Mini Review

# Induction technology of vascular networks within bioengineered tissues

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Tissue engineering research has shown rapid progress; however, techniques for effectively inducing blood capillary formation within fabricated grafts have not been well-established. Therefore, the development of such technologies remains a primary target in this field. Several methods have previously been examined and can be generally classified into those that promote host angiogenesis or those that are related to the development of grafts with angiogenic potential.

Methods that involve the promotion of host angiogenesis include gene transfection or treatment with angiogenesis-promoting factors. Interestingly, modified transplantation techniques have successfully created over 1 mm thick grafts *in vivo*. Thus, concepts involving *in vivo* tissue fabrication techniques to create thicker tissue constructs possessing intact microvascular networks have recently been established.

To promote graft angiogenic potential, several approaches including fabrication of microcapillaries *in vitro*, and the inclusion of either endothelial progenitor cells or bone marrow derived cells within the grafts have also been examined.

Moreover, recently we have reported that engineered myocardial tissue grafts containing active endothelial cells in a network-like formation maintain the ability for neovascularization during culture and transplantation due to the use of cell sheet technology. Additionally, the amount of vessels in grafts *in vivo* is affected by the ratio of endothelial cells *in vitro*.

Considering clinical applications, techniques that can create tissue grafts with functional vessels are likely required for many patients. Therefore, new technologies for the establishment of vascular networks withing bioengineered tissues *in vitro* are required for the further development of regenerative medicine.

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## The field of tissue engineering and its overriding issues

In the treatment of tissue and organ defects or loss of function, the field of regenerative medicine has received significant interest. In particular, tissue engineering with the goal of creating a supply of organs and tissues fabricated in laboratories, has

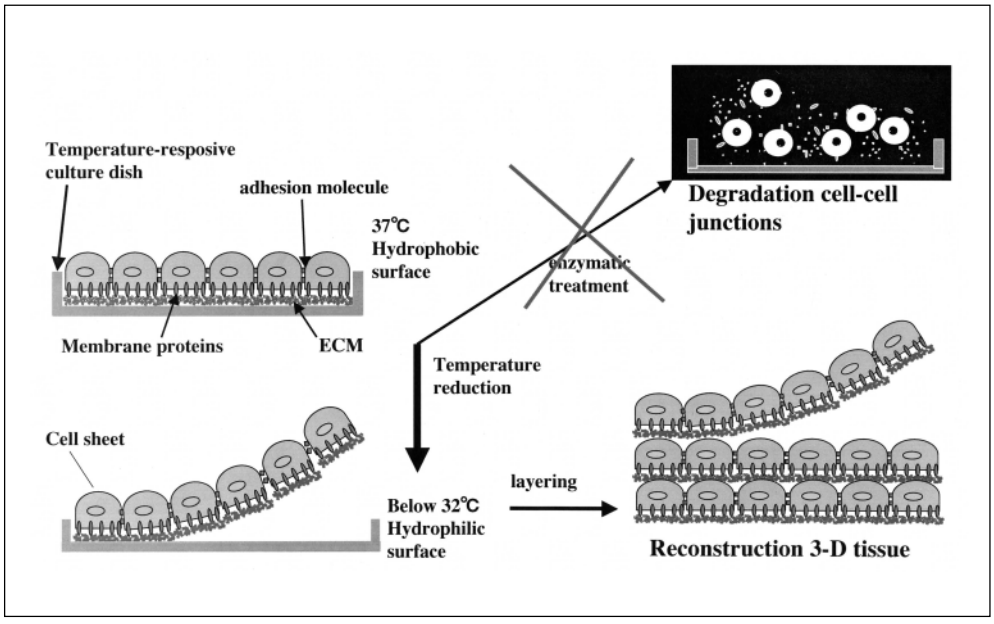


Fig.1 Schematic diagram of cell sheet technology employing temperature-responsive culture dishes. The non-enzymatic harvest of intact cell sheets along with deposited extracellular matrix and adhesive proteins allows for the fabrication of 3-D tissues by the layering of individual cell sheets.

progressed significantly[1-3].

