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REVIEW

Potential of Engineering Methodologies for the Application to Pharmaceutical Research

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Current engineering methods and their potential for use in cell-based research are reviewed. The basis of the suggested engineering methods is that real cellular responses can be assessed when the cells are under the same conditions as *in vivo*. Providing various conditions for this various engineering methodologies can be adopted. Three major factors should be considered when we apply bio-mimetic conditions to cells under *in vitro* culture conditions. They are the surface pattern and stiffness of the substrate, physical stimuli and neighboring cells. Various outcomes affected by those factors are introduced and reviewed. In particular, those outcomes from stem cell research have been reported. Even though some limitations of adopting those factors alone or combined still exist, the potential is now widely being recognized. The readers are kindly asked to consider those methodologies in relation to pharmaceutical research.

Key words: Substrate properties, Physical stimuli, Neighboring cells, Bio-mimetic

INTRODUCTION

This review article aims to introduce engineering-based experimental methodologies to current areas of biological research. Specifically, current methods will be reviewed followed by addressing the necessity of new methodologies and their potential. Also, the authors would like to ask readers and/or pharmaceutical researchers to consider their potential.

Any investigation of a system begins by characterizing its components. Consequently, most biological or medical research for diagnosis or treatment of a disease begins with cells. Dramatic improvements in the hardware, software and agents available for the measurement and analysis of cellular processes *in vitro* have been developed in recent decades.

However, in most *in vitro* experiments, isolated cells or cell lines are cultured or treated on plates, which may be surface-modified. This culture environment (i.e., cells on plates) has changed little. Recently, many reports have demonstrated the importance of culture environments (Engler et al., 2006; Discher et al., 2009; Lutolf et al., 2009). Furthermore, the culture environment can be used for controlling stem cell differentiation.

Therefore, this article will address the importance and potential of current *in vitro* culture environments.

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BASELINE FOR THE PROVISION OF ALTERNATE CULTURE ENVIRONMENTS

The best condition for growth of any cell type is termed a 'bio-mimetic microenvironment'. Every type of cell in the human body grows in a different environment. However, cells isolated from the body do not experience conditions identical to those *in vivo*, especially when cultured on a plate. Consequently, we may question whether the *in vitro* responses of cells are those in which we are interested. An example of this is presented graphically in Fig. 1.

Smooth muscle cells in blood vessels are known to be circumferentially aligned due to fiber orientation. When these cells are isolated and put on culture plates for *in vitro* investigation, they are under no restriction. Therefore, changes in cell morphology are unavoidable

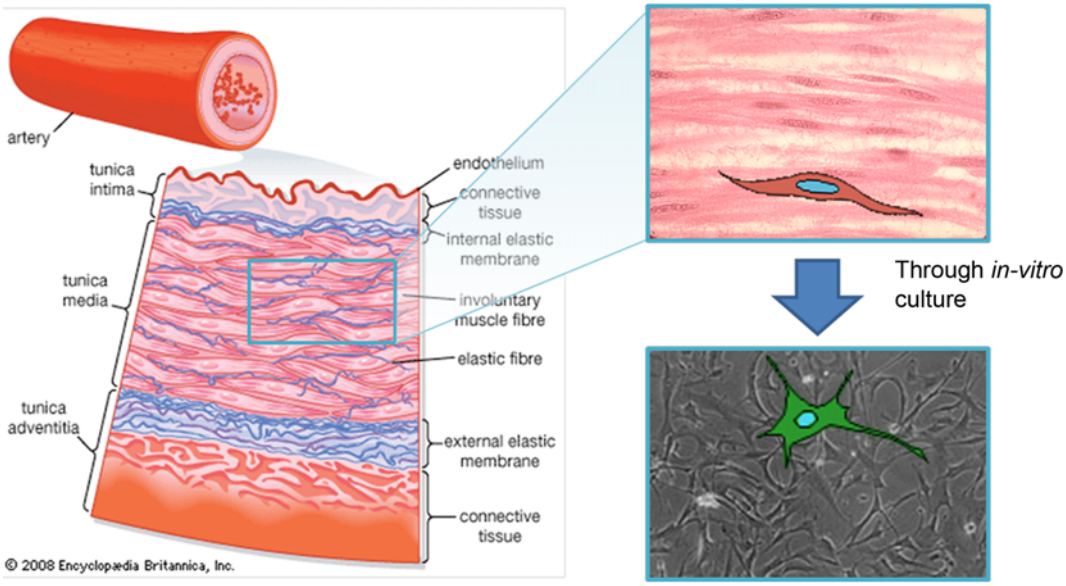

Fig. 1. This picture illustrates the typical change in smooth muscle cell morphology after isolation.

