

## Concomitant Angiogenesis/Myogenesis in the Regenerative Heart

a report by

**Professor Peter K Law**

Founder Chairman and Chief Executive Officer, Cell Therapy Research Foundation (CTRF) and Cell Transplants International, LLC (CTI)



Professor Peter K Law is Founder, Chairman and Chief Executive Officer of the Cell Therapy Research Foundation (CTRF) and Cell Transplants International, LLC (CTI). Founded in 1991, CTRF focuses on developing biologic therapies in treating hereditary, debilitating and fatal diseases. He pioneered and holds world patent rights to myoblast transfer therapy (MTT), the only human genome therapy in existence. In July 1990, Professor Law published in *The Lancet* the world's first correction of a human gene defect using MTT on Duchenne muscular dystrophy. He has published in international scientific journals and has been an invited speaker to the most prestigious campuses and pharmaceutical companies worldwide. Professor Law is a member of a wide array of biomedical societies and has served on many National Institutes of Health and National Science Foundation ad hoc committees. A former Professor of Neurology/Physiology and Biophysics at The University of Tennessee, Memphis, and of Vanderbilt University, Professor Law received his BSc with honours from McGill University, his MSc and PhD from the University of Toronto and his postdoctorate from McMaster University, Canada.

### Summary

Bioengineering the regenerative heart may provide a novel treatment for heart failure. On 14 May 2002, a 55-year-old man suffering ischemic myocardial infarction received 25 injections carrying 465 million current Good Manufacturing Practice (cGMP)-produced pure myoblasts into his myocardium after coronary artery bypass grafting.

As of 28 August 2002, his electrocardiogram reading has been normal and has shown no arrhythmia. His ejection fraction has increased by 13% and he no longer experiences shortness of breath and angina as he did before the treatment.

Three myogenesis mechanisms were elucidated with 17 human/porcine xenografts using cyclosporine as an immunosuppressant. Some myoblasts developed to become cardiomyocytes; others transferred their nuclei into host cardiomyocytes through natural cell fusion. Some myoblasts formed skeletal myofibres with satellite cells. *De novo* production of contractile filaments augmented the heart contractility.

Human myoblasts transduced with the vascular endothelial growth factor (VEGF)<sub>165</sub> gene produced six times more capillaries in porcine myocardium than the placebo. Xenograft rejection was not observed for up to 20 weeks, despite cyclosporine discontinuation at six weeks.

In this article, the pros and cons of autografts versus allografts are compared in order to guide future development of heart cell therapy.

### Introduction

Heart muscle degeneration is the leading cause of debilitation and death in humans. It results in loss of live cardiomyocytes, contractile filaments and heart function. Cardiomyocytes do not regenerate

significantly because the telomeric DNA repeats<sup>1</sup> in these terminally differentiated cells are minimal.

The degenerative heart transmits biochemical signals to recruit stem cells in order to repair the muscle damage. Being pluripotent, embryonic or adult stem cells exhibit uncontrolled differentiation into various lineages to produce bone, cartilage, fat, connective tissue and skeletal and heart muscles (see *Figure 1*).

The damaged myocardium needs additional live myogenic cells to deposit contractile filaments to regain heart function, preferably before fibroblast infiltration, which leads to scar formation. Until scientists can define the specific transcriptional factors and pathway accurately in order to guide stem cell differentiation into cardiomyocytes, the use of stem cell injection into the human heart would have a risk-benefit ratio higher than with the use of myoblasts.

As young cardiomyocytes and myoblasts become committed to myogenicity and differentiate from stem cells, they are similar in that they are mononucleated cells without contractile filaments (see *Figure 1*). In the presence of neurotrophic factors, myoblasts fuse to become myotubes that develop into myofibres. Under the influence of heart hormones, the young cardiomyocytes fuse to become mature cardiomyocytes. Cardiomyocytes and myofibres are myogenic cells that produce contractile proteins to provide for contractility.

