different strategies to evaluate their quality.

Conventional cell quality evaluation technologies, such as RT-PCR or immuno-stainning, require cell-destruction processing such as cell-lysis or cell-fixations. If the quality of the cells can be evaluated without destruction process, both examined and non-examined cells can be used for therapies. Therefore, the development of such kind of non-invasive cell quality evaluation technologies has been awaited (Takagi, M. 2010).

4.1 Problems in cell therapy and their technological assessment

The safety and efficacy of cellular products, such as human cells, tissues, and cellular and tissue-based products or HCT/P, processed for cell therapy, are currently regulated by individual nations. However, the level and scope of regulation differs greatly among countries. Frequently referred references are Current Good Tissue Practice and 21 Code of Federal Regulations (CFR), Parts 1270 and 1271 of U.S. Food and Drug Administration (FDA) (FDA homepage, 2010).

Commonly, regulations attempt to limit the unique risks associated with cellular products. These regulations focus on the followings: (1) limiting the risk of transmission of communicable disease from donors to recipients, (2) establishing manufacturing practices that minimize the risk of contamination, and (3) requiring an appropriate demonstration of safety and effectiveness for cells and tissues that present greater risks due to their processing or their use. The regulations suggest that there are two major risks associated with HCT/Ps. One is the non-cellular contaminant risk, and the other is the risk from the cells themselves. Regulations have been established to assure sterility and/or the aseptic nature of the cellular product. However, there are still few criteria or regulations to control cell quality. Especially with stem cells, cell quality per se is not covered by licenses or regulations.

Cell quality issues in cell therapy include the health of the cells, and their ability to grow and differentiate as required without risk of tumorigencity. Historically, chromosomal tests have been conventionally used to assess tumorigenic risk. Animal implant tests are commonly used in safety tests for chemical-based pharmaceutical drugs. Biomarker tests, which assess specific tumorigenic marker genes/proteins by RT-PCR or flow cytometry are the most frequently used molecular biological techniques. However, as explained below, the availability of those conventional techniques do not help clinicians overcome the four major problems which inhibit the use of cell therapies. Those problems are as follows.

The most fundamental problem is guaranteeing the non-invasiveness of the engineered cells. Cells prepared for therapeutic use should also be high in viability, since artificial manipulation could trigger cellular abnormalities. Also, autologous cells from a patient are

commonly very limited in number due to the limitations of the source. For safety, fluorescent staining or gene transfer should also be avoided.

The second problem is a need for complete and exhaustive characterization of the engineered cells. In the case of conventional pharmaceuticals, the uniformity of products can be strictly controlled by regulation of production. However, with human cells, strict regulation of processing does not guarantee uniformity of the product since there are huge individual variations. Therefore, sampling does not always assure the quality of total cellular products.

The third problem is the time limitation inherent in sample characterization. A sample undergoing processing should be assessed repeatedly, until the day of therapy to assure the highest level of safety. However, currently utilized assays such as sterilization test take a few weeks to show the safety properties of cell-products.

The fourth and final problem is instability of the cell quality throughout the culture process. Accordingly, the cell quality of the cell-based products should be assessed just before operation, though conventional evaluation techniques require a few days or even weeks for the assessment of cell-products.

Here, we introduce image-based cell quality prediction technique which might be able to overcome all the problems listed above. Image-based cell quality prediction enables non-invasive, complete, on-time, and predictive evaluation of cells.

Image cytometry is an exciting new area in cell research (Kim, JS. *et al*., 2010) and could support cell quality evaluation of tissue-engineered products. Given the advances in hardware and software, there are commercially available analysis systems for image cytometry, such as the IN Cell Analyzer (GE Healthcare, Chalfont St Giles, Buckinghamshire, United Kingdom) (GE Healthcare home page, 2011). Image cytometry provides exhaustive high content information characterizing intact/fixed cells. It can also provide very detailed data describing the localization or expression of molecules and organelles in cells. However, current image cytometry analysis is based on fluorescence. Although there are many "less-damaging" technologies for fluorescent labelling of cells, they still give some changes to the cells;, thus, incompatible to assess patient cells in clinical tissue engineering.

In contrast, there are developing technologies that evaluate/estimate cellular activities by "non-invasive" measurement technologies (Takagi, M. 2010). Takagi reviewed these non-invasive cell imaging technologies, and indicated that cellular activities can be estimated by cellular morphologies. Recent non-invasive technologies have also been applied to the cell quality evaluation of three dimensionally cultured tissue engineering products (Kino-oka *et al*., 2008).

