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Practical Application of Confocal Laser Scanning Microscopy for Cardiac Regenerative Medicine

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1. Introduction

Heart failure (HF) is an insidious disease in developed countries. Despite recent medical progress, the number of patients with HF continues to increase, with the mortality of HF as high as that of cancer. The only radical treatment for HF is cardiac transplantation, although the shortage of donor hearts poses a serious problem [1]. To overcome this unmet medical need, innovative technology is required. Specifically, cell transplantation therapy with regenerative cardiomyocytes is expected to eventually replace cardiac transplantation as the treatment for severe HF.

It was believed that, after the neonatal stage, heart cells could no longer proliferate and regenerate. However, recent evidence demonstrates the regenerative capacity of cardiomyocytes obtained from several different cell sources, such as mesenchymal stem cells (MSCs), cardiac progenitor cells (CPCs), and neural crest derived stem cells (NCSCs) [2-5]. In addition, pluripotent stem cells (PSCs), such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), seem to be potential cell sources of regenerative cardiomyocytes. Thus, basic *in vivo* and *in vitro* studies have evolved into translational research focused on stem cell therapy for severe HF.

Without the development of innovative scientific technology enabling the precise observation and analysis of individual cells, these recent advances in regenerative medicine would not have been possible. In such basic studies, the cells are often marked with green fluorescent protein (GFP) or red fluorescent protein, and co-stained with various cell-specific markers, such as α -actinin, MF20, and cardiac troponin in the case of cardiomyocytes. Fluorescence-activated cell sorting (FACS) enables population analysis of both stem cells and differentiated

cells, and fluorescent microscopy visualizes regenerative cardiomyocytes in culture, as well as in tissues. However, for either technique to be useful, it is necessary to collect detailed information at the level of individual cells.

Confocal laser scanning microscopy (CLSM) has emerged as a new high-tech method for exploring the stem cell field in cardiovascular medicine. CLSM enables the kinetics of single stem cells and differentiated cells to be studied both *in vivo* and *in vitro*, and so has opened up a new world, revealing the regenerative potential of stem cells. In this chapter we explain how CLSM has contributed to new scientific findings in cardiac regenerative medicine.

2. Technical advantages of CLSM for the investigation of stem cells in cardiovascular medicine

2.1. Serial optical thin sections

Fluorescent immunohistochemistry is important for studies into the topography of stem cells. Samples can be double stained with different markers, with the resulting fluorescent images enabling visualization of the co-localization of the different signals. It is impossible to distinguish overlapping signals using conventional fluorescent microscopy because this technique detects signals in both the field of focus and all the unfocused signals. The distinctive feature of CLSM is a pinhole that permits focusing on a small focal point compared with conventional microscopy. This technology underpins one of the advantages of CLSM, namely spatial resolution via the acquisition of a series of images called the Z-stack (Figure 1). CLSM will detect signals only at the focal point in thick samples, and can thus distinguish overlapping signals that cannot be differentiated using conventional microscopy. Another advantage of CLSM is multiple track detection, which contributes to the exclusion of signal crosstalk. In single track detection, multiple lasers excite multiple fluorescent probes simultaneously and all the fluorescent signals are emitted at the same time. In this case, each signal from each of the fluorescent probes cannot be completely delineated because the spectral wavelengths of the probes overlap. In contrast, in the case of multiple track detection, the excitation lasers stimulate the sample sequentially, eliminating signal crosstalk among fluorescent signals. This technology has made a considerable contribution to stem cell research.

2.2. Three dimensional imaging and multidimensional views

The acquisition of Z-stack images using the CLSM enables reconstruction of three-dimensional (3D) images, which make it easier to sterically analyze an object [6]. Using CLSM, signals from multiple cells can be distinguished from overlapping signals within single cells. The multidimensional view afforded by CLSM also helps researchers to understand tissue organization, particularly *in vivo*.

