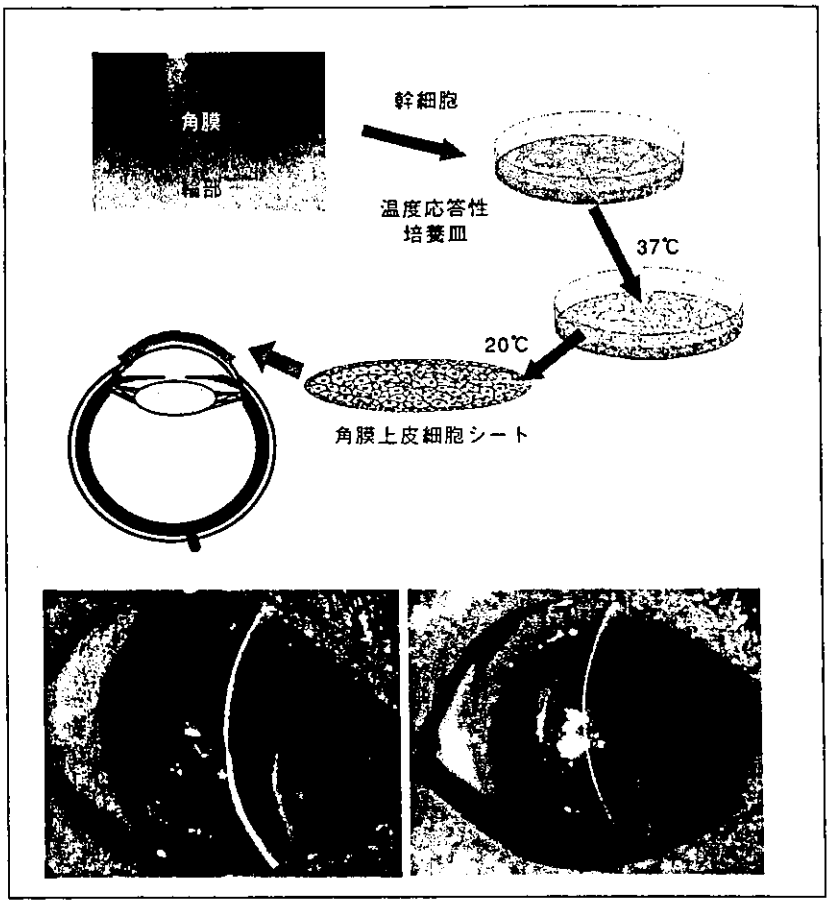

図　細胞シート工学による角膜再生
輪部に局在する角膜上皮幹細胞を単離し，温度応答性培養皿上で培養する。温度を20℃に下げ，細胞シートとして回収し，これを移植に供する。術前（下段左）ではザルツマン変性による角膜上皮幹細胞疲弊症のため重度の角膜混濁と血管の進入が認められるが，術後３ヶ月（右）で透明な角膜が再生している。
（臨床例の写真は大阪大学眼科西田幸二先生の御提供）

■ おわりに

以上概観したように，生分解性高分子製の足場を利用することなく，温度応答性培養皿から非侵襲的に回収した細胞シートを積層することで組織構造を再構築する本技術は，次世代組織工学の中核的技術として大きな期待を集めている。

文　献

1) Hirose M, Kwon OH, Yamato M, Kikuchi A, Okano T, Creation of designed shape cell sheets that are noninvasively harvested and moved onto another surface. Biomacromolecules. 1: 377-381, 2000.
2) Yamato M, Utsumi M, Kushida A, Konno C, Kikuchi A, Okano T, Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without dispase by reducing temperature. Tissue Eng. 7: 473-480, 2001.
3) Nishida K, Yamato M, Hayashida Y, Watanabe K, Maeda N, Watanabe H, Yamamoto K, Nagai S, Kikuchi A, Tano Y, Okano T, Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded *ex vivo* on a temperature-responsive cell culture surface, Transplantation. 77: 379-385, 2004.
4) Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M, Okano T, Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ Res. 90: e40, 2002.
5) Shiroyanagi S, Yamato M, Kikuchi A, Yamazaki Y, Toma H, Okano T, Urothelium regeneration using viable cultured urothelial cell sheets grafted on demucosalized gastric flaps, BJU Int. 93: 1069-1075, 2004.

Overview

血管再生医工学

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近年，欠損あるいは傷害を受けた組織・臓器に対する新たな治療法として再生医療が注目されている．すでにさまざまの組織・臓器に対する再生の研究が行われており，一部臨床応用されるに至っている．なかでも四肢末梢動脈や冠動脈の狭窄・閉塞に伴う虚血性の疾患に対する血管新生療法は国内でも数多く臨床応用されており，その有効性が示されつつある．一方，これら毛細血管レベルの再生に対し，組織工学的手法を用い3層構造を有する血管組織の再生も追究され，先天性の心血管疾患に対する臨床応用が始まっている．さらに，肝臓や心筋を含む多くの組織再生の研究においては，そのスケールアップを目的に再生組織内にいかに血管のネットワークを形成するかが課題となっており，新たな組織工学的手法の開発が期待されている．

このような背景から，血管の再生は再生医療においてきわめて重要なものとなっているが，実際，人体において血管は酸素・栄養・代謝物など生命維持に必要な物質の輸送経路として人体に欠かせない組織である．その全長は地球の2周半，約10万kmにも及び，すべての臓器において血管のネットワークが形成されている．直径約3cmの大動脈に始まり，末梢組織では数μmの毛細血管となる．構造的には，単層の内皮細胞からなる内膜—多層の平滑筋細胞と，弾性線維からなる中膜—線維芽細胞と，弾性・膠原線維からなる外膜という3層を有する血管から，分岐を繰り返して1個の内皮細胞とそれを覆う周皮細胞のみからなる毛細血管へと至る．また，末梢組織へ向かう動脈と心臓へ戻る静脈とでは，壁厚や弾性線維などの構造に相違が認められる．これは，血管内を流れる血流のメカニクスに起因すると考えられる．機能的には動静脈が心臓から拍出される血液を末梢組織まで循環するための通路として血圧など血行動態の維持に寄与し，毛細血管はさまざまの物質交換が行われる場として組織代謝の維持に貢献している．

以上のような特徴を有する血管の再生において，まず重要となるのは血管の細胞ソースである．これに関しては，近年の幹細胞生物学の目覚ましい発展が大きく貢献している．細胞ソースとして骨髄単核球細胞や血管内皮前駆細胞はすでにその採取法が確立し，虚血

Overview

性疾患に対する血管新生療法や再生血管移植に臨床応用されている．また，ES細胞からは血管内皮細胞のみならず平滑筋細胞の分化誘導も実現しており，将来的な利用の可能性も示されている．次に，これらの細胞が血管を再構築するにあたっては種々の増殖因子が必須である．生体内においては移植した細胞そのものあるいは周囲の組織からもこれらの増殖因子が分泌されるが，さらに外部から増殖因子やその遺伝子を投与することにより血管新生を促進できるものと考えられる．近年のドラッグデリバリーシステム(DDS)の発展により，これらの蛋白や遺伝子投与の有効性も向上しており，細胞との組み合わせによりさらに血管新生を加速することも可能であろう．一方，口径の大きな血管の再生に関しては，細胞の足場として，細胞外マトリックスの代わりに生体吸収性の生体材料を用いる組織工学的手法が必須となっている．より細胞接着の良い材料，生体血管と同様のコンプライアンスを持つ材料が追究されている．組織工学の研究では，血管のバイオメカニクスを考慮した設計の重要性が示され，またバイオリアクターを用いた拍動下での血管組織の環流培養により2,000 mmHgに耐える血管組織も再生されており，工学的アプローチの重要性が増している．

前述したように，血管組織はそれぞれの部位において異なる構造と機能を有するため，これまでの血管再生研究は毛細血管～細血管を対象とした場合と，中～大血管を対象にした場合とで異なるアプローチで異なる研究者が行ってきたが，再生医療という学際的な研究領域において，それぞれの知識や技術に接点も生まれ共同研究も増えつつある．また，今後は細～中血管の再生や毛細血管網を伴った組織・臓器の再生が新たな再生医療のターゲットとなりつつあり，将来的には統合的に毛細血管網から太い血管まで伴った組織・臓器の再生が必要になってくると予測される．そういった観点からも血管の再生に関わる研究者が相互理解を含め，連携・融合していくことが肝要である．

本特集では上記の内容をふまえ，血管再生の細胞ソース，増殖因子とデリバリー法，組織工学的な血管新生，太い血管の再生，また再生血管のバイオニクスといった内容で各専門家による研究・臨床の最先端の知見が紹介されている．本特集が読者の血管再生医療に関する現状と全体像の把握の一助となるとともに，本研究フィールドにおける連携・融合への端緒になれば幸いである．

Stem Cell Transplantation as a Mode of Regenerative Medicine

JMAJ 46(8): 333–338, 2003

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Abstract: Advances in stem cell biology have made it possible for organ regeneration to become a reality, and this new technique is poised to enter the field of clinical medicine. The stem cells used in regenerative medicine are classified as embryonic or adult. Neurons, vascular endothelial cells, skeletal muscle cells, cardiomyocytes, osteoblasts, and chondroblasts have already been obtained from stem cells in the laboratory setting. Embryonic stem cells are amenable to mass culture and have versatile pluripotency but tend to be associated with problems in clinical application, including tumorigenesis, immunological rejection, and ethical issues. Since adult stem cells are obtained from the bone marrow of the patient, problems related to donors, ethics, rejection, and tumorigenesis do not apply. However, techniques for the isolation and *in vitro* amplification of adult stem cells have yet to be established, raising issues that await future solutions. For stem cells to be used in the clinical setting, regeneration at the tissue level is necessary, requiring the combined resources of tissue engineering and material science. Regenerative medicine is expected to play a leading role in 21st century medicine. However, the integration of studies from various scientific fields seems necessary for success in this area.

