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Review

Myocyte and myogenic stem cell transplantation in the heart

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Abstract

Cellular transplantation is emerging as a potential mechanism with which to augment myocyte number in diseased hearts. To date a number of cell types have been shown to successfully engraft into the myocardium, including fetal, neonatal, and embryonic stem cell-derived cardiomyocytes, skeletal myoblasts, and stem cells with apparent cardiomyogenic potential. Here we provide a review of studies wherein myocytes or stem cells with myogenic potential have been transplanted into the heart. In addition, issues pertaining to the tracking and functional consequences of cell transplantation are discussed.

Keywords: Cellular transplantation; Cardiomyocytes; Skeletal myoblasts; Stem cells; Heart failure

1. Introduction

Cell transplantation is emerging as a potential therapy with which to treat heart failure. Initial efforts in the field focused on the transplantation of cardiomyocytes as well as skeletal myoblasts. More recently, stem cells with apparent cardiomyogenic potential have also been transplanted. Positive results from animal studies have prompted several preliminary clinical trials to ascertain the safety of skeletal myoblast (reviewed in other articles in this issue, see also Ref. [1]) and bone marrow stem cell [2] transplantation into heart failure patients.

Although there has been considerable experimentation with cardiomyocyte and skeletal myoblast transplantation, there are comparably fewer studies wherein multipotent stem cells have been transplanted into the heart. Accordingly, we have opted to start our review with a summary of the literature describing cardiomyocyte and skeletal myoblast transplantation into the heart. We then describe studies that report transplantation of cardiomyogenic stem cells. It is hoped that the cited cardiomyocyte and skeletal myoblast transplantation literature will provide a useful database against which to compare the efficacy of cardiomyogenic stem cell transplantation. The remainder of the manuscript provides our view on issues which influence the interpretation of cell transplantation studies, with specific discussions on methodologies for tracking donor (or homing) cells, mechanisms by which cell transplantation can enhance cardiac function, and strategies which might be useful for increasing the capacity of donor cells to integrate into the host myocardium. Where appropriate, examples of studies from our laboratory are provided as illustrations.

2. A review of the myocytye and myogenic stem cell transplantation literature

Table 1 provides a summary of studies wherein fetal, neonatal, or adult cardiomyocytes were transplanted into normal or injured hearts. Studies that describe the transplantation of cardiomyocyte cell lines are also listed. Studies for each of these categories are listed in chronological order. These studies utilized donor cells isolated from a number of species (including humans), and

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Table 1
Cardiomyocyte transplantation

Donor cell	Species: donor/host	Tracking	Heart injury	Function improvement/assay	Angiogenesis	Intervention	Refs.
F	M/M	T	Normal	ND	ND	N	[3]
F	D/D	G	Genetic	ND	ND	N	[4]
F, C	H&P/P	H	Normal	ND	+	N	[5]
F	H&R/R	D	Occlusion	ND	ND	N	[6]
F	R/R	Ca	Cryoinjury	+/L-B	ND	N	[7]
F	R/R	D	Cryoinjury	ND	ND	N	[8]
F	M/M	V	Normal	ND	ND	N	[9]
F	R/R	D	Cryoinjury	ND	ND	N	[10]
F	R/R	Li	Occlusion	ND	ND	N	[11]
F	M/M	V	Normal	ND	ND	N	[12]
F	R/R	CH	Reperfusion	+/E	ND	N	[13]
F	R/R	V	Cryoinjury	ND	+	N	[14]
F	R/R	H	Cryoinjury	+/L-B	ND	N	[15]
F	M/M	T, CH	Chemical	+/E	ND	N	[16]
F, N, C	P/P	H, I	Occlusion	ND	+	N	[17]
F	R/R	H	Cryoinjury	+/L-B	ND	N	[18]
F, N, A	R/R	M	Cryoinjury	ND	ND	N	[19]
F	R/R	M	Cryoinjury	+/L	+	Y, Matrix	[20]
F	R/R	D	Occlusion	+/E	+	Y, Matrix	[21]
F	R/R	H, I	Occlusion	+/E	ND	N	[22]
F	R/M	M	Normal	ND	ND	Y/CTLA	[23]
F	R/R	M, V	Occlusion	+/E	ND	N	[24]
F	R/R	M	Cryoinjury	ND	ND	N	[25]
F	R/R	D	Occlusion	+/E, M-B	ND	N	[26]
F	R/R	D	Occlusion	+/E, M-B	ND	N	[27]
F	R/R	D	Occlusion	+/E, M-B	+	Y/bFGF	[28]
F	D/D	G	Genetic	ND	ND	N	[29]
F	M/M	T	Cryoinjury	+/E, F	ND	N	[30]
F	M/M	T	Normal	+/L-F	ND	N	[31]
N	R/R	H	Occlusion	ND	ND	N	[32]
N	R/R	I	Occlusion	ND	ND	N	[33]
N	R/R	M	Cryoinjury	ND	ND	Y/c-FLIP	[34]
N	R/R	D	Cryoinjury	ND	ND	Y/Akt, HS	[35]
N	R/R	I	Occlusion	+/E	+/HGF	Y	[36]
N	R/R	G	Occlusion	+/M	ND	N	[37]
N	R/R	G	Normal	ND	ND	Y/Casp Inh	[38]
N	R/R	H, I	Normal	+/F	+	Y, Matrix	[39]
N	R/R	H	Normal	+/F	+	Y, Matrix	[40]
A	R/R°	M	Cryoinjury	+/L-B	ND	N	[41]
A	HM/HM	M	Genetic	+/L-B	ND	N	[42]
A	P/P°	M	Occlusion	+/SPECT, M	ND	N	[43]
A	R/R	M	Cryoinjury	+/L-B, M	+/VEGF	N	[44]
C	C/M	I	Normal	ND	ND	N	[45]
C	C/M	I	Normal	ND	ND	N	[46]

