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MYOCARDIAL REPAIR WITH SATELLITE CELL IMPLANTATION

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March, 1997

**A Thesis Submitted to the Faculty of Graduate Studies and Research
in Partial Fulfilment of the Requirements of the Degree of
MASTER OF SCIENCE (M.Sc.)**

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**TO MY PARENTS AND MY WIFE, DIANA, FOR THEIR LOVE AND
UNCONDITIONAL SUPPORT.**

ABSTRACT

Injured adult mammalian cardiac muscle does not respond with significant regeneration. Skeletal muscle, on the other hand, has an efficient regeneration mechanism attributable to myogenic stem (satellite) cells. This study investigated a hypothesis which states that skeletal muscle satellite cells (SCs), if grafted into an injured heart, can form muscle tissue and repair the damaged myocardium.

To investigate this hypothesis 2 experimental studies on dogs (n=31) and isogenic Lewis rats (n=64) were performed.

In the first study SCs obtained from 28 dogs were isolated, purified and cultured. Percoll density gradient and preplating methods for the purification of SC culture were investigated. Beta-galactosidase (β -Gal) labelled SCs underwent autografting into acutely cryoinjured myocardium. Three control dogs received only sham implants into the cryoinjured heart. Two (n=6), 4 (n=16) and 6 (n=6) weeks after SC implantation, hearts with SC graft sites were procured and processed for histological examination, as well as for detection of a β -Gal marker. Control specimens were obtained at 2 (n=1), 4 (n=1), and 6 (n=1) weeks after sham implantation and were processed as described above.

In the second study on rats, SCs were obtained from donor animals (n=30). An intramuscular bupivacaine injection method to increase SC yield was adopted. Purified, cultured, and β -Gal labelled SCs were implanted either into acutely cryoinjured myocardium (n=15), or into the mature cardiac scar (n=15) of isogenic donor animals. Myocardial specimens were obtained at 1 month after SC implantation. Techniques of rodent SC labelling in vitro with human alkaline phosphatase genes were also explored.

In 2 specimens from dogs obtained at 4 weeks after SC grafting, the β -Gal marker was detected in cells residing near implant channels. In 2 other specimens recovered at 4 and 6 weeks post-implantation, striated muscle fibres surrounded by a dense scar were found. Morphologically they resembled skeletal muscle, but showed no β -Gal activity.

There were neither muscle fibres nor β -Gal activity detected in SC implant sites obtained from the myocardium of the surviving rats.

This work demonstrates that implanted cells obtained from labelled SC culture can survive within the cryoinjured myocardium. The presence of muscle fibres around the implant channels supports the hypothesis that SCs can be used to repair the injured heart muscle.

To interpret these and previously reported findings (Marelli *et al.*, 1992) we proposed the confluence degree hypothesis, which states that SC confluence reached prior to implantation influences the phenotypic expression of intramyocardially grafted SCs.

RÉSUMÉ

Le muscle cardiaque endommagé d'un mammifère adulte ne se régénère pas de façon marquée. Par contre, le muscle squelettique est doté d'un mécanisme de régénération efficace, attribuable aux cellules souches (satellites) myogènes. Cette étude visait à vérifier si la greffe de cellules musculosquelettiques satellites à un coeur malade peut entraîner la formation de tissu musculaire et la réparation du myocarde endommagé.

Afin de vérifier cette hypothèse, on a mené deux études expérimentales chez des chiens (n=31) et des rats de Lewis isogéniques (n=64).

Lors de la première étude, on a isolé, purifié et mis en culture des cellules satellites (CS) prélevées chez 28 chiens. On a examiné le gradient de densité de Percoll ainsi que les méthodes précédant l'étalement en boîte de Petri servant à la purification des cultures. Les cellules satellites bêta-galactosidases (β -Gal) se sont auto-greffées dans un myocarde très endommagé par le froid. Trois chiens témoins ont reçu des implantations fictives. Deux (n=6), quatre (n=16) et six (n=6) semaines après l'implantation des cellules satellites, on a prélevé des échantillons des sites de greffe et on les a soumis à un examen histologique et à une épreuve visant à déceler un marqueur β -Gal. On a obtenu des prélèvements chez les témoins deux (n=1), quatre (n=1) et six (n=1) semaines après l'implantation fictive et on les a analysés de la même façon.

Lors de l'étude sur les rats, on a obtenu des cellules satellites des donneurs animaux (n=30). On a injecté de la bupivacaïne par voie intramusculaire pour augmenter le rendement des CS. On a implanté les CS purifiées, mises en culture et marquées au β -Gal dans un myocarde très endommagé par le froid (n=15) ou une cicatrice cardiaque à maturité (n=15) de donneurs animaux isogéniques. On a prélevé des échantillons du myocarde un mois après l'implantation des CS. On a également examiné les techniques de marquage in vitro des CS des rongeurs avec des gènes humains de phosphatase alcaline.

Dans deux échantillons prélevés chez les chiens 4 semaines après la greffe de CS, on a décelé le marqueur β -Gal dans les cellules résidant près des réseaux d'implantation. Dans deux autres échantillons prélevés 4 et 6 semaines après l'implantation, on a noté des fibres musculaires striées entourées d'une cicatrice dense. Sur le plan morphologique, elles ressemblaient au muscle squelettique, mais ne présentaient aucune activité de β -Gal.

On n'a décelé ni fibres musculaires ni activité β -Gal aux points d'implantation des CS dans le myocarde des rats survivants.

Ce travail montre que les cellules implantées par la culture de CS peuvent survivre au sein d'un myocarde endommagé par le froid. La présence de fibres musculaires autour des réseaux d'implantation corrobore l'hypothèse selon laquelle on peut utiliser les CS pour réparer le muscle cardiaque endommagé.

Pour interpréter ces résultats et les données existantes (Marelli *et al.*, 1992), nous avons proposé l'hypothèse du degré de confluence selon laquelle la confluence des CS, atteinte avant l'implantation, influence l'expression phénotypique des CS greffées dans le myocarde.

PREFACE

This work represents the overview of the rapidly emerging field of myocardial repair with cell implants, as well the thesis author's contribution to it. It has to be acknowledged that the work presented in this thesis is a continuation of a series of studies, which tested this hypothesis earlier in the Surgical Research Lab at the Montreal General Hospital Research Institute under the supervision of Dr. R.C.-J. Chiu.

The Introduction Chapter delineates clinical relevance of the hypothesis and objectives of the study.

The development of skeletal and cardiac muscles as well as their adaptive responses to injurious factors is presented in Chapter II. In addition, a panorama of regulatory factors and mechanisms responsible for myogenesis is overviewed. The chapter is concluded with a review of previous attempts to restore lost cardiac muscle either by gene therapy or muscle cell graft methods. Some parts of this chapter have been presented before (Zibaitis *et al.*, 1997a).

In Chapter III, a hypothesis of this project is presented along with an experimental design and techniques which were employed in animal studies.

The input of previous investigators into creation of the experimental design and its techniques is being acknowledged. Dr. D. Marelli has adopted the skeletal muscle extirpation, SC isolation and culture, as well as SC implantation into the cryoinjured myocardium techniques. Satellite cell labelling with the β -Gal plasmid technique was adopted in the lab by Dr. D. Greentree. Detailed descriptions of these steps can be found in publications emanating from the Surgical Research Lab (Marelli *et al.*, 1992; Chiu *et al.*, 1995). However, for the purpose of providing a general understanding, a brief description of each step will be outlined in Chapter III, with the main emphasis on modifications in this project introduced by the thesis author.

Chapter IV outlines the findings and results obtained from experiments.

Interpretation of gathered data, evaluation of the techniques and the experimental design is displayed in Chapter V. Also, comparison between the data of this thesis and research results from previous investigators is displayed.

To explain different findings obtained from the series of experiments performed in the lab, a confluence degree hypothesis will be proposed in Chapter V. Moreover, this chapter will survey the currently available knowledge on phenotype flexibility which is relevant to skeletal muscle, as well as to the newly proposed hypothesis. A confluence degree hypothesis has been described earlier (Zibaitis *et al.*, 1996). Some of the data providing a biological basis for the hypothesis are reproduced from a more extensive review presented elsewhere (Zibaitis *et al.*, 1997b).

Finally, a brief summary and the conclusions arising from the experimental data are submitted in Chapter VII.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Ray C.-J. Chiu who was to me not only my best supervisor I ever had but also a very caring man who taught me great lessons of life, altruistically shared secrets of successful research and had the single greatest influence on my career.

Mrs. Minh Duong was an irreplaceable team member whose tireless work and technical expertise made a great input into this project.

I would like to acknowledge the contribution of the following people:

Dr. Race L. Kao, Dr. Ren-Ke Li, Dr. Hope McArdle, and Dr. Mohamed El-Alfy for help and advice on experimental design and techniques.

Dr. John Lough, Dr. Ming-Sound Tsao, and Dr. Juan Viloria for help and assistance with specimen preparation and microscopic evaluation.

Dr. Yoshio Misawa, Dr. Joong Hwan Oh, Dr. Edward W. Wong, Mr. Jay Fong for technical assistance in the operating room.

Dr. Lloyd A. Culp and Dr. Wen-chang Lin for providing pCMVlacZ and pCMVALP plasmids.

Mrs. Dianne Murray-Desbiens, Mr. Daniel Yee, Mr. Gordon Yee, Miss Rosalie Wilkinson, and Mr. Luc Viens for assistance in animal experiments and animal care.

I am indebted to Mrs. Emma Lisi for her excellent assistance in writing of papers.

Also for numerous sound advice, help and constant support I would like to thank my friends Dr. Carlos M. Li, Dr. Christopher M. Oung, Dr. Nikolay Tchervenivanov, Mr. Allan Lee, M.Sc., and Dr. Songyang Yuan.

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LIST OF ABBREVIATIONS

AT-1	- atrial tumour cell line 1;
BCIP	- 5-bromo-4-chloro-3-indolyl phosphate;
CHF	- congestive heart failure;
cDNA	- complementary deoxyribonucleic acid;
ECM	- extracellular matrix;
FGF	- fibroblast growth factor;
aFGF	- acidic fibroblast growth factor;
bFGF	- basic fibroblast growth factor;
β -Gal	- β -galactosidase;
GM	- growth medium;
HLH	- helix-loop-helix;
bHLH	- basic helix-loop-helix;
HSPG	- heparan sulfate proteoglycan;
Id	- inhibitor of differentiation;
IGF	- insulin-like growth factor;
IGF-I	- insulin-like growth factor type I;
IGF-II	- insulin-like growth factor type II;
<i>lacZ</i>	- <i>E. coli</i> gene encoding for bacterial β -galactosidase
MEF-2	- myocyte enhancer-specific factor 2;
MRF-4	- myogenic regulatory factor 4;
myf-5	- myogenic factor 5;
myf-6	- myogenic factor 6;
MyoD	- myogenic determination factor;
NBT	- nitro blue tetrazolium;
N-CAM	- neural cell adhesion molecule;
PAP	- placental alkaline phosphatase;
PDGF	- platelet-derived growth factor;
RSV	- Rous sarcoma virus;
SC	- satellite cell;
SV40	- simian virus 40; simian vacuolating virus;
TGF- β	- transforming growth factor β ;
VCAM-1	- vascular cell adhesion molecule 1.
X-Gal	- 5-bromo-4-chloro-3-indolyl- β -D-galactoside;

Chapter I

INTRODUCTION

CLINICAL RELEVANCE AND THE AIM OF THE EXPERIMENTAL STUDY

Ischemia-reperfusion and other deleterious events can damage cardiomyocytes which then undergo coagulative necrosis and are replaced with connective tissue cells. Resulting scarring of the myocardium imposes additional strain on remaining myocytes which have to maintain an established cardiac output. Compensatory mechanisms, such as cardiomyocyte hypertrophy, are then initiated and lost muscle mass along with contractile potential are compensated. However, long-standing hypertrophy in itself will lead to further loss of myocardium and eventually exhaust compensatory capacity, resulting in congestive heart failure (CHF).

Congestive heart failure is a widespread clinical entity in developed nations. It is estimated that approximately 300,000 Canadians as well as 2.3 million Americans suffer from this serious disease (Cecere *et al.*, 1995, Schocken *et al.*, 1992). With relentlessly increasing annual incidence accompanied by the aging of the Western world population, CHF becomes the leading diagnosis for patients over 65 years (Kannel *et al.*, 1991; Francis and Cubo, 1989). However, the most devastating feature of this syndrome is the grave prognosis it carries. The Framingham Heart Study revealed the 5-year survival rate in CHF patients to be as low as 50% (McKee *et al.*, 1971). Other studies present an even more dramatic picture, with a 50% mortality rate reached as early as 2 years after the establishment of the CHF diagnosis (Smith, 1985).

Therefore, CHF presents a major challenge in providing an efficient treatment option. Impressive advances in the medical management of CHF patients, such as introduction of angiotensin-converting enzyme inhibitors, prolonged the survival and slowed down the progression of heart failure (The CONSENSUS Trial Study group, 1987). Mechanical assist devices are still considered a temporary measure reserved for deteriorating patients waiting on a transplantation list for a suitable heart donor (Cecere *et al.*, 1995). A new procedure, dynamic cardiomyoplasty, where a skeletal muscle flap wrapped around the heart and contracting in synchrony, has brought new promise to a group of patients not suited for heart transplantation due to medical or other reasons (Chiu, 1995). Currently, heart transplantation is the ultimate option of treatment for CHF patients. However, lack of donor hearts in conjunction with risks of immunosuppression and its complications is an inherent burden of this treatment option.

The ultimate method of treatment for CHF should be based on a process or a procedure which would enable to increase the number of myocytes within the failing heart. This way the pathophysiological ground of CHF would be amended.

Many organs within the human body possess stem cells which have a purpose to renew tissues following cell death due to apoptotic and necrotic events. Unfortunately, mammalian myocardium is devoid of such stem cells. Therefore, it does not regenerate after an injury. Adult skeletal muscle, on the other hand, contains myogenic stem (satellite) cells which avidly participate in skeletal muscle regeneration.

This study was driven by the hypothesis which proposes that cultured and labelled satellite cells (SCs), when implanted into the injured myocardium, may survive and differentiate into cardiac muscle under the influence of their new environment, and consequently repair the damaged heart.

An objective of the study is to demonstrate that SC implantation is a feasible method in restoring damaged myocardium.

To achieve this goal, an experimental design was composed where SCs, isolated from either adult canine or rodent muscles, were implanted into acutely cryo-injured myocardium. To avoid tissue incompatibility in a study on dogs, autologous SCs were grafted into the cryo-injured cardiac muscle. In a study on rats, SCs were implanted into the cryo-injured myocardium of isogenic animals.

From a clinical point of view, myocardial repair with SC implants would have an advantage over other cell transplantation methods due to the availability of the SC source. As the dynamic cardiomyoplasty procedure has demonstrated, there are few skeletal muscles in the human body, which can be removed without an appreciable handicap to a patient. Furthermore, implantation of autologous primary myogenic cells would eliminate the risk for allograft or xenograft immune rejection.

Chapter II

SKELETAL AND CARDIAC MUSCLE DEVELOPMENT, DIFFERENTIATION AND EXPERIMENTAL ATTEMPTS TO REPAIR MYOCARDIUM: A LITERATURE REVIEW

SKELETAL AND CARDIAC MUSCLE FORMATION

Development of Cardiac Muscle and Myocardial Adaptive Response to Injury

At the beginning of the third week of human embryo development, some mesenchymal cells from the primitive streak migrate along the notochordal process towards the cranial end of the embryo. Migrating cells meet at the cranial end of the notochordal process where they form a cardiogenic area which becomes the origin of the future heart tissue. At the end of the third week, cardiac myocytes form two parallel endothelial tubes which fuse to form a primitive tubular heart. Soon afterwards, cardiac myoblasts joined by specific desmosomal adhesion sites, gap junctions, form a synchronously beating cardiac tissue. Endocardium is formed by endothelial cells which rest on the cardiac jelly layer and cover the primitive heart lumen. Gradual disappearance of cardiac jelly, which is interposed between endothelial cells and the myocardium, eventually brings endothelial cells in close contact with the inner layer of the myocardium. The spaces among cardiac myocytes are then invaded by mesenchymal cells which form intramyocardial connective tissue (Manasek, 1970). Epicardium is formed by mesodermal cells migrating from the sinus venosus region over the entire surface of the myocardium. This process takes place during the periods of cardiac looping and septation (Icardo, 1984).

Embryonic cardiac myocytes demonstrate a distinct ability to simultaneously combine mitosis and differentiation events. Light and electron microscopy as well as radioautographic studies have demonstrated that cardiac muscle cells with cytoplasmic myofilaments undergo mitotic cell division. However, a few weeks after birth, mitotic activity in ventricular cardiac myocytes ceases and only hypertrophic growth of myocytes, along with hyperplasia of non-muscle cells, accounts for the postnatal growth of the heart (Zak, 1973).

However, not all adult heart muscle cells seem to be under nature's ban on cell division. Radioactive thymidine incorporation studies have shown that adult atrial myocytes, if challenged with an overload due to an injury of the ventricle, enter the mitotic cycle and respond to the increased work demand by myocyte hyperplasia (Rumyantsev, 1977). The simian virus 40 (SV40) large tumour antigen is also capable to initiate a proliferation program in atrial myocytes in vitro and in vivo (Field, 1988; Koh *et al.*, 1993b).

Some animals standing on the evolution ladder beneath mammals appear to have no restrictions regarding the proliferative potential of mature ventricular myocytes. Amphibians, particularly frogs and newts, are capable of restoring functional myocardium following an injury, which in some cases is as drastic as crushing or amputation of part of a ventricle (Rumyantsev, 1973; Oberpriller and Oberpriller, 1974).

Mammalian ventricular myocytes respond to an increased load by nuclear division and cytoplasmic growth of the pre-existing myocytes. Such processes lead to the appearance of polyploid muscle cells with increased cytoplasmic volume. The degree of ploidy increases in mammalian heart muscle with age, and approximately 80% of cardiomyocytes in an adult organism are polyploid (Zak, 1973). Unfortunately, for yet unknown reason, enduring hypertrophy of the heart exhausts compensatory mechanisms and eventually evolves into congestive heart failure.

It is of interest that cardiac myocytes, if stimulated by different stimuli such as overload, adrenergic agents, angiotensin II, endothelin-1, and growth factors, will begin to hypertrophy by recapitulating a certain embryonic developmental program. This leads to the ventricular expression of embryonic isoforms of contractile proteins as well as the expression of atrial natriuretic factor (Lang *et al.*, 1985), which in the adult heart is produced only by the atria (Parker *et al.*, 1990; Schneider *et al.*, 1991).

Regenerating skeletal muscle, which rebuilds itself from SCs, also expresses embryonic protein isoforms (Matsuda *et al.*, 1983). These observations suggest that certain parts of the embryonic developmental pathway are still available for differentiated tissues, such as striated muscle. Therefore, it is of interest to explore whether it can be possible to manipulate these processes, and alter phenotypic expression of striated muscle cells which could have possible therapeutic applications.

Development of Skeletal Muscle and Skeletal Muscle Myogenic (Satellite) Cells

Skeletal muscle is formed from embryonic mesodermal cells. In humans, at approximately twentieth day of embryogenesis, mesodermal cells aggregate into the first pair of somites. Cells within the lateral mesoderm follow further division into two planes of somatic and splanchnic mesoderm. The latter will eventually undergo

compartmentalization and form three layers dubbed as dermatome, myotome and sclerotome. It appears that induction of mesodermal cells to form skeletal muscle is influenced by nearby structures, such as the notochord and neural tube (Kimelmann *et al.*, 1992). Fibroblast growth factor (FGF) and transforming growth factor beta (TGF- β) families are potent inducers of embryonic cells as well (Green *et al.*, 1990). Cells within a myotome are already committed to the myogenic lineage and soon acquire an elongated shape. Limb buds subsequently are populated by myoblasts migrating from the lateral parts of the myotome. There is a mechanistic subdividing of embryonic skeletal muscle cells into primary and secondary myoblasts. Following a few rounds of division within their destination area, primary myoblasts commence a muscle differentiation program, initiate expression of structural muscle isoforms, and fuse to form a sparse network of primary myotubes. This is followed by the secondary myoblast adherence to the primary myotubes, which act as templates for building a bulk of secondary myotubes surrounding primary ones in a concentric fashion. Despite the close contact between both types of myoblasts, there is a specific recognition method in place which favours fusion of the muscle cells allocated to the same subtype (Rosen *et al.*, 1992). Although at this period of development a group of cells is intensely dividing and creating a large pool of muscle cells to join myotubes, some myoblasts, for yet undiscovered reasons, appear to escape the terminal differentiation phase. These cells, which presumably are destined to become SCs, adhere to the myotubes and remain in a quiescent stem cell phenotype for the remaining time of muscle development (Armand *et al.*, 1983; Ordahl and Le Douarin, 1992).

SATELLITE CELL: A REGENERATIVE CELL OF SKELETAL MUSCLE

Skeletal myogenic (satellite) cells were first described by Mauro in 1961 (Mauro, 1961). Electron microscopy revealed that they are situated on the sarcolemma of the mature skeletal muscle fibre and sheltered with the basal lamina from outside. Satellite cells are small cells, ca 25-30 μ m in length, with a small amount of cytoplasm, and have a relatively high nuclear/cytoplasmic ratio (Banker and Girvin, 1971). The paucity of cytoplasmic organelles in SCs suggests their dormant status. Rough endoplasmic reticulum is not prominent, suggesting a low level of ribosomal function. The Golgi complex is poorly expressed as well. Nuclei of SCs are somewhat more heterochromatic than nuclei propria of the muscle fibre (Campion, 1984). Another distinct morphological feature of these cells is the presence of caveoli on their plasma membrane, which

participate in the uptake of ions and other small molecules. Moreover, these structures are thought to be rich with Ca^{++} pump proteins, and most likely play a role in tyrosine phosphorylation signaling (Anderson *et al.*, 1992; Chang *et al.*, 1994). Activated SCs dramatically increase the quantity and volume of cytoplasmic organelles, which are essential for these myogenic cells to multiply and repair injured muscle.