The traditional methods of tissue engineering involve reconstructing organs or tissues with scaffolds composed of biodegradable polymers, into which living cells can be seeded. Over time after implantation, deposited extracellular matrix (ECM) molecules that are produced by the cells present within the grafts, replaces the scaffolding materials and results in the re-creation of tissue-like structures[4-7]. While these popular approaches using biodegradable scaffolds have succeeded in the bioengineering of some tissues, a major obstacle remains the difficulty in fabricating tissues that have complex native structures, such as the heart and liver. Using these methods, it often becomes difficult to create constructs of sufficiently high cell density.

Therefore a new technique has been developed that allows for the harvest of cultured cells as intact sheets using temperature-responsive culture dishes. This method, which uses cell sheet technology, has recently become a popular technique for tissue reconstruction and now allows for a favorable alternative in numerous areas of tissue regenerative therapies. Temperature-responsive culture dishes are created by the grafting of the temperature-responsive polymer poly(*N*-isopropylacrylamide) (PIPAAm) onto ordinary tissue culture surfaces, allowing cells to be harvested as intact sheets by simply reducing the incubation temperature, without the need for traditional enzymatic treatments[8,9]. Because of the presence of adhesive proteins and ECM that are maintained on the basal side of the cell sheets, three-dimensional tissue-like structures can be reconstructed via the layering these cell sheets.

However, a major obstacle that currently limits all tissue engineering approaches is the inability to maintain thick, viable tissues due to the lack of functional vascular network formation within the engineered constructs. Vascular network are organized throughout almost all tissues and support normal organ function by the supply of nutrients such as oxygen and glucose, as well as the removal of various metabolic wastes. In particular, within cardiac muscle, blood vessels occupy approximately 10% of the total heart volume and these vascular networks are present at a high density throughout the tissues[10]. Similarly, as cell density is increased within engineered tissues, the need for blood capillaries also increases. Thus, the need to create blood capillary adjuncts for thicker tissues has become a matter of highest priority in the field of tissue regeneration.

## Technologies for the induction of blood vessels within engineered tissues

Previously, various techniques to induce blood vessel formation within bioengineered tissues have already been attempted. Currently, the most popular methods involve the induction or

promotion of vessel outgrowth from the host vasculature either prior to or at the time of transplantation. In order to induce vessel formation, treatment with angiogenesis promoting factors by direct treatment or more complex methods such as gene delivery have become commonly used methods[11,12]. VEGF and b-FGF and HGF and other growth factor are candidates of application for clinical therapeutic vascular growth[13]. However, many growth factors related to angiogenesis and vasculogenesis are involved in complex physiological processes, and a major concern remains the need to control the normal activities of these signaling cascades. Recently, transplantation of controlled release systems using angiogenic factors at a time of graft implantation has also been examined[14,15].

As described above, the complex signal transduction pathways associated with the induction of blood capillary formation present the possibility of angiogenic abnormalities after addition of blood-vessel inducing factors. Therefore another approach that has been reported utilizes normal host angiogenesis reactions to create thick myocardial tissue grafts with blood capillaries, by the application of modified transplantation procedures.

In a recent study, cell sheet technology has been applied to create myocardial tissues possessing 3-D structures[16]. Moreover, Haraguchi et al. demonstrated that bilayer cardiomyocyte sheets coupled electrically with slight delays 34±2min (mean±SEM) after layering[17]. Appropriately, the next aim for myocardial tissue reconstruction is to increase the overall thickness of the bioengineered grafts. However, due to the lack of proper vascularization, unlimited stacking of additional cell sheets is still prevented by mass transport problems. Using simple layering techniques, myocardial cell sheets fabricated with neonatal rat cardiomyocytes have a tissue thickness restricted to ~ 80 $\mu$m (equivalent to 3 cell sheet layers), when implanted *in vivo* in the subcutaneous tissues[16]. To overcome these limitations, a plausible approach is the transplantation of triple-layer grafts performed at appropriate intervals (1-2 days) to allow for host vascularization to occur within the implanted grafts, prior to subsequent triple-layer graft transplantation[18]. Using this method of polysurgery, approximately 1 mm-thick, cell-dense myocardial tissue grafts can be fabricated, however requirements for repeated transplantation remain difficult to apply clinically. Similarly, new methods utilizing the *in vivo* host environment as a living incubator have also recently been examined[19].