Key words: Embryonic stem cells; Adult stem cells; Regenerative medicine; Cell transplantation; Cardiomyocytes

Introduction

This paper outlines the current status and future prospects of regenerative medicine, particularly with regard to the use of stem cells. This new field of medicine has attracted a great deal of attention and is at the cutting edge of 21st century medicine. It is well-known that when a limb or tail of a newt or lizard is cut off, the missing part is regenerated from the stump. This occurs because cells at the cut edge can dedifferentiate into immature, pluripotent stem

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The Japanese text is a transcript of a lecture originally aired on December 13, 2001, by the Nihon Shortwave Broadcasting Co., Ltd., in its regular program "Special Course in Medicine".

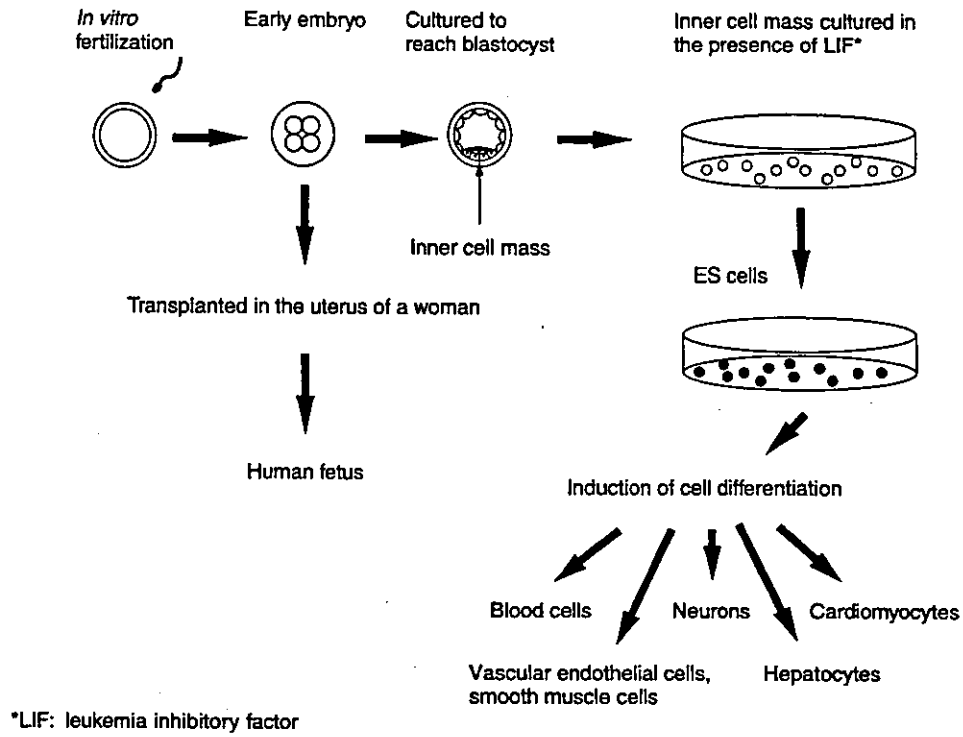

Fig. 1 Establishment of ES cells and their application to regenerative medicine

cells, and then differentiate again into the target cells after cell division and proliferation. Although this regenerative phenomenon does not occur in mammals, it does not necessarily mean that humans and other mammals lack regenerative capacity. Human somatic cells also include pluripotent stem cells, which are capable of proliferating and differentiating to repair tissue when an impairment or defect has occurred that leads to dysfunction of the organ. The aim of regenerative medicine is to treat disease and injury by making use of this capability.

Heart transplantation, which has been the main treatment option for severe heart failure, provides an example. Although heart transplantation is an excellent treatment, it is not widely employed because of the need for a donor and the possibility of rejection after transplantation. In contrast, regenerative medicine uses stem cells to induce the formation of cardiomyocytes, which are then transplanted to the impaired heart of the patient.

Stem cells used in regenerative medicine

Stem cells that can be used for regenerative medical therapies are broadly divided into two groups: embryonic stem cells (ES cells) obtained from early-stage embryos and adult stem cells that still are present in the adult body. These two types of stem cells have their own particular advantages and disadvantages. Whether one type is superior to the other remains controversial, depending on the type of tissue to which they are to be transplanted. A method of culture has already been established for ES cells, and their particular advantage is that they are capable of differentiating into any type of cell within the body.

Current status and problems of regenerative medicine using ES cells

At present, the regeneration of neurons,

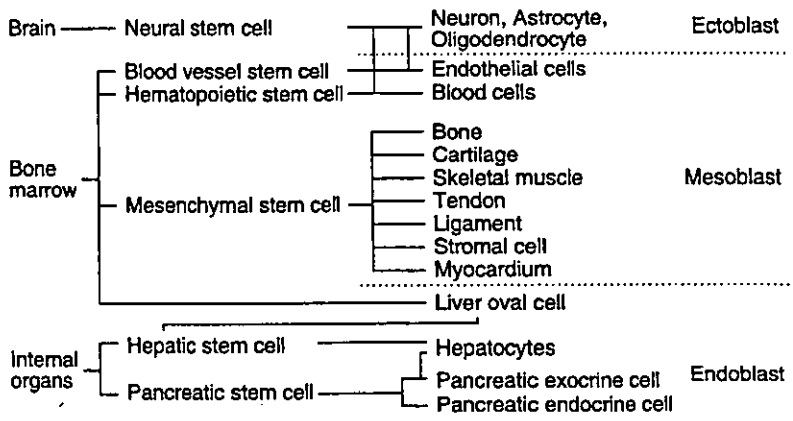

Fig. 2 Classification of stem cells in adults

blood cells, vascular endothelial cells, cardiomyocytes, pigment cells, osteoblasts, and islet cells is feasible or trying to be developed (Fig. 1). In theory, ES cells can differentiate into any type of cell in the body. However, in the actual setting, cells that occur in the early stage of development are fairly easy to obtain, whereas those that have undergone advanced differentiation in the late prenatal or postnatal period are more difficult to obtain. In addition, among neurons, large cells such as spinal cord ventral horn cells are considered easier to obtain from ES cells than from adult stem cells.

Although ES cells represent a very attractive tool from the viewpoint of tissue regeneration, they are associated with a number of drawbacks. First, because the ES cells used in allogeneic transplantation are obtained from others, rejection reactions cannot be avoided. Immunosuppressive therapy is required to manage the rejection, but such therapy may cause the quality of the patient's life to deteriorate and lead to high, ongoing medical expenditures. Second, if undifferentiated cells are present among the transplanted cells, it is possible that a malignant tumor, teratoma, may form in the tissue undergoing transplantation. In general, rejection reactions are more likely to occur in organs where blood flow is abundant, leading to a higher possibility of cellular infiltration. On the other hand, from clinical experience with the transplantation of fetal midbrain obtained through artificial termination of pregnancy into the nigrostriatum in patients with Parkinson's disease, it has been found that about one month of immunosuppressive therapy is sufficient for cases of transplantation of allogeneic nerve cells into the brain, because lymphocytes cannot cross the blood-brain barrier. Thus, ES cells are presumed to be superior for the regeneration of nerve cells in the central nervous system.

Application of adult stem cells to regenerative medicine

Let us now turn to the other type of stem cells, adult stem cells. To begin with, stem cells are known to be characterized by their capacity for self-replication, proliferative potency, and pluripotency.

Stem cells are ranked from high to low in terms of the diversity of their ability to differentiate. For example, ES cells, which can differentiate into any type of cell, are given the highest rank, whereas hematopoietic stem cells are ranked in the middle, and cutaneous stem cells are considered low-ranking stem cells or precursor cells. Somatic tissues are formed from endoblasts, mesoblasts, or ectoblasts in the

fetal stage (Fig. 2). Skin and nerve tissues are derived from ectoblasts, and the stem cells of these tissues are therefore present in local areas. More specifically, cutaneous stem cells are present in the granular layer of the dermis, and neural stem cells are present around the cerebral ventricle of the cerebral hippocampus, where they are responsible for the regeneration of the respective tissues. Visceral organs such as the liver and pancreas are derived from endoblasts, and the stem cells of these tissues are present in the respective organs. If the liver is excised, oval cells or small hepatocytes in the remaining liver proliferate to regenerate the liver.

