Modifying Stem Cells for Clinical Regenerative Applications: A Practical Approach to a Fundamental Problem in Translational Research

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Various stem cell types are being evaluated for the potential regeneration of heart, bone, cartilage, nerve, and other tissues. While unmodified cells may prove satisfactory in some applications, the ability to introduce into the cell a bioactive molecule, usually a gene construct, greatly increases the potential of the cell-based approach. This concept has been stated in a number of recent publications and commentaries.

1. Ian Phillips, et al. reported that the tolerance of mesenchymal stem cells (MSC) transplanted to ischemic hearts could be enhanced by upregulating heme oxygenase.[1]

   In an accompanying editorial,[2] Victor Dzau et al. commented, "Genetic modification of stem or progenitor cells may represent an important strategic advancement in regenerative medicine. By combining gene with cell therapy, one may be able to enhance stem cell function and viability."

2. Meyer et al. reported clinical data from the BOOST Study and concluded " ... a single dose of intracoronary BMCs [bone marrow cells] did not provide a long term benefit on LV systolic function after AMI compared with a randomized control group."[3]

   In a commentary on this report,[4] Losordo and Welt discussed the lessons learned, and the comment included the following statements, "What have we learned? ... Another question is whether the use of unselected bone marrow mononuclear cells is optimal—the overwhelming evidence would argue that this is inconceivable ... We can phenotypically characterize cells that work versus those that don't and ultimately modify the cells to enhance their potency for specific indications."

3. MSCs have been used in regenerative applications requiring bone formation, where bone morphogenic protein 2 (BMP-2) leads to higher quality bone, which is not desirable for all soft tissue repairs.

   Dan Gazit et al. recently reported results with a rat Achilles tendon model. In an elegant paper in the *Journal of Clinical Investigation,* they state that transfection with a second genetic construct, encoding for Smad8, " ... inhibits the osteogenic pathway induced by BMP-2 and promotes the tendon/ligament differentiation route."[5]

The cell-loading system to be described here can introduce multiple molecules into a cell during a single processing step.

Many more examples can be given, showing that cell modification can affect cell targeting, differentiation, angiogenesis, and other important functions. Though the concept that cells used for regenerative applications will require some modification, some "engineering," the tools for accomplishing these changes have been wanting. While viral vectors and chemical transfection agents are useful tools for research at the bench level, translation into the clinic requires that a number of challenges be addressed, including safety concerns associated with viral vectors and potential toxicity of chemical agents. Electroporation has been shown to be an effective and safe method for loading cells with bioactive molecules, but instrumentation created for laboratory use has limited volume capacity (usually < 1 mL), operates in an open system with attendant sterility concerns, and these units use electrodes fabricated from materials with toxicity potential. The problems with such systems are:

- Limited volume (~ 106 – 108 cells), not scalable
- Inconsistent process, non-GMP
- Uses undefined chemical / biological agents
- No regulatory support
- Lack of validation in clinical setting

On the other hand, the system we describe here has addressed and dealt with these problems and is intended

for clinical use. What is needed for regenerative applications and is provided by the system presented:

- Can process $10^5 - 10^{11}$ cells; $5 - 50 \times 10^8$ cells processed / minute
- Robust processing; closed, sterile, GMP compliant
- Simple, chemically defined media – no added biological agents
- Master File at CBER/FDA
- 3 IND (FDA) and 1 CTA (Health Canada)

Experience with the System

The data listed below were generated using the MaxCyte cell-loading system, which was designed to enable cell-based applications from benchtop to the clinic.[6] This system is electroporation-based, but cell handling is a computer-controlled flow approach that allows processing of volumes from 20 µL to 1 L, utilizes non-toxic electrode materials, can be configured as a closed, sterile, disposable processing unit, and is consistent and reproducible. A wide range of molecules (low molecular weight fluorescent probes, albumin, IgG, 500 kD MW dextran, DNA plasmids, mRNA, and RNAi) have been loaded effectively into >50 types of cells, including primary cells and cell lines. This system produces transient transfection, with the expression of the gene product for days to months, depending upon the nature of the gene construct. This is satisfactory for most regenerative applications and is preferable, from a safety perspective, to systems that result in genomic integration of the transgene. The system has been accepted by the Food and Drug Administration (FDA) for clinical studies, and there are Master Files documenting its performance at CBER/FDA and at Health Canada.