On the other hand, methods to create vascular networks *in vitro* or to accelerate vessel reconstruction within engineered tissues using cell biotechnology have also been explored. Since endothelial progenitor cells (EPC) and bone marrow derived cells (BMC) both have shown the ability to differentiate into endothelial cells, grafts containing these cells with the potential to become mature endothelial cells present the possibility for angiogenesis after transplantation. Moreover, since these cells can be easily obtained from nearly all patients, the possibility of immunological rejection can theoretically be avoided[20,21]. However, with the use of EPC or BMC as a source of endothelial cells, issues still remain regarding the differentiation and survival of these cells over time.

## Vascular induction techniques within cardiac tissue grafts engineered with cell sheet technology

As described above, 3-D myocardial tissue grafts have been previously engineered with cell sheet technology, with the ultimate goal of clinical applications to treat patients with severe heart failure. As the thickness limitation remains a primary problem, the induction of blood capillary formation within the grafts at the time of fabrication is a highly desirable approach. Therefore, the understanding of the mechanisms of tissue neovascularization after transplantation may be key milestone in the development of new methods for creating thick, blood-supplied tissues.

As described, after the implantation of bioengineered myocardial tissues with cell sheet technology, neovascularization occurs rapidly within the transplanted grafts. Therefore, we first examined the origin of the newly formed blood vessels within the myocardial tissue grafts *in vivo*, by using EGFP expressing myocardial cell sheets. The results indicated that surprisingly, all vessels in the myocardial tissue grafts originate from the grafts and spread to connect with the host vasculature[22].

These results imply that the grafts themselves have an inherent ability for angiogenesis, which leads to the observed neovascularization. Indeed, our myocardial tissue grafts contain active endothelial cells and show the expression of autocrine angiogenesis promoting factors. Due to non-invasive cell harvest using cell sheet technology, endothelial cells that have organized into network-like formations maintain this structure during *in vitro* layering, as well as transplantation *in vivo*. The transplantation of these intact endothelial cell networks may be the reason that bioengineered grafts are able to rapidly reconstruct vessels *in vivo*.

To directly confirm this hypothesis, we tried to control the ratio of endothelial cells in the myocardial tissue grafts *in vitro* prior to graft transplantation. Results showed that the amount of reconstructed vessels is affected by the ratio of endothelial

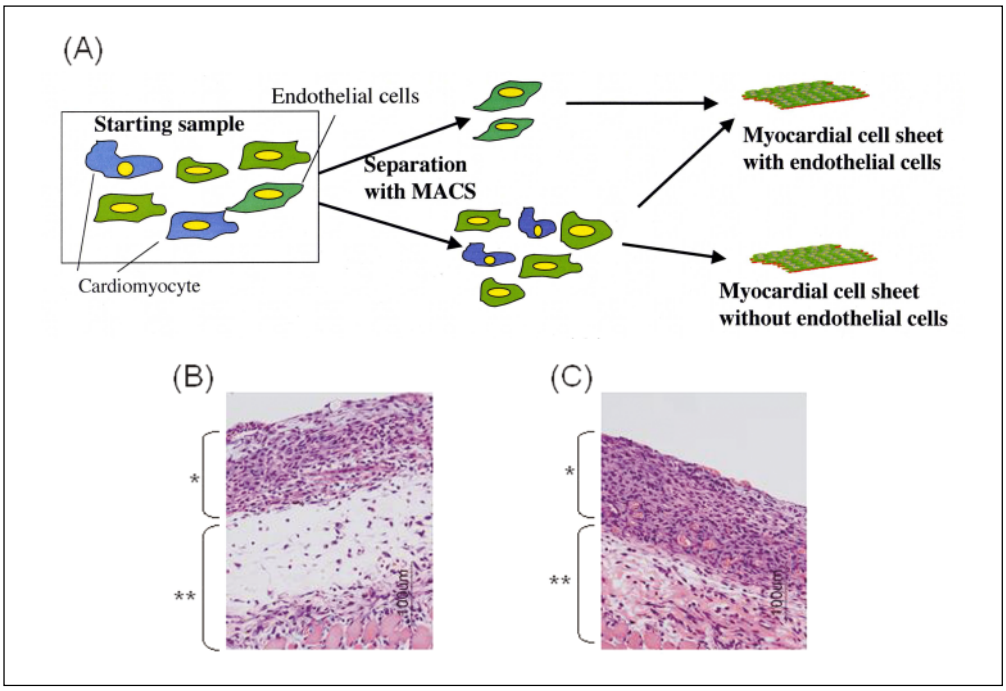


Fig.2 Methods for controlling vascularization within the engineered myocardial tissue grafts

(A) Schematic diagram of the controlling the ratio of endothelial cells *in vitro* with MACS (magnetic cell sorting). Histological analysis 3 days transplantation show (B) myocardial tissue grafts fabricated without endothelial cells and (C) myocardial tissue grafts with endothelial cells present. *indicates the areas of the transplanted grafts and **indicates the host tissue areas.