Various other cells of the body, such as bone, cartilage, fat, ligament, tendon, skeletal muscle, myocardium, and smooth muscle, are derived from mesoblasts. Among the stem cells of these tissues, some exist in muscle, for example, satellite cells, which are low-ranking stem cells found in the skeletal muscle. However, recent studies have revealed that the stem cells of these tissues are present in the bone marrow. As is well known, bone marrow consists mainly of hematopoietic stem cells and other cells of the blood cell series, but cells which are not blood cells are also present in bone marrow. Called bone marrow stromal cells, these cells are known to secrete various cell growth factors and cytokines that control the proliferation and differentiation of the blood cell series.

In recent years, it has become apparent that mesenchymal stem cells in the bone marrow with pluripotent capacity are present among marrow stromal cells. It had been reported by the early 1990s that mesenchymal stem cells differentiate into osteoblasts, chondroblasts, and adipocytes, and these cells began to be referred to as mesenchymal in the sense of mesoblast-derived stem cells. Since mesenchymal stem cells are stem cells for mesoblast-derived cells, we wondered whether they could differentiate to become cardiomyocytes, and we carried out studies along this line. We demonstrated that cardiomyocytes that beat regularly by themselves could be obtained from mesenchymal stem cells. It has also been reported that mesenchymal stem cells can differentiate into mesoblast-derived tissues such as tendon and ligament.

At this point, it is important to determine to what extent these stem cells in bone marrow are pluripotent and how far they can differentiate. Results were reported in the U.S. last year of an autopsy case of leukemia in a female patient who died after the transplantation of bone marrow from a male donor. In this patient, cells possessing the Y chromosome derived from the male donor were found in liver, skeletal muscle, and the intestinal tract. Thus, it became apparent that adult stem cells can differentiate not only into mesoblast- but also into endoblast-derived organs. A more recent study demonstrated that mesenchymal stem cells can differentiate into ectoblast-derived nerve cells. Therefore, the expression "mesenchymal" is no longer accurate, and these cells have been called adult stem cells because they can differentiate to become tissues derived from any germ layer.

Current status of regenerative medicine using adult stem cells

What procedures, then, are necessary for enticing these stem cells to differentiate into the target cells? The procedures naturally vary according to the cells that are desired. For instance, differentiation into osteoblasts that produce bone can be induced by adding dexamethasone, ascorbic acid, and β-glycerophosphite to the culture medium. To obtain differentiation into chondroblasts, the presence of insulin, transferrin, proline, and sodium pyruvate is required. Thus, selective differentiation can be induced by using known growth factors, biological substances, or even chemical substances in some cases. For those cells for which an established procedure does not currently exist, the use of differentiation inducers is now an option.

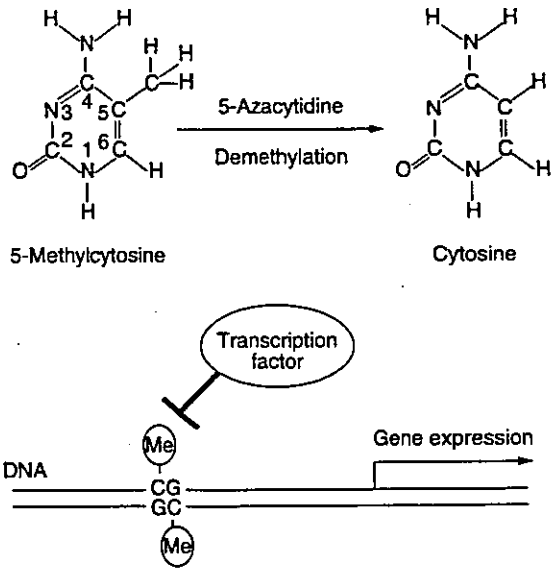

Fig. 3 Demethylation of DNA and differentiation of stem cells

When cytosine radicals on DNA are methylated, transcription factors cannot be bound to them, and, therefore, gene expression is inhibited. If the methylated cytosine radicals are demethylated by 5-azacytidine, various genes are activated, leading to differentiation of stem cells.

Me: Methyl radical

Although various differentiation-inducing agents are now available, we prefer a demethylating agent of DNA, 5-azacytidine. Details of its mechanisms of action, however, will not be discussed here. With this agent, it is possible to induce stem cells to differentiate into various directions through the random activation of transcription factors. From among the cells that have differentiated along various lines, those suitable for the particular purpose should be selected and utilized for the regenerative medicine procedure (Fig. 3).

Another method is to infuse stem cells directly into the target organ or tissue, to cause tissue regeneration. In each tissue, paracrine cytokines and growth factors are secreted from the surrounding cells, and tissue-specific cell adhesion factors and extracellular matrix are also expressed. Differentiation is induced by placing stem cells in such situations. Such a local environment is called a "niche". It is expected that the niche will induce the infused stem cells to differentiate in the same direction as the surrounding cells.

Adult stem cells are advantageous in that they are present within the bone marrow and their collection causes no organ loss to the donor. Further, the already well-developed bone marrow bank system theoretically makes possible HLA-compatible transplantation. Another advantage of adult stem cells is that the ethical issues surrounding them are far fewer than those related to ES cells. In addition, if the bone marrow of the same patient is used, there is no posttransplantation rejection or any need for immunosuppressive drug therapy, providing another great advantage.

Problems associated with adult stem cells

One problem associated with adult stem cells is that they are present in small numbers in the body, occurring at a rate of one in several hundred thousand marrow cells. The success of regenerative medicine using adult stem cells depends on whether they can be collected efficiently and proliferated under *in vitro* conditions while maintaining their pluripotent capacity.

Second, the problem of how to induce stem cells to differentiate into target cells is an important issue. Aside from particular cells for which the method of differentiation of stem cells is already known, further close investigation is necessary to establish methods of differentiation into various other cells present in the body. Another area of further investigation is to determine to what extent it is possible to induce differentiation in the niche by direct transplantation of stem cells in the target organ. Although the use of the niche is feasible for some organs such as myocardium and skeletal muscle, it is extremely difficult at present in the case of complexes of multiple types of cells, such as those comprising liver, lung, and kidney.

Use of tissue engineering and material science

Finally, it should be stressed that regenerative medicine has a very close relationship with tissue engineering and material science. Even if target cells can be obtained by using stem cells, they will need to possess a form consistent with their purpose when transplanted into the patient's body. For example, even if vascular smooth muscle cells are regenerated, blood vessels are not necessarily formed. For the cells to take the form of a blood vessel, a scaffold should be created from high-molecular-weight compounds that would dissolve slowly in the body. The cells should be placed on the scaffold and incubated to achieve the form of a blood vessel. The development of good materials for such scaffolding is another important aspect of the regenerative medicine.

Conclusion

Regenerative medicine has become an important focus of the medical profession in the 21st century. However, the success of this type of medical care will require the cooperation of various fields of science, including molecular biology, developmental biology, embryology, anatomy, tissue engineering, and material science. People have high expectations of regenerative medicine. It is therefore important that basic research and translational research that applies the results of basic research continue to make progress.

REFERENCES

1) Makino, S., Fukuda, K., Miyoshi, S. *et al.*: Cardiomyocytes can be generated from marrow stromal cells *in vitro*. *J Clin Invest* 1999; 103: 697–705.
2) Hakuno, D., Fukuda, K., Makino, S. *et al.*: Bone marrow-derived cardiomyocytes (CMG cell) expressed functionally active adrenergic and muscarinic receptors. *Circulation* 2002; 105: 380–386.

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Review

Application of mesenchymal stem cells for the regeneration of cardiomyocyte and its use for cell transplantation therapy

Keiichi FUKUDA

<Abstract> We have isolated a cardiomyogenic cell line (CMG cell) from murine bone marrow mesenchymal stem cells. The cells showed a fibroblast-like morphology, but the morphology changed after 5-azacytidine exposure. They began spontaneous beating after 2 weeks, and expressed ANP and BNP. Electron microscopy revealed a cardiomyocyte-like ultrastructure. These cells had several types of action potentials; sinus node-like and ventricular cell-like action potentials. The isoform of contractile protein genes indicated that their muscle phenotype was similar to fetal ventricular cardiomyocytes. They expressed α_{1A}, α_{1B}, α_{1D}, β_1, and β_2 adrenergic and M_1 and M_2 muscarinic receptors. Stimulation with phenylephrine, isoproterenol and carbachol increased ERK phosphorylation and second messengers. Isoproterenol increased the beating rate, which was blocked with CGP20712A (β_1-selective blocker). These findings indicated that cell transplantation therapy for the patients with heart failure might possibly be achieved using the regenerated cardiomyocytes from autologous bone marrow cells in the near future.

Key words : bone marrow stroma, mesenchymal stem cell, cardiomyocyte, differentiation, regenerative medicine, adrenergic receptor, muscarinic receptor

[HUMAN CELL 16(3) : 83 - 94, 2003]

Introduction

Heart transplantation is the ultimate therapy for the treatment of severe heart failure. However, it has not been widely examined, because it requires donor hearts. The inadequate supply of donor hearts is often a major problem everywhere in the world. As a result, the current challenge in cardiology is how to reserve pump failure by cell transplantation or regenerative medicine. Recent studies have shown that transplanted fetal cardiomyocytes can survive in heart scar tissue and that the transplanted cells limit scar expansion and prevent post-infarction heart failure. Transplantation of cultured cardiomyocytes into damaged myocardium has been proposed as a future method of treating heart failure[1),2)]. This revolutionary concept remains unfeasible in clinical settings because of the difficulty of obtaining donor fetal hearts. Thus, a cardiomyogenic cell line has long been awaited, and such a line might be capable of substituting for fetal cardiomyocytes in this therapy.