The data presented here, generated in the course of several research projects, were chosen to demonstrate the potential utility of this system for regenerative applications.

TABLE 1. RESULTS OF TRANSFECTING CD34+ HEMATOPOIETIC STEM CELLS

Efficiency is percentage of cells showing fluorescence at 24 to 48 hours post processing. *Viability* was determined by propidium iodide exclusion. All assays performed by flow cytometry.

TARGET	MOLECULES	EFFICIENCY	VIABILITY
FITC-Dextran	(500kD)	>95%	>95%
siRNA	(FITC-label)	>95%	>95%
mRNA	(eGFP)	>85%	>95%
cDNA	(eGFP)	>60%	>70%

Studies with mesenchymal stem cells (MSCs)

A) Mouse-derived cells were transfected by loading with a DNA plasmid encoding for the muscle differentiation factor MyoD. After incubation for 10 days, test cells demonstrated fibrillar morphology consistent with muscle cells and stained positively for myosin; control cells (null vector) showed no change.

B) Rat and human MSCs were studied for a potential anti-neoplastic application. Initial optimization studies were with DNA plasmid encoding for green fluorescent protein (GFP) with efficiencies of 75 to 85 percent and a viability rate of 80 percent. After loading with DNA plasmid encoding for IL-12, the cytokine was expressed at levels sufficient for biological activity. MSCs are being widely studied for cardiac, orthopedic and other regenerative uses. Some results have suggested that cells modified to upregulate factors affecting apoptosis improved the functional results.

Studies with CD34+ cells

Cells obtained via leukopheresis from mobilized donors (provided by Dr. C. Dunbar, NHLBI, Bethesda, Maryland) were loaded with various agents to demonstrate the capability of the system. Results are displayed in Table 1, which shows efficiency as the percentage of cells displaying the marker, and viability as the percentage of cells excluding propidium iodide (PI), assayed via flow cytometry. The lower values obtained in studies with DNA plasmids reflect the well-known toxic effects of DNA in primary cells. This problem can be addressed by using mRNA in place of DNA (to be discussed in more detail below). CD34+ cells are being studied for cardiac regeneration, and control of differentiation may be a critical element for such work.

Studies with endothelial progenitor cells (EPC)

These cells have recently been studied intensively and are felt to have great potential for cardiovascular regen-

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erative applications. Pulmonary arterial hypertension (PAH) is a serious disorder of the pulmonary microvasculature with no satisfactory therapeutic approach. The cell therapy being developed by Stewart et al. at the University of Toronto, transfects autologous EPC with a plasmid, carrying full length cDNA encoding endothelial nitric oxide synthase (eNOS). The cells are infused into a peripheral vein and a significant percentage remain in the pulmonary vessels and secrete nitrous oxide (NO), which is a vasodilator and an angiogenic stimulant. Data from a rat model of the disease (induced by the administration of monocrotoline (MT) are shown in Figures 1 and 2.[7]

FIGURE 1. REVERSAL OF PAH IN RATS USING ENGINEERED CELLS.
Right ventricular systolic pressure measures pulmonary hypertension induced by MT. Twenty-one days post MT, rats are infused with either naïve cells or cells transfected with eNOS. Difference in RVSP obvious at 35 days post MT.