It has been estimated that SCs comprise, on average, 4% of all nuclei in the muscle fibres (Schmalbruch and Hellhammer, 1976). Although these myogenic cells inhabit the whole myofibre, the majority of them reside at neuromuscular junction sites where they are in close contact with motor-end plates (Kelly, 1978).

Satellite cells remain in a quiescent state until their host muscle fibre is challenged with an injury or other factors (cytokines, exercise, etc.) (Darr and Schultz, 1987; Olwin *et al.*, 1994). Such a disturbance of the environment prompts SCs to enter the mitotic cell cycle. The regenerative capacity of SCs is approaching that of true stem cells. It is estimated that one skeletal myogenic cell has the potential to produce up to 10^{17} progeny cells (Webster and Blau, 1990).

Localized injury to the myofibre activates SCs positioned not only in close vicinity to the damaged area, but also on distant myofibres. Studies on myogenic transcription factors have shown that, in case of injury to skeletal muscle, the appearance of these early myogenic markers precedes the mitotic activity of SCs. Furthermore, these factors become expressed even in the SCs residing further away from the injured site (Grounds *et al.*, 1992). Some controversies still persist regarding the ability of SCs to cross the basal lamina which encloses them and thus, it is believed, interferes with the possible myoblast contribution to other myofibres than its own. However, there is a growing collection of data arguing that SCs possess the migratory ability which can be a valuable feature in recruiting neighboring myogenic cells to repair injured skeletal muscle fibres (Watt *et al.*, 1987; Hughes and Blau, 1990).

SCs can extend their "repairing" ability beyond postnatal myofibres. Satellite cells and embryonic myoblasts recognize each other and, through the process of fusion, form mixed myotubes in vitro (Jones, 1982.). Earlier studies also suggested that myoblasts have a certain degree of willingness to form heterokaryonic muscle fibres. Studies on patients with Duchenne muscular dystrophy showed that skeletal myoblasts of heterogenic origin can fuse with the host's myofibres and contribute to the expression of

structural proteins (Gussoni *et al.*, 1992). When myoblasts of genetically different animal species were co-cultured in vitro, muscle cells of both species contributed to the formation of "mosaic" myofibres (Yaffe and Feldman, 1965). The above mentioned and other observations prompted ideas for exploring SC regenerative potential in restoring damaged cardiac muscle (Chiu *et al.*, 1995).

FACTORS INFLUENCING DIFFERENTIATION OF STRIATED MUSCLE CELLS

Transcriptional Factors Involved in Control and Regulation of Skeletal Muscle Differentiation

The major breakthrough in determining the control of the skeletal muscle program came upon discovery of myogenic regulatory factors. So far there are four members known that belong to this family of nuclear transcription proteins. The first member, MyoD, was discovered in 1987 when Davis and colleagues (1987) introduced a specific cDNA sequence into 10T1/2 fibroblasts, and showed that this genetic maneuver can convert fibroblasts into cells exhibiting a skeletal muscle phenotype. The genomic sequence used was determined by the screening of a distinct mRNA present in 10T1/2 fibroblasts treated with 5-azacytidine, an antitumour agent affecting the DNA methylation process and a subsequent gene expression (Taylor, 1993).

The other discovered members of the myogenic determination factor (MyoD) family are myogenin (Wright *et al.*, 1989), myf-5 (Braun *et al.*, 1989), and MRF-4 (Rhodes and Konieczny, 1989), also known as myf-6 or herculin. All four proteins belong to the ubiquitously present group of helix-loop-helix (HLH) proteins. These molecules have a particular structure which consists of two amphipathic helices within the core region which are joined by a loop segment (Anthony-Cahill *et al.*, 1992). All MyoD family proteins, as well as some other members of the HLH family, have an additional structure, a "basic region", in their N-aminoterminal end. Therefore, HLH proteins possessing a basic region are referred to as basic-helix-loop-helix (bHLH) factors. The basic region of bHLH factors can bind DNA, which gives these molecules the capacity to act as nuclear transcription factors, e.g., activate specific gene transcription programs. Along with the capacity to control muscle cells, myogenic bHLH proteins established themselves as being able to alter the phenotypic fate of non-muscle cells as

well. A variety of cells other than muscle respond to the driving force of MyoD factors, which can convert them into the muscle cells (Weintraub *et al.*, 1991).

MyoD members have conserved sequences among vertebrates and many invertebrates which support the ancient ancestry of these genes. Myogenic regulatory genes originating from invertebrates are able to initiate the myogenic program in mammalian cells as well. Sea urchin myogenic factor 1, SUM-1, as well as CeMyoD, a nematode *Caenorhabditis elegans* myogenic protein, when introduced into mammalian 10T1/2 fibroblasts, effectively converted them into muscle cells which subsequently fused and formed myotubes (Venuti *et al.*, 1991; Krause *et al.*, 1992). This shows that the muscle gene activation pathway has been well preserved throughout the evolution process.

Basic HLH proteins, including MyoD family members, interact with DNA through association between their basic region and a distinctive DNA site, termed E-box. This DNA sequence, targeted by basic HLH proteins, is present in skeletal muscle genes (muscle creatine kinase, acetylcholine receptor α -subunit genes, etc.), as well as in other otherwise unrelated genes (Lasar *et al.*, 1989; Piette *et al.*, 1990; Murre *et al.*, 1989). It was discovered that all bHLH proteins are able to bind E-boxes, but only the MyoD family member interaction with these DNA structures can activate the myogenic program.

Myogenic activity of MyoD factors is affected by dimerization with other HLH molecules. Some of them, referred to as E2-proteins, are ubiquitously expressed products of the *E2A* gene. Similar to the MyoD factors, E2-proteins belong to the bHLH family. Dimerization between bHLH peptides involves joining the helix regions which evokes a conformational change in both participating proteins, bringing basic regions together, and creating an interface ready for recognition and binding of the E-box (Murre *et al.*, 1989). Although MyoD factors can bind DNA on their own, the affinity of such an interaction is ten-fold lower when compared to similar DNA binding by the MyoD and E2-protein complex (Weintraub *et al.*, 1991).

However, not all the members of the HLH group play the role of MyoD enhancers. Inhibition of myogenesis is in part attributed to an association of bHLH factors with another HLH member, inhibitor of differentiation (Id). This molecule displays helix-loop-helix structure, but lacks a DNA-binding basic region. Since it has a functional HLH domain, it can form heterodimers with E2-proteins. Subsequently, the

lack of a basic region on one side abolishes the ability of such a complex to recognize and interact with the E-box. Id proteins, if present in abundance, compete for E2-factors, and in this way heterodimerization partners for MyoD transcriptional factors become depleted. In agreement with this, high levels of Id factor have been detected in proliferating myoblasts, but as differentiation goes on, the expression of this protein becomes silenced (Jen *et al.*, 1992). Therefore, Id might contribute to retaining an undifferentiated phenotype in myoblasts.

The question regarding the purpose of extensive redundancy in the myogenic program remains unanswered. A complex view of interrelation among myogenic factors emerges where MyoD members can up-regulate each other's expression as well as its own.

The earliest factor appearing in somitogenesis is *myf-5*. The polymerase chain reaction technique detected *myf-5* mRNA expression even prior to the formation of a myotome. Myogenin and MyoD appearance follow shortly *myf-5*, and is accompanied by MRF-4 production which then becomes the most abundant myogenic factor in skeletal muscle (Ott *et al.*, 1991; Hannon *et al.*, 1992). Of interest are data that forced expression of *myf-5* in transgenic animals can result in ectopic formation of skeletal muscle tissue (Santerre *et al.*, 1993). All these findings argue that *myf-5* is the earliest muscle determination factor influencing expression of other MyoD members.

Genetic knockout experiments in mice revealed that despite an appreciable redundancy in the performance of the MyoD family regulators, each member seems to play a distinct role in myogenesis. Homozygous *MyoD* mutants demonstrated increased *myf-5* expression which led to normal formation of skeletal muscle (Rudnicki *et al.*, 1992). Likewise, transgenic mice with null mutations of the *myf-5* gene were able to form muscle tissue as well, perhaps under the influence of intact *MyoD* (Braun *et al.*, 1992). The remaining factors, myogenin and MRF-4, in *myf-5* null mice were also absent until the appearance of the MyoD protein which conceivably stimulated their expression (Braun *et al.*, 1994). However, in double knockout experiments, mice lacking both *MyoD* and *myf-5* showed no morphological signs of skeletal muscle, and no muscle messenger RNAs along with their encoded proteins were detected (Rudnicki *et al.*, 1993). Similar experiments in mice carrying mutations in the myogenin gene did not affect myotome formation, and skeletal myoblast appearance proceeded as usual. Nevertheless, the skeletal muscle formation was dramatically affected due to a disturbance in secondary myotube genesis. *MRF-4* knockout impedes morphogenesis of ribs, but does not interfere with the

generation of skeletal muscle (Braun and Arnold, 1995). These discoveries led to the more complex myogenesis concept in which MyoD and myf-5 are interchangeable, but either one is absolutely required for the commitment of embryonic mesodermal cells to a myogenic lineage. Myogenin emerged as an essential factor controlling later steps of myogenesis (Rudnicki and Jaenisch, 1995).

There are many more myogenic factors which do not belong to the MyoD nuclear regulators' family, but can co-operate in MyoD directed muscle induction through a different pathway than the one which involves E-box. One such representative is myocyte enhancer factor 2 (MEF-2). It is a member of the RSRF (related to serum response factor) family ubiquitously expressed in mammalian cells (Chambers *et al.*, 1992; Yu *et al.*, 1992). Structural muscle gene transcription is regulated by MyoD factor-dependent E-box binding. In addition, binding sites for MEF-2 regulator protein were detected in a few structural muscle genes (muscle creatine kinase, myosin light chain 1/3) as well, and it is most likely to participate in the activation of these tissue-specific genes (Gossett *et al.*, 1989). Some muscle-specific genes in cardiac and skeletal muscles are influenced mainly by MEF-2 site, but not E-box binding (Nakatsuji *et al.*, 1992). Furthermore, MEF-2 binding sites were found within the regulatory elements of MyoD members, myogenin and MyoD (Buchberger *et al.*, 1994; Kaushal *et al.*, 1994). Since these sites and other DNA binding sequences are within close range of each other, they might contribute to the stability of E-box-dependent binding (Buckingham, 1994). These data support the view that the muscle differentiation program is under much more complex control involving more nuclear transcription factors than those of the MyoD family alone.

Transcriptional Factors Contributing to Differentiation of Cardiac Myocytes: Similarities and Differences with Skeletal Muscle

Although both types of striated muscle (skeletal and cardiac) share many common features, their differentiation programs appear to embrace different pathways.

Contrary to skeletal muscle, where MyoD family transcription factors initiate the myogenic program, the developmental fate of cardiac myocytes seems not to be controlled by these myogenic regulators. Analysis of promoters responsible for determining the cardiac muscle phenotype supports this view. It was demonstrated that different regions

of the same promoter are required to produce identical contractile protein isoforms in cardiac myocytes as compared to skeletal muscle (van Bilsen and Chien, 1993). Studies performed so far demonstrated that MyoD proteins are not expressed in cardiac myocytes. Thus, there should be alternative ways for cardiac myocytes to achieve a morphological structure similar to that of skeletal muscle.

Some of the nuclear factors participating in the transcription of structural muscle genes are the same in both types of striated muscle. The MEF-2 factor is thought to play a role in the activation of the skeletal muscle program, accomplishing this by co-operating with the bHLH regulatory factors. The muscle isoform of phosphoglycerate mutase (PGAM) enzyme involved in glycolytic reactions in striated muscle is controlled solely by the MEF-2 factor in cardiac as well as in skeletal muscle cells (Nakatsuji *et al.*, 1992). In addition, MEF-2 participates in transcriptional regulation of cardiac structural genes, such as α -cardiac myosin heavy chain and β -myosin heavy chain (Adolph *et al.*, 1993).

Homeobox transcription factors which control embryo body plane specification, as well as migration, differentiation and proliferation processes in a variety of cells, are also involved in heart muscle genesis (McGinnis and Krumlauf, 1992; Niehrs *et al.*, 1993). Of interest is MHox/Phox homeobox protein which is involved in creatinine kinase M gene regulation, and activates this gene in cardiac and skeletal muscle cells through co-operation with myogenic HLH transcription factors (Cserjesi *et al.*, 1992).

Despite the lack of MyoD-dependent muscle regulatory pathway, some experimental data provide a hint for the possible involvement of basic HLH proteins in cardiomyocyte development. Complexes composed of the MyoD family members with E2-proteins bind DNA E-boxes which participate in the expression of the structural skeletal muscle genes. Similar complexes were found to be able to bind cardiac α -actin promoter, implying the existence of functional E-boxes within this cardiac regulatory sequence (French *et al.*, 1991). Another group of investigators demonstrated that antibodies raised against the MyoD1 helix segment reacted with an unknown protein expressed early in the developing chick heart. Moreover, this protein was able to recognize the muscle creatinine kinase DNA sequence containing an E box (Litvin *et al.*, 1993). Therefore, despite broad differences in regulatory mechanisms of myogenic programs, heart and skeletal muscles might have similar principles and share similar regulatory factors involved in the implementation of muscle formation.

Growth Factors and Differentiation of Striated Muscle

The development, growth and differentiation processes of both, cardiac and skeletal muscle cells are tremendously influenced and directed by the effect of the growth factors present in their environment. Experiments on myogenic cells in vitro suggest that a number of mitogens affect the developmental fate of myoblasts. Studies in the mid-1980s showed that crushed adult muscle is a potent mitogen for SCs (Bischoff, 1986). Chen and colleagues (1994) attempted to fractionate crushed muscle extract into potential mitogenic factors. They demonstrated that crushed mouse muscle extract contains such mitogenic factors as transferrin, basic fibroblast growth factor (bFGF), insulin-like growth factor I (IGF-I), platelet-derived growth factor (PDGF), and an uncharacterized heparin-binding factor which was able to stimulate muscle cell proliferation as well.

Fibroblast growth factors, both acidic (aFGF) and basic, are shown to stimulate myogenic cell division and repress or delay the terminal differentiation phase. Growth factors that stimulate DNA synthesis as well as protein and muscle-specific myosin accumulation in myogenic cell cultures have been purified from the avian skeletal muscle (Kardami *et al.*, 1985). This finding supports earlier reports about the release of myogenic growth factors from damaged skeletal muscle fibres (Bischoff, 1986). Since bFGF is highly mitogenic for adult SCs it is considered to play an important role in SC activation and regeneration of the skeletal muscle (Allen *et al.*, 1984). Experiments with a mouse muscle SC line (MM14) have shown that when MM14 myoblasts enter the G1 phase in the presence of bFGF, they replicate DNA without further expression of myosin heavy chain (Clegg *et al.*, 1987).

The cloning of the MyoD family enabled investigators to look at the influence of growth factors on the bHLH transcriptional activity. Experiments with myogenin and myogenin mutants revealed that bFGF exerts its influence on the MyoD family proteins through phosphorylation of threonine-87 and some other nearby located amino acids which compose the myogenic recognition motif. Although phosphorylation of this residue does not affect DNA binding, it completely abolishes the transcriptional potential of the myogenic bHLH proteins (Li *et al.*, 1992). In the same study it was demonstrated that inactivation of muscle-specific transcription factors by bFGF most likely occurs through involvement of protein kinase C (PKC). This kinase plays an important role in the transduction of signals from the cell surface to the nucleus (Bell and Burns, 1991; James and Olson, 1992).

Myoblasts grown in medium devoid of serum differentiate very rapidly (Davis *et al.*, 1987). It was postulated that differentiation in this case is related to the withdrawal of bFGF and other mitogens which act as suppressors of the transcriptional activity of MyoD members (Yutzey *et al.*, 1990; Brennan *et al.*, 1991b). Also, there are some data demonstrating that the inhibitory effect of serum might be mediated by Id protein (Benezra *et al.*, 1990).

Of particular interest are data that a growth factor strongly mitogenic for chicken skeletal myoblasts has been isolated from rat, chicken, sheep and cow atrial and ventricular heart tissue extracts. This mitogen has been identified as bFGF (Kardami and Fandrich, 1989). It was noted that bFGF was localized in close relationship with specific structures of cardiac myocytes, such as nuclei, basal membranes and intercalated discs.

Another member of the FGF family, aFGF, is also expressed in abundance in cardiac myocytes during embryogenesis. It has been shown that aFGF is produced by heart muscle cells themselves and deposited within the surrounding extracellular matrix where it can act in a paracrine fashion. Data demonstrating that aFGF expression is highest in the fetal to early neonatal ventricle supports its role in capillary angiogenesis, as well as ventricular maturation and remodeling in cardiac development (Engelmann *et al.*, 1993).

Transforming growth factor beta is known as a potent inhibitor of myoblast differentiation. When added to the myogenic cell culture it inhibits myoblast fusion into myotubes and other biochemical parameters of differentiation such as creatine kinase activity, appearance of acetylcholine receptors, synthesis of myosin heavy chain and skeletal α -actin (Florini *et al.*, 1986; Olson *et al.*, 1986). However, the inhibitory effect of TGF- β is a reversible one and, after removal of this growth factor from cell culture, myoblasts resume their characteristic morphology and fuse into myotubes. The process of fusion is accompanied by the loss of TGF- β receptors on myotubes (Ewton *et al.*, 1988). Therefore, it has been postulated that TGF- β inhibits the commitment to myoblast terminal differentiation, but once differentiation has occurred, TGF- β has no effect on the differentiated functions expressed by the myotube (Hu and Olson, 1990). The inhibitory effect of TGF- β on MyoD proteins, myogenin in particular, is thought to act through mechanisms which do not involve Id proteins nor DNA binding by muscle-specific regulatory factors. Brennan and colleagues (1991b) have raised the hypothesis that the inhibitory effect of TGF- β on muscle differentiation might be mediated through

action on a third, yet undetermined co-factor protein which is thought to participate in the formation of MyoD-DNA complex. Another possible way of inhibition of muscle differentiation could lead through TGF- β induced expression of inhibitory extracellular matrix molecules (Heino and Massague, 1990). Koff and colleagues (1993) have shown that TGF- β arrests cells in the G1 phase, and that its growth inhibition characteristic is related to the ability to interfere with cyclin-dependent protein kinases (cdks).

There are 3 isoforms of mammalian TGF- β and all of them are expressed in the heart. Cardiac myocytes express three TGF- β receptors in *Xenopus laevis*, and all three TGF- β s play a very important role in the organogenesis, induction of the mesodermal layer and, finally, the expression of function of the cardiac muscle cells (MacLellan *et al.*, 1993). It has been shown that TGF- β induces cardiac formation events in the explanted amphibian mesoderm (Muslin and Williams, 1991). Likewise, in murine ES-5 embryonic cells as well as in P19 embryonal carcinoma cells, members of TGF- β family were shown to induce formation of cardiac muscle (Slager *et al.*, 1993; van der Eijnden-van Raaij *et al.*, 1991). Administration of TGF- β antisense oligonucleotides was shown to interfere with endocardial cushion and valve formation (Potts *et al.*, 1991).

Hemodynamic load is known to induce hypertrophic growth of mature cardiac myocytes along with the fetal cardiac muscle isoform expression. It was shown that TGF- β plays an important role in the cascade of events initiated by the overload (Villarreal and Dillmann, 1992). Of interest are data revealing that in cardiac myocytes TGF- β activates the whole group of fetal cardiac genes, such as skeletal α -actin, β -myosin heavy chain, atrial natriuretic factor, and smooth muscle α -actin. However, contrary to that, TGF- β inhibits myogenic gene activation in skeletal myoblasts and halts these cells in the G1 phase of the cell cycle (Komuro and Yazaki, 1993; MacLellan *et al.*, 1993).

Eghbali *et al.* (1991) has demonstrated that cultured cardiac fibroblasts from adult animals acquired some muscle-specific properties when treated with TGF- β . In particular, they expressed sarcomeric α -actin mRNA and changed their shape from the typical star-like to the elongated one. Furthermore, it has been noticed that TGF- β treated cardiac fibroblasts exhibited decreased staining with anti-vimentin (an intermediate-sized filament present in nonmuscle cells originating from mesenchyme) as compared to the control non-treated cardiac fibroblasts.

Most cells produce TGF- β and have the receptors for its action. It has been shown that TGF- β acts on the cells through both autocrine and paracrine pathways. The hypothesis of indirect TGF- β effect involving an autocrine pathway has been supported by data presented by Battegay *et al.* (1990). He demonstrated that TGF- β action on human arterial smooth muscle cells has induced platelet derived growth factor (PDGF) synthesis. Furthermore, administration of anti-PDGF antibodies has led to the suppression of TGF- β mediated DNA synthesis. Thus, PDGF might play an important role by mediating the proliferation and differentiation events evoked by TGF- β . It is conceivable that TGF- β could appear after injury to skeletal muscle when platelets following degranulation release TGF- β (Assoian and Sporn, 1986).

Insulin-like growth factors type I (IGF-I) and type II (IGF-II) play a very important role in activation of the myogenic program and muscle cell proliferation as well as differentiation. C2C12 myoblasts transduced with the IGF-I gene driven by the skeletal α -actin promoter demonstrated enhanced expression of myogenic transcription factors as well as contractile proteins. This resulted in augmented myotube formation (Coleman *et al.*, 1995). In vivo administration of antibodies directed against IGF-I decreased the numbers of regenerating muscle fibres following injury (Lefaucheur and Sebillé, 1995).

In vitro studies showed that IGF type I implements its action through insulin receptor substrate 1 (IRS-1). This protein transmits a signal to molecules with SH2 domains and is able to activate Ras proteins downstream (Pruett *et al.*, 1995). The performance of IGFs type I and type II is modulated by insulin growth factor binding proteins (IGFBP) that are produced by differentiated myotubes and can inhibit the myoblast differentiation process initiated by either of the IGFs (Bach *et al.*, 1994; Silverman *et al.*, 1995). Ewton and colleagues (1994) showed that IGF-I has a greater mitogenic activity than IGF-II and insulin. It was also noted that both mitogenic and myogenic actions of IGF-I are moderated by the IGF type I receptor. Authors also suggested that IGF-I is a more potent mitogen than a stimulator of myogenesis.