Various studies have demonstrated that cardiomyocytes can differentiate from multipotent stem cells such as embryonic stem (ES) cells[3)] and embryonic carcinoma (EC) cells[4)]. ES cells are an attractive cell source in regenerative medicine, but the recipients must take immunodepressant drugs

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throughout their lives because the transplanted ES cells are allogeneic. Use of these reagents impairs the quality of life of the recipients, and transplantation of undifferentiated ES cells often causes teratocarcinoma. In addition, the establishment of human ES cells involves ethical problems and is not allowed in every country. Because of these circumstances, the regeneration of cardiomyocytes from adult autologous stem cells has been awaited.

Recent reports have demonstrated the existence of pluripotent stem cells in adult tissues. Roy et al. reported the existence of neural stem cells in the brain that can differentiate into neurons, oligodendrocytes, and astrocytes *in vitro*[5]. Marrow stromal cells have been shown to possess many characteristics of mesenchymal stem cells[6], and pluripotent progenitor marrow stromal cells can differentiate into various types of cell types, including osteoblasts[7,8], myocytes[9], adipocytes, tenocytes, and chondroblasts[10]. We recently reported the differentiation of mesenchymal stem cells into cardiomyocytes after exposure to 5-azacytidine and the establishment of cell line CMG (CardioMyoGenic) that differentiates into cardiomyocytes *in vitro*[11]. CMG cells exhibit spontaneous beating and express atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), and they may provide a useful and powerful tool for cardiomyocyte transplantation after further characterization of their cardiomyocyte phenotype.

This paper describes the characteristics of bone marrow-derived regenerated cardiomyocytes and discusses the possibility of using them for cardiovascular tissue engineering. The expression and function of adrenergic and muscarinic receptors in CMG cells is also described, because these receptors play a critical role in modulating cardiac function[12].

Mesenchymal marrow stem cells as a possible source of cardiomyocytes: the cardiomyogenic (CMG) cell?

Fig. 1 shows the classification of the stem cell system of adults[13]. Bone marrow stromal cells were previously used as a feeder layer to culture hematopoietic stem cells, and are known to be of mesodermal origin and produce various cytokines and growth factors. In late 1990's, a number of papers reported that bone marrow stromal cells contain multipotent stem cells for non-hematopoietic tissues,

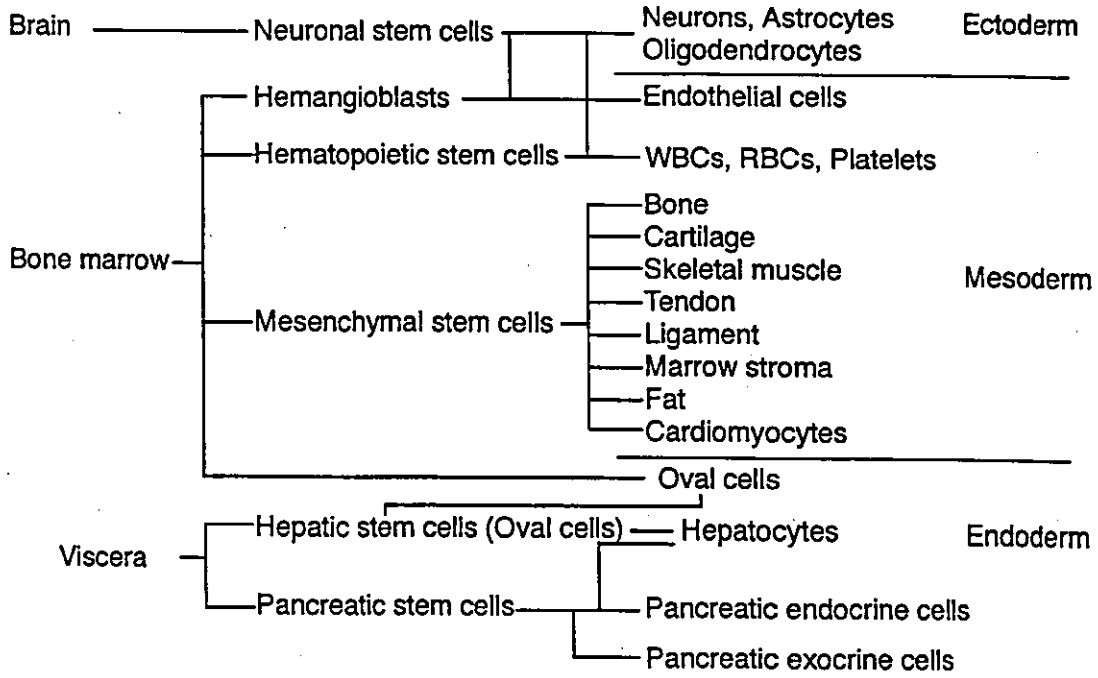

Fig. 1: Classification of pluripotent stem cells in adult tissues
Bone marrow contains various kinds of stem cells. Mesenchymal stem cells may differentiate into various mesoderm-derived cells, such as osteoblasts, chondroblasts, adipocytes, skeletal muscle cells and cardiomyocytes.

called "marrow mesenchymal stem cells", that could differentiate into osteoblasts, chondroblasts, and adipocytes. All of these cells were known to be of mesodermal origin. If mesenchymal stem cells are multipotent, we hypothesized that they might have the ability to differentiate into cardiomyocytes and instituted this study. We also thought that bone marrow cells could be obtained from patients themselves and that autologous cells would not be rejected after cell transplantation.

Method of establishing bone-marrow derived cardiomyocytes

Female C3H/He mice were anesthetized with ether, their femora were excised, and primary culture of the marrow cells was performed according to Dexter's method. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum and penicillin (100 μ g/ml)/streptomycin (250 ng/ml)/ amphotericin B at 33°C in humid air containing 5% CO_2. After a series of passages, immortalized cells were obtained by frequent subculture for more than 4 months. Cell lines from different dishes were subcloned by limiting dilution. To induce cell differentiation, cells were treated with 3 μ mol/L of 5-azacytidine for 24 hours. Subclones that included spontaneously beating cells were screened by microscopic observation (first screening), and cells surrounding spontaneous beating cells were subcloned with cloning syringes. Subcloned cells were maintained, exposed to 5-azacytidine again for 24 hours, and clones that showed spontaneous beating most frequently were screened (second screening). The clonal cell line thus obtained was named the CMG cell.

As a result of repeated rounds of limiting dilution, we succeeded in isolating 192 single clones, several of which differentiated into cardiomyocytes and showed spontaneous beating. The experiments were reproducible, but the percentage of cells that differentiated into cardiomyocyte differentiation was specific to each clones. Phase-contrast photography and/or immunostaining with anti-sarcomeric myosin antibodies were used to identify the morphological changes in the CMG cells. CMG cells showed a fibroblast-like morphology before 5-azacytidine treatment (0 week), and this phenotype was retained through repeated subculturing under non-stimulating conditions. After 5-azacytidine treatment, however, the morphology of the cells gradually changed (Fig. 2). Approximately 10-30% of the CMG cells gradually

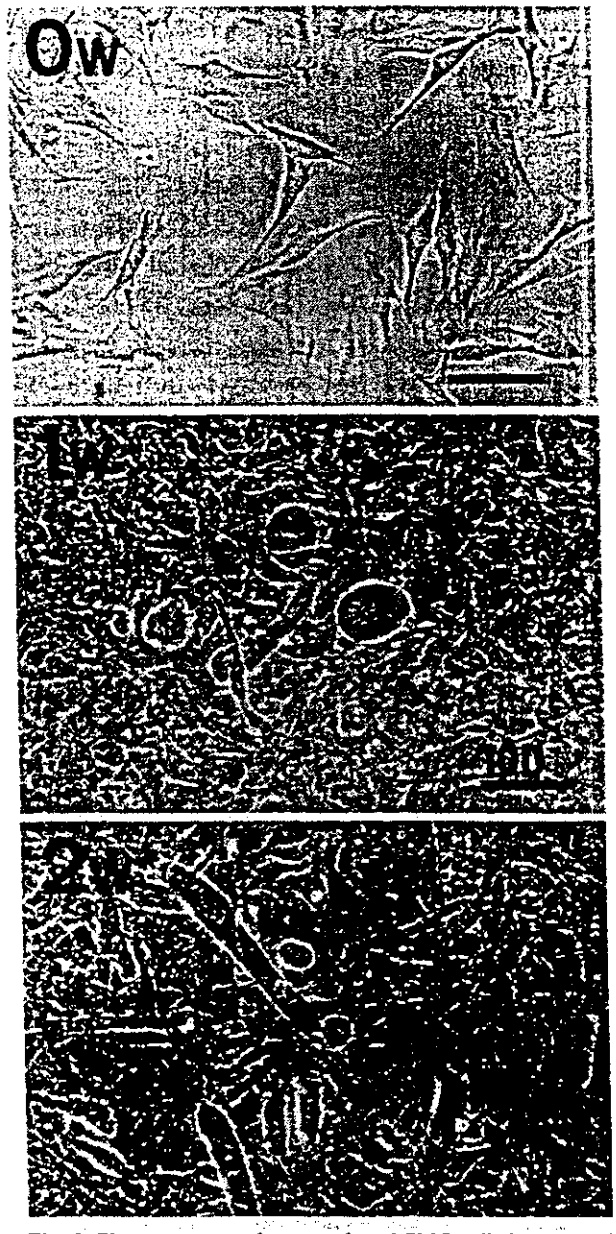

Fig. 2: Phase-contrast photographs of CMG cells before and after 5-azacytidine treatment

(Upper panel) CMG cells have a fibroblast-like morphology before 5-azacytidine exposure (0 week). (Middle panel) One week after treatment, some cells gradually increased in size, and developed a ball-like or stick-like appearance. (Lower panel) Two weeks after exposure, the ball-like or stick-like cells began spontaneous beating. Bars indicated 100 μm.

increased in size at 1 week, and they formed ball-like appearance, or had lengthened in one direction to exhibit a stick-like morphology. Most of the other non-myocytes had an adipocyte-like appearance.