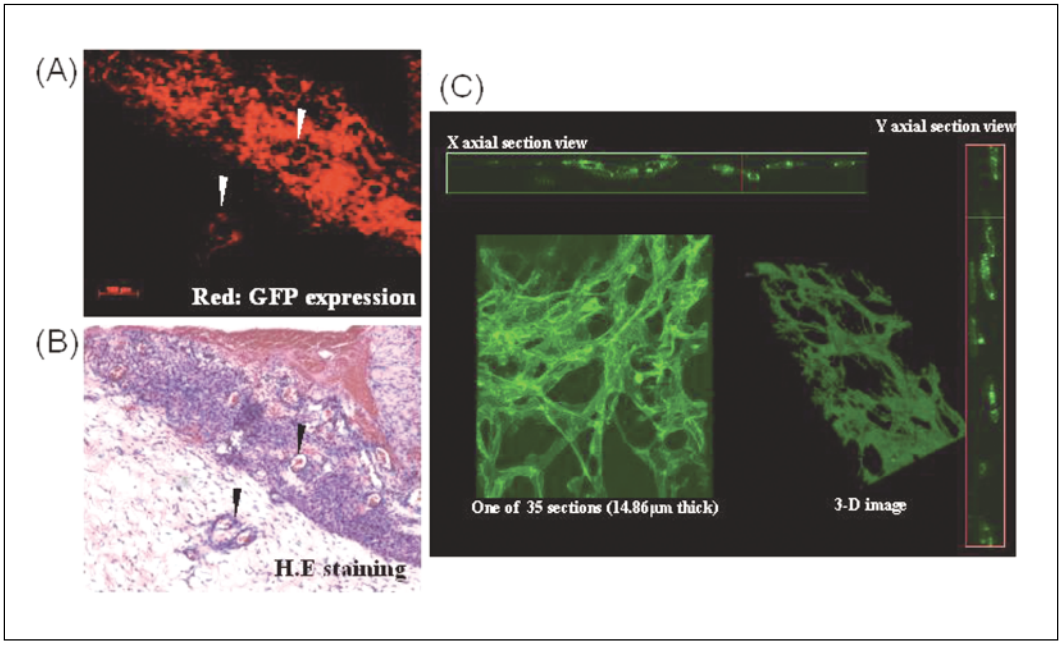


Fig.3 Vascularized myocardial tissue grafts with graft-derived endothelial cells. GFP expressing myocardial tissue grafts from GFP-Tg neonatal rat were transplanted into dorsal subcutaneous tissues of normal rats

(A) shows EGFP expression and (B) shows H.E. staining of a serial section. Transplanted grafts could be detected as areas of EGFP expression. Almost all vessels within the grafts (arrowheads) expressed EGFP. (C) shows of myocardial tissue grafts stained with anti-CD31 antibody and analyzed by confocal microscopy. Tubular formations of endothelial cells could be observed in x or y axial section views of about triple-layer myocardial tissue grafts. However continuous tubular structures could not be seen.

cells present within the myocardial cell sheets *in vitro*[22]. Thus, our vascular induction technology involves the inclusion of endothelial cells within the engineered myocardial grafts, the activation of endothelial cells *in vitro*, and finally followed by maintenance of newly formed endothelial cell networks both *in vitro* and *in vivo*, after cell sheet harvest. The advantage of this method, we can rapidly induce vessels at the limitation area and it may be low-potential the affect of the around healthy area. For the future clinical application, we should research the cell source.

## Conclusion

While the formation of endothelial cell networks and their subsequent development into microvascular networks can be controlled *in vivo*, with the present conditions these maturation processes cannot be performed under *in vitro* culture. Thus, newer and innovative culture technologies allowing for the formation of mature blood vessels, with methods such as the addition of physical stimuli or shear stress, are still required.

In the future, *in vitro* engineering of various 3-D tissues with blood vessels that can be easily connected to host vessels will enable the reconstruction of higher-order tissues for various regenerative therapies.

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