Like cardiomyocytes, myoblasts are differentiated cells that are destined to become muscles. Unlike cardiomyocytes, myoblasts have long telomeric DNA subunits and are capable of extensive mitosis. The ability to undergo mitosis and to fuse is conserved in mononucleated satellite cells that are essentially myoblast reserves in adult muscles. Satellite cells are differentiated cells; they are not stem cells.

1. F Ishikawa, M J Matunis, G Dreyfuss and T R Cech, "Nuclear Proteins that Bind the Pre-mRNA 3' Splice Site Sequence r(UUAG/G) and the Human Telomeric DNA Sequence d(TTAGGG)<sub>n</sub>", *Molecular Cell Biology*, 13, 1993, pp. 4,301-4,310.
2. P K Law, et al., "World's First Human Myoblast Transfer into the Heart", *Frontiers in Physiology*, 2000, p. A85.

In cell culture, satellite cells divide and exhibit all characteristics of myoblasts. Myoblasts survive and proliferate in intercellular fluid when implanted into the human body. Their survival and development into myoblasts does not depend on vascularisation or nerve innervation.

The first human myoblast transfer into the porcine heart revealed that it was safe to administer one billion myoblasts at  $100 \times 10^6/\text{ml}$  through the Myostar catheter of the NOGA™ system (Biosense Webster, Inc.) using 20 injections at different locations inside the left ventricle.<sup>2</sup> It was determined that 0.3ml to 0.5ml would be the optimal volume per injection.

### Materials and Methods

In the myogenesis study, cultured myoblasts derived from satellite cells of human rectus femoris biopsies were transduced with a retroviral vector carrying a Lac-Z reporter gene. A porcine heart model of chronic ischemia (control = 3, myoblast-implanted = 6) was produced by clamping an ameroid ring around the left circumflex artery. Four weeks later, the heart was exposed by left thoracotomy.

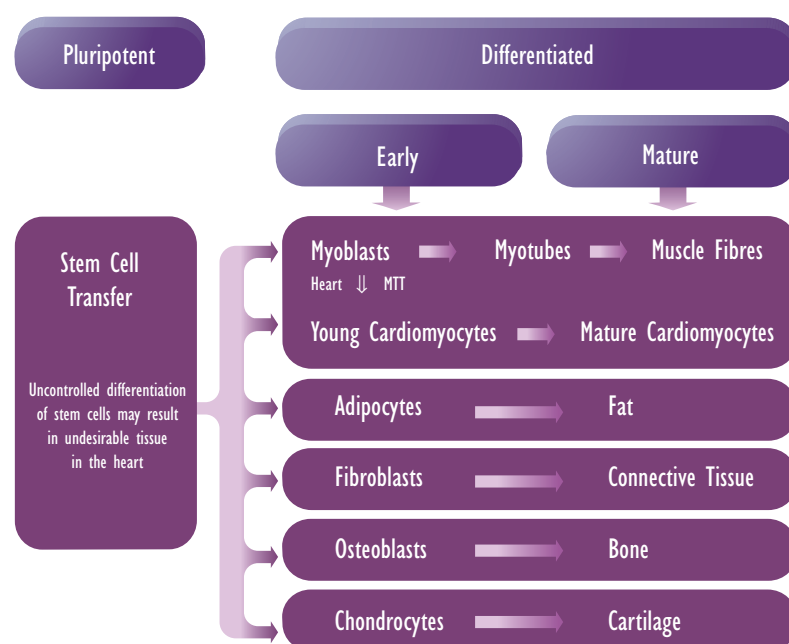
Twenty injections (0.25ml each) containing 300 million myoblasts or 5ml total volume of basal Dulbecco's Modified Eagle's Medium (DMEM) as control were injected into the left ventricle intramyocardially. Left ventricular function was assessed using MIBI-Tc<sup>99m</sup> single photon emission computed tomography (SPECT) scanning one week before injection to confirm myocardial infarction and at six weeks after injection.