due to the geometrical changes of the environment. These changes in morphology surely induce changes in cytoskeleton arrangement (Fraley et al., 2010). The cytoskeleton is known to play an important role in signaling and alterations in its arrangement can affect various cellular functions (Yourek et al., 2007; Mammoto and Ingber, 2009). Therefore, insight into the question asked above is easily obtained.

Moreover, the mechanical environment is no less important. Most human organs are continuously exposed to external mechanical stimuli. Therefore, the basic elements, cells, are also exposed to them. For example, endothelial cells in blood vessels experience shear stresses and tensile stresses due to blood flow in addition to the compliance effect from pulsatile blood pressure (Fig. 2; Ohashi and Sato, 2005). Cardiac cells are obviously exposed to various mechanical stimuli. Chondrocytes in articular cartilage are predominantly exposed to a compressive environment and more or less to tensile and shearing forces (Fig. 3A; Mansour, 2003).

Bone cells are exposed to compressive conditions as well as shear stresses due to blood flow in lacunae during daily activities (Fig. 3B; Turner et al., 1994; Klein-Nulend et al., 2005).

Meanwhile, most cells in tissues are in contact with other cell types, and thus, we cannot neglect the effects of neighboring cells.

Therefore, other than the biological/biochemical, three major factors must be considered to reproduce the *in vivo* environment: substrate, mechanical stimuli and neighboring cells.

We review the current research on these factors and address their potential applications in cell-based research.

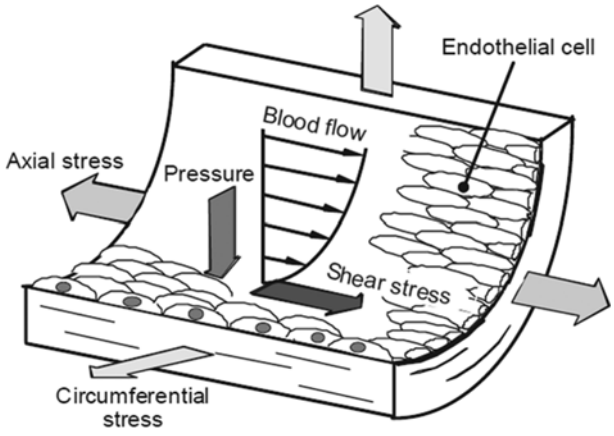

Fig. 2. Cells in blood vessels are continuously experiencing mechanical stimuli. Due to the blood flow and compliance effect, the endothelial cells are experiencing shear stress and circumferential stretch, respectively.

IN VITRO SUBSTRATES

The substrate upon which cells grow shall be investigated in terms of its stiffness and geometry.

Stiffness, an engineering terminology, can be used to describe how hard a material is. Its units of measurement are Pascals (Pa) or Newtons per square meter. For example, the stiffness of human cortical bone is 14-22.8 GPa (Cuppone et al., 2004). A material of stiffness 1.0 MPa can be stretched by 0.1% under 1.0 kPa tension.