Regenerated cardiomyocytes display a fetal ventricular phenotype

Various cardiac contractile protein isoforms are differentially expressed in cardiomyocytes at different developmental stages and in different chambers. At around the time of birth there is a developmental switch in the ventricular muscle of small mammals from expression of β-myosin heavy chain (MHC), which is the predominant fetal form, to expression of α-MHC. There is also a developmental switch from expression of α-skeletal actin, which is the predominant fetal and neonatal form, to that of α-cardiac actin, the predominant adult form. We investigated the contractile protein isoforms of bone marrow-derived CMG cells to characterize their phenotype as cardiomyocytes. Table 1 summarizes the results. Fetal, neonatal, and adult ventricle and atrium were used as controls[13]. Expression of both α- and β-MHC was detected in differentiated CMG cells by RT-PCR, but β-MHC expression was overwhelmingly greater than that of α-MHC. CMG cells expressed both α-cardiac and α-skeletal actin, but the α-skeletal actin gene was expressed at markedly higher levels than the α-cardiac actin gene. Interestingly, CMG cells expressed the myosin light chain (MLC)-2v gene, but not the MLC-2a gene. MLC-2v is specifically expressed in ventricular cells, while MLC-2a is specifically expressed in atrial cells. Skeletal muscle cells do not express either α-MHC or MLC-2v. These results indicated that differentiated CMG cells possess the specific phenotype of the fetal ventricular cardiomyocytes[11].

Developmental stage of undifferentiated and differentiated CMG cells

Various cardiac specific transcription factors have been cloned, and their genes are serially expressed in the developing heart during myogenesis and morphogenesis. Fig. 3 shows the time course of the expression of cardiomyocyte-specific transcription factors in fetal developing heart and CMG cells. The genes coding Nkx2.5[14] (homeobox type transcription factor specifically expressed beginning in the early developing heart), GATA4[15] (GATA-motif-binding Zinc finger type transcription factor expressed beginning in the early stage developing heart), HAND1/2 (basic HLH type transcription factor expressed in the heart and autonomic nervous system), and MEF2-B/C[16] (muscle enhancement factor: a MADS box family transcription factor expressed in the myocytes) were

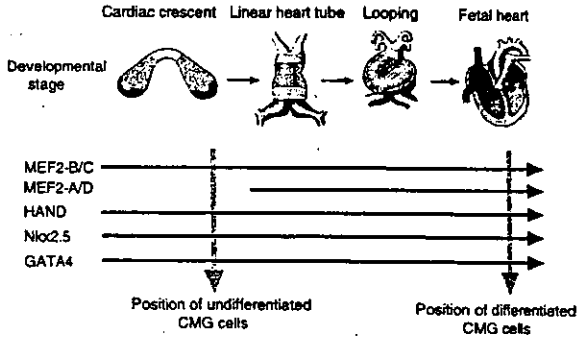

Fig. 3: Expression of cardiac-specific transcription factors in the developing heart and in CMG cells

The horizontal arrows indicate the time course of the expression of cardiac-specific transcription factors in the developing fetal heart. The dotted vertical arrows indicate the expression of these factors in undifferentiated and differentiated CMG cells. CMG cells expressed MEF2-A and MEF2-D after 5-azacytidine exposure, when they acquired a cardiomyocyte phenotype.

Table 1. Isoforms of the contractile proteins in differentiated CMG cells

Developmental stage	Atrium		Ventricle			CMG
	Fetus	Adult	Fetus	Neonate	Adult	
α-actin	skeletal	cardiac	skeletal>cardiac	skeletal	cardiac	skeletal>cardiac
myosin heavy chain	$\alpha > \beta$	α	$\beta > \alpha$	$\alpha > \beta$	α	$\beta > \alpha$
myosin light chain	2a	2a	2v	2v	2v	2v

expressed in the early stage of heart development, and MEF2A and MEF2-D in the middle stage. The CMG cells already expressed GATA4, TEF-1[17] (transcription enhancement factor 2), Nkx2.5, HAND, and MEF2-C before exposure to 5-azacytidine, and they expressed MEF2-A and MEF2-D after exposure to 5-azacytidine. This pattern of gene expression in CMG cells was similar to that of developing cardiomyocytes *in vivo*[17], and indicated that the developmental stage of the undifferentiated CMG cells is close to that of cardiomyoblasts or the early stages of heart development. We estimated that the stage of differentiation of the CMG cells lies between the cardiomyocyte-progenitor stage and the differentiated cardiomyocyte stage.

Serial changes in action potential shape in CMG cells simulate those of fetal ventricular cardiomyocytes *in vivo*

CMG cells exhibit at least two types of distinguishable morphological action potentials: sinus-node-like potentials (Fig. 4-A) and ventricular myocyte-like potentials (Fig. 4-B)[11]. The cardiomyocyte-like action potential recorded from these spontaneous beating cells is characterized by [1] a relatively long action potential duration or plateau, [2] a relatively shallow resting membrane potential, and [3] a pacemaker-like late diastolic slow depolarization. Peak-and-dome-like morphology was observed in ventricular-myocyte-like cells. Fig. 4-C shows the time course of the percentages of the sinus node-like and ventricular-myocyte-like action potentials. All action potentials recorded from CMG cells until 3 weeks were sinus-node-like action potential. The ventricular-myocyte-like action potentials were first recorded after 4 weeks, and their percentage gradually increased thereafter.

The observation of several distinct patterns of action potential in CMG cells may reflect different developmental stages. Yasui et al. studied action potentials and the occurrence of one of the pacemaker currents, I(f), by the whole-cell voltage and current-clamp technique at the stage when a regular heartbeat is first established (9.5 days post coitum) and at 1 day before birth[18]. They showed a prominent I(f) in mouse embryonic ventricles in the early stage, and that it decreased by 82% before birth in tandem with the loss of regular spontaneous activity by the ventricular cells. They concluded that the I(f) current of the sinus node type is present in early embryonic mouse ventricular cells. Loss of the I(f) current during the second half of embryonic development is associated with a tendency for the ventricle to lose pacemaker potency. Our findings in CMG cells may reflect the developmental changes in the action potentials that occur in embryonic ventricular cardiomyocytes.

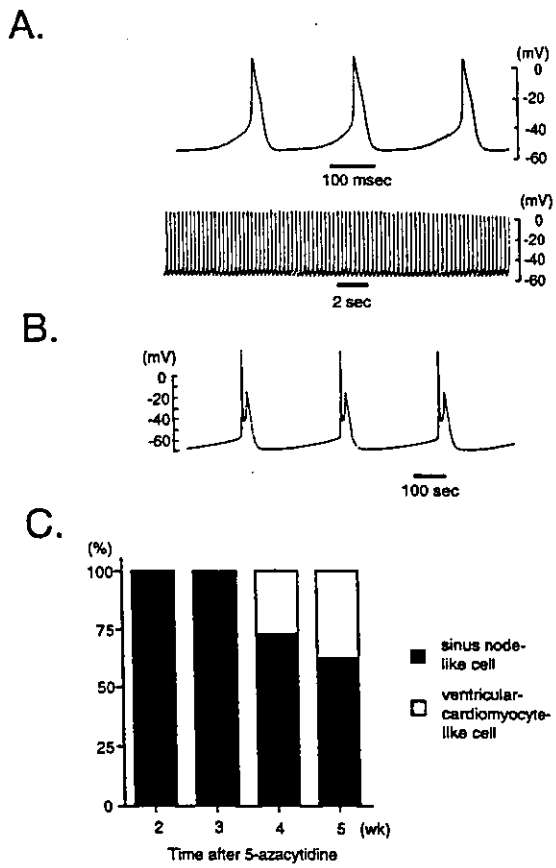

Fig. 4: Representative tracing of the action potentials of CMG myotubes

(A, B) Action potential recordings from spontaneous-beating cells were obtained with a conventional microelectrode at day 28 after 5-azacytidine exposure. The action potentials were classified into two groups: (A) sinus-node-like action potentials and (B) ventricular-cardiomyocyte-like action potentials.