IGF type II accumulates during myoblast differentiation. This growth factor has the ability to down-regulate the IGF-I receptors and to blunt in this way the mitogenic effect of IGF-I, thus diverting muscle cells towards differentiation pathway (Rosenthal and Brown, 1994). High concentrations of serum inhibit myogenesis most likely by

blocking autocrine secretion of IGF-II (Magri *et al.*, 1994). Therefore, it appears that the IGF-I receptor can mediate action of both types of IGFs.

Extracellular Matrix and Muscle Differentiation

The extracellular matrix (ECM) provides a substrate for cell attachment as well as signal transduction through specific receptors to a cell. Likewise, spatial and temporal expression of different components of ECM is thought to dramatically influence organogenesis. Griffith and Sanders (1991) have demonstrated that multipotent mesenchymal cells derived from chick tail bud can differentiate *in vitro* into a variety of tissues if exposed to various ECM components. It was shown that collagen type I was able to promote both, myogenesis and chondrogenesis in undifferentiated mesenchymal cells. Collagen type IV, on the other hand, directed cells into the myogenic lineage only. Laminin was shown to promote differentiation of neural crest cells into neurons, melanocytes and neuroepithelial cells.

Some authors suggest that muscle basement membranes and their components serve as scaffolds for regeneration of injured skeletal muscle by SCs (Alameddine *et al.*, 1991). Other studies, in concert to the former, demonstrate that in degenerating and regenerating muscle fibres, type IV collagen and laminin are preserved on the basement membrane, in spite of the muscle fibre phagocytosis (Narukami *et al.*, 1991).

Laminin is an important glycoprotein of the muscle basal lamina. It has been shown that cultured myoblasts on laminin migrate much faster than the ones grown on fibronectin or type I collagen (Ocalan *et al.*, 1988). Experiments with MyoD transduced fibroblast cell lines revealed that laminin secretion by these cells has a temporal relationship with the expression of myogenic transcription factors (Kroll *et al.*, 1994). Laminin is an approximately 800kDa glycoprotein composed of three subunits- one heavy and two light chains. So far there are 5 subunits found which can form 4 heterotrimer-combinations of laminin (Engvall, 1993). Electron microscopy revealed that laminin has a cross-shape with three short arms and one long arm. Each arm has an end globular domain (Cioce *et al.*, 1993). It was established that the region located on the long arm near to the joint of all four arms is crucial for *in vitro* cell attachment to laminin (Cioce *et al.*, 1993). Experiments *in vitro* with blocking antibodies against the globular (G) domain have shown that this domain plays a central role in promoting myoblast adhesion

and spreading. However, it is not involved in laminin self-assembly (Yurchenco *et al.*, 1993).

Immunofluorescence studies unveiled that most of the basement membranes have only one tissue-specific subunit of laminin. A member of the laminin family, merosin, was shown to be present only in the basal lamina of skeletal and cardiac muscles (Engvall *et al.*, 1990). Immunofluorescence studies revealed that laminin colocalizes within the muscle basement membrane with another important muscle protein, dystrophin, which was implicated in protecting muscle fibres from damage and degradation (Dickson, 1992).

Collagen type IV is one of the main composites in the basement membrane. Three intertwined polypeptide chains, classically, two $\alpha 1(\text{IV})$ and one $\alpha 2(\text{IV})$, with the globular (G) domain constitute a single collagen type IV monomer. These molecules are prone to dimerization. The dimers form a branching collagen network by joining to each other at the globular domains (Brown and Timpl, 1995). Currently there are 6 collagen isoforms known which have a tissue-specific arrangement (Hudson *et al.*, 1993). Isoforms of type IV collagen, $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$, have been found localized in the basement membrane of muscle fibres almost exceptionally at neuromuscular synapses. Other areas of the basement membrane did not show expression of these particular isoforms. Ubiquitous $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ isoforms were present in much smaller quantities at the neuromuscular junction as compared to other areas of muscle basement membrane (Sanes *et al.*, 1990). Non-muscle cells are also capable of producing basement membrane components. In particular, cardiac fibroblasts manufacture type IV collagen (Eghbali, 1992).

Collagen type VIII was found to be expressed during cardiogenesis in mice and it was implicated to be involved in the differentiation of cardiac myocytes and the formation of cardiac valves (Iruela-Arispe and Sage, 1991).

The family of proteoglycans is another ubiquitous component of basal laminae. The most extensively examined group of proteoglycans is heparan sulfate proteoglycans (HSPG). Together with collagen IV and laminin they form support structures in basement membranes. Furthermore, HSPG act as ligands for laminin (Yurchenco and O'Rear, 1993). It has been shown that HSPG are present not only in the ECM but are expressed on the cell surface as well, where they act as an integral part of FGF receptors (Olwin *et al.*, 1994).

Entactin (or nidogen) is also a composite part of basement membranes. It is a small glycoprotein that is anchored to one of short arms of laminin. A putative role for entactin is bridging and forming stable complexes of laminin and collagen IV, as well as laminin and HSPG in the basement membrane (Aumailley *et al.*, 1993).

Tenascin is a six-armed glycoprotein which constitutes a part of basement membranes as well. It is thought to be involved in organogenesis during fetal development. A novel member of tenascin family, tenascin-X, is predominantly expressed in both types of striated muscle and is shown to be associated with the ECM (Matsumoto *et al.*, 1994).

Fibronectin is an ECM glycoprotein composed of two chains. The fibronectin molecule has three variably spliced functional domains. The central domain containing RGDS adhesive sequence can bind to the $\alpha_5\beta_1$ integrin which is expressed on fibroblasts, monocytes and other cells of mesenchymal origin. The IIICS variable domain binds cells expressing $\alpha_4\beta_1$ integrin (Limper and Roman, 1992). This particular integrin is expressed on myoblasts, and blocking of this integrin results in impaired fusion of muscle cells (Rosen *et al.*, 1992). Cells of mesenchymal origin, including fibroblasts, synthesize and secrete fibronectin. The ECM sites rich in fibronectin serve as guides for migrating embryonic cells during organogenesis. In adult mammals production of fibronectin is increased following injury to tissues. Therefore, it appears that fibronectin plays an important role in the tissue regeneration process (Mosher, 1984).

Expression of another ECM component, thrombospondin, which is absent at birth, progressively increases during the first month of neonatal life in rodent endomysium. Muscle cells in vitro manufacture thrombospondin only at the myotube stage, which might correlate with the expression of the myogenic program (Hantai *et al.*, 1991).

Many of the cell receptors for the ECM glycoproteins belong to the integrin family. Integrins are found not only on the cell surface, which is in contact with the ECM proteins, but also at the cell-cell interface. They mediate interaction of ECM components with the actin cytoskeleton of cells (Albeda and Buck, 1990). Of particular interest are integrins from the β_1 subgroup expressed on the surface of myoblasts. Alpha7 beta1 was identified on the surface of differentiating muscle cells and has been shown to bind laminin. The L-14 lectin (lactoside-binding protein) exists as a dimer and has been

localized on a variety of cells, in association with the ECM. Affinity chromatography and immunoblotting revealed that $\alpha_7\beta_1$ binds to fibronectin and to L-14 as well (Gu *et al.*, 1994). Following myoblast differentiation and fusion in vitro, L-14 is secreted by muscle cells into the extracellular space where this protein associates with laminin in the myofibre basement membrane. The presence of L-14 inhibits spreading and fusion of myoblasts seeded on laminin. Moreover, L-14 through binding to laminin abolishes cell-substratum adhesion. Therefore, this lectin, by competing with $\alpha_7\beta_1$ integrin for bonds with laminin, appears to induce myoblast detachment from laminin during differentiation. This process might facilitate myoblasts fusion into myotubes (Cooper *et al.*, 1991). Reverse transcriptase-polymerase chain reaction revealed the existence of alternatively spliced α_7 integrin mRNA in rodent skeletal and cardiac muscle (Ziober *et al.*, 1993).

Another member of integrin family, $\alpha_6\beta_1$ is expressed on myotomal precursor cells during somite division into the dermomyotome and sclerotome. This integrin remains on myoblasts and differentiated myofibres at all stages of muscle development. Laminin has been shown to be the ligand of the $\alpha_6\beta_1$ integrin (Bronner-Fraser *et al.*, 1992).

Integrin $\alpha_4\beta_1$ and its ligand, vascular cell adhesion molecule 1 (VCAM-1), are expressed on secondary myoblasts and are implicated in the formation of secondary myotubes. Satellite cells that lay on the mature myofibre retain expression of VCAM-1 even after the formation of muscle fibres (Rosen *et al.*, 1992).

Integrin $\alpha_5\beta_1$, similarly to other members of the integrin family, is also developmentally regulated during myogenesis. Enhanced binding of this integrin and its ligand was shown to interfere with the cell migration and morphogenesis of myotubes (Boettiger *et al.*, 1995). Following differentiation of the muscle cells the localization pattern of this integrin is changed which permits muscle cells to acquire structural changes as myogenesis proceeds (Enomoto *et al.*, 1993).

Other cell adhesion molecules, such as neural cell adhesion molecules (N-CAM), are found at cell-cell contacts as well. These molecules moderate cell-cell adhesion in a calcium-independent pathway. Neural cell adhesion molecules are detected in somites very early, even prior to formation of the myotome compartment. Cells in myotomes as well as adult myoblasts express two major isoforms on myoblasts; the 180-kD and 140-

kD transmembrane N-CAM isoforms (Lyons *et al.*, 1992). Overexpression experiments showed that both isoforms promote myoblast fusion (Peck and Walsh 1993).

Cell-cell interaction during embryogenesis is regulated tremendously by cadherins, which are calcium dependent cell adhesion molecules. Cadherins display a homotypic molecular recognition mechanism. One particular member, M-cadherin, has been isolated from myoblasts and has been shown to be upregulated in the differentiating myotubes (Donalies *et al.*, 1991). During embryogenesis it is localized in the somites and confined only to the desmin positive (i.e., muscle) cells. Temporal expression of M-cadherins shows that this cell adhesion molecule appears simultaneously with myogenin and cardiac α -actin. M-cadherin production is down regulated after birth. However, this protein reappears on regenerating myoblasts and fades away after myotube formation (Moore and Walsh, 1993). Double immunostaining of developing myoblasts revealed that M-cadherins are localized in cell-cell contact areas, whereas laminin positive areas, i.e., areas of cell contact with extracellular matrix, were devoid of M-cadherins (Rose *et al.*, 1994).

Another member of the cadherin family, N-cadherin, also plays a role in the development and innervation of skeletal muscle fibres. It is suggested that N-cadherin might mediate fusion of myoblasts into primary myotubes. This molecule also participates in the development and stabilization of synapses on muscle fibres. Recently, it was described that N-cadherin appears not only on the entire surface of denervated muscle fibres, but in the basal lamina and ECM as well (Cifuentes-Diaz *et al.*, 1994). Therefore, it appears that various cell adhesion molecules mediate myoblast self-recognition events at very early stages, as well as at much later processes of myofibre formation.

EXPERIMENTAL ATTEMPTS TO RESTORE LOST CARDIAC MUSCLE TISSUE

Molecular Cardiomyoplasty: Gene Transfer into Cardiac Cells

It is an attractive idea instead of replacing the whole heart with a mechanical device to bring back to life contractile heart cells lost to disease. Some attempts in this field concentrate on the awakening of myocardial ability to re-enter the cell cycle, and thus create a larger pool of muscle cells. There is a strong feeling in the research community that cardiac myocytes have not lost their competence for cytokinesis, but this feature is

under the suppressive control of yet undiscovered factors. Experiments with transgenic mice demonstrated that under specific conditions it is possible to induce cytokinesis of some myocardial cells in vivo. The simian virus 40 (SV40) large tumour antigen gene, which has a capacity to stimulate DNA replication (Fanning and Knippers, 1992), was coupled to a promoter sequence of atrial natriuretic factor that is constitutively expressed in atrial myocytes. Following the introduction of this genetic construct into fertilized mice eggs, investigators obtained mice that developed atrial hyperplasia. Morphologically, muscle cells looked similar to the normal atrial myocytes, and there was no local tumour outgrowth (Field, 1988). Similar results in rat ventricles were obtained when ventricular myocytes were targeted with another genomic construct producing SV40 protein (Katz *et al.*, 1992). Although these experiments have demonstrated that cardiac muscle cells can be induced to enter the cell cycle, the possibility of uncontrolled growth of oncogene-stimulated myocytes poses a serious risk for a patient and therefore, some other methods to activate a replication program should be sought.

Another direction of research concentrating on revitalizing heart muscle has been called "molecular cardiomyoplasty". The idea of this approach is to turn back the altered ratio of connective-to-muscle tissue in favour of cardiac muscle cells. With the discovery of the myogenic regulatory factors, there has been much interest in the introduction of myogenic master genes into non-muscle cells of the heart. If successful, such an attempt would transform non-contracting cells into desirable contractile tissue. In vitro studies have already been done where the MyoD gene coupled to a retroviral vector sequence was introduced into primary cardiac fibroblasts obtained from newborn rat ventricles. Non-muscle cells transduced by this method altered their shape into an elongated one, and following serum withdrawal, fused and formed multinucleated myotubes, a morphological feature specific to skeletal muscle. Immunocytochemical evaluation of the MyoD-converted cardiac fibroblasts demonstrated the appearance of MEF-2, another myogenic transcription factor, as well as expression of myosin heavy chain protein (Tam *et al.*, 1995). Despite the promising results, some questions regarding this approach still remain. There is no knowledge yet of which genes play master roles in directing the cardiac myogenic program. Therefore, there is no suitable candidate yet that can be used for cardiac fibroblast conversion into cardiac muscle cells. Furthermore, a gene delivery system targeting only non-muscle cells has yet to be developed.

Cellular Cardiomyoplasty: Muscle Cell Implantation into the Heart

The "cellular cardiomyoplasty" procedure has the goal to supply cardiac muscle with myocytes derived from outside the heart. It is hoped that these muscle cells will eventually be able to contribute to the contractile performance of the failing organ.

Since fetal cardiac tissue is still in the stage of vigorous cell cycling, it offers the prospect of being used as a source of neocardiomyocytes. Indeed, Field's group, working with mice embryonic cardiac muscle cells, brought a true excitement into the field of myocardial regeneration (Soonpaa *et al.*, 1994). They showed that fetal cardiac myocytes grafted into adult mice formed stable long-term implants. Moreover, electron microscopy revealed that implanted cardiomyocytes joined host muscle cells by intercalated disks, thus establishing the likelihood that the implanted fetal myocytes may join the myocardial electrical network. Although technically this approach is very promising, it has serious drawbacks for clinical use, such as the need to obtain human embryonic cardiac tissue and the potential for immunorejection.

To avoid the problem of implant tissue availability, myogenic cell lines have also been employed in attempts to restore cardiac muscle. The AT-1 cell line is a cardiac muscle cell line derived from SV40-transformed atrial myocytes. When implanted into syngeneic rat myocardium, these cells have developed into structural cardiac muscle. However, as measured by radioactive thymidine uptake, AT-1 cells retained the ability for uncontrolled proliferation and were not coupled through gap junctions to the syncytium of host cardiac myocytes (Koh *et al.*, 1993b). Similarly, a mouse SC line (C2C12) was also used in pursuing the objective of restoring cardiac myocytes. Of interest is that these transformed muscle cells formed stable muscle grafts within syngeneic mice myocardium for a prolonged period of observation (3 months). Cells expressed skeletal myosin heavy chain isoform and, following myotube formation, seemingly stopped dividing. Although C2C12 cells were juxtaposed to the host cardiac myocytes, there was no evidence of electrical coupling to the native myocardium (Koh *et al.*, 1993a). The finding that skeletal muscle cells can survive within the heart is encouraging, and it clearly demonstrates that both types of striated muscle can coexist in the same tissue. Nevertheless, it is more desirable to use primary muscle cells for the intramyocardial implantation since, contrary to the myogenic cell lines, they are devoid of risk of developing into tumourigenic tissue (Rando and Blau, 1994).

The third approach in cellular cardiomyoplasty is implantation of SCs, which are primary myogenic cells. This approach takes the advantage that SCs are undifferentiated stem cells of myogenic lineage. The cellular cardiomyoplasty stream examines the possibility for SCs to alter their phenotypic expression under the influence of the myocardial milieu and to become a source of neomyocardium.

Chapter III

HYPOTHESIS AND EXPERIMENTAL DESIGN

MYOCARDIAL REPAIR HYPOTHESIS

Adult cardiac muscle possesses virtually no regenerative ability after injury. Contrary to myocardium, SCs have the main purpose to restore skeletal muscle fibres there is an injury. Therefore, we investigated the proposed hypothesis that SCs when implanted into the injured myocardium can survive and repair it. Furthermore, we hypothesized that under a cardiac environment influence, implanted SCs could transdifferentiate and assume a cardiomyocyte-like phenotype that could enable them to join native cardiomyocytes in their contractile performance.

INTRODUCTION TO EXPERIMENTAL DESIGN

To test this hypothesis, we employed the following experimental design. Two similar studies were performed on dogs (n=31) and isogenic rats (n=64). In the dog study, 28 animals were allocated to the experimental group and 3 served as a control. Each study was divided into several consequent steps, namely, skeletal muscle extirpation from donor animals' legs, SC isolation from procured skeletal muscle tissue, SC plating and maintenance in culture dishes in vitro, SC labelling in vitro, cultured and labelled SC implantation into freshly cryo-injured myocardium of the same host animal they were obtained from. Following a certain period of time post-implantation, animals were sacrificed and hearts with SC implants were processed for gross, histological and histochemical evaluation of cell graft sites.

All animals received humane care, and all experiments were performed according to "Guide to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care. All experiments on animals received an approval from the Animal Care Committee of McGill University.

EXPERIMENTAL DESIGN OF A STUDY ON DOGS

Operative Techniques Used in Experiments on Dogs

Skeletal Muscle Procurement

Under general anesthesia and mechanical ventilation, an incision was performed over the anterior tibial muscle. The muscle was excised under aseptic conditions and placed in ice cold normal saline solution before undergoing SC isolation. The operative wound was closed in layers according to established surgical techniques. For the post-operative observation and care, animals were placed in the allocated post-operative room for the first 24 h period. Four to 6h post-operation, the animals were extubated. Adequate analgesia was provided every 6-8h. The animals showed no appreciable handicap following the recovery period.

Satellite Cell Implantation Technique

Cryo-injury followed by SC implantation into the injured myocardium was done according to Kao (1989). Briefly, under general anesthesia, a left lateral thoracotomy approach was used to expose the left ventricle (LV) in 21 dogs. Cryo-injury on the LV free wall was inflicted by a cryoprobe cooled to -160°C which was applied to the myocardium for 20 to 25min. Autologous SCs were collected from culture dishes and implanted with a 16 gauge intravenous teflon catheter (Criticon Inc., Tampa, FL) into acutely cryodamaged heart muscle. The number of implanted cells ranged from 1.0 to 12.5×10^6 (mean- 5.1×10^6) which were resuspended in 0.5ml of M199 solution (Addendum 1). A 6-0 prolene suture (Ethicon Ltd., Peterborough, Ont.) was placed within the implantation channel to mark the SC graft site. Entrance and exit apertures of the channel were closed with 2 separate 4-0 prolene sutures in a purse-string fashion.

In 3 animals allocated to the control group, a sham implantation into cryo-injured myocardium was accomplished with M199 solution void of cells. Closure of the wound in both animal groups was done in a surgically established manner.

Procurement of Heart Specimens with Satellite Cell Implants

The animals were sacrificed at 2 (n=6), 4 (n=16) and 6 (n=6) weeks following intramyocardial SC grafting. The control group animals (n=3) were also sacrificed at 2 (n=1), 4 (n=1) and 6 (n=1) weeks.

Under general anesthesia, a midline sternotomy approach was used to expose the heart. Just prior to the removal of the heart, the animals were overdosed with pentobarbital administered intravenously. Venous blood return was then impeded by snaring the superior and inferior venae cavae along with the azygous vein. When the heart was empty, the ascending aorta and the pulmonary trunk were clamped. Immediately thereafter, intracoronary perfusion of the heart with an ice-cold isotonic solution supplemented with 80mEq of potassium was initiated. Both auricles of the atria were cut open to decompress the heart and allow cardioplegia solution to fill the arterial coronary system. When full cardioplegia was achieved, the cardioplegia solution was substituted with cold Karnovsky's fixing solution. Such intracoronary fixation of the heart ensured adequate and prompt fixation of the whole cardiac muscle. Following intracoronary fixation, the SC implant areas were identified by a prolene suture left during the SC grafting procedure as a marker.

Techniques Used for Manipulation of Satellite Cells in Vitro

Satellite Cell Isolation from Skeletal Muscle Technique

Satellite cells were liberated from extirpated skeletal muscle through a few denoted sequential steps described bellow. The muscle was minced mechanically and processed with proteolytic enzymes [1% collagenase, 0.2% type 1-S hyaluronidase and 1% pronase (all from Sigma, St. Louis, MO)] to release the SCs contained on myofibres by the basal lamina. After brief digestion periods, proteolytic enzymes were quenched by fetal bovine serum (Gibco, Grand Island, NY) to prevent damage to the SCs. Following the SC release, supernatants containing cells were subjected to a few centrifugation steps with the goal of removing debris from the cells. Washed and resuspended cells were counted on a hemacytometer and the proportion of viable cells was assessed. Cell viability, as judged by the trypan blue exclusion test, was approximately 84% (Addendum 2). The number of required culture dishes was calculated according to the obtained cell quantity and

respecting an initial plating density of $5.0-7.5 \times 10^5$ cells per 60mm diameter tissue culture dish (Corning Inc., Corning, NY) with a cell culture area of 21cm^2 . Cell growth medium (GM) change was carried out every 24-48h.