Several recent studies have demonstrated that sub-

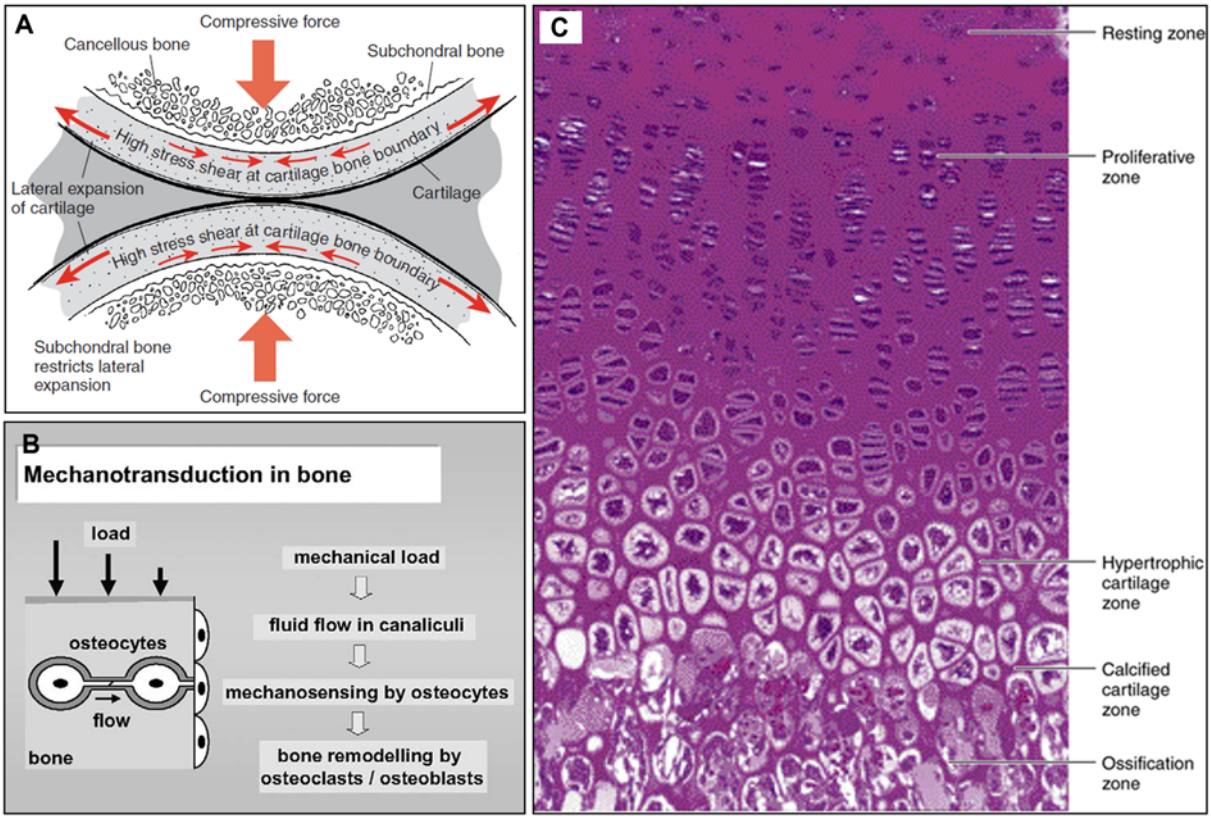

Fig. 3. (**A**) Chondrocytes in articular cartilage experience compressive force and shear stress as well. (**B**) There are compressive forces and shear forces mechanotransduction in bone cells. Bone remodeling is known to be closely related to these physical stimuli. (**C**) Cross sectional photomicrograph of articular cartilage. There are five distinctive layers where different morphology of chondrocytes can be found.

strate stiffness affects cellular responses in various ways (Even-Ram et al., 2006; Rowlands et al., 2008; Zajac and Discher, 2008; Reilly and Engler, 2010; Zhang et al., 2011). This reflects the current method of culturing on plates with or without a coating. Is it appropriate to culture a cell isolated from a site of different stiffness on a plate?

The importance and potential effect of substrate stiffness is now being recognized and reported. Therefore, by taking advantage of stiffness control techniques, researchers can control stem cell differentiation (Engler et al., 2006; Discher et al., 2009; Lutolf et al., 2009) and cell migration (Lo et al., 2000; Wong et al., 2003; Cheung et al., 2009). One report (Buxboim et al., 2010) even suggested that cells can sense substrate thickness.

Another report (Engler et al., 2006) of the effect of substrate stiffness on differentiation of mesenchymal stem cells is the example to which many researchers refer. Data suggested that mesenchymal stem cells seeded onto high (>34 kPa), intermediate (8-17 kPa), and low (0.1-1 kPa) stiffness changed their morphology within 4 h of seeding, and eventually differentiated into osteo-, smooth muscle-, and neuronal-like cells, respectively. Surprisingly, no growth factors were used in this experiment. The mechanism explaining these outcomes has not been clarified even though many researchers suggested and examined various hypotheses. Engler et al. suggested NMMIIs (nonmuscle myosin IIs) as a critical factor. They reported NMMII is likely to be involved in sensing matrix elasticity through focal adhesions. Also, they found that inhibition of NMMII blocked all elasticity-directed lineage specifications without affecting other aspects of cell function and shape.