(C) Percentages of CMG cells exhibiting sinus-node-like and ventricular- cardiomyocyte-like action potentials after 5-azacytidine exposure. A ventricular cardiomyocyte-like action potential was first recorded 4 weeks after 5-azacytidine exposure, and it rapidly became more prevalent thereafter.

Expression and function of α_1-adrenergic receptors in CMG cells

In the heart *in vivo*, α and β adrenergic receptors play a key role in modulating cardiac hypertrophy and cardiac function, such as heart rate, contractility, and conduction velocity. CMG cells expressed all the α_1 receptor subtypes (α_{1A}, α_{1B}, and α_{1D}) before 5-azacytidine exposure (Fig. 5-A)[12], and their expression in undifferentiated CMG cells may be explained by

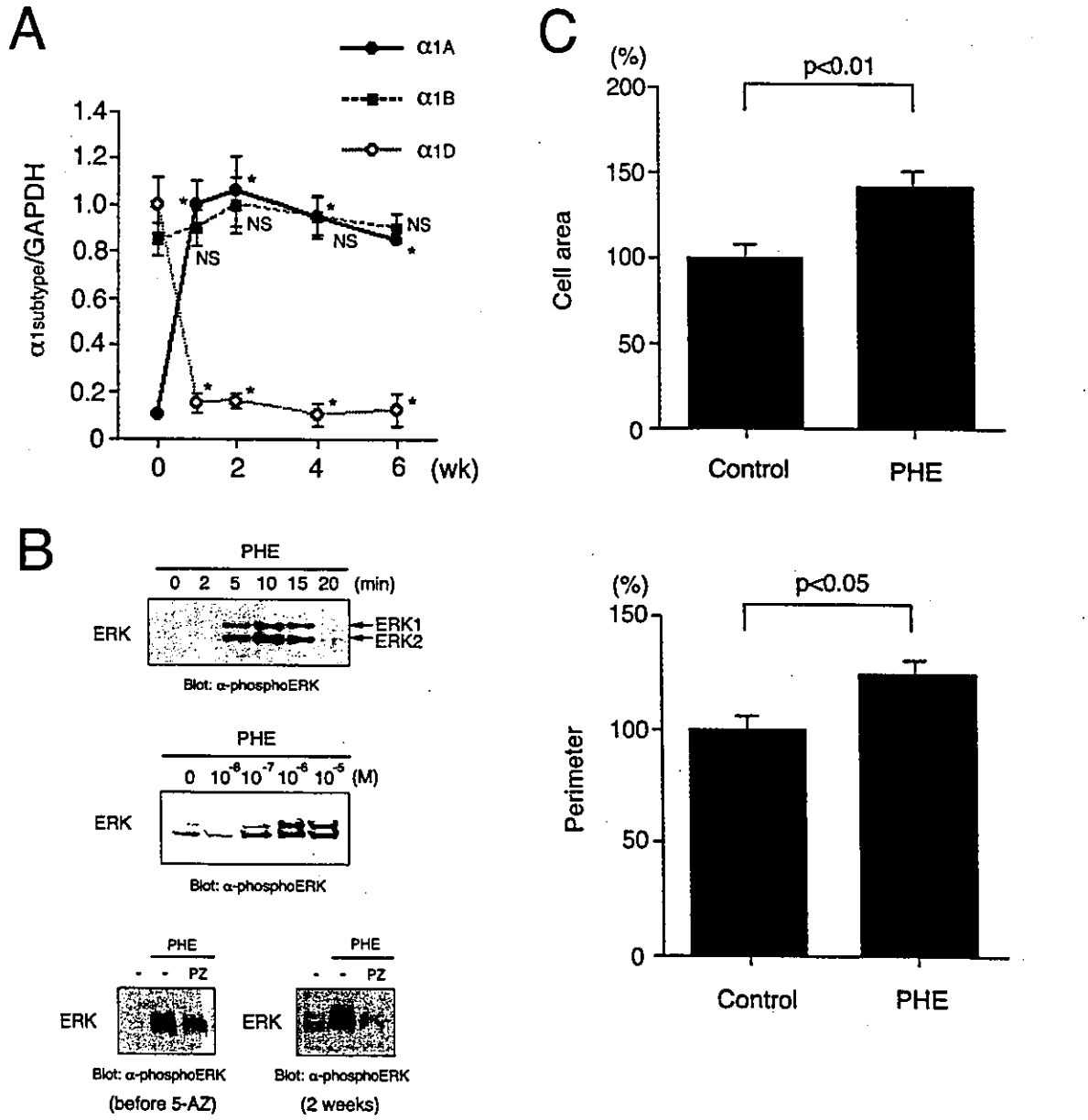

Fig. 5: Expression and function of α_1-adrenergic receptor subtype in CMG cells
A; Densitometric analysis was performed, and the ratio of the RT-PCR product of α_1 subtype (α_{1A}, α_{1B}, α_{1D}) receptors to that of GAPDH is shown. Data were obtained from 5 separate experiments and are shown in arbitrary units compared to the controls. Values are mean±SE. *:p<0.01 vs. controls (before 5-azacytidine exposure). NS: not significant. B; (Upper panel) Cells at 2 weeks after 5-azacytidine exposure were stimulated with phenylephrine (10^{-4} mol/L), and Western blot analysis was performed to detect phosphorylation of ERK1/2. (Middle panel) Cells were stimulated with phenylephrine (10^{-7} - 10^{-5} mol/L) for 10 minutes, and phosphorylation of ERK was detected. (Lower panel) Prazosin (10^{-4} mol/L) was added to cells 20 minutes before stimulation with phenylephrine (10^{-4} mol/L). PHE: phenylephrine, PZ: prazosin. C; Cells were serum depleted for 24 h, stimulated with phenylephrine for 24 h, and stained with anti-sarcomeric myosin antibody. Cell area and perimeter were quantitated with NIH Image software. (n=100) *: p<0.01 vs. control.

their ubiquitous or wide expression *in vivo*[19]. A low level of expression of α_{1A} was observed before 5-azacytidine exposure, and it increased markedly after exposure. Expression of α_{1B} was unaffected by 5-azacytidine. A high level of expression of α_{1D} was detected before 5-azacytidine exposure, but it decreased considerably after exposure. This transcriptional switch may be attributable to the CMG cells having acquired the cardiomyocyte phenotype. The ventricular cardiomyocytes *in vivo* mainly expressed α_{1A} and α_{1B}, and expressed a low level of α_{1D} receptor. The temporal changes in expression of α_1 adrenergic receptor subtypes in CMG cells are very similar to the postnatal changes observed in neonatal rat heart[20,21].

ERK1/2 was activated by phenylephrine, an α_1 stimulant, within as little as 5 minutes, and the activation peaked at 10 minutes. The phenylephrine-induced phosphorylation was completely inhibited by prazosin (Fig. 5-B). Phenylephrine increased the cell area and perimeter of the CMG cardiomyocytes (Fig. 5-C). These findings indicated that CMG cells express functionally active α_1-adrenergic receptors[12].

Expression and function of β_1- and β_2-adrenergic receptors in CMG cells

The cardiomyocytes of the mammalian hearts express both β_1 and β_2-adrenergic receptors, the β_1 receptor being the predominant subtype (approximately 75 - 80% of total β receptors)[22]. CMG cells did not express β_1 and β_2 receptor transcripts before 5-azacytidine exposure, but RT-PCR showed expression of their mRNAs after 1 week (Fig. 6-A)[12]. CMG cells expressed β_1 and β_2 mRNA after acquiring the cardiomyocyte phenotype. The temporal pattern of expression of these receptors differed from that of α_1.