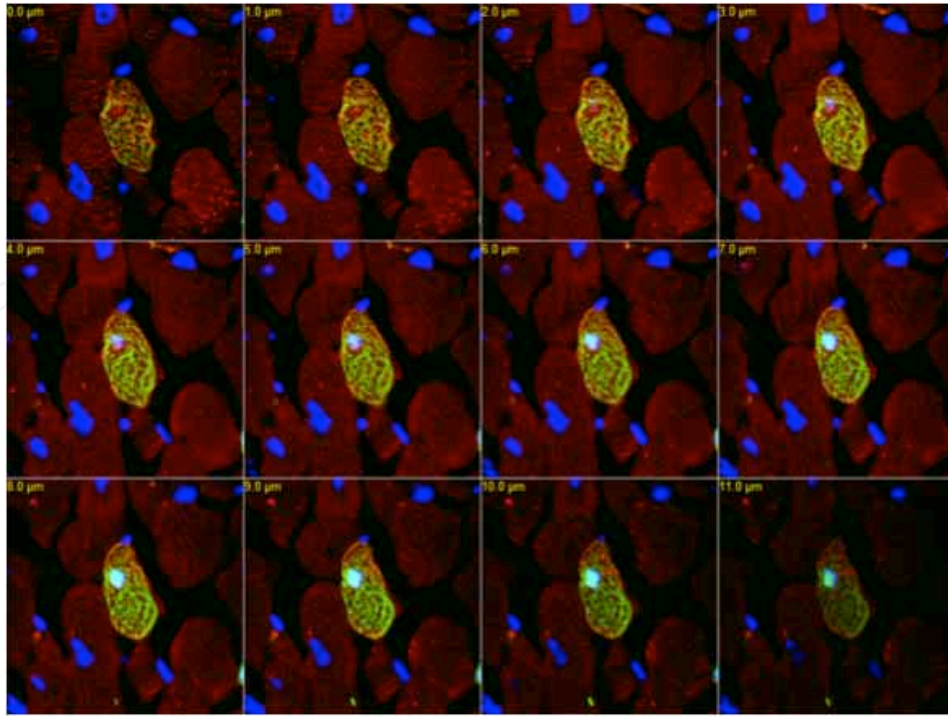


Figure 1. Sequential images of a green fluorescent protein (GFP)-positive bone marrow stem cells (BMSC)-derived cardiomyocyte were acquired with confocal laser scanning microscopy (CLSM). Red, α -actinin; blue, nuclei; green, GFP.

2.3. Region of interest scanning

By using CLSM, it is easy to focus on a region of interest (ROI). If the ROI cannot be scanned specifically, as may be the case using conventional microscopy, an all-field picture must be taken and the ROI analyzed later using imaging software. In this case, the image acquired contains too much extra information and the file that needs to be saved is very big. If the field of focus is a single cell or a part of a cell, such as the nuclear membrane or organelles, it is often difficult to obtain clear images using conventional microscopy. Focusing on the ROI also helps prevent the loss of signals in other parts of the field.

2.4. Emission fingerprinting and multifluorescence imaging

The spectral imaging (SI-) CLSM system developed by Carl Zeiss is the most innovative technology in this field. The SI-CLSM system simultaneously detects spectral curves on the fluorescence wavelength (λ). Conventional filter systems cannot distinguish closely adjacent signals, such as those of GFP (peak emission wavelength; 509 nm) and fluorescein isothiocyanate (FITC) (peak emission wavelength; 525 nm), but the SI-CLSM system uses a grating mirror and a 32-channel array detector to separate close emission spectra. In addition, the maximum number of available signals using conventional filter systems is usually four; in contrast, more than four colors are available in the SI-CLSM system. Another advantage of the SI-CLSM system is its ability to distinguish specific wavelengths of an object against non-specific background signals. The SI-CLSM system has significantly increased the reliability of data obtained in regenerative medicine [7-10].

2.5. Two-photon laser scanning microscopy and time-lapse imaging

One of the major disadvantages of CLSM is sample damage caused by the laser. Furthermore, if the signal is very weak, the laser power must be high. This can result in photobleaching, and the subsequent disappearance of signal. It is also difficult to take pictures of living cells using CLSM. These problems have been overcome by two-photon laser scanning microscopy. The infrared laser used in two-photon laser scanning microscopy causes less damage to living cells than visible light lasers, allowing time-lapse images of living cells to be acquired using LSM [11]. Time-lapse images are important for analyzing stem cell behavior *in vitro* and the differentiation process of pluripotent stem cells [12]. Furthermore, two-photon laser scanning microscopy enables the detection of signals from deeper within tissues because the infrared laser tends to reach greater depths within specimens compared with ultraviolet and visible light [13].

Overall, the development of CLSM and two-photon laser scanning microscopy has been essential for advances in cardiovascular regenerative medicine.

3. Bone marrow stem cells

3.1. Bone marrow stem cell-derived cardiomyocytes

Bone marrow stem cells (BMSCs) consist of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), and MSCs have been shown to have the potential to develop into cardiomyocytes both *in vitro* and *in vivo* [2, 8, 14].

In an early study, Makino et al. established an MSC cell line (cardiomyogenic [CMG] cells) that stably developed into cardiomyocytes [2]. The cardiomyocytes derived from this cell line exhibited the same functional properties as native cardiomyocytes [15]. In a later study, CMG cells with cardiac-specific promoter (myosin light chain-2v [MLC-2v])-derived GFP were generated. Transplantation of MLC-2v-GFP CMG cells *in vivo* demonstrated the successful delivery of MSC-derived cardiomyocytes into the murine heart [7], with CLSM clearly showing the GFP signals of the donor cardiomyocytes.

However, the origin of the bone marrow (BM)-derived cardiomyocytes (i.e., HSC or MSC) remained contentious. To investigate this issue, we generated BM transplantation models with HSCs, whole BM, and MSCs [14]. Myocardial infarction (MI) was induced in these BM-transplanted mice, and the BM cells were mobilized with granulocyte colony-stimulating factor (G-CSF). In contrast with results obtained following transplantation of whole BM, HSC-derived cardiomyocytes were very rare and, on the basis of these observations, it was concluded that BMSC-derived cardiomyocytes were of MSC origin [14].