Another point to be considered is the structural geometry of the substrate. Structural geometry is sometimes addressed in two- or three-dimensional cultures. In general, cells reside in a three-dimensional structure. As noted above, changes in cytoskeletal structure after isolation and culture are unavoidable. Other than this, changes in phenotype are well recognized in chondrocytes of articular cartilage. As shown in Fig. 3C, chondrocytes in articular cartilage are in a three-dimensional structure and show differing shapes, depending on their location (Junqueira and Carneiro,

2005). Various reports have suggested that freshly isolated chondrocytes lose their phenotype when cultured on a plate (Bonaventure et al., 1994; Lee et al., 2003), showing decreased expression of collagen type II. However, the phenotype is maintained when cultured in a three-dimensional structure (Lee et al., 2003; Park et al., 2005; Yamaoka et al., 2006; Marsich et al., 2008; Seda Tigli et al., 2009). Alginate beads are widely used for three-dimensional culture of chondrocytes. This alginate, one of the hydrogel materials, is highly permeable, thus enabling easy supply of nutrients (Barbetta et al., 2009; Eiselt et al., 2000).

Besides the encapsulation technique, the scaffold concept has also been introduced for three-dimensional *in vitro* culture. A scaffold is a three-dimensional structure within which cells reside. It provides a physical space for growth, along with biocompatible characteristics. Tissue engineering adopts this scaffold as a carrier of cells. The cells in the scaffold can be cultured three-dimensionally. The cell-containing scaffold replaces the damaged tissue and sometimes the scaffold is composed of biodegradable materials (Gunatillake and Adhikari, 2003; Engineer et al., 2011).

Various techniques for the manufacture of three-dimensional scaffolds have been developed and introduced in the area of tissue engineering (Sacholos and Czernuszka, 2003; Annabi et al., 2010). These can also be used for three-dimensional culture of cells *in vitro*. The key point is to provide physical space for cells to reside in three dimensions. Two commonly used techniques are salt leaching (Liao et al., 2002; Hou et al., 2003) and gas forming (Tai et al., 2007). In the salt leaching technique, the pore size is dependent on the salt particle size (Mikos et al., 1993). However, complete removal of salt from the polymerized scaffold is not easy on thicker scaffolds (>2.0 mm, approx.) (Cao et al., 2006). Therefore, it raises doubt as to whether this technique is applicable in fabricating three-dimensional scaffolds with large defects. The gas forming technique utilizes high pressure CO_2 gas processing. The porosity is dependent on the amounts of gas dissolved into the polymer. Also, the structure is affected by the diffusion rate of gas molecules through the polymer. Moreover, this technique sometimes results in a closed cellular structure within the scaffold (Chen et al., 2002). In summary, each technique has its own limitations. The morphology of pores (spaces) inside the structure is hard to control. Also, the interconnectivity among pores is poor, which hinders the supply of nutrients. Also, some difficulty may exist in retrieving cells for later analyses. Images of a typical scaffold made using the leaching technique are shown in Fig. 4.

To overcome these limitations, the so-called rapid prototype (RP) technique has been introduced. This has been widely adopted in manufacturing engineering to produce prototypes or sample prospective products.

The desired product is designed with the help of computer-aided-design (CAD). The interface technique between the CAD software and machine enables manufacturing of the same product as was designed on the computer. Specifically, the three-dimensional structural scaffold is designed with CAD software; polymer in a syringe-type cylinder is then extruded. Strand diameter is controlled by syringe gauge size and extrusion pressure, even to the micro level. The pore size is also controllable; consequently, interconnectivity can also be adjusted (Sachlos and Czernuszka, 2003; Fedorovich et al., 2007). Since this technique is applicable to most biopolymers, any biochemical agent may be added to the polymer solution and so control scaffold stiffness (Park et al, 2005; Slaughters et al., 2009).