Animals were maintained on cyclosporine at 5mg/kg body weight from five days before until six weeks after cell transplantation. The animals were euthanised at six weeks to five months post-operatively, and the hearts were processed for histological, immunocytochemical and ultra-structural studies.

Laser nuclear capture, together with single nucleus reverse transcription polymerase chain reaction (RT-PCR), was performed to delineate host and donor nuclei. *In situ* hybridisation using fluorescent DNA probes specific to human Y-chromosomes and chromosomes 1 and 10 for pigs were used.

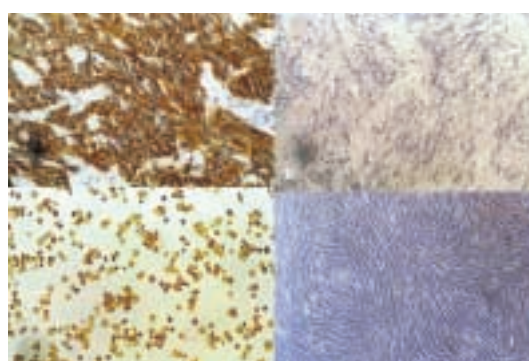
In the angiogenesis study, the human myoblasts were transduced with retroviral and adenoviral vectors carrying Lac-Z and human VEGF<sub>165</sub> genes, respectively. The cells were characterised for VEGF<sub>165</sub> transduction and expression efficiency by immunostaining, enzyme-linked immunosorbent assay (ELISA), immunoblotting and RT-PCR.

**Figure 1: Advantages of Using Myoblasts over Stem Cells in Treating Heart Failure**



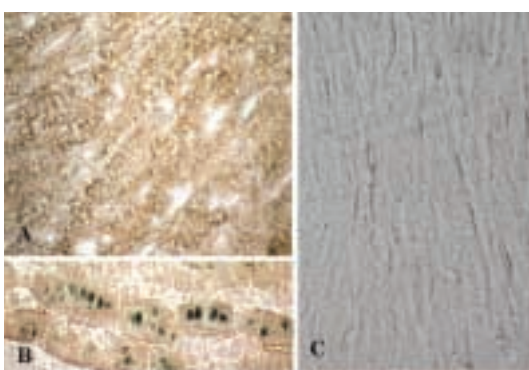
MTT = myoblast transfer therapy.

**Figure 2: Human Desmin Immunostain for Myoblast Purity**



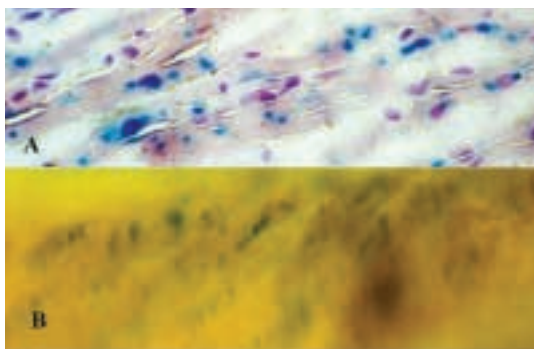
(A) Positive control of leiomyosarcoma, desmin staining brown. (B) Negative control. (C) Pure human myoblasts immunostained with desmin. (D) Pure human myoblasts in culture.

**Figure 3**



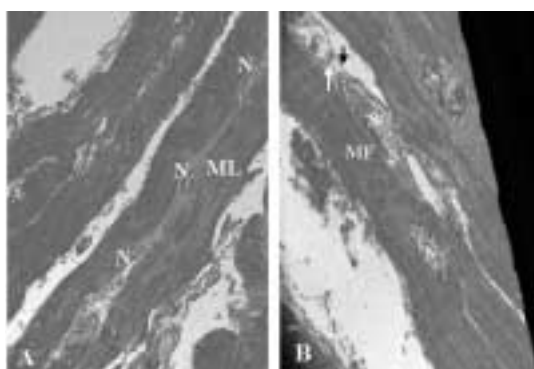
(A) Brownish immunostain of human myosin in porcine myocardium 12 weeks after human myoblast injection. (B) Cardiomyocytes with Lac-Z positive nuclei and human myosin stain, indicative of donor or myoblastic in origin. (C) Negative immunostain (grey) of human myosin in porcine myocardium sham-injected without myoblasts.