Here, we review some important aspects of basic image analysis strategy, especially designed for “cell quality prediction system” in tissue engineering and cell therapy, and show examples of image-based cell quality prediction technology.

4.2 Basics of cell image analysis for cell quality prediction

For image-based cell quality prediction, there are four major steps (Fig. 2): (1) image data collection, (2) image processing, (3) experimental data collection, and (4) data analysis. By adding a fifth step, (5) Prediction, conventional correlation analysis (in-sample analysis) is extended to achieve predictive performance (out-of sample analysis) for the evaluation of new samples.

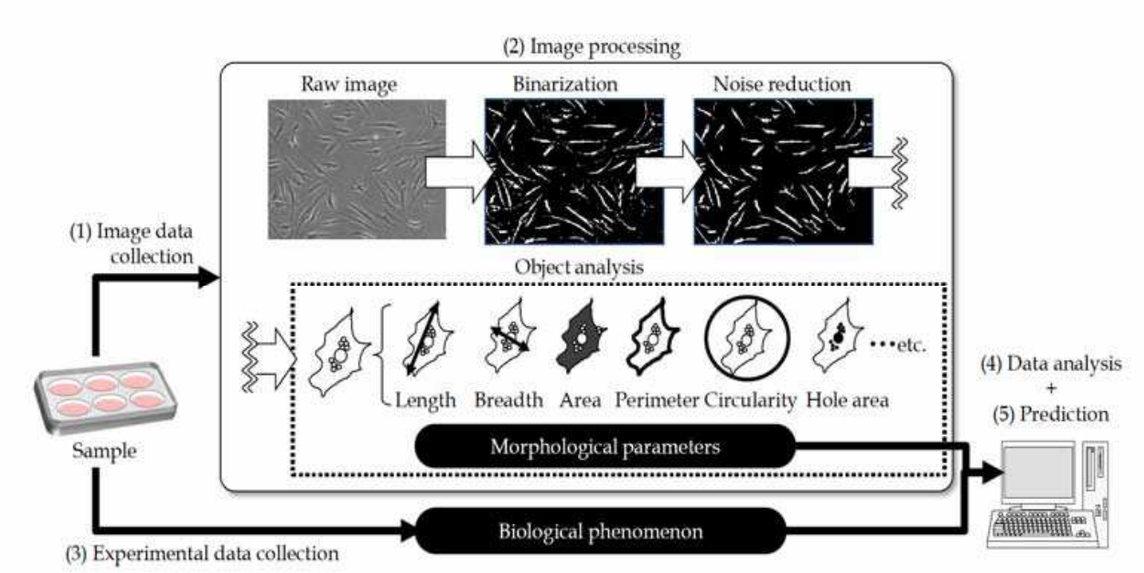

Fig. 2. Work flow of non-invasive image-based cell quality prediction

4.2.1 Image data collection

To assess cellular quality by digital imagery, a large number of detailed images should be collected. There are reports of various types of cellular images applied to non-invasive image analysis, such as phase-contrast microscopy, differential interference contrast microscopy, light-field microscopy, phase-shifting scanning confocal laser microscopy, etc.
In our laboratory, phase contrast microscopic images are obtained by BioStaition CT (Nikon Corporation, Tokyo, Japan), a fully-automatic cell monitoring system.

4.2.2 Image processing

After collecting data, raw images can be processed by image processing software, such as ImageJ, CellProfiler, Scion Image, Metamorph, CellClassifier, etc. The image processing scheme for cell image analyses combines (1) binarization, (2) noise reduction, and (3) object analysis.
There is no gold standard threshold for image analysis. Based on the type of images, different processing filters and orders should be examined to gain the best processing result.
For the analysis, object analysis is the most informative and essential process, that is the conversion of cellular images into numerical morphological parameters. The objects in the images that correspond to cells are individually labelled and their morphological parameters are measured. Although there are some variations in morphological parameters calculated by software, the basic parameters are length, breadth, area, perimeter, centroid, inner/outer radius, and area of holes of cells.

4.2.3 Experimental data collection

To find out the correlation between the cellular morphology and cell quality, cellular images should be compared with the data from biological experimentals. Therefore, the samples are evaluated by conventional biological techniques after the image acquisition.

4.2.4 Data analysis

Choosing the best approach to data analysis depends on the goal. Bioinformatics, the computational and statistical science that analyzes data from molecular biology, offers many effective solutions for the analysis of image data and biological experimental data. If the aim is evaluation of cell quality, regression analysis is an effective solution. Regression analysis could be easily introduced in laboratories by commercially available statistical software, such as SPSS. Free statistical programming platform "R" and BioConductor (R Project homepage, 2011) provide appropriate applications.