Apart from a full three-dimensional culture, cells may be cultured on patterned plates. This is known as a two-dimensional culture. A controlled surface morphology is easily produced with the aid of microelectromechanical systems (MEMS) techniques and among these, photolithography is widely used (Camelliti et al., 2006). This precise patterning technique enables coating of different constituents on specific areas. In addition, this platform can assist in investigations of single cell responses (Chen et al., 1997; Song et al., 2011). When a transparent substrate is adopted, investigation of cellular responses becomes possible with the

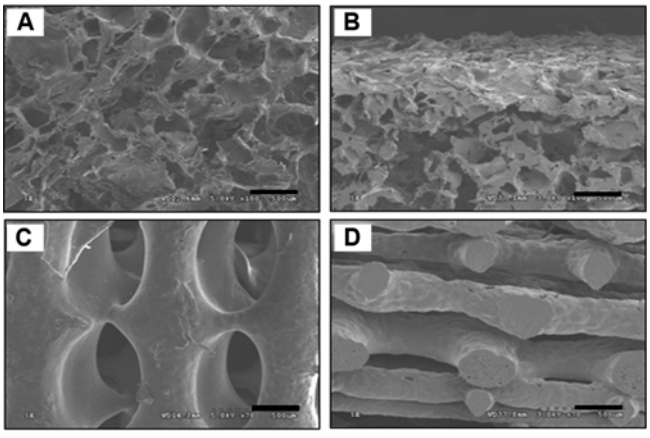

Fig. 4. Polymeric scaffold manufactured by the salt leaching method (**A**, **B**) and rapid prototype method (**C**, **D**). (**A**) Top view before seeding the cells; (**B**) Cross sectional view after cell culture; (**C**) Top view before seeding the cells; (**D**) Cross sectional view after cell culture. Note that cells cannot penetrate through the thickness due to poor interconnectivity as shown in (**B**).

aid of staining and confocal microscopic observations (Kraning-Rush et al., 2011).

Cells in muscles or ligaments are closely related to fiber bundles *in vivo*. Therefore, culturing those cells in fiber bundles appears to be promising. One technique to produce micro- or nano-sized fibers is electrospinning. This has been used in the fabric industry for a long time, especially in the area of fabricating fine textiles. A simple system is shown in Fig. 5. It consists of polymer solution in a syringe, a collection part and a high electric field generated by a power supply. When the electric field is set between the syringe and collector, the polymer solution is easily spun into a fine fiber to the collector. By adjusting voltage and the distance between the collector and syringe tip, the diameter and/or density of spun fibers can be controlled. When a rotating cylinder is used as a collector, spun fibers can be aligned depending on the rotating velocity. An SEM image of spun fibers is shown in Fig. 6 (random, aligned). Various studies reported the electrospinning technique (Shin et al., 2006; Mauck et al., 2009; Shin et al., 2009; Yu et al., 2009; Cui et al., 2010; Russo and Lamberti, 2011). A report (Lee et al., 2005) that combined electrospun fibers with mechanical sti-

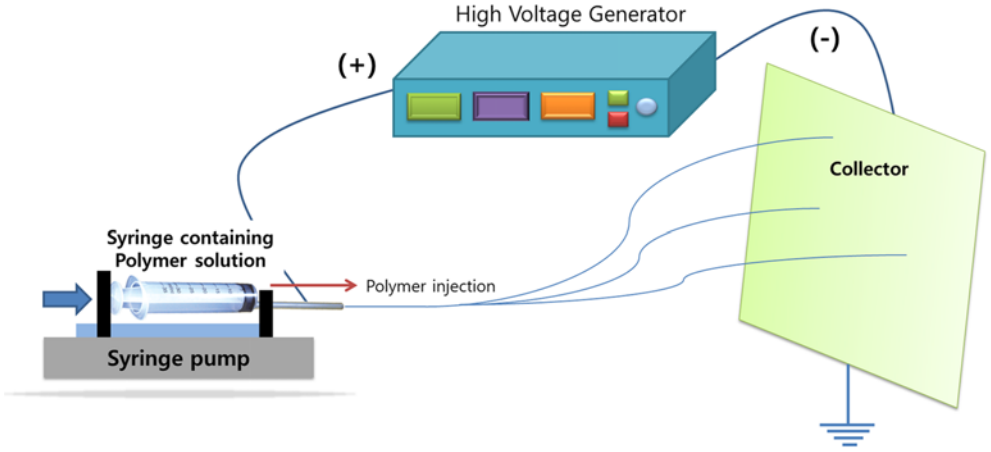

Fig. 5. Schematic diagram showing an eletrospinning system.