Satellite Cell Isolation, Culture and Labelling Techniques: Differences from Previously Employed Protocols

Myogenic Cell Culture Enrichment Techniques

The earlier myocardial repair studies performed in the Surgical Research lab at The Montreal General Hospital Research Institute did not focus on the issue of increasing the SC yield in cell culture prior to initial plating (Marelli *et al.*, 1992). Nevertheless, it is likely that SC culture purity is a valid aspect in the myocardial repair project.

To increase the relative number of SCs in the initial culture, two additional techniques which addressed this issue were adopted for the previously described protocol of SC isolation.

The first technique is known as a density centrifugation method which was shown to be effective in SC culture purification in chicken (Yablonka-Reuveni and Nameroff, 1987). This method is based on a differential mass and size of cells.

In brief, a cell suspension is layered on the viscous 20% Percoll (Sigma, St. Louis, MO) which itself rests on an even more concentrated (60%) Percoll layer. The cells layered in this way are centrifuged at $15,000g$ for 5min. Erythrocytes, which are the smallest cells in this cell suspension, travel down to the very bottom of a test tube during centrifugation. The movement of large non-muscle cells as well as muscle fragments is hindered by the viscous Percoll solution. Therefore, they remain on the top of a 20% Percoll layer. It has been shown that the highest proportion of the SCs can be recovered from the 20 and 60% Percoll concentrations interface (Yablonka-Reuveni *et al.*, 1987). In our experiments, SCs collected from this interface were pooled and used for further propagation on laminin-coated (Sigma, St. Louis, MO) culture plates (Fig.1).

A second step, which was used for SC culture enrichment, was done according to Jones (1979). Before the final seeding SCs were preplated on collagen-coated culture

dishes at 37°C in an atmosphere of 5% CO₂ air. After 20min, all non-attached cells were gently collected and transferred onto laminin-coated culture dishes which were maintained in a 37°C humidified atmosphere of 95% air with 5% CO₂.

To evaluate the effectiveness of the 2 methods for myogenic cell enrichment, the quantity of myotubes was compared among the Percoll density gradient pre-treated, preplated and control SC cultures. The amount of myogenic cells within these groups was estimated by counting visible myotubes in 5 randomly chosen high magnification fields of 7 day-old non-passaged cultures (5 culture dishes x 5 fields = 25 fields in each group).

Satellite Cell Labelling with Recombinant Plasmids

To label SCs, we used the lipofection method described by Felgner *et al.* (1987). Cultured SCs were labelled with a recombinant DNA plasmid pCMVlacZ which was generously provided to us by Dr. L.A. Culp (Lin and Culp, 1991). This genetic construct is of 10.9kb in size and has two genes of interest embodied in its DNA. One genetic sequence is known as the *lacZ* gene, which is an *E. coli* gene coding for bacterial β -galactosidase (β -GAL). This gene has the role of a marker gene since its product can be readily detected by a specific histochemical reaction. A second gene, *neo^R*, is a representative of bacterial genes situated on transposons Tn601 and Tn5. The presence and expression of *neo^R* confers cell a resistance against cytotoxic aminoglycoside antibiotic G418, also known as Geneticin®. The *lacZ* gene in this plasmid is driven by the cytomegalovirus (CMV) promoter and the *neo^R* gene by the simian virus 40 (SV40) early promoter (Fig.2).

To obtain a sufficient amount of plasmids for cell labelling, *E. coli* bacteria harbouring these recombinant DNA plasmids were multiplied. Then plasmids were released from bacteria and collected with the use of a QIAGEN plasmid kit (QIAGEN, Germany).

On day 4 following SC initial plating cells were lipofected with the pCMVlacZ plasmids. SC transfection was done according to the manufacturer's (Gibco BRL, Grand Island, NY) guidelines. In brief, cultured SC labelling was accomplished when cells reached 30 to 40% confluence. For each tissue culture dish (60mm in diameter), 1 to 2 μ g

of plasmid DNA was diluted into 100µl of serum-free medium. Similarly, 20µl of Lipofectin®, a synthetic cationic liposome formulation (Gibco BRL, Grand Island, NY), was diluted within 100µl of serum-free medium. Then both solutions were combined and kept at room temperature for 10-15min. During this period, positively charged liposomes spontaneously formed complexes with negatively charged DNA (Felgner *et al.*, 1987). Following this, SC culture was overlaid with the mixture and incubated for 18h to allow cells to uptake DNA.

Seventy-two hours following the transfection, cytotoxic antibiotic G418 (Gibco BRL, Grand Island, NY) was added to the final concentration of 400µg/ml to the growth medium of the SCs. This aminoglycoside-related antibiotic is known to interfere with the function of 50S and 80S ribosome subunits, thus blocking protein synthesis in eukaryotic cells. Cells which do not possess *neo^R* gene in their DNA are subjected to the toxic effects of G418 (Hanchett *et al.*, 1992).

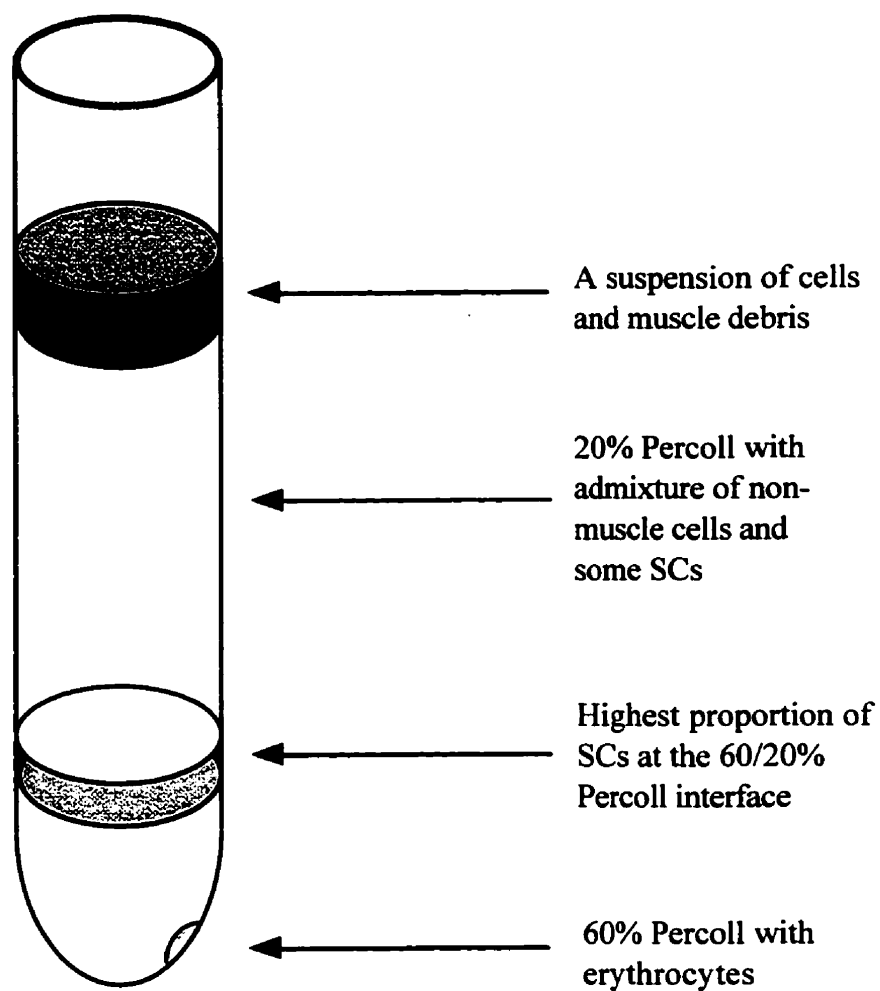


Fig. 1.
Schematic drawing of a tube containing Percoll column
composed of 20 and 60% concentrations

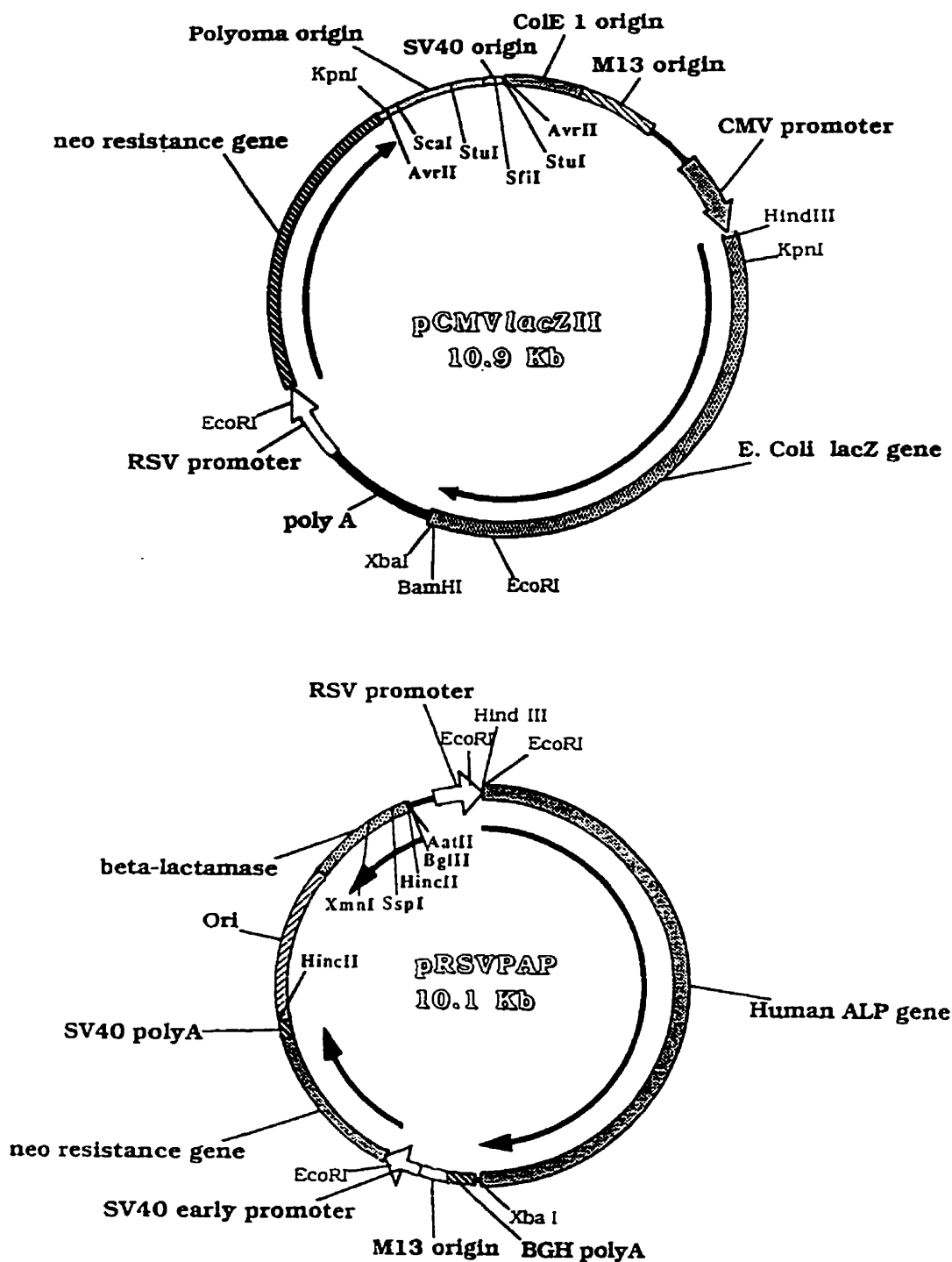


Fig.2.

Structural maps of pCMVlacZ and pRSVPAP plasmids used for SC labelling.
Courtesy of Dr. L. A. Culp.

Modifications of Cell Culture Conditions Implemented by the Satellite Cell Labelling Technique

The use of a DNA plasmid-based marker introduced a few alterations from the SC culturing protocol utilized by previous investigators in Dr. Chiu's lab. For the SCs to efficiently uptake plasmid DNA, we left transfected cells undisturbed for the first 3 days after the transfection. Moreover, to avoid mechanical damage of cells which were subjected to selection medium we chose to retard SC passaging to 4 day intervals. Even when transfection was done on 30 to 40% confluent cells, such modification of the protocol resulted in a higher degree of SC confluence prior to implantation as compared to cell confluence in previous experiments.

After 2 weeks of selection, surviving SCs were collected by brief trypsinization and centrifugation at 775g for 10min. The cells were then resuspended and the number of recovered cells was counted before they were implanted shortly thereafter into acutely cryo-injured myocardium.

Specimen Staining with X-Gal and Preparation for Histology

Satellite cell implant sites along with immediate adjacent areas were sectioned into approximately 3mm-thick slices and fixed for 4-6h duration in Karnovsky's solution at 4°C. Following the fixation, the specimens were rinsed with ice-cold PBS and stained according to Sanes *et al.* (1986) to reveal the product of the *lacZ* marker gene. Briefly, the muscle tissue was submersed in a stain solution composed of 20mmol/l potassium ferricyanide, 20mmol/l potassium ferrocyanide, and 2mmol/l MgCl₂ (Fisher Scientific, Fair Lawn, NJ). To increase tissue permeability for these reagents 1/100 volume of 2% Nonidet P-40 and the same volume of 1% sodium deoxycholate (Sigma, St. Louis, MO) was added to the stain solution. To provide a substrate for β -Gal, 1/40 volume of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Gibco BRL, Grand Island, NY), also known as X-Gal, was admixed to the stain solution. Tissue specimens were stained in this compound at 30°C overnight. The following day tissue samples were brought to the Department of Pathology of The Montreal General Hospital for routine histology. Hematoxylin-Eosin as well as Masson trichrome stains were used to delineate the morphological structure of the examined tissue. Specimens obtained from sham implants were processed in an identical manner as described above.

Techniques for Transfected Satellite Cell Staining In Vitro

Detection of cultured SCs exhibiting the β -Gal marker was done by cell culture staining with X-Gal as described above. The only difference between these 2 techniques was the length of the fixation period. Cultured cells were fixed for only 10min before staining with X-Gal. Following the staining procedure, labelled cells were identified under light microscopy by their particular bluish-green colour. Fixed and stained SC culture was stored in 0.02% sodium azide at 4°C.

EXPERIMENTAL DESIGN OF A STUDY ON RODENTS

There were 3 experimental groups formed which comprised a total of 64 inbred Lewis (LEW/CrlBR) rats weighing 275-300 g. Subjects in the first group were selected as donors (n=30) of skeletal muscle SCs. Rats in the second group were recipients (n=30) of SC implants. The recipient group was further subdivided into 2 subgroups (n=15 each) as described below. Skeletal muscle obtained from rats allocated to the third group (n=4) was used for an in vitro study, the goal of which was to explore the possibility of using human placental alkaline phosphatase as a SC marker (Fig.3).

Cultured SCs were implanted into the myocardium of animals allocated to the SC recipient group. To investigate the influence of different environmental conditions upon the differentiation of implanted SCs, the recipient group was further subdivided into 2 subgroups.

In the first subgroup, further referred as an "acute injury" subgroup, the SCs were implanted into the myocardium during the first thoracotomy procedure, immediately after the heart was damaged by the cryoprobe. Animals designated to the second subgroup, termed as a "mature scar" subgroup, underwent 2 thoracotomy procedures. During the first operation, a cryo-injury upon the myocardium was accomplished. The animals were then allowed a 1 month recovery period, during which their injured hearts healed by scarring. Following this quiescent period, the rats were subjected to a second thoracotomy during which the SCs were grafted into an already formed myocardial scar.

All operative procedures were performed under aseptic conditions and general anesthesia. Four weeks following SC implantation into either the acutely cryodamaged myocardium ("acute damage" subgroup) or the homogeneous myocardial scar ("mature scar" subgroup), the experimental animals were sacrificed under general anesthesia. Hearts with the cell implant sites were histologically evaluated for the presence of myofibres within the SC graft sites.

All animals received humane care, and all experiments were performed according to "Guide to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care. All experiments on the animals received an approval from the Animal Care Committee of McGill University.

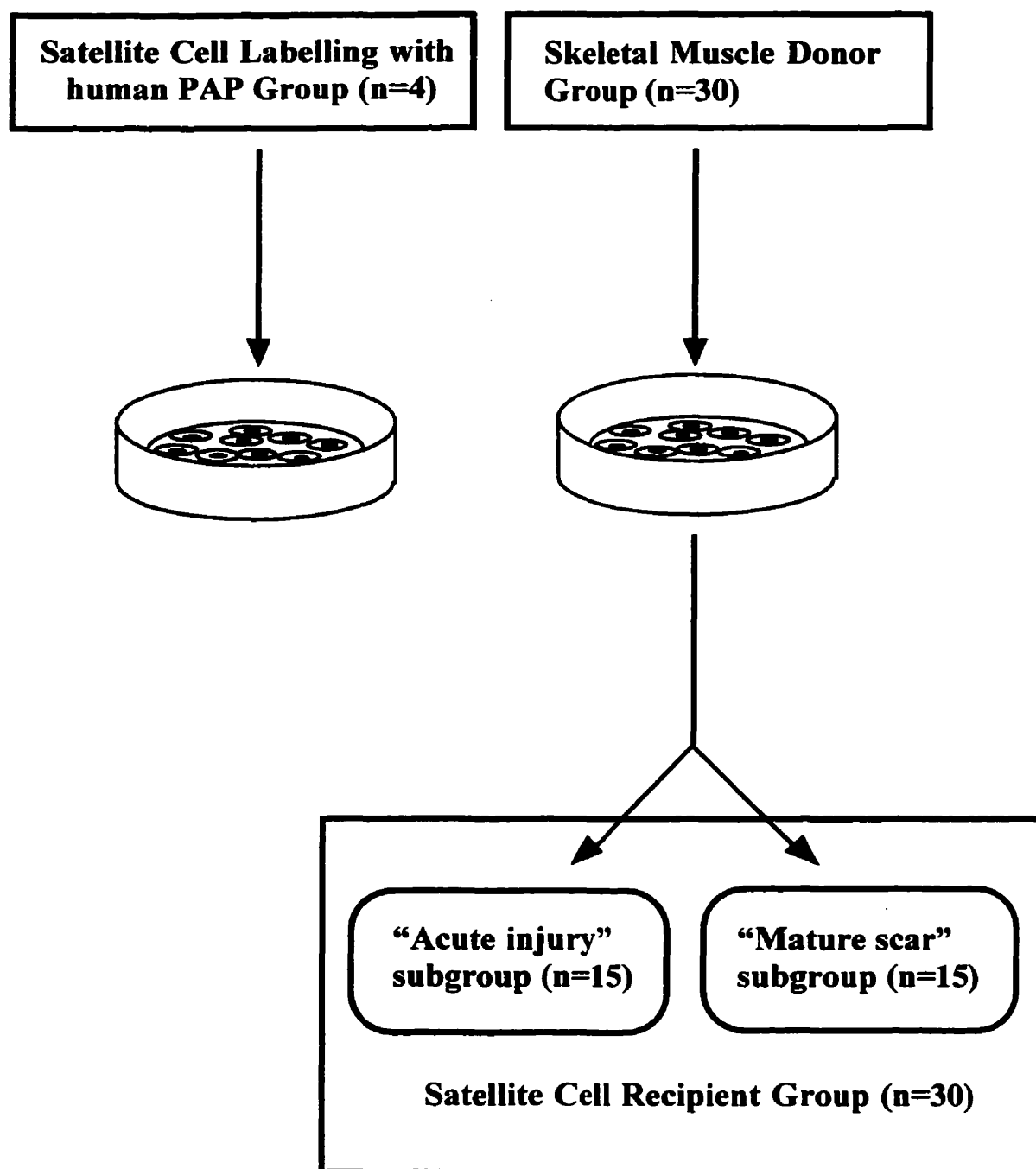


Fig. 3.

An experimental design of the rodent study

Operative Procedures on Rats

Skeletal Muscle Injury with Bupivacaine Technique Prior to Muscle Extirpation

To increase SC yield, skeletal muscle prior to its extirpation was subjected to myonecrosis caused by the local anesthetic bupivacaine. Briefly, skeletal muscle donor rats were premedicated with atropine sulfate (0.04mg/kg S.Q.) and general anesthesia was induced with ketamine (90mg/kg) and xylazine (5-8mg/kg) intraperitoneally. This was followed by several intramuscular bupivacaine-hyaluronidase injections. A mix of 0.5ml of 0.5% bupivacaine and 15IU of hyaluronidase was prepared immediately before injection. Femoral biceps, gastrocnemial, and anterior tibial muscles were injected bilaterally with this solute. The procedure was administered for 3 consecutive days before skeletal muscle removal. Twenty rats out of 30 allocated to the skeletal muscle donor group were treated with the bupivacaine-hyaluronidase mix. The skeletal muscle from the remaining 10 rats was obtained without the induction of myonecrosis.

Procurement of Rodent Skeletal Muscle as a Source of Satellite Cells

The skeletal muscle donor rats were anesthetized as described above. The chest and all four limbs were shaved and the operating field was then prepared subsequently with providone-iodine topical solutions. Incisions on the skin were performed under aseptic conditions to expose the latissimus dorsi, brachial triceps, femoral biceps, gastrocnemius, and anterior tibial muscles bilaterally. Bleeding from the incision sites was stopped with high temperature cautery. Major arteries and veins supplying the above mentioned muscles were divided and ligated with 5-0 silk sutures. Extirpated skeletal muscles were immediately transferred into a phosphate-buffered solution (PBS), which had been kept on ice. Following the removal of the skeletal muscles, the donor animals were sacrificed under general anesthesia with intramyocardial injection of 20mEq of potassium chloride solution.