The basic strategy for image-based cell quality prediction is to select the proper regression type and construct a model by using a dataset containing both morphological data and experimental data. In the regression analysis, the algorithm searches for the best mathematical function ("the model") that can explain the correlation between a change in morphological data (input data) and biological data (output data). Hence, once a model that links input and output data with good accuracy has been established, future results could be predicted using image data alone. This is analogous to arriving at a prognosis using gene expression from microarray studies. SPSS (IBM, New York, U.S.A.) and the R platform provide multiple optional functions for parameter selection.

4.2.5 Prediction

Prediction is the final process for practical image-based cell quality assessment. The prediction process is similar to the process of data analysis. Briefly, after the completion of regression analysis, the resultant model can function as a "prediction model". However, compared to the analytic process that uses all data (known as "in-sample analysis"), partitioned data are separately used for "model construction" and "model test" (known as "out-of-sample analysis") for evaluating the generalization performance. In practice, the total data are commonly partitioned into training data and test data. Using the regression model constructed only with the training data, the error rate between the actual output values of test data and the predicted values calculated from the input values of test data are compared. When the average error rate for all test data is small, the constructed model could be considered a "reliable model" to use with new data. In such a data model validation process, leave-one-out or several-fold cross validation is commonly used. Since this final process requires the partitioning of total data, the total data pool should be large and varied. When the quantity of data is too small, the constructed model will have limited prediction value with new data. Also, when data variations are very biased to certain conditions, such as with only one lot of cells, the constructed model may perform well for the cell lot examined, but would not work with new cell lots.

4.3 A prediction system based on cell image analysis which supports clinical tissue engineering

Here, we introduce practical examples of non-invasive cell quality prediction to support clinical tissue engineering.

4.3.1 Prediction of cell yield

In clinical tissue engineering, cell yield on the day of surgery is a critical factor. Seeding cell density or cell number is commonly critically defined in most cell therapy protocols to assure a certain therapeutic effect. Therefore, the surgery schedule is projected based on the

experienced observation of cellular behaviour in the culture flasks. However, such scheduling is very tentative and there are cases in which the cell yield is insufficient on the scheduled day of surgery.

To overcome such problems in clinical tissue engineering, we tried to predict the future cell yield (14 days later) from early cellular images (images one to three days into the culture period). For image data, we collected total 270 phase contrast microscopic images (4x) of cultured primary dermal fibroblasts, obtained from 10 healthy volunteers (3 males, 7 females, 29 - 72 years old). Informed consent was obtained according to a protocol approved by the ethics committee of Nagoya University Hospital. Biological data (cell growth rate) within 14 days was obtained by manual cell count. All cells were prepared using passage three or four from the primary expansion in modified Eagle's medium (DMEM) containing 10% FBS at 37°C in the presence of 5% CO_2. A schematic image is provided in Fig. 3.