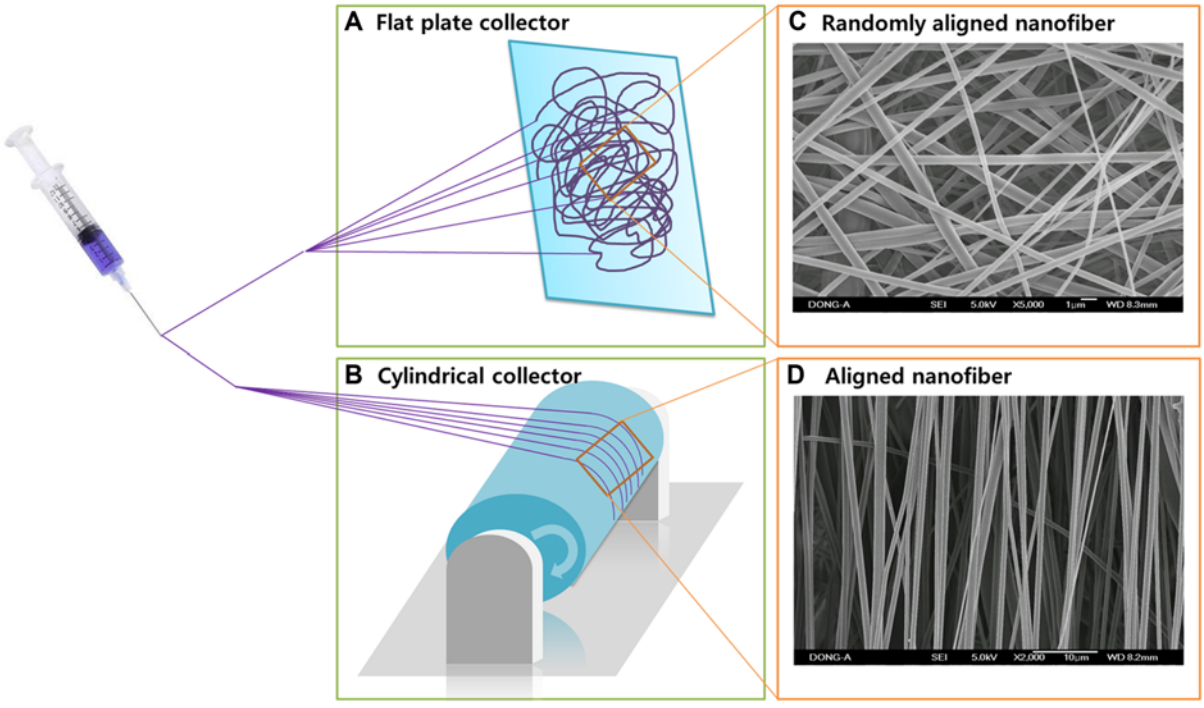

Fig. 6. Depending on collector type, various textures of the spun sheet can be obtained.

mulation showed the potential of bio-mimetic environments in *in vitro* research.

PHYSICAL STIMULI

Most organs, tissues and related cells in the human body are continuously experiencing physical stimuli, which may be either voluntary or passive. Typical examples are illustrated in Figs. 2-3B. This stimulation can be classified into three types from a mechanical engineering point of view: tensile, compressive and shear. Compression is sometimes referred to as negative tension.

Cells in muscle, ligament, or cardiac tissue usually experience tensile force during daily activities and cardiac function. A typical example of compression can be found in articular cartilage tissue (Fig. 3A). Meanwhile, endothelial cells in blood vessels are mainly exposed to shear stresses due to blood flow.

What happens to cells in the absence of mechanical stimuli? Retrogression or degeneration occurs. When an old person loses one or more teeth due to aging or other causes, he/she needs dentures. Two main reasons exist for having dentures. One is to help with chewing food. Another is to prevent resorption of alveolar tissues. Chewing causes alveolar bones to experience mechanical (compressive) stimuli via the teeth. Without such stimuli, the alveolar bones degenerate and are eventually resorbed. This phenomenon can be explained by the theory of use and disuse, which can be easily seen in athletics.

How can such mechanical stimuli be applied to cells *in vitro*? Recently, various equipment for the application of mechanical stimuli to cells or tissues have been developed and marketed. Some examples are shown in Fig. 7 (Taesan Solutions Ltd., Flexcell International Corp., and Synthecon Inc.). Use of such equipment has facilitated many research outcomes (O'Connor et al., 2002; Park et al., 2006; Kim et al., 2008). This equipment is even being used in stem cell research (Kim et al., 2007, 2009; Lee et al., 2007; Jang et al., 2011).