Implantation of Cultured Satellite Cells into Left Ventricle of Donor Rat

The cryo-injury on the myocardium and the SC implantation into the heart was done according the technique described by Li (1996). Premedication, general anesthesia

and operative field preparation were done as mentioned in the skeletal muscle procurement section. Anesthetized rats were then intubated with a 16-gauge I.V. teflon catheter and connected to the Harvard rodent ventilator (model 683) to ensure adequate air exchange in the operated animal's lungs (settings: respiratory rate- 100+/min, volume- 1.5-3.0ml, respiratory gas- room air). Under aseptic conditions, a thoracotomy was performed over the sixth intercostal space on the left. The intercostal muscles were divided and, after opening of the pericardium, the left ventricle was exposed. A 70mm in diameter cryoprobe cooled in liquid nitrogen was applied to the free wall of the left ventricle (Fig.4). The duration of each application ranged from 2 to 3 minutes. Each cryo-injury procedure consisted of 5 such applications (Fig.5). The injured area of the heart could be readily distinguished by a dark purple appearance (Fig.6). In the "acute injury" subgroup, 75 μ l of SC suspension was then injected into the cryo-injured area with a 28-gauge needle (Fig.7). Care was taken not to enter the cavity of the left ventricle that could have resulted in the loss of delivered SCs. Following cell implantation, the sixth and seventh ribs were approximated with a 4-0 vicryl suture and a 14-gauge I.V. teflon catheter was used as a chest tube. This tube was connected to a vacuum suction to evacuate air and fluid from the pleural cavity. The operative incision was closed in layers with a 4-0 vicryl suture in a "running" fashion. The chest tube was left in place until the animal resumed spontaneous breathing. Then the tube was removed and its channel closed with an earlier placed suture. After disconnection from the ventilator the animal was closely monitored in the operating room until spontaneous breathing was resumed and maintained before being returned to the animal care facilities. Postoperative pain was controlled by buprenorphine (0.1-0.5 mg/kg S.Q.) every 10-12hrs for a 24-48h period. Sutures from the skin were removed on the 10-12 postoperative day.

Animals allocated to the "mature scar" group were subjected to a second thoracotomy during which the SCs were implanted into a mature myocardial scar. This procedure was performed in the same fashion as described above.



Fig. 4.

The cryoprobe cooled in liquid nitrogen is applied on to the left ventricle.



Fig. 5.

Frozen myocardium immediately after the removal of the cryoprobe.



Fig. 6.

Acutely cryo-injured myocardium can be distinguished by its dark-purple colour.

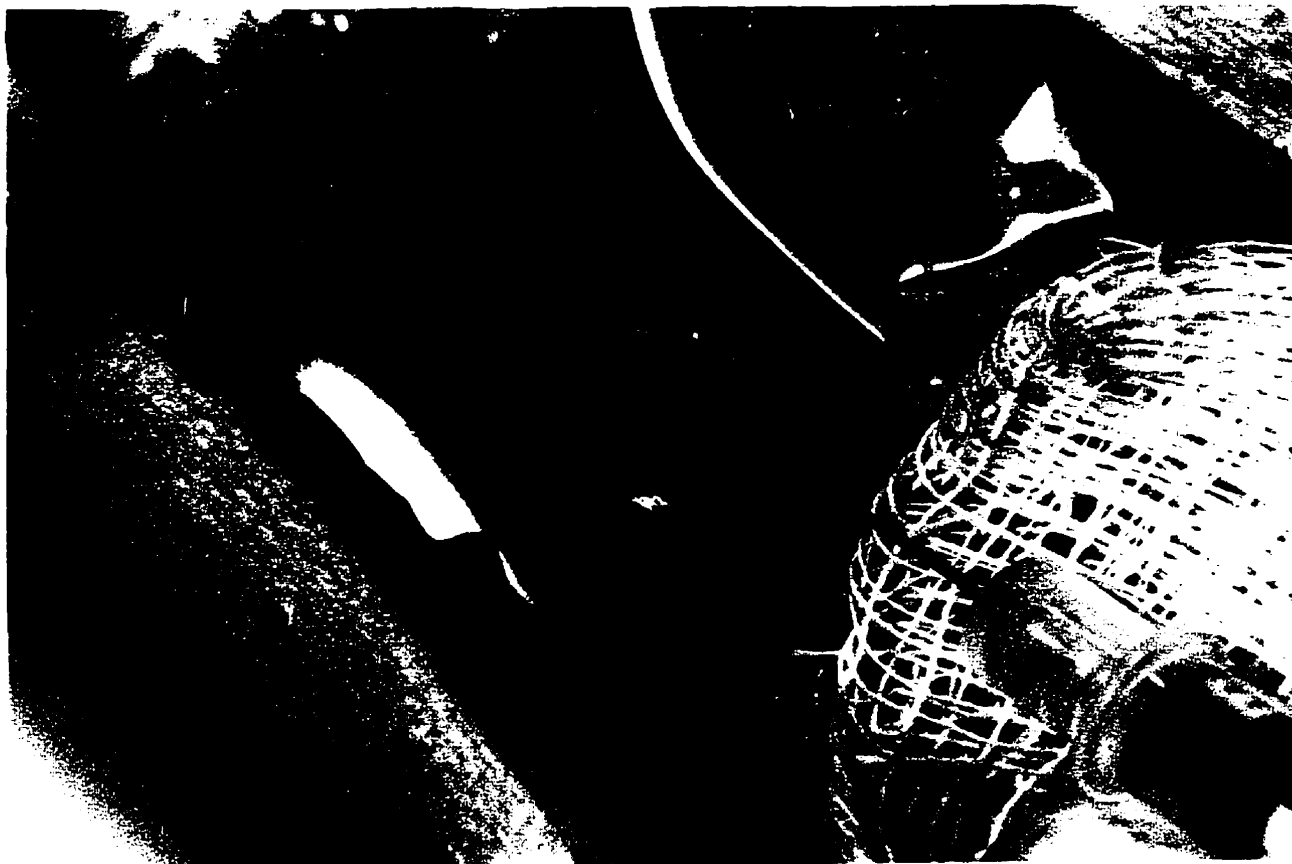


Fig. 7.

Satellite cells are implanted into the cryo-injured wall of the left ventricle.

In Vitro Procedures on Rat Satellite Cells

Satellite Cell Isolation from Adult Rat Skeletal Muscle

Myogenic cells were isolated from rat skeletal muscles according to a method described by Bischoff (1974). Briefly, explanted muscle was washed several times in ice-cold Hank's BSS. Then fascia and connective tissue were removed and the muscle minced into small fragments. To digest connective tissue, the muscle suspension was incubated in 0.1% Pronase solution for a 1hr duration at 37°C with constant agitation. Digested muscle fragments underwent 2 sequential steps of sedimentation at 500g followed by resuspension. During the next step, muscle fragments were passaged repeatedly through a wide mouthed pipette for 1/2hr. Procured suspension was divided into aliquots. By sedimentation of aliquoted volumes, supernatants were obtained which contained SCs. This fluid with cells was pooled and subjected to further sedimentation at a higher speed of 1500g. Cells that were precipitated during this step were resuspended within GM and cell culture was enriched with myogenic cells as described below.

Percoll Density Gradient and Preplating Techniques for Satellite Cell Culture Purification

Isolated rat SC culture was enriched for myogenic cells by applying either Percoll gradient or preplating techniques. These procedures were performed in the same manner as described for SC isolation and purification from canine skeletal muscles. Following myogenic culture enrichment, the SCs were counted and plated at a density of 500,000 cells/ 60mm laminin-coated cell culture plate.

Satellite Cell Labelling with Recombinant DNA Plasmids

Myogenic cells that were intended for implantation into rat myocardium were labelled with pCMVlacZ plasmids (Fig.2). The transfection technique, as well as selection against non-labelled cells, was identical to that described in the Experimental Design section which outlined technical aspects of canine SC labelling.

To investigate the possibility of SC tagging with marker genes of mammalian origin, myogenic cells obtained from 4 animals allocated to the in vitro only experimental group (Fig.3) were transfected with recombinant DNA plasmids, containing human placental alkaline phosphatase (PAP) gene. The human PAP genes in these plasmids were used as marker genes and their expression was under the transcriptional control of a Rous sarcoma virus (RSV) promoter. The neomycin resistance gene was driven by a simian virus 40 (SV40) early promoter (Fig.2). Since human PAP is a mammalian gene, it was anticipated that its incorporation into a mammalian cell DNA would be more efficient than that of the bacterial gene, *lacZ*. Transfection of 30-40% confluent cells was performed as described earlier.

To detect cultured cells that express human PAP, we employed the following protocol (courtesy of Dr. L.A. Culp from Case Western Reserve University).

To decrease the endogenous alkaline phosphatase activity within non-transfected cells, the culture was heated at 65°C for 30min before staining. Cells fixed in formaldehyde were incubated at 37°C with 0.1M Tris-HCl solution as well as with alkaline phosphatase substrate which contained 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) salt in 40mg/ml concentration. After the 30min period, all staining solutions were removed, cells were rinsed in PBS, and preserved in 0.02% sodium azide. Under light microscopy, cells expressing human PAP were identified by their purple-black colour.

Tissue Specimen Preparation for Histological Examination

Four weeks after SC implantation into their myocardium, the animals were sacrificed with xylazine and ketamine overdose. The heart chambers were flushed with ice-cold PBS and the left ventricular wall was cut open at the anticipated location of the SC graft. Whole heart specimens were placed into Karnovsky's fixative solution immediately thereafter. Tissue fixation was done at 4°C for 4-6h period. Following this, specimens were stained overnight at room temperature with X-Gal to unveil the presence of bacterial β -Gal within the tissue. Staining was done as described earlier in the Experimental Design section which outlined the details of this procedure as it was applied to tissue specimens obtained from canine hearts.

Embedding of the tissue into paraffin blocks as well as tissue sectioning and staining with Hematoxylin-Eosin and Masson trichrome was accomplished in the Department of Pathology at The Montreal General Hospital.

Chapter IV

RESULTS

RESULTS FROM IN VITRO EXPERIMENTS ON CANINE SATELLITE CELLS

Myogenicity Enhancement in Percoll-Treated and Preplated Satellite Cell Cultures

Cell cultures which underwent treatment with either Percoll differential density centrifugation or preplating prior to initial seeding of the SCs, had formed more myotubes (Fig.8) and presumably had a higher myogenic potential than cell cultures which were not treated in such a way and had a higher proportion of fibroblast-like cells (Fig.9). The difference in the number of myotubes between preplated and control cultures was statistically significant at a 5% level ($p < 0.05$). The same was true for the comparison of the Percoll-treated cultures versus the control where statistical significance of the difference between these 2 groups was at a 1% level ($p < 0.01$). There was no statistically significant difference ($p = 0.627$) in the amount of myotubes formed in the Percoll-treated and the preplated cultures (Addendum 3).

Canine Satellite Cell Labelling with Recombinant DNA Plasmids

SC labelling experiments in vitro showed that canine SCs, being adult primary cells, can be successfully transfected with recombinant DNA plasmids carrying *E. coli* β -Gal, and this marker gene was expressed in culture (Fig.10). Moreover, gene labelling of the SCs appears not to interfere with the internal muscle formation program in vitro (Fig.11).

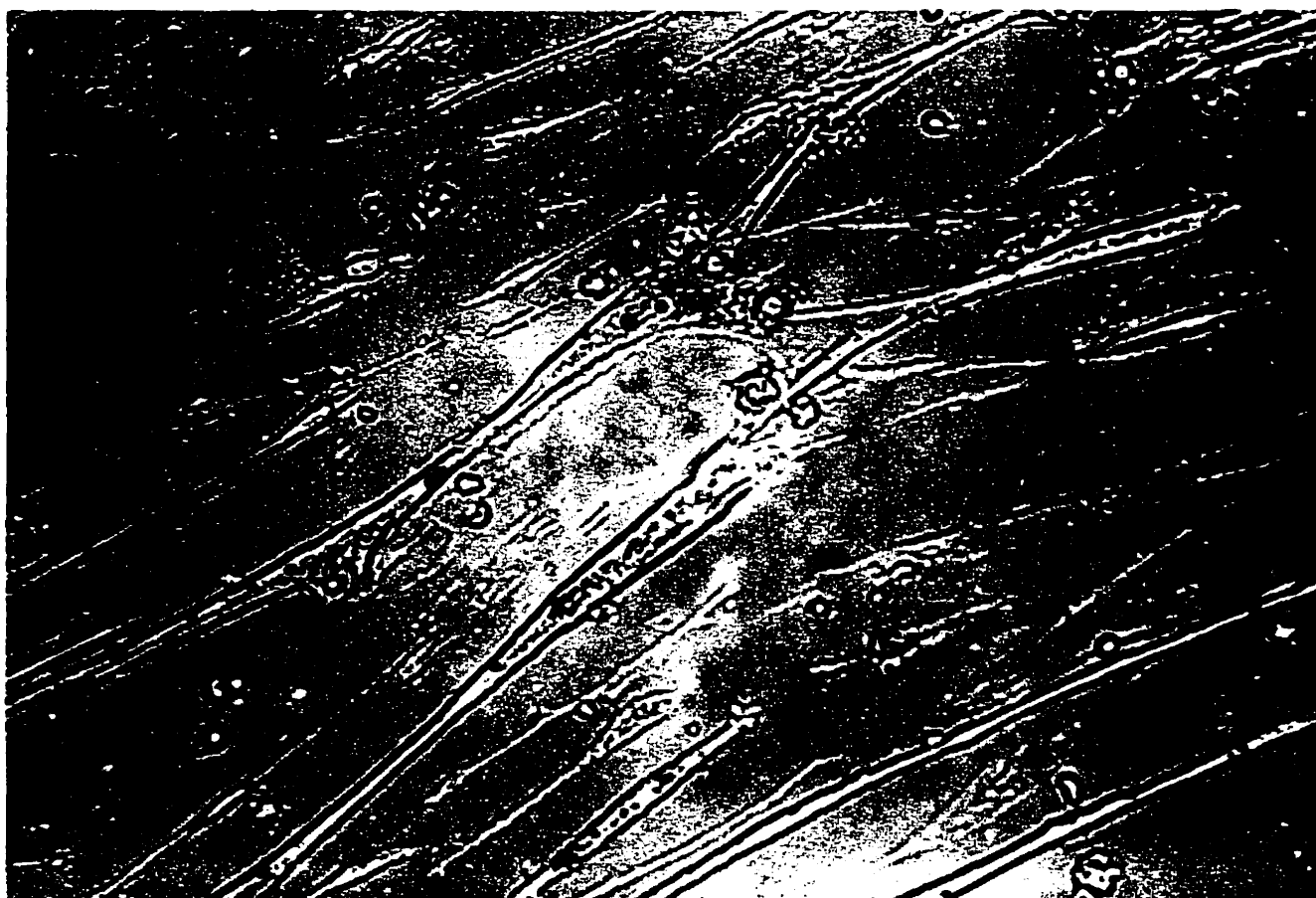


Fig. 8.

Percoll-treated cell culture rich in skeletal muscle myotubes.

Magnification x400.



Fig. 9.

Non-purified culture of SCs with a high proportion of fibroblast-like cells. Only a few spindle-like cells are seen which might be of myoblast origin.

Magnification x400.



Fig. 10.

A cluster of β -Gal positive SCs in vitro.

X-Gal stain; magnification x320.



Fig. 11.

Myotubes formed from β -Gal labelled SCs.

X-Gal stain; magnification x400.

FINDINGS FROM IN VIVO EXPERIMENTS ON DOGS

A total of 31 dogs were operated upon. There were no infectious complications as well as no peri- or postoperative mortality in neither the experimental (n=28) nor the control (n=3) group. Four dogs in the experimental group required intraoperative resuscitation due to ventricular fibrillation following SC implantation into the cryo-injured myocardium. In addition, 3 of these 4 animals required open heart massage of short duration. No positive findings were detected in neither of these 4 animals. The possible influence of these intraoperative complications upon the experimental findings will be contemplated in the Discussion chapter.

Gross and histological evaluation confirmed that cryo-injury on the heart created transmural homogenous fibrous scars. Areas of SC grafting were identified by prolene marker sutures (Fig. 12).

Gross observation of specimens obtained from the hearts of 2 animals sacrificed at 4 weeks following gene-labelled SC implantation, revealed areas of reddish discolouration surrounded by a scar (Fig.13). Histochemical staining of these sections with X-Gal demonstrated the presence of bacterial β -Gal (Fig.14). Light microscopic examination of these areas disclosed clusters of aligning cells which expressed β -Gal (Fig.15). Some β -Gal positive cells were seen in the proximity of an implant tract (Fig.16). However, in these particular specimens no muscle fibres identifiable by light microscopy were noted.

In specimens from 2 more animals sacrificed at 4 and 6 weeks post-implantation, muscle fibres were identified within the implant areas. These fibres were organized in longitudinal fascicles with elongated nuclei which resembled skeletal muscle (Fig.17). In a specimen obtained 4 weeks after cell grafting, a muscle island was partially surrounding a tract left by a prolene marker suture which was placed directly in the SC implant channel (Figs.18 and 19). Unfortunately, no β -Gal expression was noted within these skeletal-like muscle fibres.

No myofibers or β -Gal expressing cells were detected in specimens obtained from animals sacrificed at 2 weeks (n=6) following SC grafting.



Fig. 12.

Left ventricle with the 4 week-old scar created by the cryo-injury. Note that the scar traverses through the entire thickness of the left ventricle. Prolene suture can be seen within the SC graft site.

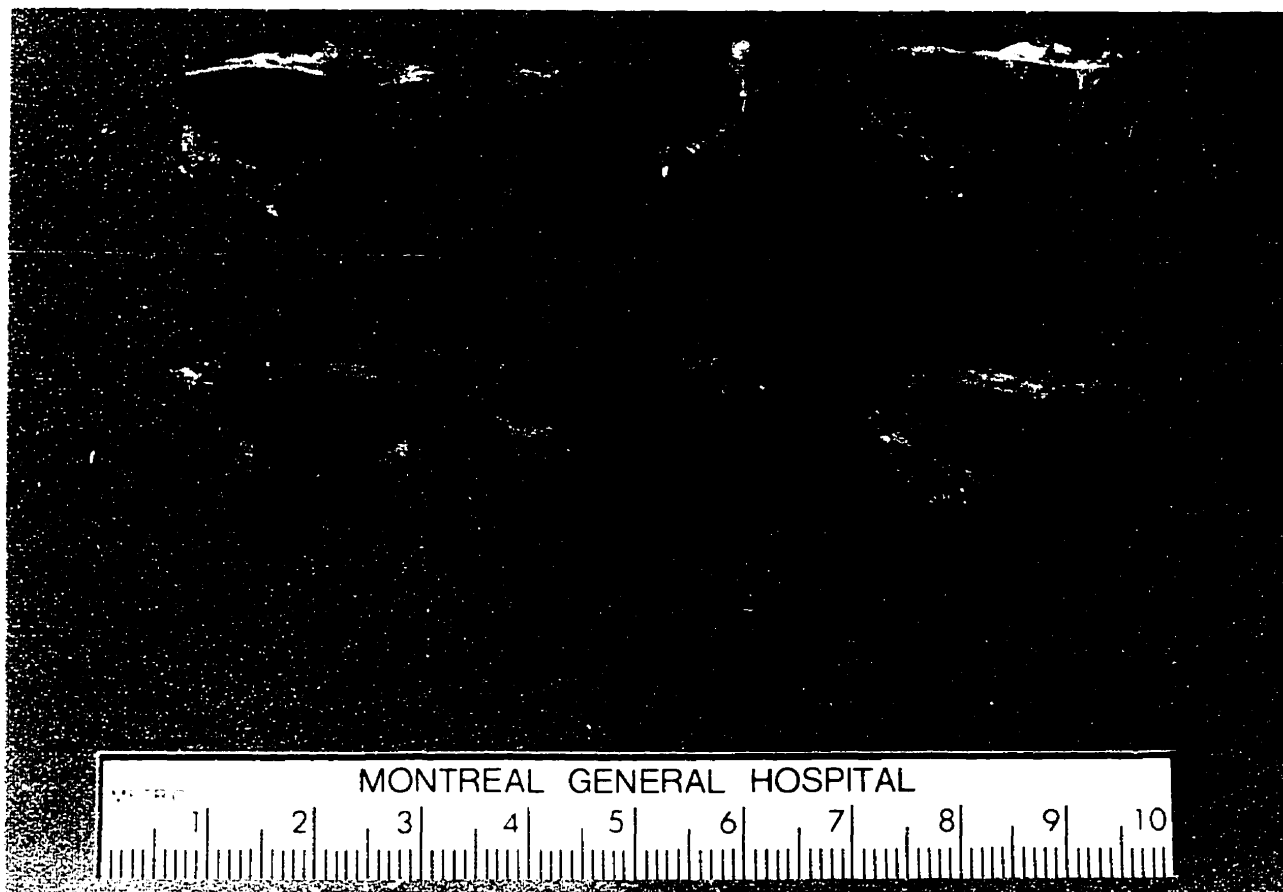


Fig. 13.

Serial sections of a specimen obtained 4 weeks after SC implantation. Note the heterogeneous appearance of the tissue within the scar.