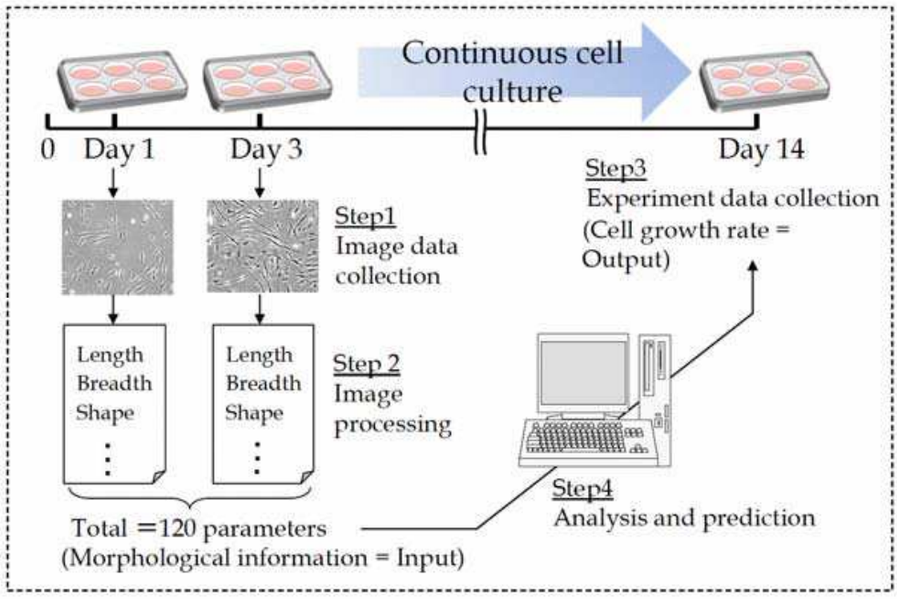

Fig. 3. Schematic diagram of cell yield prediction

The cell attachment rates and growth curves differed greatly among individuals (data not shown). However, by multiple-regression analysis (MRA) with parameter selection, the predicted growth rates had very small error rates compared to the actual growth rates (Fig. 4). Among 120 parameters extracted from the image data, three parameters, such as (P1) change rate of the variation of elliptical rate (1st day to 3rd day), (P2) Size of the inner radius on the third day, and (P3) cell number on the first day, were found to be the best combination of cell culture parameters to predict future cell yield (average squared error = 0.14) (Fig. 4C). It is interesting that other parameters, intentionally selected by cell culture experts badly correlated with the cell yield (Fig. 5). By comparing all the data, we arrived at three conclusions: (1) morphological cell information is informative for cell growth prediction, (2) objectively selected parameters are more effective in cell growth prediction, and (3) multiple combinational parameters work better than a single parameter in cell growth prediction.

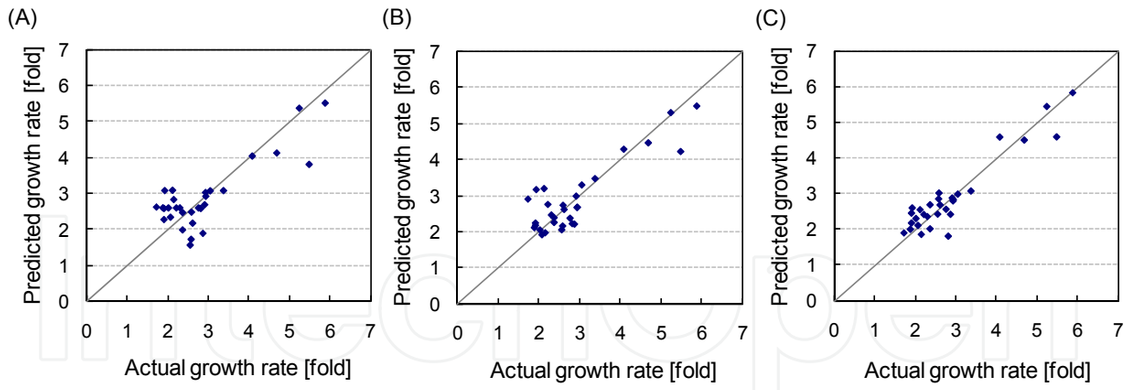

Fig. 4. Accuracy of cell yield prediction models. (A) one-parameter prediction model (P1 only), (B) two-parameter prediction model (P1 and P2), (C) three-parameter prediction model (P1, P2, P3). P1, change rate of the variation of elliptical rate (first day to third day); P2, size of the inner radius on the third day; P3, cell number on first day

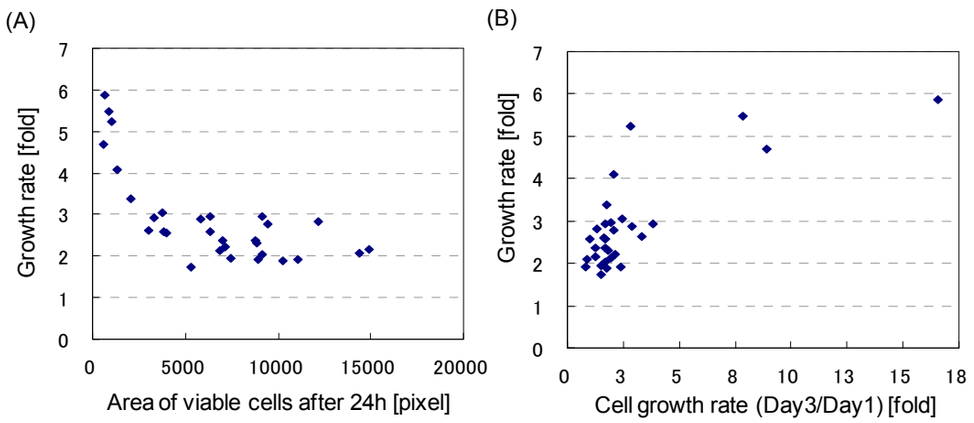

Fig. 5. Correlation plot with the image-derived parameter selected by cell culture experts. (A) Correlation with area of viable cells after 24 h, (B) Correlation with total cell growth rate (day three/day one).