In pressure overloaded HF models (i.e. hypoxia-induced pulmonary hypertension-induced right ventricular hypertrophy and transverse aortic constriction-induced left ventricular hypertrophy), many BMSC-derived cardiomyocytes were mobilized with ventricular pressure by both cell fusion and transdifferentiation [8]. These GFP-labeled BMSC-derived cardiomyo-

cytes are clearly visible using CLSM (Figure 2), with their signals clearly distinguished from non-specific background signals (Figure 3).

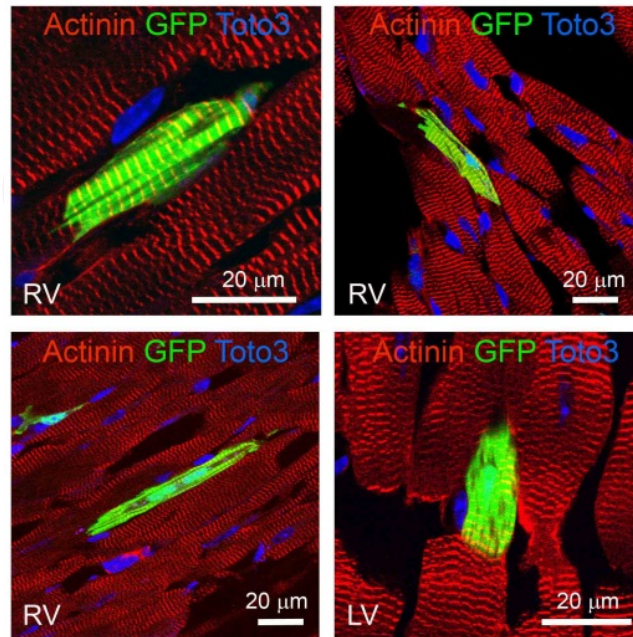


Figure 2. Mobilization of GFP-positive BMSC-derived cardiomyocytes after transplantation in the host heart. The red periodic striations represent expression of the myocyte marker, α -actinin, in cardiac muscle. RV, right ventricle; LV, left ventricle; Toto3; nuclear marker. (Reproduced with permission from Endo et al. [8].)

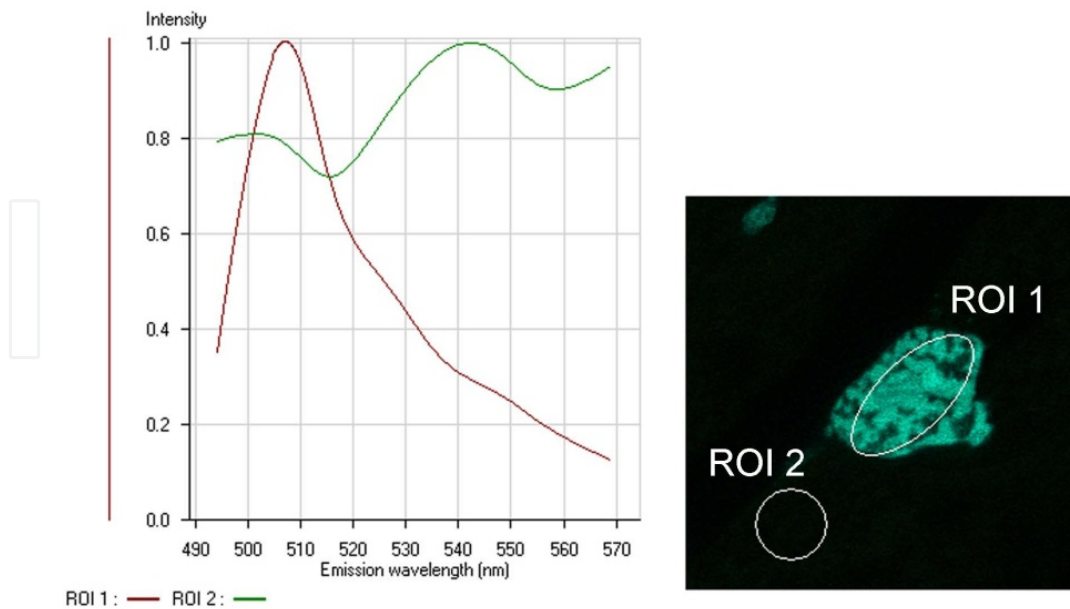


Figure 3. Emission profile of the GFP signal in BMSC-derived cardiomyocytes. The image was acquired with the Zeiss spectral imaging (SI)-CLSM system. Note that the cardiomyocyte on the right clearly shows the emission wavelength of GFP (left panel). ROI, region of interest. (Figures are modified from Endo et al. [8].)