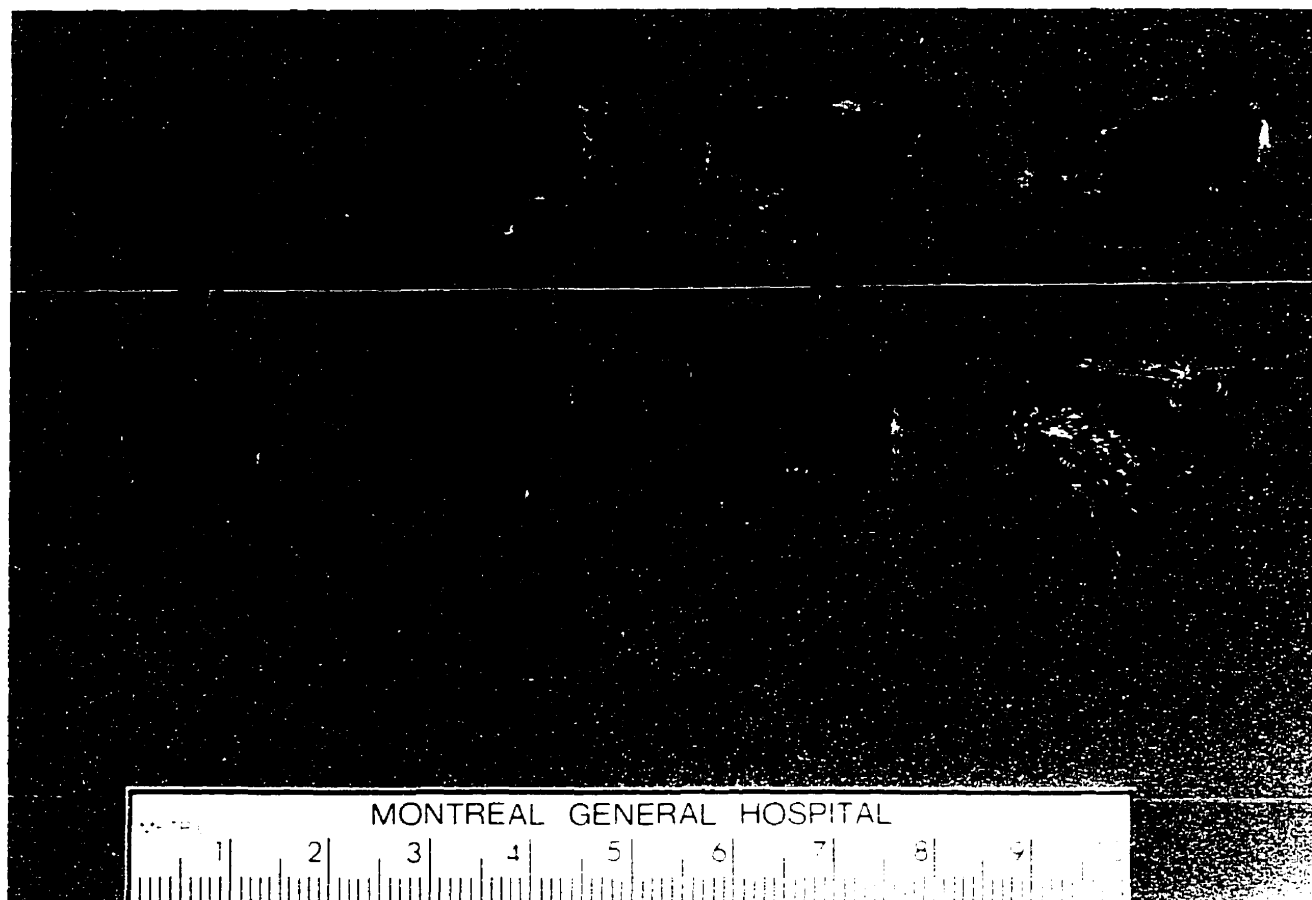


Fig. 14.

The same specimen as in Fig.13 stained for the presence of β -Gal. Bluish green discolouration of the tissue demonstrates the presence of *lacZ* gene-labelled cells.

X-Gal stain.

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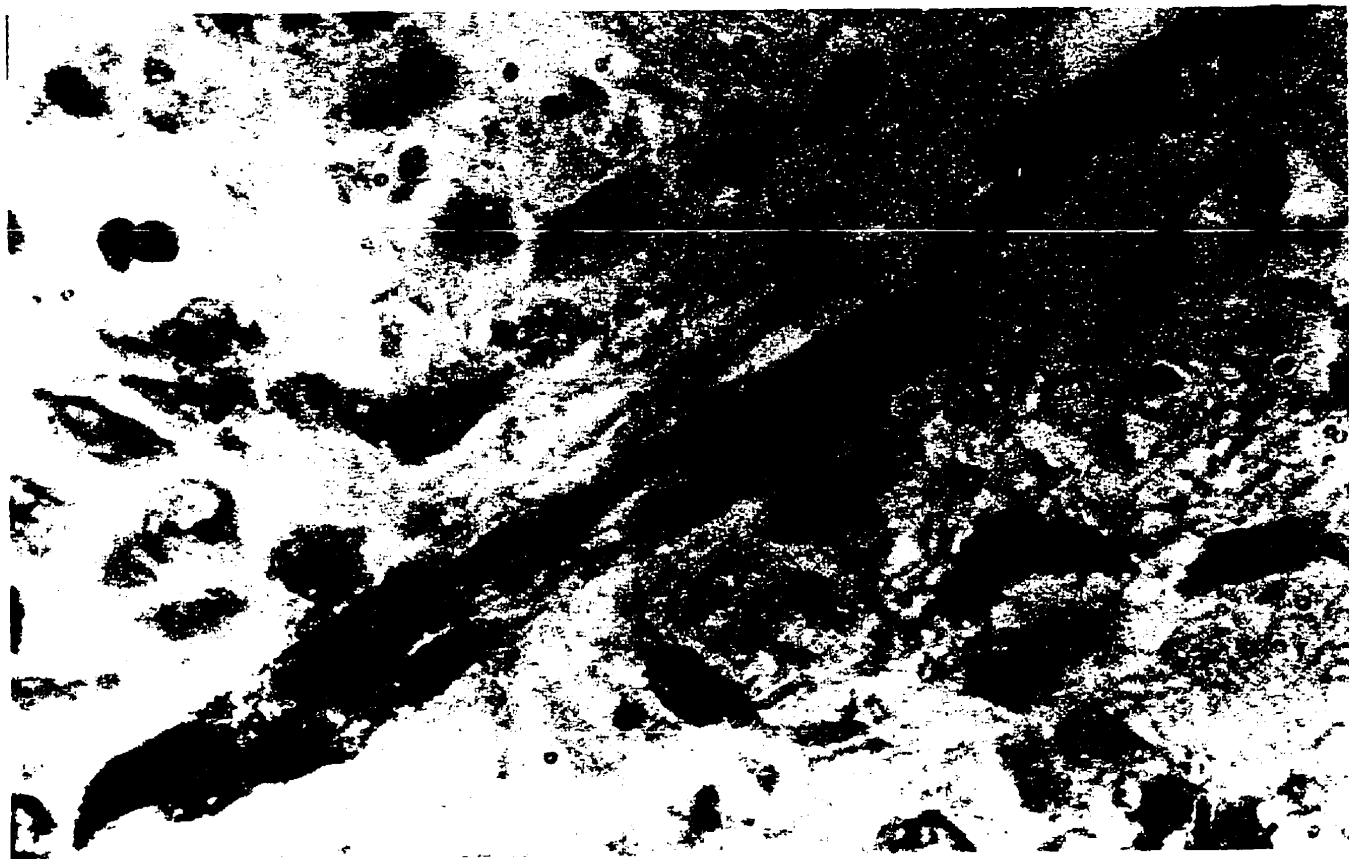


Fig. 15.

High power magnification of a histology slide from the specimens showed in Figs.13 and 14. A few aligned cells, surrounded by a dense connective tissue, express β -Gal.

X-Gal stain with Masson trichrome counterstain; magnification $\times 1,000$.

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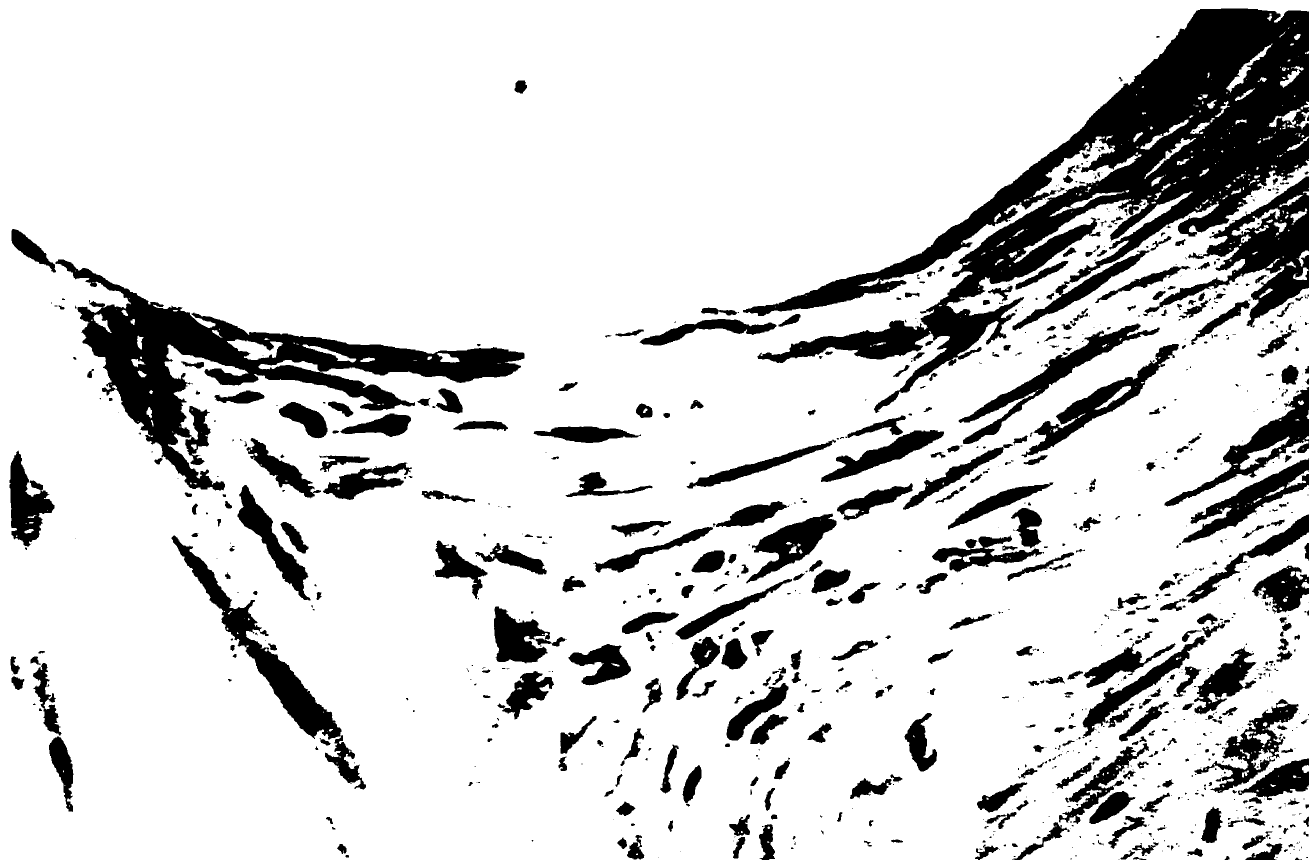


Fig. 16.

High power magnification of β -Gal positive cells in the proximity of the channel left by a prolene suture which was left within the SC implantation site as a marker. Specimen was obtained 4 weeks post-implantation.

X-Gal stain with Masson trichrome counterstain; magnification x400.



Fig. 17.

High power magnification of muscle fibres found within a SC implant site 6 weeks post-implantation. Note the absence of intercalated disks and fasciculated appearance of the fibres which is characteristic to skeletal muscle. Masson trichrome stain; magnification x400.



Fig. 18.

Low power magnification of an area around the SC implant site. The channel left by prolene marker suture is partially surrounded by muscle fibres. Specimen was obtained 4 weeks after SC implantation into the cryo-injured myocardium. Hematoxylin and Eosin stain; magnification x100.

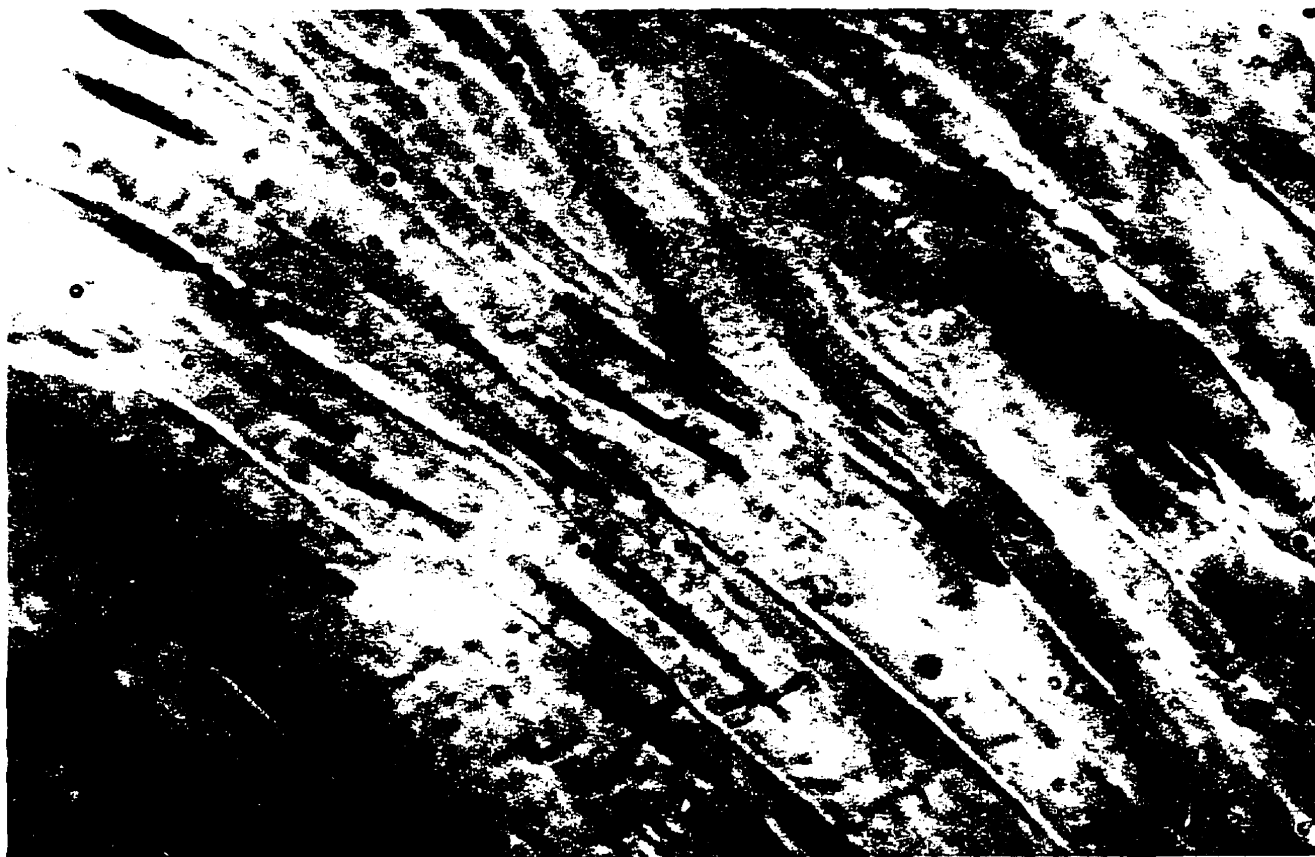


Fig. 19.

High power magnification of the specimen shown in Fig.18. Muscle around the implant channel demonstrates elongated nuclei in the periphery of the fibres. Intercalated disks are not observed. These morphological features suggest that this could be an island of skeletal muscle.

Hematoxylin and Eosin stain; magnification x400.

RESULTS FROM THE RODENT STUDY

Satellite Cell Labelling with Human Placental Alkaline Phosphatase Gene

Experiments in which rodent SCs were labelled with pRSVPAP plasmids demonstrated that primary myogenic cells can be tagged with mammalian reporter genes. The proportion of SCs expressing human PAP in culture was comparable to that reported in experiments with β -Gal (Fig.20). SC preheating before staining significantly reduced native alkaline phosphatase activity and background staining, while human PAP performance remained unaffected in transfected cells (Fig.21).

Bupivacaine-Related Myonecrosis and Satellite Cell Yield

Isolated cells from donor rats were counted on a hemacytometer before Percoll density sedimentation and preplating to evaluate the effect of bupivacaine injury on cell yield. Data on this subject are presented as a number of isolated cells with regard to the obtained muscle mass (Addendum 4). SC yield from the bupivacaine-treated muscles was approximately 4.5 times higher than the yield from non-injured muscles. Statistical comparison of these 2 groups with the t-test showed a significant difference between the groups denoted by p value of less than 0.01.

Results From Satellite Cell Implantation into Cryo-Injured Rat Myocardium

A total of 30 inbred (Lewis) rats were allocated to both SC recipient groups. Seven animals from the "acute injury" group (n=15) expired immediately post-op after undergoing SC implantation into a freshly cryo-injured myocardium. The combined perioperative and early postoperative mortality rate in this group was 47%. The hearts of the remaining 8 animals were processed for histological evaluation as well as detection of the β -Gal marker.

There were 2 immediate post-operative deaths during the first step, i.e., cryo-injury procedure, among the rats allocated to the "mature scar" experimental group (n=15). Moreover, 4 more animals died in this group during the following step, the SC implantation into a scarred myocardium. The combined perioperative and early

postoperative mortality after both operative procedures in the "mature scar" experimental group was 40%. Nine animals in the "mature scar" group survived both operative procedures and their hearts were processed for histological and histochemical evaluation.

The specimens from both groups demonstrated varying degrees of fibrosis within the cryo-injury areas that accepted the SC implants (Fig.22). However, no muscle fibres or β -Gal positive cells were identified within the SC implant sites.

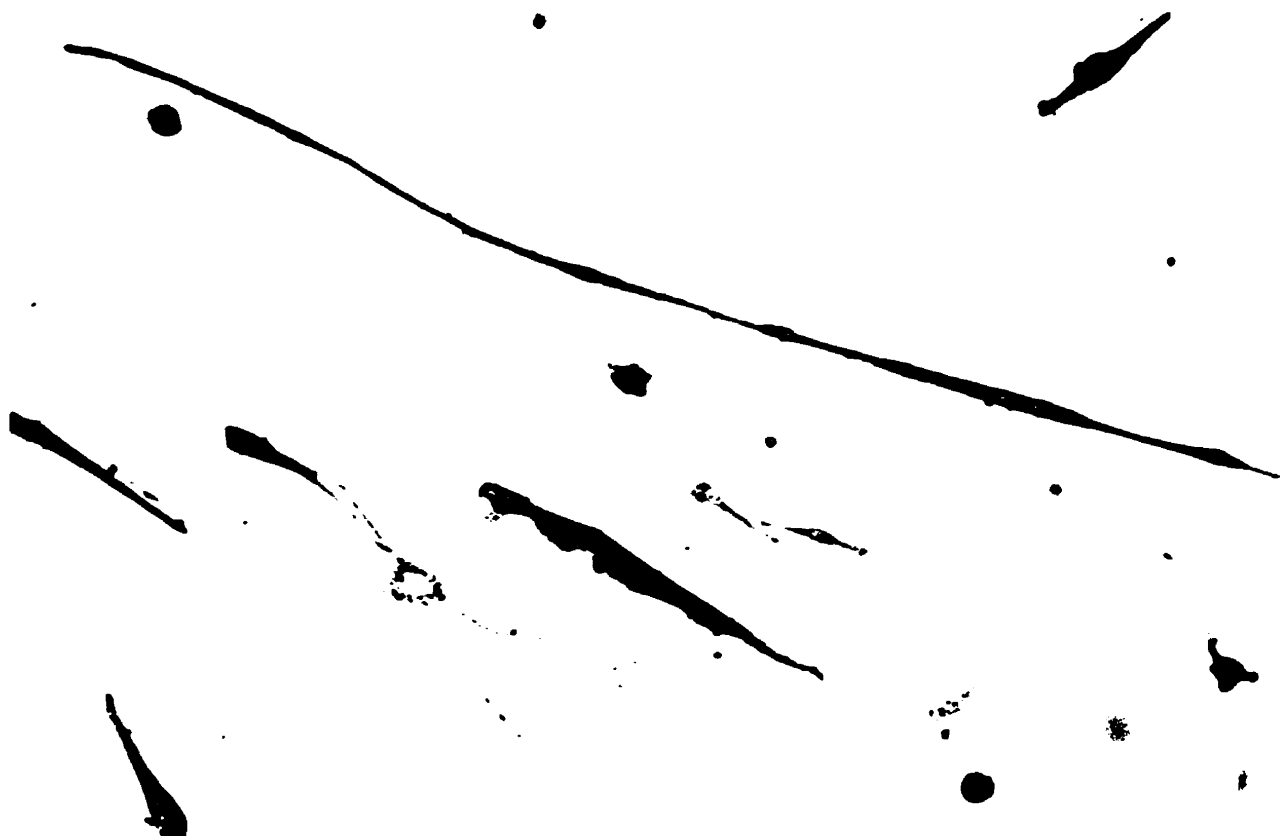


Fig. 20.

Satellite cells in vitro transfected with human PAP gene.

Stained with BCIP and NBT; magnification x320.



Fig. 21.

High power magnification of a myotube expressing human PAP gene in vitro. This culture was subjected to heating at 65°C to eliminate endogenous alkaline phosphatase activity. Background staining after such treatment is negligible while the activity of human PAP is unchanged. Stained with BCIP and NBT; magnification x600.