4.3.2 Predicting the level of osteogenic differentiation

In stem cell therapy for bone regeneration, the extent of differentiation critically affects the *in vivo* bone formation after implantation. However, good early markers that predict the future differentiation level of cells have been unavailable. If the differentiation level of cells could be predicted in advance, scheduling of surgery could be set to optimize therapeutic outcome.

As a prediction model of cell differentiation ability, we attempted to predict the alkaline phophatase (ALP) activity of human mesenchymal stem cells (hMSCs). For image data, we collected 1,170 phase contrast microscopic images (4x) of cultured commercial hMSCs (three lots; 20 years old male, 22 year old male, and 19 year old male). Cells at the same passage number were cultureed in the differentiation medium (Agata *et al.*. 2009) at 37°C in the presence of 5% CO_2. For biological phenomenon data, we collected the ALP activity of all

the samples using the method described previously (Agata *et al.*, 2009). We compared the two groups of induction positives and negatives. The protocol is described in Fig. 6.

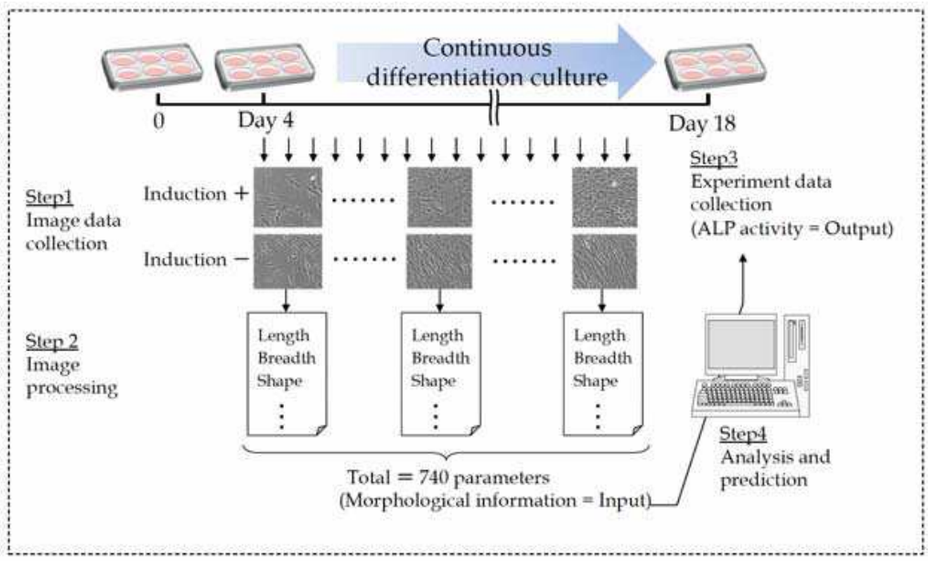

Fig. 6. Schematic diagram of osteogenic differentiation prediction

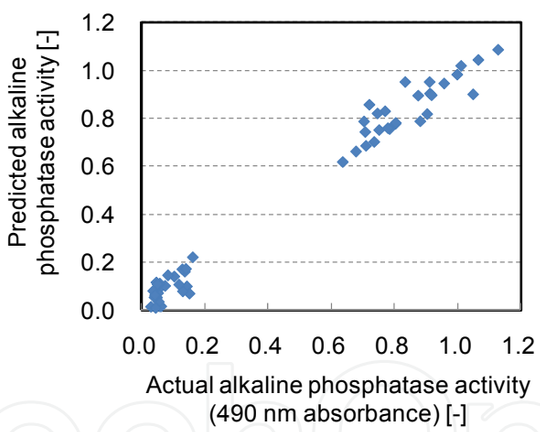

Fig. 7. Prediction accuracy of osteogenic differentiation model

By using ten morphological parameters selected by MRA, we found that ALP activity could be predicted with high accuracy (Fig. 7). Although there were slight differences in differentiation ability among the examined MSCs, the regression model could be extended to respond to any MSCs. In other words, patient differences could be compensated for by such a model-based analysis.

5. Conclusions

Despite rapid progress in treatment technologies using tissue engineering and regenerative medicine, those novel therapies have yet to be extended to standard medical practice and only limited numbers of patients have thus far benefited from these less invasive and

potentially efficient therapies. The development of the technologies described here should facilitate general medical acceptance of tissue engineering. Increased availability of tissue engineering should contribute to the quality of life of patients with a wide range of diseases.

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Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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