3.2. BMSC-derived vascular cells

It has been reported that vascular progenitor cells (VPCs) can also be derived from BMSCs [16, 17]. Cytokine therapy is a useful method of mobilizing BMSC-derived VPCs to ischemic areas and inducing angiogenesis in ischemic limbs. The combination of G-CSF and hepatocyte growth factor (HGF) was shown to increase the number of BMSC-derived endothelial and smooth muscle cells, and to promote angiogenesis [18]. The induction of angiogenesis was greater following G-CSF than HGF treatment. CLSM clearly showed the colocalization of endothelial markers, such as von Willebrand Factor, CD31, and α -smooth muscle actin (Figure 4).

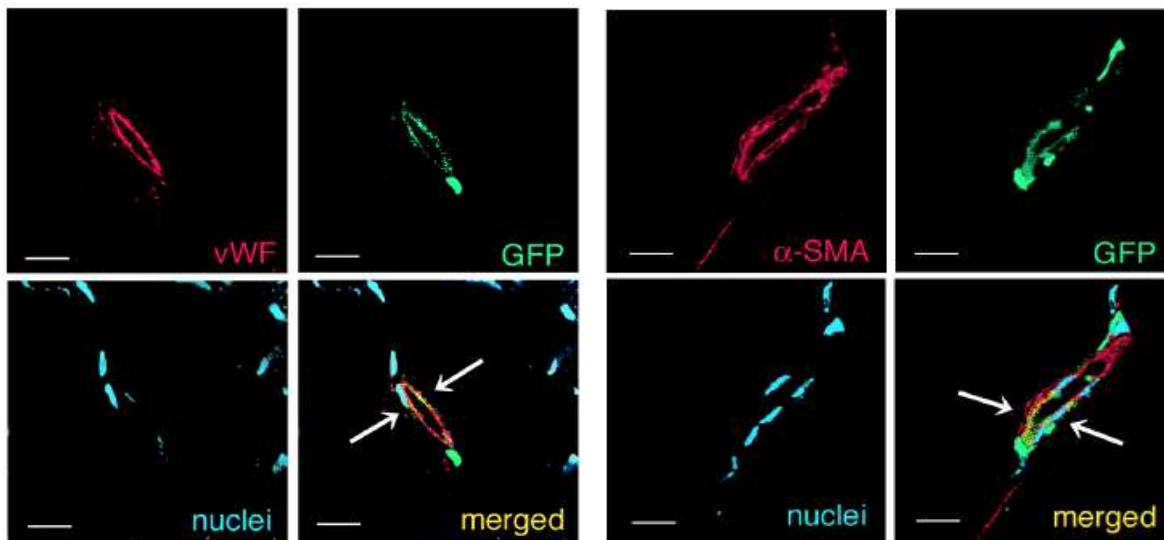


Figure 4. BMSC-derived endothelial and smooth muscle cells were recruited to the ischemic limb with cytokine therapy. vWF, von Willebrand factor. Bars, 10 μ m. (Reproduced with permission from Ieda et al. [18].)

3.3. BMSC-derived cells in pulmonary hypertension

BMSC-derived cells are involved in the vascular remodeling of pulmonary arteries and the progression of pulmonary hypertension. In one study, whole BM cells from GFP transgenic mice were transplanted into wild-type mice [9] and pulmonary hypertension was induced in the BM-transplanted mice by placing them in a hypoxic chamber. A considerable number of GFP-positive BMSC-derived cells were found to be involved in pulmonary artery remodeling [9], which was confirmed by CLSM using a grating mirror and a 32-channel array detector (Figure 5).

3.4. BMSC-derived myofibroblasts in MI

In wild-type mice transplanted with GFP-positive BM, the administration of G-CSF improved cardiac function, prevented cardiac remodeling, and improved survival after MI [14], although the presence of BMSC-derived cardiomyocytes was not enough to explain the beneficial effects of G-CSF therapy after MI. In a subsequent study, we found that G-CSF mobilized a considerable number of BMSC-derived myofibroblasts in the MI scar [10]. The BM-derived GFP-

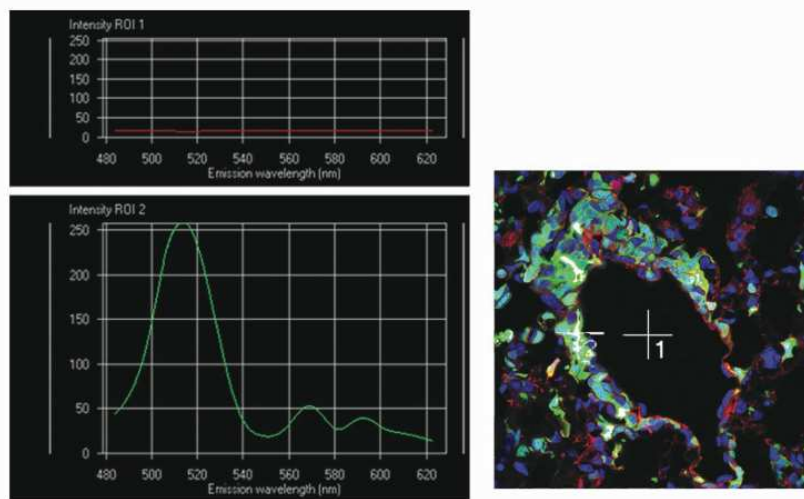


Figure 5. CLSM confirmation of the contribution of BMSC-derived cells to pulmonary artery remodeling in pulmonary hypertension. The image was acquired with the Zeiss SI-CLSM system. The GFP signal from the BMSC presented in the right micrograph (bottom left graph) was clearly distinguished with non-specific background (top left graph). (Reproduced with permission from Hayashida et al. [9].)