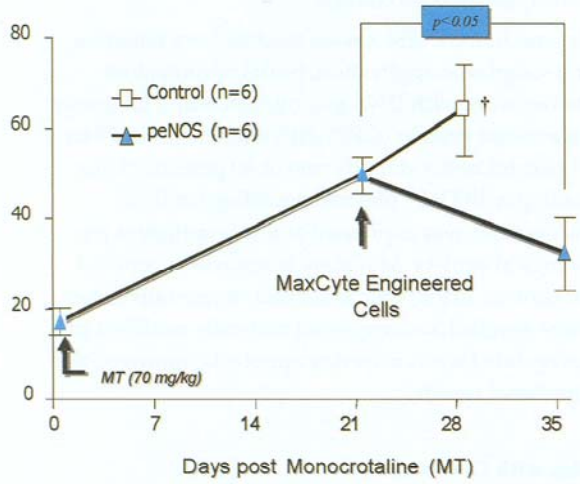
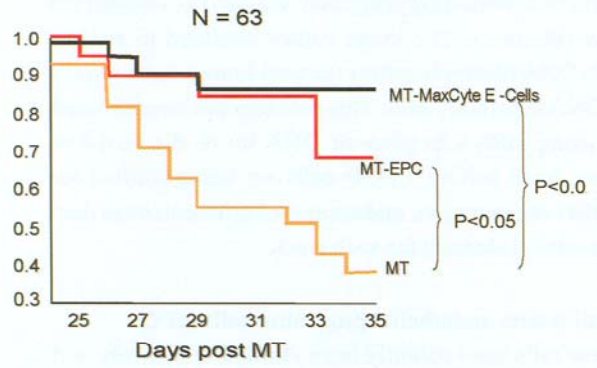


FIGURE 2. SURVIVAL IS INCREASED BY ENGINEERED CELL THERAPY.
There is incremental improvement in survival with naïve cells, and further increase in survival with engineered cells.



The animal data were used to support a clinical trial protocol and that trial is underway, with encouraging early results.[8] This study has been presented in recent months at the Third International Conference on Cell Therapy for Cardiovascular Diseases ("PHACeT Trial: The First "Engineered" Cell Study for Patients with Idiopathic Pulmonary Hypertension, presented by Dr. Duncan Stewart," in New York in January 2007) and at the annual meeting of the American Society for Gene Therapy in Seattle, June 2007.

Studies to demonstrate transfected cells can deliver a therapeutic protein.

Data have been obtained with a number of models, including animal tumors and cytokines. Mouse fibroblasts were transfected with DNA plasmid carrying full-length cDNA encoding erythropoietin, then these cells or control cells (null vector) were injected subcutaneously into normal mice (n = 5). At 5 days, the test hematocrit was 50 percent, while the control hematocrit was 43 percent. The hematocrit difference between treated and control mice persisted until the animals were sacrificed, at 25 days, according to protocol. This is a simple example that suggests a number of possible applications, such as the use of cells, with or without matrix support, to provide bone morphogenic protein (BMP) for orthopedic use.

Other proteins that can be upregulated using this system include those that affect stem cell differentiation (in other words, allow stem cells to proliferate without differentiating—Oct4, HoxB4), angiogenesis (VEGF, HIF-1), and targeting (CXCR4). An example of modulating differentiation by cell engineering is shown in Figure 3.

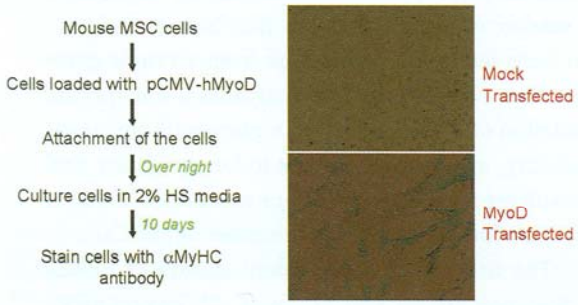
Conclusions

The computer-controlled, flow electroporation system described can load a wide range of biologically active molecules into primary cells and cell lines. It can do this with consistency, without added chemical or biological

reagents, using a closed and sterile processing assembly that can be integrated into a cGMP manufacturing process. The system can expedite the translation of regenerative applications from the bench to commercialization by avoiding regulatory hurdles associated with other methods.

As mentioned above, DNA is toxic, especially to primary cells. As seen in Table 1, the transfection of CD34+ stem cells with GFP is more efficient and is accomplished with better cell viability when mRNA encoding GFP is used, rather than the corresponding DNA plasmid. Electroporation is the method of choice for transfection with mRNA, and the MaxCyte system enables this approach for clinical therapeutics.

FIGURE 3. MOUSE MSC INDUCED TO DIFFERENTIATE INTO SKELETAL MUSCLE.
Transfection of mouse MSC with DNA plasmid, using the MaxCyte system, results in cells that stain for myosin and show myofibrillar form.



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FOOTNOTES

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