Isoproterenol, a β stimulant, increased the cAMP content of CMG cells, and propranolol completely inhibited the isoproterenol-induced cAMP accumulation (Fig. 6-B,C). Isoproterenol was applied to the cells to determine whether it would increase the spontaneous beating rate (Table 2), and the results showed that it increased it significantly to 48% over the rate in the control cells[12]. Preincubation with propranolol (non-selective β blocker), CGP20712A (β_1-selective blocker) strongly reduced the isoproterenol-induced increase in beating rate, and preincubation with ICI118551 (β_2-selective blocker) only slightly

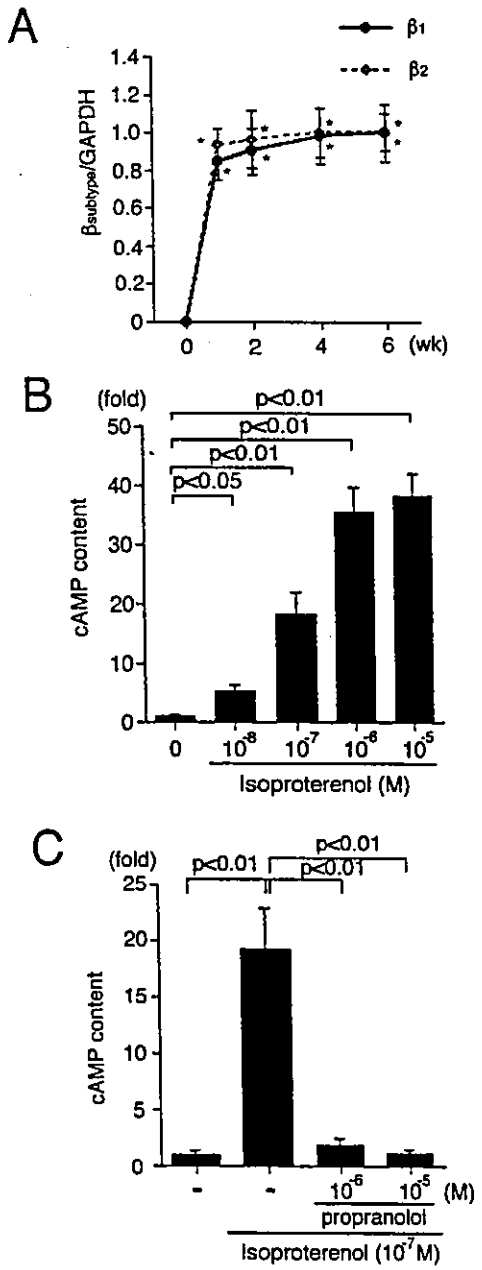

Fig. 6: Expression and signal transduction of β_1-and β_2-adrenergic receptor subtype in CMG cells

A; Densitometric analysis was performed, and the ratio of the RT-PCR product of β subtype (β_1 and β_2) receptors to that of GAPDH is shown. B; Effect of isoproterenol on cAMP accumulation in CMG cells at 2 weeks after 5-azacytidine exposure. C; Cells were preincubated with propranolol (10^{-4} or 10^{-5} mol/L) for 20 minutes and stimulated with isoproterenol (10^{-7} mol/L) for 10 minutes. Data were obtained from 5 separate experiments and are shown as arbitrary units compared with the controls. *:p<0.01, **:p<0.05 vs. controls.

decreased the beating rate. The increase in beating rate was similar to that of adult murine cardiomyocytes and ES cell-derived cardiomyocytes.

We also investigated the effect of isoproterenol on the contractile function of CMG cells and found that it increased cell motion distance, %shortening, and contractile velocity. The isoproterenol-induced increase in contractility was almost completely inhibited by both propranolol and CGP20712A. Collectively, these results indicated that the β_1 and β_2-adenergic receptors expressed in CMG cells are functional, and that the isoproterenol-induced increase in spontaneous beating rate and contractility is mainly mediated by β_1 receptors. The β_1 receptor was the predominant subtype that mediated changes in the beating rate in CMG cells, and the beating rate and the contractility were significantly increased by isoproterenol, and completely inhibited by propranolol and CGP20712A. β_1-Receptors played a critical role in mediating the isoproterenol-induced signaling in differentiated CMG cells. This expression pattern was consistent with that of cardiomyocytes *in vivo*.

CMG cells express muscarinic receptor mRNA after 5-azacytidine exposure

Heart rate, conduction velocity, and contractility were negatively regulated by the parasympathetic nervous system in cardiomyocytes, and muscarinic (cholinergic) receptors play an important role in mediating this function. To date, 5 subtypes (M_1-M_5) of muscarinic receptors have been cloned. The expression of the muscarinic receptors is tissue-specific, and cardiomyocytes mainly express M_2 receptors in the mouse and human[25]. The M_1 receptor subtype is also expressed in murine neonatal and adult cardiomyocytes. Fig. 7-A shows the temporal expression pattern of M_1 and M_2 receptor mRNA. Neither receptor was detected prior to 5-azacytidine exposure. CMG cells began to express these receptors when they acquired the cardiomyocyte phenotype.

M_1 receptors coupled to Gq/G_{11} and activated phospholipase Cβ via Gqα, leading to IP_3 production, and M_2 receptors coupled to Gi/G0/Gz and activated phospholipase Cβ via Gi$\beta\gamma$, leading to IP_3 production[20,25]. Carbachol, an acetylcholine homologue, increased the content of a second messenger, IP_3 (inositol triphosphate), in CMG cells (Fig. 7-B), and

Table 2. Isoproterenol increased the spontaneous beating rate and contractility of CMG cells, mainly via β_1 receptors

	Control	Isoproterenol (10^{-7}mol/L)			
		Vehicle	Propranolo (10^{-7}mol/L)	CGP20712A (10^{-7}mol/L)	ICI118551 (10^{-7}mol/L)
%increase in beating rate	–	47.6±8.4*	10.0±1.9†	13.8±2.4†	37.6±1.9‡
cell motion (μm)	5.0±0.3	6.8±0.7*	5.6±0.8‡	5.3±0.6‡	ND
%shortening (%)	6.9±0.5	8.5±1.2*	7.2±0.8‡	5.6±0.6‡	ND
contractile velocity (μm/s)	71.1±5.2	100.9±11.0*	71.3±8.8‡	70.6±6.6‡	ND

CMG cells at 4 weeks after 5-azacytidine exposure were initially exposed to prazosin (10^{-4}mol/L) for 30 minutes to block α_1-adrenergic receptors. Cells were then preincubated for 20 minutes with vehicle (PBS), propranolol, CGP20712A, or ICI118551, and then stimulated with isoproterenol. The beating rate was counted 3 minutes after stimulation. Contractile parameters were analyzed 90 seconds after stimulation. Each contractile parameter value was calculated as the mean of 3 randomly selected beats in one cell. PBS was added to the control. Values are means±SE (n=100, each). *: $p<0.05$ vs. control, †: $p<0.01$ vs. vehicle (isoproterenol only), ‡: $p<0.05$ vs. vehicle, ND: not determined.

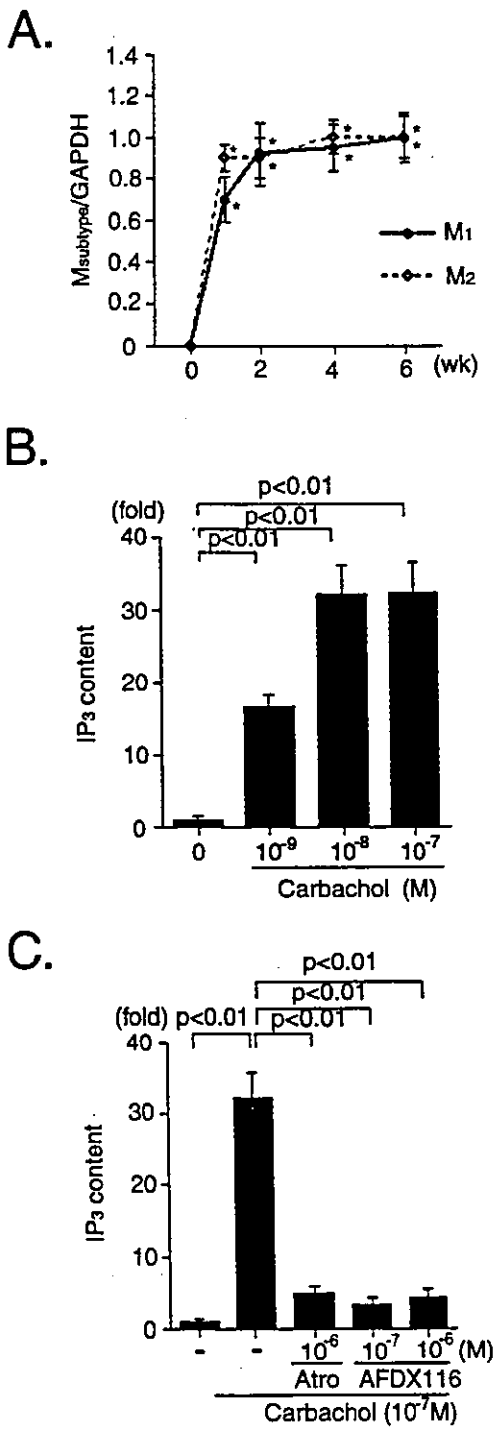

Fig. 7: Expression and function of M_1- and M_2-muscarinic receptors in CMG cells

A; The ratio of the RT-PCR product of muscarinic subtype to that of GAPDH is shown. Data were obtained from 5 separate experiments and are shown as arbitrary units over controls. *: $p<0.01$ vs. controls B; Effect of carbachol on IP_3 production in CMG cells at 2 weeks after 5-azacytidine exposure. C; Effect of atropine (10^{-4} mol/L) and AFDX116 (10^{-7} or 10^{-6} mol/L) on carbachol-induced IP_3 production. Data were obtained from 5 separate experiments and are shown as arbitrary units compared with the controls. *:$p<0.01$ vs. controls. Atro: atropine

preincubation with atropine (non-selective muscarinic blocker) and AFDX116 (M_2-selective blocker) inhibited the carbachol-induced IP_3 production (Fig. 7-C). These findings indicated that muscarinic receptors can transduce their signals, and that M_2 receptors play a critical role in this carbachol-induced IP_3 production in CMG cells. This expression pattern is similar to that of cardiomyocytes *in vivo*.