positive cells were co-stained with vimentin, α -smooth muscle actin, and a nuclear marker. Fluorescent signals were detected using a 32-channel array detector and, although the emission signals for Toto3 and Alexa 660 were highly overlapping, they were clearly separated using a grating mirror and a 32-channel array detector (Figure 6).

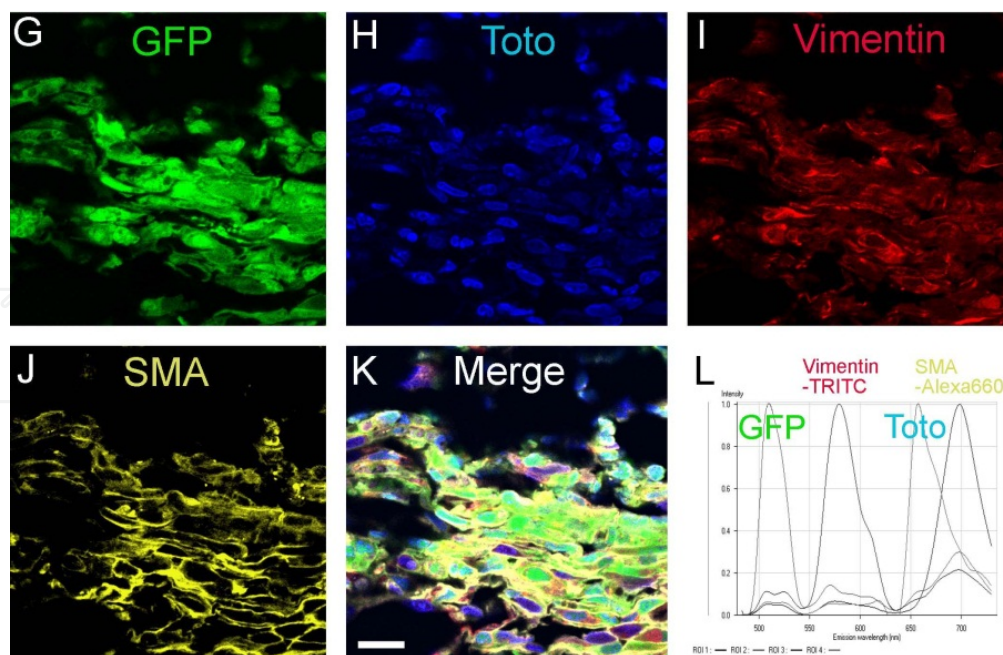


Figure 6. Migration of BMSC-derived cells into the infarcted area and their differentiation into myofibroblasts after myocardial infarction (MI). SMA, α -smooth muscle actin; GFP, green fluorescent protein; TRITC, tetramethylrhodamine-5-(and 6)-isothiocyanate. Bar, 20 μ m. (Reproduced with permission from Fujita et al. [10].)

4. Cardiac development and cardiac neural crest stem cells

Embryonic development is instructive for regenerative medicine. Specifically, heart development is a textbook for cardiomyocyte differentiation from stem cells, because effective differentiation depends on a precise process. CLSM is a powerful tool with which the localization of small groups of cells in small tissues (e.g. murine embryonic hearts) can be observed.

4.1. Cardiac neural crest-derived cardiomyocytes

During development of the mammalian heart, neural crest-derived stem cells (NCSCs) migrate to the developing heart and differentiate into several types of cells, including cardiomyocytes [4]. The number of NCSC-derived cardiomyocytes increases during postnatal growth. The NCSCs in a heart can be cultured as a cardiosphere and will develop into neurons, smooth muscle cells, and cardiomyocytes *in vitro*. They can also migrate into a heart after the induction of MI and develop into cardiomyocytes [19]. CLSM has contributed to observations of NCSC-derived cells in the heart (Figure 7).