**Figure 4**



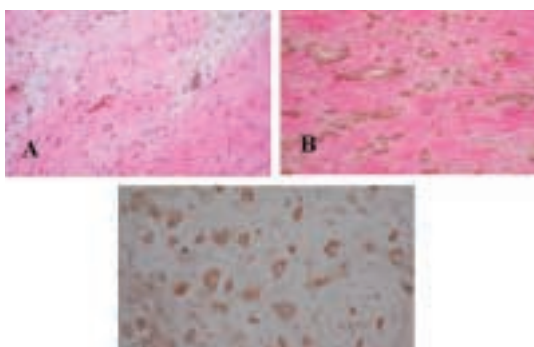
(A) Heterokaryons derived from fusion of porcine cardiomyocytes and human myoblasts showing Lac-Z positive human myoblast nuclei (bluish green) and porcine cardiomyocyte nuclei (purple) in the heterokaryotic syncytium. (B) These heterokaryons expressed human myosin heavy chain.

**Figure 5**



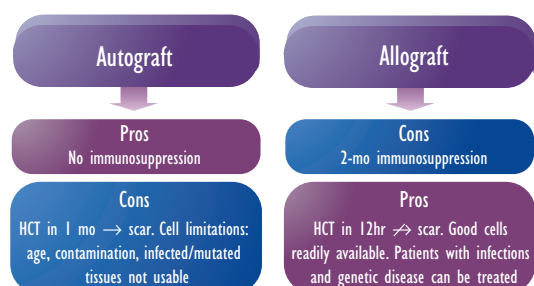
Electron microscopy of the myoblast-injected porcine myocardium showing (A) myotubes with central nuclei and myofibril (ML) deposits, and (B) skeletal myofibre with satellite cell (SC) and nucleus (N). The satellite cell was located between the basement membrane (black arrow) and the plasma membrane (white arrow). Sarcomeres showed proper alignment of newly formed contractile filaments.

**Figure 6**



(A) Control myocardium immunostained for vWF VIII and counterstained with eosin to show capillaries. (B) VEGF<sub>165</sub>-transduced myoblasts produced increased vascular density. (C) As in B but without eosin counterstain.

**Figure 7: Autograft versus Allograft for the Regenerative Heart**



A porcine heart model of infarction was created in eight female swines by left circumflex artery ligation. The animals were grouped as control (n = 3) and myoblast-implanted (n = 5). Angiography was performed to ensure complete occlusion of the blood vessel. Infarction was confirmed with MIBI-Tc<sup>99m</sup> SPECT scanning.

Four weeks later, 5ml basal DMEM with or without 3 x 10<sup>8</sup> human myoblasts carrying VEGF<sub>165</sub> and Lac-Z genes were injected into the left ventricle intramyocardially. The animals were maintained on cyclosporine (5mg/kg body weight) for six weeks post-operatively. Hearts were then explanted and processed for immunocytochemical studies.

**Results**

Human myoblasts cultured using patented technology yielded purity of 99% by human desmin immunostaining (see Figure 2). About 75% of the myonuclei were transduced successfully with a retrovirus vector carrying a Lac-Z gene. Trypan blue stain revealed >95% cell viability immediately before injection.

Histological examination of myoblast-injected myocardium showed cardiomyocytes containing Lac-Z positive nuclei (of donor origin) after 12 weeks (see Figure 3b). More than 80% of the Lac-Z positive cardiomyocytes immunostained positively for human myosin heavy chain (see Figure 3a). The control heart without myoblast injection did not show Lac-Z positive myonuclei nor human myosin (see Figure 3c).

Triple stain of myoblast-injected myocardia demonstrated multinucleated heterokaryons containing human and porcine nuclei with expression of human myosin (see Figure 4). Electron microscopy demonstrated human myotubes and skeletal myofibres with satellite cells in the porcine myocardium (see Figure 5).