Significance of expression of adrenergic and muscarinic receptors in CMG cells

Cardiomyocytes *in vivo* respond to stimulation by both sympathetic and parasympathetic nerves, and such stimulation alters the heart rate, conduction velocity, and contractility, enabling the cells to adapt to rapid changes in systemic oxygen demand. To date, and to our knowledge, ES cells and mesenchymal-stem-cell-derived CMG cells are the only possible candidates for regeneration of cardiomyocytes. We have already transplanted these cells into normal adult mouse hearts, and have observed that transplanted cells survived in recipient hearts for at least several weeks. Regenerated cardiomyocytes must express functional adrenergic and muscarinic receptors to be useful for transplantation, and although we did not investigate all signaling pathways and their functions. CMG cells are potential candidates for cardiomyocyte cell transplantation, because they possess such receptors.

Cell transplantation therapy for the treatment of heart failure

We have already transplanted CMG cells into normal adult mouse hearts, and observed that the transplanted cells could survive in the recipient heart for at least several months. Fibroblasts, smooth muscle cells, and skeletal muscle cells were the first cells used for transplantation into scar tissue secondary to experimental myocardial infarction in the heart *in vivo*. While transplantation of these cells into scar tissue might improve cardiac remodeling or diastolic function, it is unlikely to improve systolic function. Transplantation of cardiomyocytes, however, might rescue systolic function. The only potential sources of regenerated cardiomyocytes available to date are

embryonic stem (ES) cells and mesenchymal stem cells. ES cells differentiate into cardiomyocytes *in vitro* and have both advantages and disadvantages for cardiomyocyte regeneration. Transplanted ES cells may form teratomas if some undifferentiated totipotent cells are still present, and recipients must take immunosuppressants, because ES cells are allogeneic. By contrast, since mesenchymal stem cells do not carry any inherent risks of tumor formation and are syngeneic, it is reasonable to use autologous mesenchymal stem cell to treat heart disease. Nevertheless, there is a need to improve both the current methods for identification and culture of mesenchymal stem cells, and for induction of CMG cell differentiation, which are still inefficient and slow. Identification of specific growth factors, cytokines, or extracellular matrix factors that regulate cardiomyocyte differentiation may help to accelerate this process faster and make it more efficient.

In vivo evidence that marrow cells can generate functional cardiac tissues

Recent studies have revealed that bone-marrow-derived cells differentiate into various types of cells *in vivo*. Shimizu et al. reported that smooth-muscle-like cells (SMCs) in graft-vs-host arterial lesions could arise from circulating bone-marrow-derived precursors. They used murine aortic transplants to formally identify the source of SMCs in lesions in grafted arteries[26]. Allografts in beta-galactosidase transgenic recipients showed that intimal SMCs arose almost exclusively from host cells, and bone-marrow transplantation of beta-galactosidase-expressing cells into aortic allograft recipients demonstrated that the intimal cells included those of marrow origin.

Kocher et al. showed that bone marrow from adult humans contains endothelial precursors with phenotypic and functional characteristics of embryonic hemangioblasts and that they can be used to directly induce new blood vessel formation in the infarct-bed (vasculogenesis) and proliferation of preexisting vasculature (angiogenesis) after experimental myocardial infarction[27]. The neoangiogenesis resulted in decreased apoptosis of hypertrophied myocytes in the peri-infarct region, long-term salvage and survival of viable myocardium, reduction in collagen deposition, and sustained improvement in cardiac function.

We also observed that transplanted bone marrow cells differentiated into cardiomyocytes in the recipient heart *in vivo* (unpublished observation). These findings provided direct evidence that bone marrow cells can regenerate various types of cells in cardiac tissue. We expect cardiac tissues damaged by myocardial infarction or other diseases to be repaired by bone-marrow-derived stem cells in the near future, and the precise mechanism should be investigated to achieve this goal.

References

1) Soonpaa MH, Koh GY, Klug MG, Field LJ: Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science, 264 (1997) 98-101.
2) Delcarpio JB, Claycomb WC: Cardiomyocyte transfer into the mammalian heart. Cell-to-cell interactions *in vivo* and *in vitro*. Ann NY Acad Sci 52 (1997) 267-285.
3) Wobus AM, Wallukat G, Hescheler J: Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. Differentiation. 48 (1991) 173-182.
4) Wobus AM, Kleppisch T, Maltsev V, Hescheler J: Cardiomyocyte-like cells differentiated *in vitro* from embryonic carcinoma cells P19 are characterized by functional expression of adrenoceptors and Ca^{2+} channels. *In Vitro* Cell Dev Biol Anim 30(1994) 425-434.
5) Roy NS, Wang S, Jiang L, Kang J, Benraiss A, Harrison-Restelli C, Fraser RA, Couldwell WT, Kawaguchi A, Okano H, Nedergaard M, Goldman SA: *In vitro* neurogenesis by progenitor cells isolated from the adult human hippocampus. Nat Med 6(2000) 271-277.
6) Prockop DJ: Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276(1997) 71-74.
7) Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS, Kazhdan I: Induction of rapid osteoblast

differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. Dev Bio 161(1994) 218-228.

8) Friedenstein AJ, Chailakhyan R, Gerasimov UV: Bone marrow osteogenic stem cells: *in vitro* cultivation and transplantation in diffusion chambers. Cell Tissue Kinet 20(1987) 263-272.

9) Ferrari G, Angelis GC, Colleta M, Paolucci E, Stornaiolo A, Cossu G, Mavilio F: Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279(1998) 1528-1530.

10) Ashton BA, Allen TD, Howlett CR, Eaglesom CC, Hattori A, Owen M: Formation of bone and cartilage by marrow stromal cells in diffusion chambers *in vivo*. Clin Orthop 151(1980) 294-307.

11) Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S: Cardiomyocytes can be generated from marrow stromal cells *in vitro*. J Clin Invest 103(1999) 697-705.

12) Hakuno D, Fukuda K, Makino S, Konishi F, Tomitra Y, Manabe T, Suzuki Y, Hisaka Y, Umezawa A, Ogawa S: Bone marrow-derived cardiomyocytes (CMG cell) expressed functionally active adrenergic and muscarinic receptors. Circulation 105(2002) 380-386.

13) Fukuda K: Development of regenerative cardiomyocytes from mesenchymal stem cells for cardiovascular tissue engineering. Artificial Organs 25(2001) 183-193.

14) Linnets TJ, Parsons LM, Harley L, Lyons I, Harvey RP: Nkx-2.5: a novel murine homeboy gene expressed in early heart progenitor cells and their myogenic descendants. Development 119(1993) 419-431.

15) Arceci RJ, King AA, Simon MC, Orkin SH, Wilson DB: Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. Mol Cell Biol 13(1993) 2235-2246.

16) Edmondson DG, Lyons GE, Martin JF, Olson EN: Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. Development 120(1994) 1251-1263.

17) Chen Z, Friedrich GA, Soriano P: Transcriptional enhancer factor 1 disruption by a retroviral gene trap leads to heart defects and embryonic lethality in mice. Genes Dev 8(1994) 2293-2301.

18) Yasui K, Liu W, Opthof T, Kada K, Lee JK, Kamiya K, Kodama I: I(f) current and spontaneous activity in mouse embryonic ventricular myocytes. Circ Res 88(2001) 536-42

19) Alonso-Llamazares A, Zamanillo D, Casanova E, Ovalle S, Calvo P, Chinchetru MA: Molecular cloning of alpha 1d-adrenergic receptor and tissue distribution of three alpha 1-adrenergic receptor subtypes in mouse. J Neurochem 65(1995) 2387-2392.

20) Stewart AF, Rokosh DG, Bailey BA, Karns LR, Chang KC, Long CS, Kariya K, Simpson PC: Cloning of the rat alpha 1C-adrenergic receptor from cardiac myocytes. alpha 1C, alpha 1B, and alpha 1D mRNAs are present in cardiac myocytes but not in cardiac fibroblasts. Circ Res 75(1994) 796-802.

21) Rokosh DG, Stewart AF, Chang KC, Bailey BA, Karliner JS, Camacho SA, Long CS, Simpson PC: Alpha1-adrenergic receptor subtype mRNAs are differentially regulated by alpha1-adrenergic and other hypertrophic stimuli in cardiac myocytes in culture and *in vivo*. Repression of alpha1B and alpha1D but induction of alpha1C. J Biol Chem 271(1996) 5839-5843.

22) Rockman HA, Koch WJ, Lefkowitz RJ: Cardiac function in genetically engineered mice with altered adrenergic receptor signaling. Am J Physiol 272(1997) H1553-H1559.

23) Sharma VK, Colecraft HM, Rubin LE, Sheu SS: Does mammalian heart contain only the M2 muscarinic receptor subtype? Life Sci 60(1997) 1023-1029.

24) Nakamura F, Kato M, Kameyama K, Nukada T, Haga T, Kato H, Takenawa T, Kikkawa U: Characterization of Gq family G proteins G_{11} alpha (G_{11} alpha), G_{12} alpha (G_{11} alpha), and Gq alpha expressed in the baculovirus-insect cell system. J Biol Chem 270(1995) 6246-6253.

25) Berstein G, Blank JL, Smrcka AV, Higashijima T,