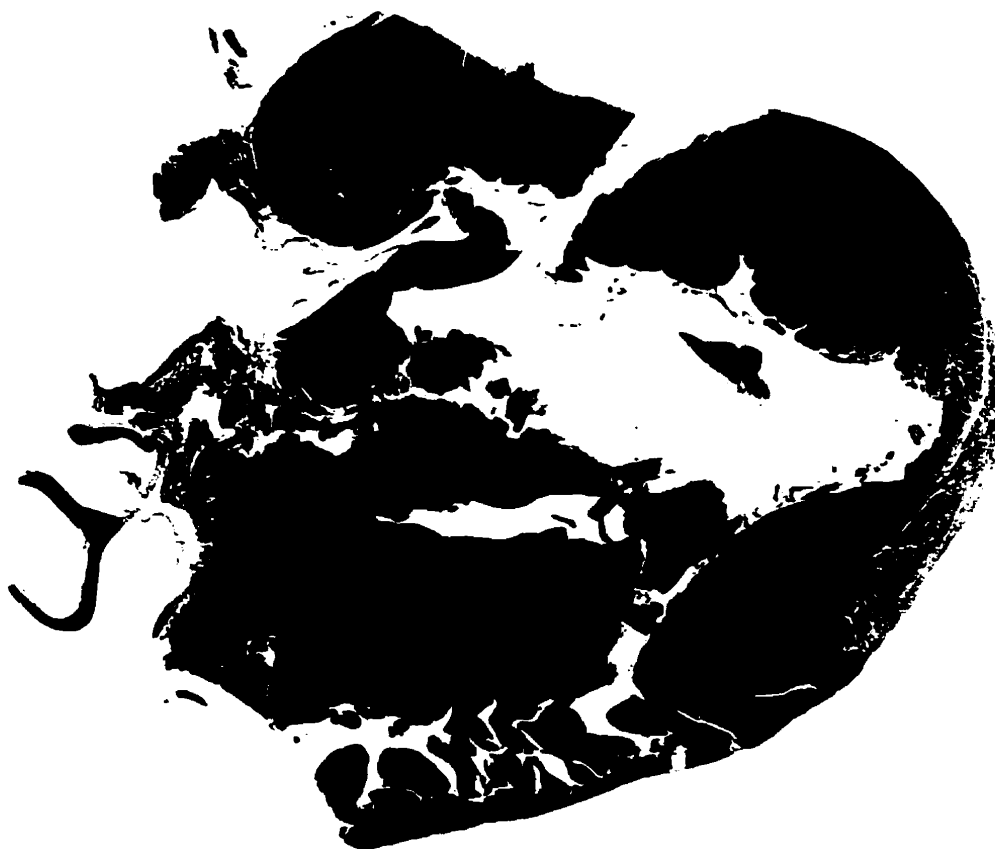


Fig. 22.

Transmural scar in the apex of the heart that healed after cryo-injury and then accepted SC graft. Specimen was obtained 4 weeks post-implantation.

Masson trichrome stain.

Chapter V

DISCUSSION: EVALUATION OF EXPERIMENTAL FINDINGS AND CONFLUENCE DEGREE HYPOTHESIS

INTERPRETATION OF RESULTS

Satellite Cell Culture Enrichment by Density Gradient Separation and Preplating Techniques

Satellite cells comprise only a small percentage of cells in the skeletal muscle. Data gathered from rodent and human muscles show that of each 100 myonuclei found in myofibres, only 5 to 8 nuclei belong to SCs. Currently, there is very little data available on the ratio of fibroblasts and other non-muscle cells to SCs in the skeletal muscle. Schmalbruch and Hellhamer (1976) reported that the fibroblast-to-SC ratio in humans is approximately 2 to 1. However, this study took into account only these fibroblasts which were in immediate proximity to basal lamina of muscle fibres. The non-muscle cells within the perimysium were excluded from the study. Therefore, it could be assumed that the fibroblast-to-SC ratio in a whole explanted muscle is much greater than described. Similarly, in muscle specimens which were used in our experiments there was a greater proportion of non-muscle cells. Therefore, even multiple steps of sedimentation during the SC isolation procedure cannot eliminate all non-muscle cells, fibroblasts in particular, from the SC suspension.

To increase the relative number of SCs in the initial culture, two additional techniques that addressed this issue were adopted for the previously described protocol of SC isolation.

Percoll medium, which is used in density gradient centrifugation, is a suspension of non-toxic silica particles coated with polyvinylpyrrolidone. Since colloidal silica suspension has quite a wide variation in the size of its particles, centrifugation of such a medium spontaneously creates a continuous range of density gradients (Pertoft *et al.*, 1968). The Percoll gradient centrifugation method for enrichment of myogenic cells was first adopted by Yablonka-Reuveni and Nameroff (1987). Their study has shown that most of the SCs accumulate at the 20 and 60% Percoll interface. Satellite cells comprised approximately 70% of all the cells in this accumulation. Cultures grown from cells which were recovered from other regions of the Percoll column had a much higher proportion of non-muscle cells.

The preplating technique stems from an observation that fibroblasts have a tendency to attach to culture dishes more rapidly than myoblasts (Jones, 1979).

Therefore, as our results indicate, a 30min period of preplating noticeably reduces the amount of fibroblasts within the SC cultures.

In our study, cell cultures which underwent Percoll gradient and preplating treatment were compared to the non-treated cultures regarding their myogenic properties. As indicated in the Results section, cultures enriched by these 2 methods demonstrated a higher proportion of myotubes than compared to the non-treated ones. Therefore, the Percoll density gradient as well as the preplating technique, appear to be efficient tools in enhancing myogenic properties of the SC culture.

Bupivacaine-Related Myoinjury as a Means to Increase SC Yield

The SCs have the ability to rapidly multiply in response to an injury. This feature was exploited to increase the proportion of SCs within explanted muscles.

It is known that the local anesthetic bupivacaine (1-n-butyl-DL-piperidine-2-carboxylic acid-2,6-dimethyl anilide hydrochloride) can cause an extensive damage to skeletal myofibres, but not to SCs. Bupivacaine-induced myonecrosis activates SCs which already after 24h show a dramatic increase in proliferative activity. This SC response to the injury peaks on approximately the third day (Saito and Nonaka, 1994). On the basis this knowledge, we adopted a skeletal muscle injury technique used by Neumeyer and colleagues (1992). Hyaluronidase was added, as described, to facilitate the diffusion of bupivacaine within the injected muscle (Neumeyer *et al.*, 1992).

As can be seen from our presented data, bupivacaine injections significantly increased SC number within the muscle tissue. Some authors report the increase in SC numbers as high as ten-fold as compared to the non-injured muscle (Cantini *et al.*, 1994). In our experiments, this difference was somewhat smaller, in the order of five-fold. This could be attributed to the amount of bupivacaine used per muscle mass. We divided 0.5 ml of 0.5% bupivacaine into 2 portions and administered each of them into the large muscle groups of both legs. In other studies, investigators injected a similar amount of bupivacaine (0.5ml of 0.5% solution). However, their target areas were small muscle groups such as tibialis anterior muscles (Neumeyer *et al.*, 1992; Cantini *et al.*, 1994). It is likely that this anesthetic injected into larger muscle areas is subjected to a dilution effect which could explain its lesser myodegenerative potential. In our studies, we chose not to

subject anesthetized rats to greater amounts of this medication, since in higher concentrations it can suppress the animal's breathing activity.

Of interest are reports which describe that bupivacaine-activated SCs form myotubes in culture more rapidly than their "non-activated" counterparts (Cantini *et al.*, 1994). We observed an additional feature attributable to such an activation process. In our studies, all bupivacaine-activated SCs needed to be cultured on extracellular matrix-coated plates. For this purpose, we used laminin-coated dishes. When bupivacaine-activated SCs were grown on plates devoid of ECM coating, their culture rapidly deteriorated and eventually died. These 2 observations taken together might suggest that bupivacaine-activated SCs are already in the progress of executing their myogenic differentiation program, which for successful completion requires interaction with specific elements of ECM.

Satellite Cell Implantation Study on Dogs

As described earlier in the Results section, positive findings within the SC implant sites were identified in 4 dogs. In specimens from 2 animals, there were cells within graft sites that expressed a marker gene, i.e., bacterial β -Gal. Despite the alignment and the proximity of these cells to each other, they had not formed morphologically identifiable muscle fibres.

In 2 more animals, striated muscle-like fibres within an otherwise homogeneous scar were identified. Under light microscopy, these fibres did not exhibit intercalated disks and had few nuclei within their sarcoplasm. Therefore, their appearance was interpreted as resembling that of skeletal muscle. However, no cell marker was present within these fibres. Speculations on the possible reasons for the appearance of such "unmarked" skeletal-like-muscle fibres are presented below.

One possibility is that these muscle fibres arose from intramyocardially implanted non-labelled SCs. In experiments on dogs, we employed bacterial β -Gal as a cell marker. However, transfection efficiency of the SC cultures was not higher than 10%, as judged by semiquantitative means. According to the transfection protocol supplied by Gibco, the most favourable time period for transfection is when cells in culture are 50 to 60% confluent. In our experience, this recommendation was in agreement with our findings

from the SC transfection as well. The SC cultures that displayed higher degrees of confluence more readily took up plasmids containing marker genes during transfection. However, this approach posed some difficulties. Namely, when cell-to-cell contact was in abundance, the SCs unavoidably withdrew from the mitotic cell cycle and by fusion with each other, formed skeletal muscle myotubes. Such cell cultures with a high content of myotubes were judged as not suitable for implantation.

Therefore, to keep the SCs from premature fusion, myoblast cultures were transfected earlier when the cells were less than 50% confluent. Such modification of the transfection protocol, imposed by avoidance of myotube formation in culture, most likely decreased the SC transfection efficiency.

Moreover, fibroblasts that comprised a small fraction of the SC culture also competed with myoblasts for lipid droplets containing marker gene plasmids. This factor could further diminish the number of plasmids available for SCs. Therefore, the SC cultures used as intramyocardial cell grafts always contained some SCs that did not carry a marker gene. The fusion of such myoblasts within the implant sites could result in the formation of "unmarked" skeletal muscle fibres.

In 2 specimens with β -Gal positive cells, there were no muscle fibres present as examined by light microscopy. However, these cells were close to each other and exhibited alignment. A similar alignment can be observed in vitro during SC fusion into a myotube. It is tempting to speculate that these cells can be labelled and implanted SCs, and the specimen was harvested just prior to their fusion into a myotube.

Conversely, it is also conceivable that β -Gal expressing cells found within the implant sites arose from labelled and implanted fibroblasts that were transferred to the myocardium along with the SCs. Perhaps, meticulous examination of these cells by electron microscopy could provide an answer to the raised questions.

A relative paucity of positive findings in the SC implantation study on dogs could be attributed to some inherent aspects of the chosen cell marker, as well as to some technical difficulties in a small number of animals that are discussed below.

Despite the fact that bacterial β -Gal is a good marker enabling one to unambivalently identify labelled cells, it bears some drawbacks that could negatively

influence the outcome of an *in vivo* study. A recent study presented by Baer and colleagues (1996) showed that *in vivo* proliferation of endothelial cells transduced with retroviral constructs containing β -galactosidase (*lacZ* gene) and the neomycin resistance genes was significantly retarded, as compared to growth of cells infected with a similar retroviral construct, but carrying the neomycin resistance gene alone. Moreover, the same trend was noted in experiments *in vivo* where *lacZ*-transduced endothelial cells, when seeded on polytetrafluoroethylene grafts, colonized significantly smaller surface areas than their non-transduced counterparts. These observations suggest that bacterial β -Gal when used as a cell marker can impede cell proliferation. The mechanism for this phenomenon is yet unknown, and perhaps may require interplay with additional factors, such as retroviral sequences. Despite the differences in cell phenotype as well as in cell transduction techniques used above and in our studies, it is conceivable that the *lacZ* gene could have hindered the proliferation of intramyocardially implanted SCs as well.

Antigenic properties of bacterial β -Gal could have contributed to the diminished survival of transfected and implanted SCs. It has been shown that mature skeletal muscle does not express class 1 and class 2 MHC gene products. Only class 1 MHC is expressed on the sarcolemma of regenerating skeletal myofibres. However, fibroblasts contrary to myoblasts or myotubes can present both, class 1 and class 2 MHC peptides (Karpati *et al.*, 1988). Since our SC cultures contained fibroblasts, a proportion of which was transfected with the *lacZ* gene, it is tempting to speculate that fibroblast presentation of β -Gal peptides to immune cells might have resulted in elimination of the rest of the *lacZ* transfected and implanted cells.

Four out of 20 dogs in the experimental group required brief cardiac resuscitation measures following SC implantation. In 2 cases, it included open heart massage of short duration. This could have its effect on the experimental results as well, since implanted SCs could have been expelled or displaced from the implantation channel to other areas of muscle during open heart massage. No positive findings were detected in any of these 3 dogs.

As noted from the presented data on SC implantation in our experiments, there was quite a wide variation in the number of implanted SCs (Addendum 1). Perhaps this could be attributed to the varying age of the experimental animals. It is known that as a subject ages, the SC numbers in skeletal muscles decline significantly along with their replication potential (Gibson and Schultz, 1983). Therefore, the use of younger animals

would be preferable in a study that requires a sufficiently large amount of muscle to be produced.

Intramyocardial Satellite Cell Grafting in Rats

This study has shown that it is feasible to perform SC implantation either into a freshly injured myocardium or into an already mature scar in the heart. The ease of handling of these animals as well as the relative inexpensiveness make this model a suitable one for investigation of SC development within the heart.

The necessity of using a few large muscles from a donor rat is based on the experimental data showing that myogenic SCs comprise only 3-8% of the total number of all the cells in skeletal muscle. In a small animal, the absolute number of cells within the muscle is fewer when compared to a larger one. Furthermore, SC purification on the Percoll gradient during cell isolation, as well as the cell preplating method inevitably carries a loss of a certain proportion of SCs. Therefore, it was necessary to have a sufficient amount of cell suspension.

Most of the perioperative and immediate postoperative mortality in the "acute injury" and "mature scar" experimental groups could be attributed to a learning curve since more than 70% of the deaths occurred within the first 1.5 months of this study.

The SC implantation technique was slightly different from the one employed in the study on dogs. Contrary to canine experiments, there was no marker left within the implantation channel and no suture was used to secure the implant channel entrance. Moreover, the distal end of the needle once it entered the left ventricular muscle was not seen and a satisfactory injection was considered when the needle was advanced only a few millimeters and no blood appeared in the syringe. Despite the care taken not to exit into the left ventricular cavity, it is however conceivable that in a small proportion of experiments, the SCs could have been injected into the blood stream of the left ventricle. This technical aspect in conjunction with the already discussed pitfalls attributable to bacterial β -Gal as a marker, might have partially contributed to the negative results in the study on rats.

In our experiments that involved SC labelling in vitro, we have shown that the SCs can be successfully labelled with 2 different recombinant DNA plasmids containing either *E. coli lacZ* gene encoding for bacterial β -Gal or a gene of human placental alkaline phosphatase (PAP). Although human PAP codes for a mammalian enzyme and, perhaps for this reason might be more readily incorporated into SC DNA, we did not use this marker for our in vivo studies. In our experience, to quench the activity of endogenous alkaline phosphatase, which is present in many cells including both striated muscles, heating of cell culture at 65°C for a period of 30min was necessary. Such treatment caused a significant coagulation accompanied by distortion of muscle tissue structure in our preliminary tests.

POSSIBLE ADVANCES FOR FUTURE STUDIES ON MYOCARDIAL REPAIR WITH SATELLITE CELL GRAFTS

We postulated that SCs when implanted into the cryo-injured myocardium might differentiate into cardiac muscle under the influence of a cardiac milieu. As a method to damage myocardium, we have chosen cryo-injury that is known to create a homogenous scar without remaining viable heart muscle tissue (Jensen *et al.*, 1987). Furthermore, it spares large blood vessels leaving a source of blood supply to the injured area. Therefore, after elimination of the native myocardium, implanted SCs should be the only striated muscle cells capable of surviving within the cryodestroyed area of the heart. However, it is conceivable that cryo-injury inflicted upon the myocardium might not be the most suitable approach for investigation of the SC development within heart muscle. In our experiments, SCs were implanted into either freshly cryodamaged myocardium (in the dog and the rat "fresh injury" studies) or a homogenous scar (in the rat "mature scar" study) created by this injury. To achieve transdifferentiation into the cardiac muscle the implanted SCs, most likely, have to have a close interaction in their immediate environment with some healthy cardiomyocytes. Perhaps it is the influence of the remaining normal myocardium that is most important, since it can serve as an example of the phenotype for the SCs to convert to.

In the acutely cryo-injured myocardium grafted SCs can certainly see native myocytes. However, it is unknown if the interaction between the SCs and the dying cardiomyocytes could be of desired value to this project. The short duration of such cell interaction (until dead cardiomyocytes are eliminated by macrophages) along with the

injurious distortion of cell-to-cell signaling might not be similar to these where the SCs can "see" and interact with healthy cardiomyocytes.

On the other hand, when the SCs are implanted into a homogeneous scar that forms as a consequence of a cryo-injury, the majority of cells surrounding newly grafted myocytes will be of connective tissue origin. Therefore, immediate cell-to-cell interaction between fibroblasts and SCs would doubtfully result in the appearance of a third phenotype.

The most appropriate as well as the most clinically relevant model for investigation of intramyocardially grafted SC development would be implantation of the SCs into the myocardium scarred by an ischemia-reperfusion injury. This model closely resembles the acute coronary blockage by thrombi formed over an atherosclerotic plaque that commonly occurs in humans. Moreover, the experimental ischemia-reperfusion injury creates a heterogeneous scar closely resembling that which develops as a consequence of myocardial infarction. Satellite cells implanted into such a scar would contact some remaining healthy cardiomyocytes that might influence the grafted muscle cells and prompt them to assume the phenotype of cardiac muscle.

The selection of such a model would require the preference of a cell marker that would unambiguously label SCs, could be faithfully transferred to and expressed by their progeny, and would not alter cell development as well as the immunogenic properties of a labelled cell. Currently, the use of a certain nontoxic cationic dye for SC DNA labelling is in the progress of investigation by Dr. Julia Dorfman (supervised by Dr. Ray C.-J. Chiu) which explores this project further.

It is important to consider the possibility of skeletal muscle appearance within the cardiac scar as well. If the SCs implanted into the heart will not be able to alter their phenotype and will form only skeletal muscle fibres, this finding can be of great importance on its own. Such skeletal myofibres can be appropriately stimulated to contract in synchrony with the rest of the heart by using an appropriate pacemaker, as it has been done in dynamic cardiomyoplasty. Therefore, creation of skeletal muscle islands within the cardiac scar could have the potential to become a valuable treatment modality for congestive heart failure.

COMPARISON OF OBTAINED MORPHOLOGICAL FINDINGS WITH OBSERVATIONS OF PREVIOUS INVESTIGATORS

In the experiments on dogs a few specimens from hearts with SC implants contained striated muscle fibres. Since these fibres did not have intercalated disks and contained a few long nuclei positioned in the periphery of the myofibres, they most closely resembled skeletal muscle (Figs.17 and 19). Therefore, based on these histological features determined by light microscopic examination, it was preliminary concluded that these structures are skeletal muscle fibres.

It is of interest to compare these findings with the data of previous investigators from Dr. Chiu's lab that explored myocardial repair.

Dr. D. Marelli reported muscle fibres within the implant area that possessed structures identified as intercalated disks (Marelli *et al.*, 1992). This finding echoed Dr. R.L.Kao's reports where he described myofibres with intercalated disks that were surrounded in perimeter by a mature scar (Kao *et al.*, 1989). Therefore, it appears that in these distinctive experiments intramyocardially implanted SCs assumed the phenotypic appearance of cardiomyocytes (Fig.23).

A particular muscle was identified in a study done by Dr. F. Ma (Zibaitis *et al.*, 1996). These muscle fibres did not have intercalated disks, but exhibited branching syncytium that is characteristic of the cardiac muscle (Fig.24). Moreover, myofibres encased two different types of nuclei. Some of the nuclei were dense, elongated and were positioned in the periphery of the myofibres. These nuclei bore resemblance to skeletal myonuclei. Nearby to these was a large, vesicular nucleus with a prominent nucleolus, which is typical of cardiac myocytes (Fig.25). Therefore, as judged by morphological criteria, this particular muscle showed features attributable to both, cardiac and skeletal muscles.

The SC isolation technique employed in the experiments described above was identical to the one used in the most recent study. The only difference between these studies was the frequency of the SC culture passaging. In experiments where cardiac-like muscle was found sandwiched within a mature myocardial scar, SCs were passaged frequently, on average every second day before implantation (Kao *et al.*, 1989; Marelli *et al.*, 1992). A slightly longer 3 day period of undisturbed culture was allowed for SCs in a

study done by Dr. F. Ma (personal communication). Finally, in our experiments, a quiescent period for cultured cells was extended up to 4 days. This deviation from the original protocol was introduced to allow transfected SCs to more efficiently incorporate plasmid genes into their DNA (Zibaitis *et al.*, 1994). As described in the Experimental Design chapter, selection for *lacZ*-labelled cells was done in the G418-containing medium for 2 weeks. The appreciable toxicity of G418 antibiotic to mammalian cells was of concern. Despite that transfected cells conferred resistance to this compound, care was taken not to passage cells frequently, since this could damage cell integrity and make such cells more vulnerable to the toxic medium.

Therefore, considering all the mentioned observations, it is tempting to suggest that varying SC culture conditions could influence different morphological findings obtained in the discussed studies. To interpret the available data, we propose a confluence degree hypothesis that will be discussed bellow.