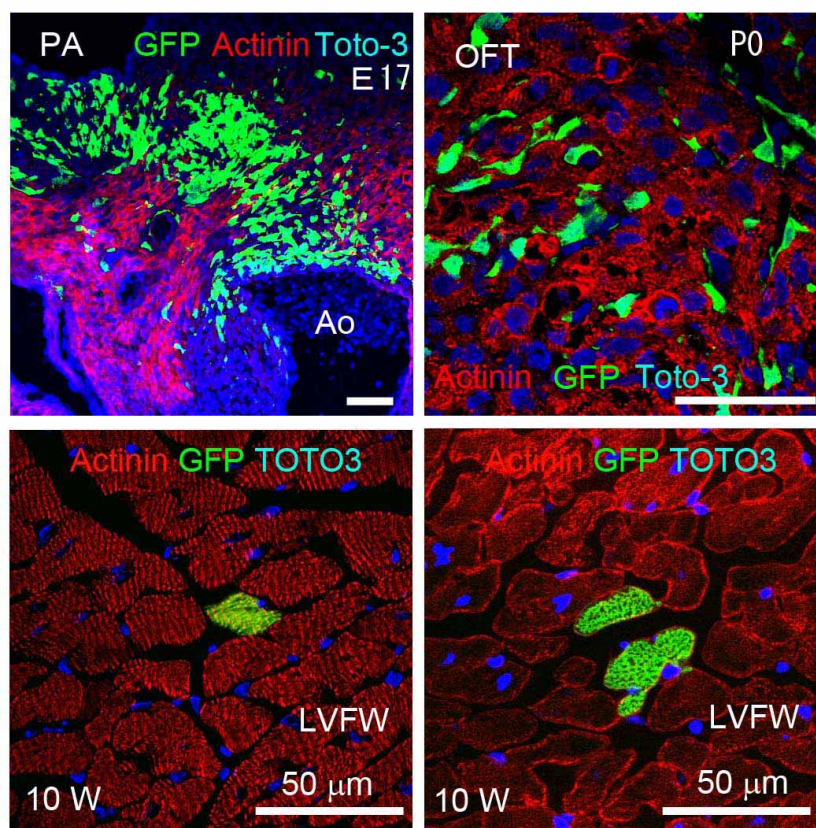


Figure 7. Neural crest stem cells migrate into the developing heart and differentiate into cardiomyocytes after MI in adult mice. PA, pulmonary artery; Ao, aorta; E17, embryonic day 17; OFT, outflow tract; P0, postnatal day 0; LVFW, left ventricular free wall; 10, 10 weeks postnatally; GFP, green fluorescent protein. Bars, 50 μm on top panels. (Reproduced with permission from Tamura et al. [19])

4.2. Heart valve development and chondromodulin-I

Heart valve formation is controlled by anti-angiogenic activity, such as that of chondromodulin-I. Downregulation of chondromodulin-I leads to neovascularization of the cardiac valves, resulting in valvular heart diseases [20]. CLSM has been used to clarify the localization of chondromodulin-expressing cells in developing embryonic hearts (Figure 8).

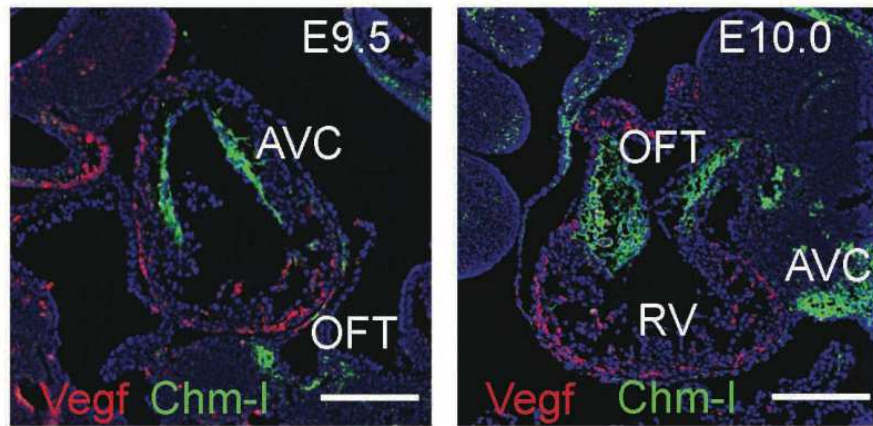


Figure 8. Expression of chondromodulin-I at the atrioventricular canal and outflow tract in the developing murine heart. AVC, atrioventricular canal; Vegf, vascular endothelial growth factor; Chm-1, chondromodulin-I; E, embryonic day; RV, right ventricle; OFT, outflow tract. Bars, 200 μ m. (Reproduced with permission from Yoshioka et al. [20].)

5. Pluripotent stem cells

In 1998, hESCs were reported as true PSCs [21]. Although the clinical application of hESCs has been hindered by ethical considerations, tumor formation, and immunological rejection, the clinical potential of hESCs as a cell source for regenerative medicine is undeniable [22]. In addition, hiPSCs were developed in 2007 following the transfection of four pluripotent factors into fibroblasts to yield PSCs with the same differentiation capacity as hESCs [23, 24]. The advantage of hiPSCs is that immunosuppressive therapy and ethical issues are not limiting factors in their clinical application (as opposed to hESCs) because hiPSCs are generated from individual patients. Both hESCs and hiPSCs have good potential to differentiate into all components of the heart, including endothelial cells, smooth muscle cells, and cardiomyocytes. Two-photon laser scanning microscopy has proved useful in observing PSCs-derived cells (Figure 9), which are expected to become a future cell source for human regenerative cardiomyocytes. Nevertheless, there are still some issues that need to be resolved before the application of cell therapy using PSC-derived cardiomyocytes. For example, teratoma formation as a result of contamination by residual PSCs is the most pressing issue, highlighting the need to purify the differentiated cardiomyocytes. To this end, GFP-labeled hESCs or iPSCs are extremely valuable in studies investigating the differentiation of undifferentiated PSCs to yield pure cardiomyocytes (Figure 10).