During recent decades, growth factors have played a major role in controlling stem cell differentiation. Recently, stem cells, especially mesenchymal stem cells (MSCs), have been found to exist in most parts of the body, and thus stem cells also experience mechanical stimuli, which will likely affect stem cell differentiation. Various reports on control of stem cell differentiation with the aid of mechanical stimuli have been published (Pavalko et al., 1998; Masuda et al., 2008; Adamo et al., 2009; Huang et al., 2009; Quaglino et al., 2009; Maul et al., 2011; Wu and Hochedliger, 2011).

The effect of tension on differentiation of mesenchymal stem cells is well recognized and depends on the magnitude of tensile strain. When mesenchymal stem

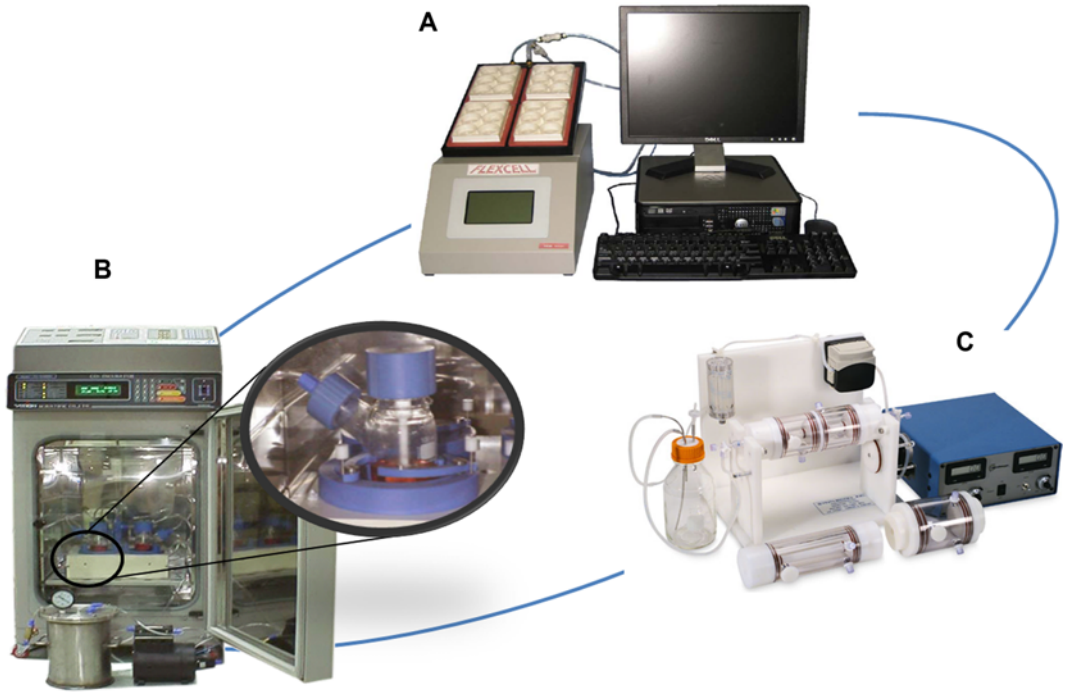

Fig. 7. Some typical bioreactors in the market for mechanical stimuli. (**A**) for stretching (Flexcell FX-5000 Tension System, Flexcell International Corp.), (**B**) or hydrostatic pressure and shear stress (TSMBI 100, Teasan Solutions Ltd., Korea), (**C**) for perfusion (RCMWTM systems, Synthecon Inc.).

cells experience both small (~3.0%) and large (~10%) strain, they differentiate into osteo- or smooth muscle-like cells, respectively (Riha et al., 2007; Kearney et al., 2010; Jang et al., 2011). Unfortunately, no concrete explanation for this mechanism of strain magnitude-dependent differentiation of MSCs has been reported. Only Kearney et al. reported that strain-induced BMP2 plays an important role in signaling of ERK and PI3 kinases which are known to be involved in the differentiation of MSCs to an osteo-like lineage.