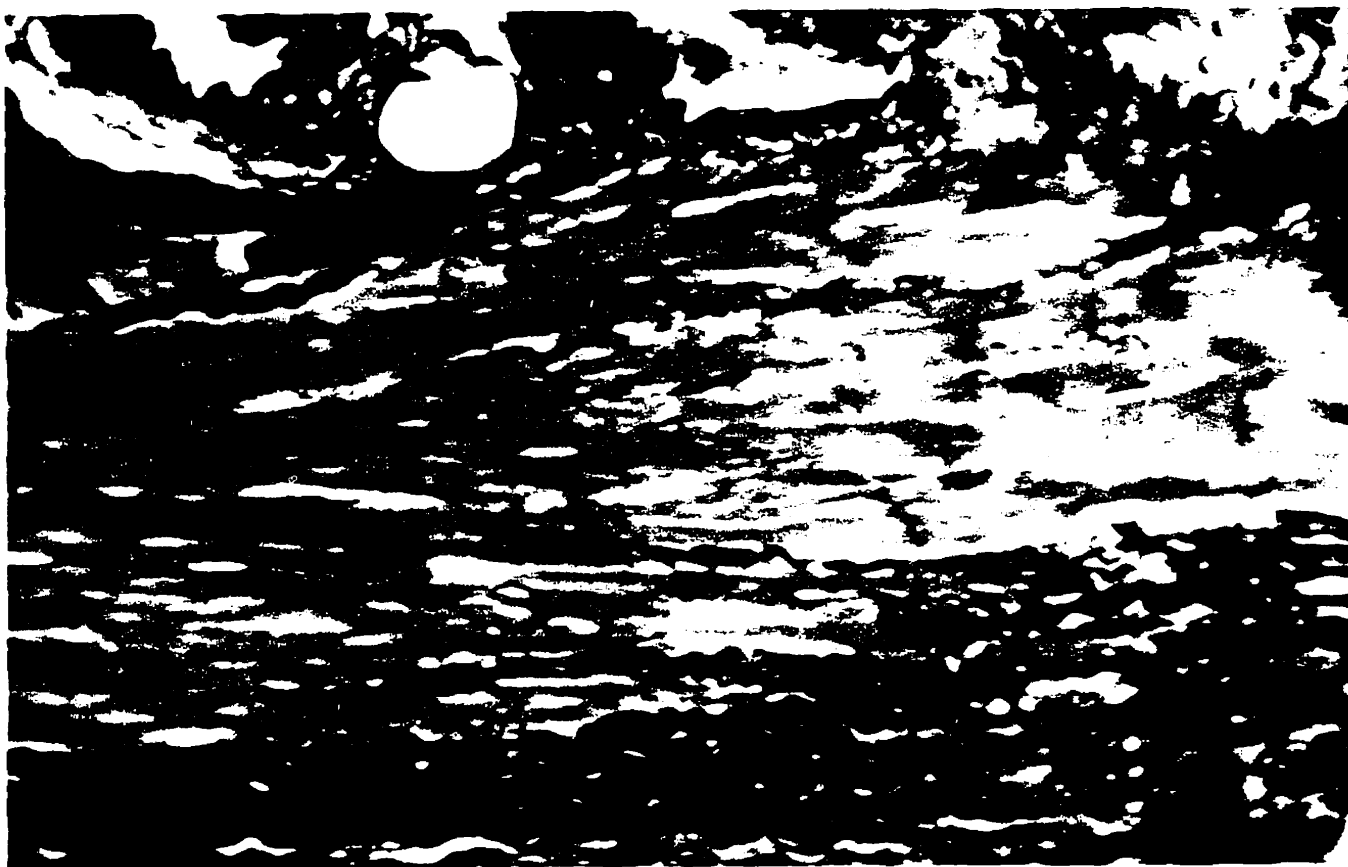


Fig. 23.

High power magnification of cardiac-like muscle fibres found within an implant site of SCs that were passaged frequently during cell culture period.

Courtesy of Dr. Race L. Kao.

Masson trichrome stain; magnification x400.

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Fig. 24.

Low power magnification of mosaic-like muscle fibres found within an implant site of SCs that reached moderate degree of confluence before implantation.

Toluidine blue stain; magnification x200.

Courtesy of Dr. Felix Ma.



Fig. 25.

High power magnification (x1,000) of mosaic-like muscle fibres presented in Fig.24. Filled arrow points to a central light nucleus with noticeable nucleolus, a feature attributable to cardiac muscle. Elongated peripheral nuclei resemble these of skeletal muscle (open arrow). Toluidine blue stain. Courtesy of Dr. Felix Ma. Reproduced with permission from Zibaitis A, Ma F, Duong M *et al.* Cellular cardiomyoplasty: results and possibilities for the future. Cardiovascular Engineering 1996;1: 55-59. ©Pabst Science Publishers.

CONFLUENCE DEGREE HYPOTHESIS

There could be 3 possible interpretations for the findings discussed. First, the tissue shown in Figure 25 could be regenerating skeletal muscle, since during normal development of skeletal muscle, contractile myofilaments are produced constantly in increasing numbers. As these filaments are laid out within the muscle fibre, they gradually displace the skeletal muscle nuclei from their central position to the periphery. Therefore, centrally located nuclei in this particular specimen could belong to the skeletal myofibre in the very early stage of development. Another possibility is that these muscle fibres are cardiac muscle fibres which require a special stain to better demonstrate intercalated disks. Finally, there is an explanation based on the milieu-dependent differentiation theory. Perhaps, these fibres express mixed skeletal and cardiac phenotypes. In other words, this could be a "mosaic" striated muscle fibre.

If the last interpretation is correct, we wish to propose a confluence degree hypothesis to explain these findings. Since the SCs in different studies were passaged at different intervals of time during the in vitro culture, they should have reached different cell-to-cell contact levels, which resulted in varying degrees of cell confluence in culture. The stage of SC commitment to skeletal muscle phenotype presumably was most advanced in the latest experiments, where the cell culture exhibited the greatest number of cell-to-cell contacts and, consequently, the highest degree of cell confluence. The opposite is true for the frequently passaged cell culture with a low level of cell confluence. Therefore, when SCs with a low level of commitment to skeletal muscle phenotype were implanted into the injured heart, they retained the potential of a stem cell, and formed cardiac muscle-like fibres under the influence of cardiac milieu. On the other hand, cells that achieved a high degree of cell-to-cell contact became committed to the skeletal phenotype, and when implanted, formed skeletal muscle-like fibres. Cells that reached an intermediate degree of cell confluence in vitro, according to this scenario, formed mosaic cardio-skeletal fibres. Therefore, SCs at various stages of commitment to the skeletal muscle phenotype in vitro, when implanted into the heart, could develop into different forms of striated muscles. It should be emphasized that this hypothesis is based on our very preliminary results, and further confirmation with the use of monoclonal antibodies and other techniques is warranted.

BIOLOGICAL BASIS SUPPORTING THE CONFLUENCE DEGREE HYPOTHESIS

There are numerous reports indicating that cell culture density indeed affects cell commitment to a certain phenotype. Studies with embryonic chick mesenchymal cells *in vitro* showed that culture density was the primary factor that determined the phenotype of cultured cells. When mesenchymal cells were plated at a high density and consequently had ample cell-to-cell contacts, they consistently produced chondrocyte-like cells that synthesized type II collagen and keratan sulfate proteoglycan. By decreasing cell culture density, cell-to-cell contact was reduced. This change in culture conditions resulted in osteoblast formation with expression of bone alkaline phosphatase as well as cell response to parathyroid hormone (Caplan, 1970; Osdoby and Caplan, 1981). Investigations with pigmented epithelial cells (PECs) of the eye also demonstrated that cell density in the culture and the abundance of cell-to-cell contacts could affect differentiation decisions of the cells. Under established culture conditions PECs assumed their usual phenotype that was marked by synthesis of melanosomes. However, when cells were plated at a very high density and grown under "crowded" conditions they began to express Δ -crystalline, which is a marker for cells representing lens phenotype (Itoh and Eguchi, 1986).

Studies with mammary gland cells revealed the complexity in environmental regulation and maintenance of a differentiated phenotype. Primary mammary epithelial cells, when explanted from pregnant mice, can survive on a two-dimensional surface in culture but fail to differentiate into their phenotype and are non-responsive to lactogenic hormone stimulation (Streuli and Bissell, 1990). When transferred on to a basement membrane layer these cells started to produce β -casein, which is a milk protein appearing early in the gestation period. However, there was no sign of production of late-appearing milk proteins, specifically, whey acidic protein (WAP). To express WAP cells required to be cultured in a three-dimensional configuration resembling alveoli-like structures (Chen and Bissell, 1989). On the basis of studies with mammary epithelial cells, Lin and Bissell (1993) developed a concept of hierarchy that is required for a proper cell differentiation. According to the proposed model, growth factors are the ground element for any cell differentiation system and their presence is an absolute requirement for the expression of basic functions of cells. For continued differentiation of cells, an interaction with the extracellular matrix is mandatory. An expression of even more complex functions requires the establishment of direct cell-to-cell communication. Finally, to achieve a complete

phenotype that enables to present all aspects of the internal cell differentiation program, an establishment of a tissue-specific spatial structure is required.

With regard to this model, of interest is a recent study by Cossu and colleagues (1995) where they evaluated the effect of neighboring cells on the induction of myogenic program in uncommitted cells from somites. These authors showed that non-myogenic somitic cells can be influenced to commit to myogenic lineage by culturing them with other cells from the trunk. Moreover, three-dimensional culture conditions as well as a defined critical number of surrounding mesodermal cells were both found to be prerequisites for initiation of the myogenic program. This suggests that cell-to-cell interaction as well as a specific spatial structure plays a great modulatory role in muscle differentiation.

SCs is a population of cells that arise from embryonic myoblasts during a late period of myogenesis, probably before the development of a basal lamina that surrounds individual myofibres (Hartley *et al.*, 1992). These cells are induced to enter myogenic lineage and are destined during postnatal life to regenerate injured skeletal muscle. This myogenic potential is faithfully expressed in vivo and even in vitro where external factors influencing normal myogenesis are eliminated.

Under usual circumstances adult SCs participate in the skeletal muscle regeneration process, where they recognize each other as well as the damaged nearby myofibres and fuse with them. This restores the continuity and function of the skeletal muscle fibre. However, the firmly planted skeletal muscle program allows certain flexibility for SCs in executing it. Experiments with adult SCs that were placed into developing embryonic limbs showed that SCs and embryonic myoblasts recognized each other and formed mixed-phenotype myotubes. If myoblasts prior to their transfer into a limb are cultured in vitro they fail to form long lasting myofibres in the developing muscles (DiMario and Stockdale, 1995). This observation also adds proof that in vitro conditions do influence certain developmental aspects of myoblasts and is consistent with our "confluence hypothesis" presented above.

Chapter VII

SUMMARY AND CONCLUSIONS

The presented work was done to explore the hypothesis which proposes that adult skeletal muscle myogenic (satellite) cells can be implanted into the damaged myocardium and can consequently repair it.

In summary, 2 specimens from hearts with SC implants contained marker gene-labelled cells. Moreover, in 2 other samples muscle fibres were found in proximity to the implant channels.

The data from in vitro experiments showed that the bupivacaine injury significantly increases SC yield within the explanted muscles, and the Percoll density gradient as well as preplating methods increased the myogenic properties of the SC culture.

The following conclusions can be drawn from the findings of this work:

1. A *lacZ* marker gene that codes the production of bacterial β -Gal was used to label SCs before their implantation into the injured myocardium. Expression of this marker within cells situated near the SC implantation channel confirms that implanted cells survived within the injured myocardium.
2. Skeletal-like muscle fibres were found in some specimens surrounded by a homogenous scar. This preliminary finding lends support to the investigated hypothesis by suggesting that implanted SCs can form myofibres within the injured myocardium. The nature of these muscle fibres should be determined by monoclonal antibody probes.
3. Myocardial cryo-injury can be successfully performed on rats. This model is an appropriate one for investigating SC differentiation within the cryo-injured heart.
4. Intramuscular bupivacaine injections significantly increase SC numbers within the donor skeletal muscles and can be used to increase SC yield for intramyocardial grafts.
5. The Percoll differential density gradient as well as the SC preplating techniques are well-suited and efficient measures in enriching cell culture with myoblasts.

6. The experimental findings presented here and the observations made from experiments of previous investigators, prompted the advent of a new hypothesis which states that SC confluence degree achieved prior to cell implantation into the injured myocardium plays a determining role in the phenotypic expression of the SCs grafted into the heart.

APPENDIX

ADDENDUM 1**Number of implanted SCs in experiments on dogs ($\times 10^6$):**

Dog#	SC quantity	Dog#	SC quantity	Dog#	SC quantity	Dog#	SC quantity
1	5.4	8	4.0	15	8.0	22	2.0
2	5.6	9	2.8	16	1.2	23	1.0
3	12.0	10	4.0	17	9.2	24	1.0
4	12.5	11	4.0	18	3.5	25	1.2
5	1.5	12	6.4	19	2.4	26	1.5
6	7.5	13	8.0	20	12.0	27	6.0
7	4.8	14	5.0	21	7.8	28	1.5

Sample size: 28; Mean: 5.1; SD: 3.5; Range: 1.0-12.5 $\times 10^6$ cells/implant.

Note: Dogs #29, #30 and #31 underwent sham implantation only.

ADDENDUM 2

Cell viability (%) as judged by the Trypan blue cell exclusion test:

Dog#	Viability	Dog#	Viability	Dog#	Viability	Dog#	Viability
1	82	8	86	15	86	22	84
2	87	9	90	16	82	23	79
3	88	10	81	17	83	24	90
4	80	11	86	18	78	25	81
5	78	12	85	19	84	26	86
6	79	13	91	20	80	27	85
7	84	14	79	21	80	28	85

Range: 78-91%; Mean: 83.5%; SD: 3.8

ADDENDUM 3

Percoll-treated, preplated and control SC culture comparison with regard to their myogenicity (myotube number/high power magnification field):

Percoll-treated group	Preplating group	Control group
2; 2; 6; 2; 1; 0; 9; 7; 0; 5; 1; 4; 8; 9; 8; 7; 3; 10; 0; 4; 6; 4; 3; 0; 0. (sample size- 25)	2; 11; 4; 0; 6; 3; 7; 2; 2; 0; 5; 0; 3; 9; 6; 5; 2; 1; 2; 8; 0; 1; 7; 3; 1. (sample size- 25)	3; 4; 0; 0; 2; 5; 0; 1; 6; 2; 0; 6; 0; 5; 1; 0; 1; 2; 4; 1; 1; 0; 1; 3; 2. (sample size- 25)

Percoll-treated group:

(myotubes/HP field)

Mean: 4.04

SD: 3.27

SE: 0.65

Preplating group:

(myotubes/HP field)

Mean: 3.60

SD: 3.08

SE: 0.62

Control group:

(myotubes/HP field)

Mean: 2.00

SD: 1.98

SE: 0.39

Run Stats 1.0 application for Macintosh (©1988 by McGraw-Hill, Inc.) was used for all descriptive analyses as well as for all statistical comparison of data.

T-test was used to determine statistical significance of difference between groups:

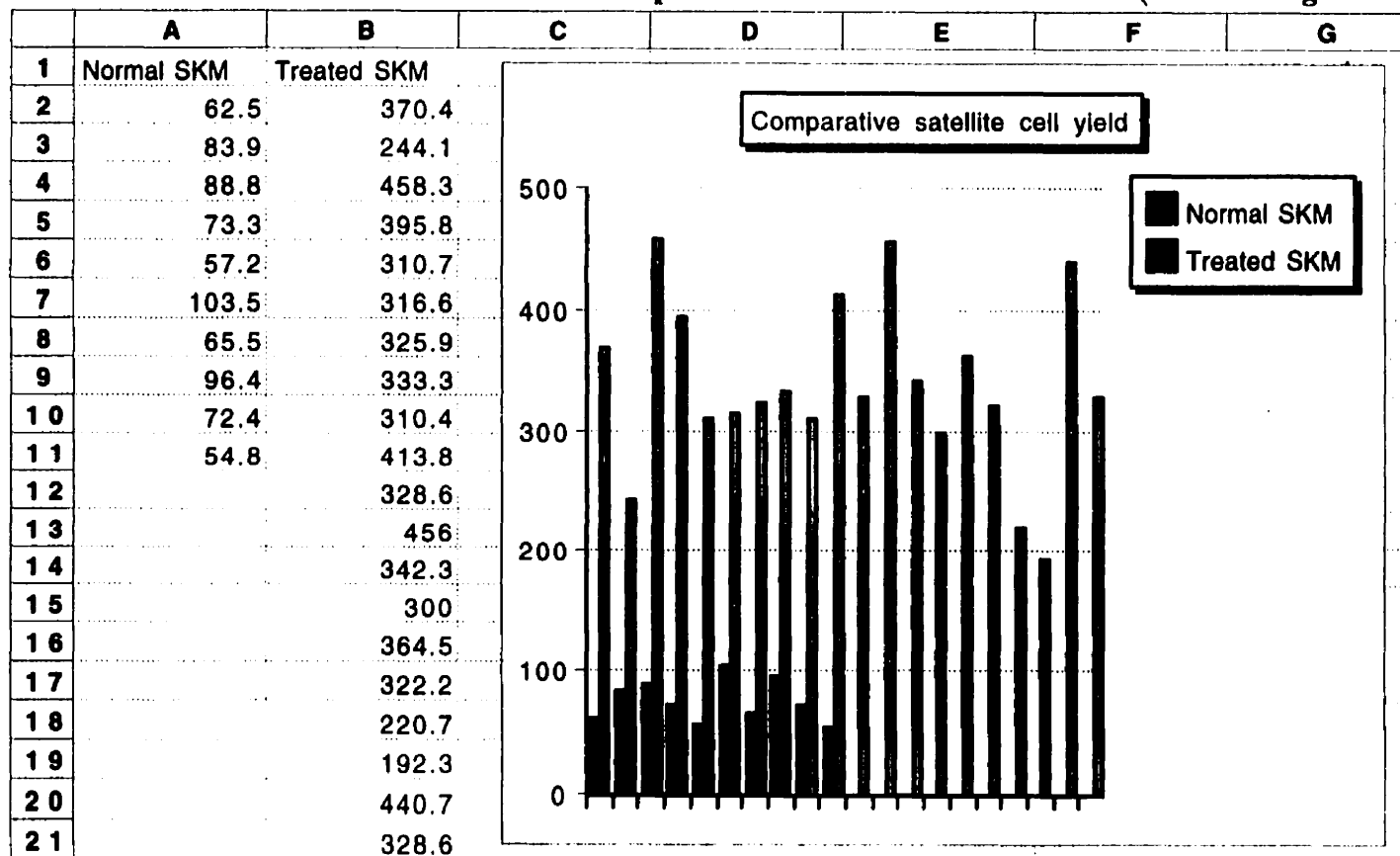
Percoll-treated vs. Preplating group: n.s., (p=0.627);

Percoll-treated vs. Control group: difference significant with p value of 0.01;

Preplating vs. Control group: difference significant with p value of 0.034.

ADDENDUM 4

Yield of satellite cells from normal and bupivacaine-treated skeletal muscles ($10^3 \times \text{cells/g muscle}$)



SC yield from non-injured muscle:

Mean: 75.8 ($10^3 \times \text{cells/g muscle}$)

SD: 16.7

Range: 54.8 to 103.5

SC yield from bupivacaine-treated muscle:

Mean: 338.8 ($10^3 \times \text{cells/g muscle}$)

SD: 71.7

Range: 192.3 to 458.3

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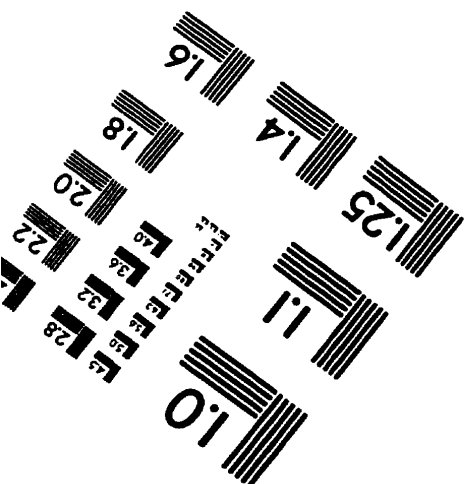
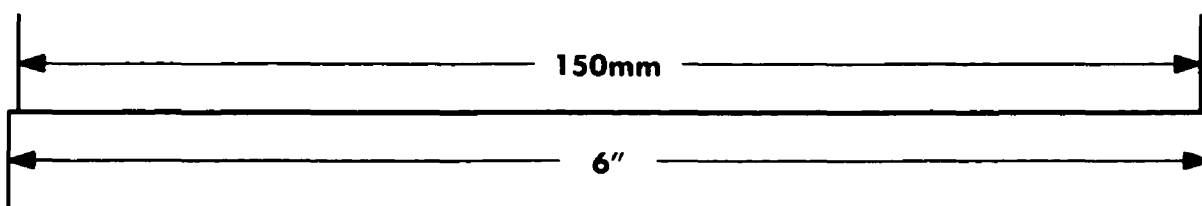
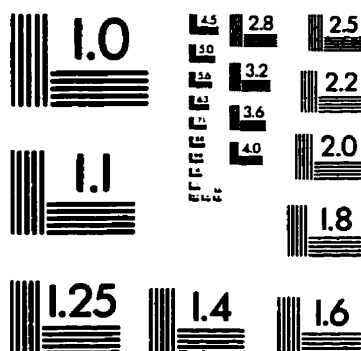
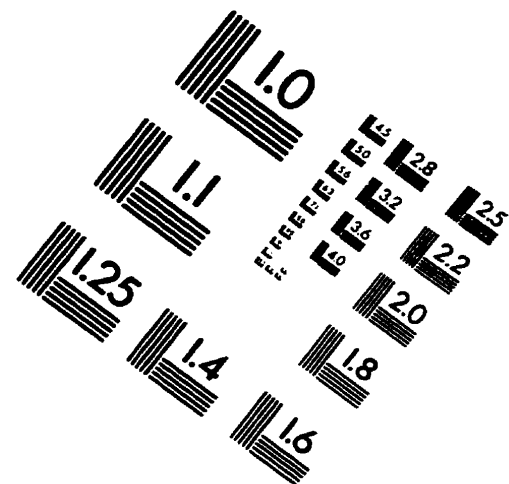
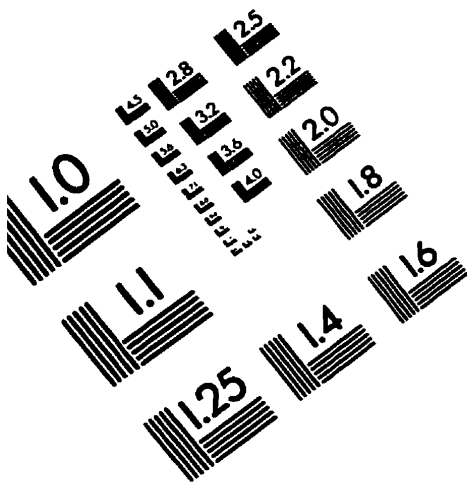
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IMAGE EVALUATION TEST TARGET (QA-3)



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