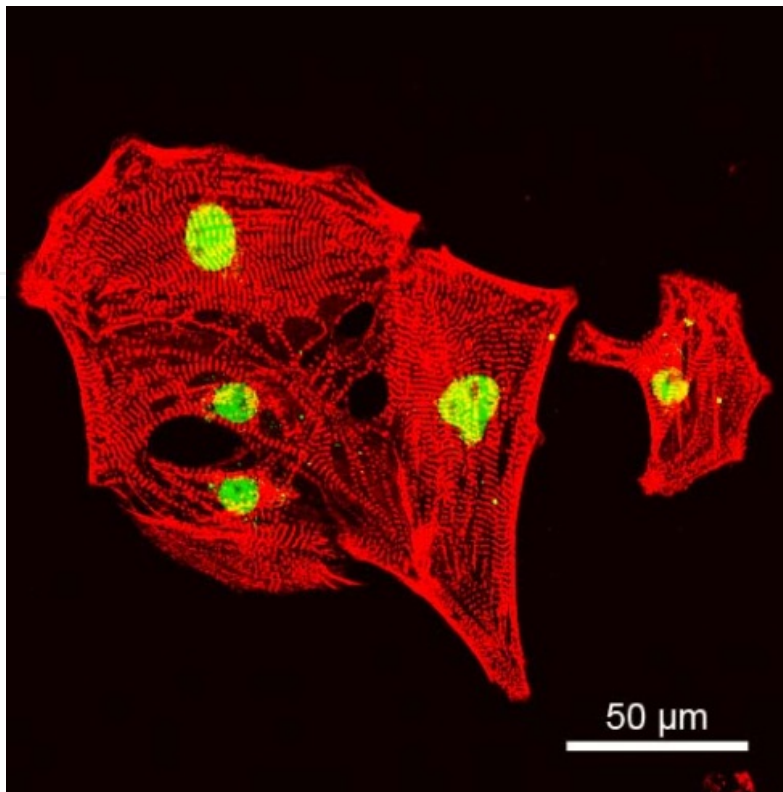


Figure 9. Human induced pluripotent stem cell (253 G4, [25])-derived cardiomyocytes photographed using two-photon CLSM. Green, Nkx2-5 (cardiac-specific transcription factor); red, α -actinin.

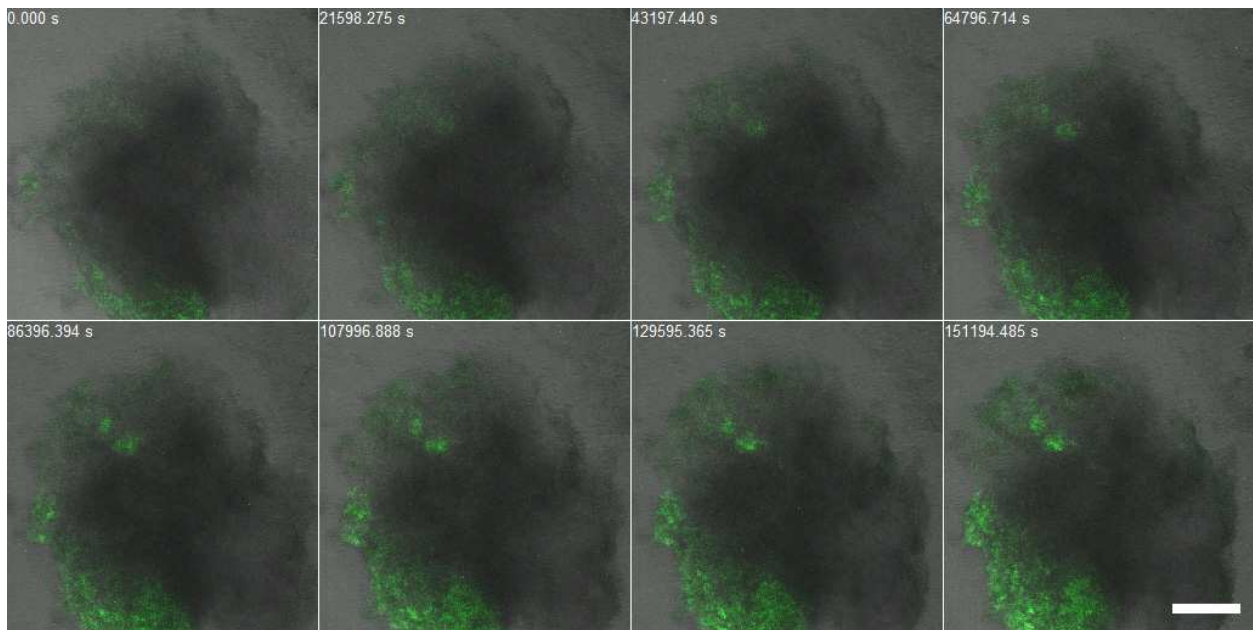


Figure 10. Time-lapse imaging of human embryonic stem cell-derived cardiomyocytes showing the formation of embryoid bodies by the H9-hTnnTZ pGZ-D2 embryonic stem cell line and increased expression of the cardiac marker troponin T-GFP in sequential time frame. Bar, 200 μ m.