To apply compression to cells in bioreactors, hydrostatic pressures are usually used (Kim et al., 2007; Kim et al., 2009), and direct compression is sometimes adopted (Lozito et al., 2009; Ma et al., 2011). Hydrostatic compression is commonly used to study chondrocytes of articular cartilage, since they are known to experience compression due to the fluid in cartilage during walking. In this case, the hydrostatic pressure is generally intermittent. In terms of intermittent pressure, the parameters are magnitude and frequency. For magnitude, more than 20 times atmospheric pressure is considered normal during walking (Beecher et al., 2007; Sowa et al., 2011). However, we may not stipulate that the magnitude of pressure cannot be identical to that *in vivo* when cells are cultured *in vitro*. Therefore, approximately two to three times atmospheric pressure has been adopted (Kim et al., 2007; Kim et al., 2009; Walter et al., 2011). Another study revealed that 0.2 MPa (~2 times atmospheric pressure) for 2 and 15 min for pressurizing and resting, respectively, increased the adhesion of chondrocytes, human vein endothelial cells and calf pulmonary arterial endothelial cells (Kim et al., 2008).

Now that the potential of mechanical stimulation has been recognized, one barrier remains to be overcome: what is the optimal stimulation pattern? That is, how long, how often, and how large should the stimulation be? Obviously, the optimal pattern depends on cell type, and unfortunately, no study has suggested an appropriate methodology.

In addition, the basic mechanism underlying the effect of mechanical stimulation has not been elucidated, despite its potential in the area of stem cell research.

Recently, with the aid of improvements in confocal microscopy, staining and imaging, study of single cells may provide answer to this question (Goffin et al., 2006; Urban et al., 2010). For example, cytoskeletal rearrangements under tension (Brangwynne et al., 2006; Felder et al., 2008; Kaunas et al., 2011) and the location of protein expression (Wang et al., 2006; Herrmann et al., 2007) were observed and quantified. Also, live cell imaging techniques will help researchers study this issue in greater depth.

NEIGHBORING CELLS

Another key principle for the production of bio-mimetic environments *in vitro* is that most cells in the human body are in direct or indirect contact with other cell types. Therefore, some communication between them likely occurs, which may be essential for proper functioning.

However, reconstructing the exact *in vivo* conditions *in vitro* may be impossible. The best available method to overcome this problem may be coculturing (Darland and D'Amore, 2001; Ball et al., 2004; Wu et al., 2005; Kasper et al., 2007; Potapova et al., 2007; Henrich et al., 2009; Kim et al., 2009; Lozito et al., 2009; Xue et al., 2009; Jing et al., 2010). We may classify coculturing into two categories: direct contact and indirect contact. As the names imply, direct contact coculture means culturing two or more types of cells in the same media. In this case, an isolation process or other post-processing is necessary to identify the responses of the designated cell type. For this, tagging is usually used. However, the effect of tagging on a cell should be investigated in advance. Alternatively, a staining technique may help researchers to identify the responses they are investigating.

To minimize these difficulties, an indirect method should be considered. Two types of indirect methods are currently used. The first is encapsulation of a cell type into a material with high permeability (Kim et al., 2009). This enables easy sorting of the designated cell type for later analysis. The second is to separate two types of cells with a transwell membrane. For example, one type of stem cell can be separated from developed cells by a transwell membrane in a culture dish, thus enabling the investigation of stem cell differentiation into the targeted cell type under various conditions (Wu et al., 2005; Ye et al., 2008).

CONCLUSIONS

The baseline of the *in vitro* methods discussed so far is that real or close responses can be induced, provided that the cells under investigation experience an environment identical to that *in vivo*. For the desired bio-mimetic environments, we have discussed current and potential methods based on engineering concepts/methods.

All engineering methods can be summarized as 'reproducible, quantitative measurements and analyses'. In fact, these are closely related.

In most biological studies, the experimental results

are presented along with those of a control group, especially when a new method is to be suggested. The need for a control group arises from the non-quantitative nature of the measurements.

Moreover, we need extremely large data sets to be accumulated for further use. More importantly, an identical experimental environment cannot be guaranteed regardless of the researcher's skill. However, quantitative measurements render the time and cost of including control groups unnecessary.

The methods discussed so far may assist in providing a consistent environment since they are controllable through available engineering knowledge.

What about the cell-based experimental paradigm in pharmaceutical research? Can we adopt the paradigms described here?

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