The transduction efficiency for Lac-Z and VEGF<sub>165</sub> was 75% to 80% and >95%, respectively. The transduced myoblasts continued to secrete VEGF<sub>165</sub> for longer than 18 days, which was significantly higher (37 ± 3ng/ml) than non-transduced myoblasts (200 ± 30pg/ml). A dye exclusion test revealed >95% cell viability at the time of injection. Histological examination showed extensive survival of the grafted myoblasts expressing Lac-Z gene in and around the infarct.

The vascular density (mean ± scanning electron microscopy (SEM)) counted in an average of 12 low power fields (x200) in control animal hearts was 4.18 ± 0.42, compared with the VEGF<sub>165</sub> myoblast-

transplanted group ( $28.31 \pm 1.84$ ) (see Figure 6). The SPECT scans showed improved perfusion in the infarcted region.

Discontinuation of cyclosporine after six weeks prompted no xenograft rejection for up to 20 weeks.

### Conclusion

The on-going clinical trial is based on unequivocal evidence of cGMP-produced pure human myoblasts and proof of concept for heart cell therapy.

Human myoblasts survived and integrated into the porcine ischemic myocardium, allowing concomitant cell therapy and gene therapy. Whereas the newly formed myofibres harbour satellite cells and impart regenerative capacity to the heart muscle, the genetic transformation of cardiomyocytes *in vivo* to become regenerative heterokaryons through myoblast genome transfer<sup>3</sup> constitutes the ultimate heart repair. The regenerative heart<sup>4</sup> also contains cardiomyocytes of myoblastic origin. In all three scenarios, new contractile filaments are deposited to improve heart

contractility. The latter can be translated into improvement in the quality of life for heart patients and in the prevention of heart attacks.

It can be concluded that pure VEGF<sub>165</sub> myoblasts, when injected intramyocardially, are potential therapeutic transgene vehicles for concurrent angiogenesis and myogenesis to treat heart failure. Immunosuppression using cyclosporine for six weeks is effective for long-term survival of xenografts or allografts. There are many advantages in developing allografts, as depicted in Figure 7. ■

### Additional Information

*The studies were conducted in the laboratory of Professor Eugene Sim under the direction of Dr Khauja Haider in the National University Hospital (NUH) of Singapore. Human myoblasts were manufactured in Cell Transplants Singapore Pte. Ltd. (CTS) under the direction of Drs Gwendolyn Fang and Florence Chua. Financial support was provided by Singapore Economic Development Board (EDB) through Innovation Development Scheme (IDS) and Initiatives in New Technology (INTECH) grants to CTS.*

3. P K Law, "Nuclear Transfer and Human Genome Therapy", Business Briefing: Future Drug Discovery, Dec. 2001, pp. 38-42.

4. P K Law, "The Regenerative Heart", Business Briefing: PharmaTech 2002, Apr. 2002, pp. 65-70.



# Pharm-O-Kin<sup>®</sup> SCREEN

## A Major Move Ahead in Genotyping

### ••• Broad spectrum for significant pharmacogenetic profiling

- parallel detection of 39 pharmacokinetically most relevant polymorphisms
- based on our leading edge chip technology

### ••• Driven by customer benefit

- starting from unprocessed blood samples
- complete results with interpretation within 2 - 3 days

### ••• Outstanding reliability based on our validated method

- reproducibility > 98%
- accuracy > 98%

### ••• Highest quality standards

- in line with GLP
- controlled process flow
- certified according to ISO 9001 : 2000

- AgroFood
- **Diagnostics**
- Science
- Production
- Basic Technology

BioChip Technologies GmbH  
Business Unit Diagnostics  
Engesserstrasse 4  
D-79108 Freiburg / Germany  
Fon: +49-761-5038-119  
Fax: +49-761-5038-240  
diagnostics@genescan.com  
www.genescan.com

••••• **GeneScan**