6. In vivo assessment of tissue engineering of heart diseases

There are three major methods for the transplantation of regenerative cardiomyocytes: direct cell injection, the use of a biological scaffold, and the use of a cell sheet [26]. The status of transplanted cells is the most critical issue before cardiomyocyte cell therapy can be realized. For example, although direct cell transplantation has traditionally been the most common way to transplant cells, < 15% of transplanted cardiomyocytes survive due to aggregation and necrosis of the grafted cells. [7, 27]. CLSM has advanced analyses of the status of transplanted cells in vivo. Following direct cell injection, CLSM could readily distinguish transplanted cells from host tissue, and the GFP signal was confirmed using a 32-channel array detector [7].

The 3D reconstruction of the myocardium is a challenge for tissue engineering applications in the field of cardiovascular therapy. Many biomaterials are available for the construction of 3D scaffolds for regenerative therapy [28]. Although creating both aligned donor cardiomyocytes and dense myocardial tissues is difficult, cell sheet technology enables the construction of myocardial tissue with aligned cardiomyocytes. Numerous basic studies have shown that Myocardial cell sheets (MCSs) effectively restore cardiac function [26]. In MCSs, the densely aligned myocardium was clearly shown by CLSM (Figure 11). The advantages associated with high-resolution CLSM aid in the analysis of dense myocardial tissue.

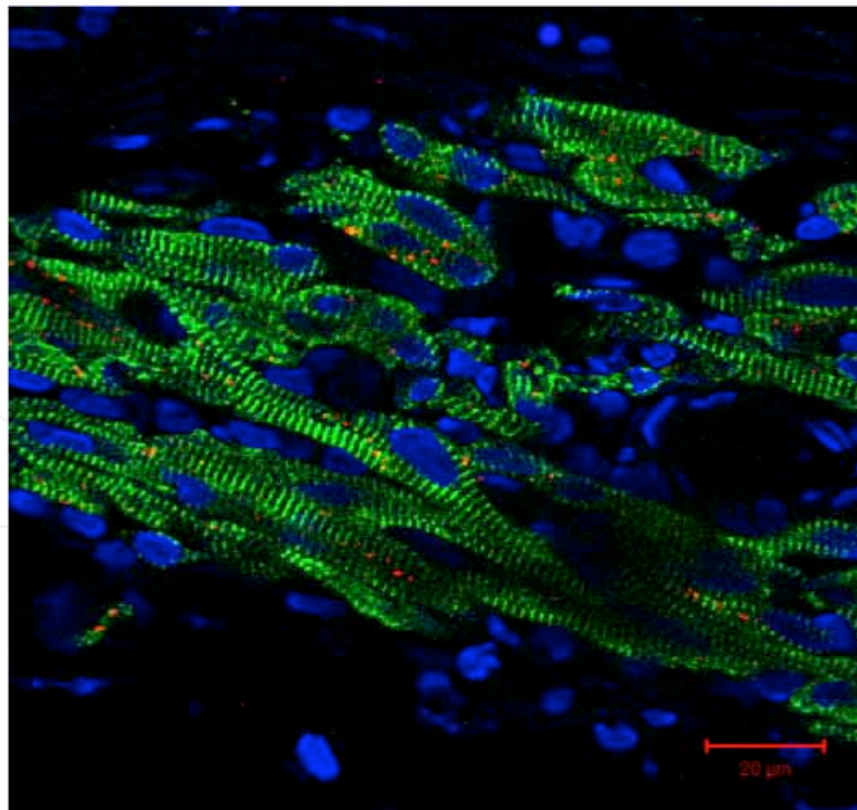


Figure 11. Graft cardiomyocytes constitute the functional myocardial cell sheets. Green, GFP; red, connexin 43; blue, nuclei. Bar, 20 μm . (Figures modified from Itabashi et al. [29, 30].)

7. Conclusion

CLSM has contributed to the advances in cardiac regenerative medicine. The precise data obtained using CLSM have been extremely valuable in confirming the potential of stem cells to differentiate into cardiomyocytes. Based on the investigations completed thus far, translational research in large, animal models and clinical studies have started. It is expected that the findings of basic research into cell therapies will be transferred successfully into clinical practice, with cell therapy ultimately becoming the standard treatment for severe HF. CLSM has thus opened up a new therapeutic field for HF with cardiac regenerative medicine.

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