Donor cell: F, fetal cardiomyocyte; N, neonatal cardiomyocyte; A, adult cardiomyocyte; C, atrial tumor cell line.
Species, donor species/host species: M, mouse; R, rat; H, human; D, dog; P, pig; HM, hamster; C, cell line; °, autologous transplant.
Tracking: D, dye; H, histology; I, immunostain; T, transgenic; CH, Y chromosome; G, donor specific gene (mdx; or sry); V, viral transfection; Ca, calcium phosphate transfection; E, electroporation; Li, liposome gene delivery; M, metabolic label.
Heart injury: Normal, Cryoinjury, Reperfusion injury, Coronary occlusion, Genetic (i.e., mdx), Chemical, Cardiotoxic agent.
Function improvement/assay: +, improved; L-B, isolated perfused Langendorff with intraventricular balloon; L-F, Langendorff with fluorescence microscopy; M, closed-chest intraventricular micromanometer; M-B, micromanometer with intraventricular balloon; M&S, micromanometer and sonomicrometer; E, echocardiography; F, in vitro force measurements; SPECT, [^{99m}Tc]MIBI single photon emission computed tomography; ND, not determined.
Angiogenesis: +, angiogenesis; ND, not determined.
Cell transplant survival intervention: Y, yes; N, no; Casp Inh, caspase inhibitor; Matrix, support matrix (i.e., scaffold).

the host animals included mice, rats, rabbits, pigs and dogs. A variety of techniques were used to track the fate of the donor cells. In some studies, the cardiomyocytes were transplanted into hearts subjected to cryoinjury, permanent coronary artery occlusion, reperfusion injury, chemical cardiotoxicity, or alternatively into hearts with genetic cardiomyopathies. There were no obvious differences in the capacity of donor cardiomyocyte seeding in the differ-

ent injury models. Several studies performed functional analyses on the hearts following cardiomyocyte transplantation, while others determined if cell transplantation resulted in enhanced angiogenesis. It is very striking that for each study in which both cardiac function and donor cell-induced angiogenesis were scored, there was a positive correlate.

Table 2 provides a summary of studies wherein skeletal myoblasts were transplanted into normal or injured hearts. Myoblasts from neonatal and adult animals, as well as from established myoblast cell lines have been studied. Once again the studies for each category are cited in chronological order. Autologous, syngeneic, allogenic and xenogenic transplants have been performed. Most of the studies performed transplantation into injured hearts; cardiac damage was induced via cryoinjury, permanent coronary artery occlusion, reperfusion injury or chemical cardiotoxicity. The vast preponderance of studies concluded that the transplanted skeletal myoblasts differentiated into skeletal myotubes. In light of this it is extremely interesting that functional improvement was observed in many of the studies.

Table 3 provides a summary of studies wherein stem cells with cardiomyogenic potential have been transplanted

Table 2
Myoblast transplantation

Donor cell	Species: donor/host	Tracking	Heart injury	Function improvement/assay	Angiogenesis	Survival intervention	Refs.
C	C/M	I	Normal	ND	ND	N	[47]
C	C/M	I	Normal	ND	+/TGF-beta	N	[48]
C	C/M	V	Normal	ND	ND	N	[49]
C, NM	C, R/R	I	Normal, Cryoinjury	ND	ND	N	[50]
C	C/R	V	Occlusion	+/L-B	ND	N	[51]
C	C/R	V	Normal	ND	ND	Y/HS	[52]
NM	P/P	H	Normal	ND	+	N	[5]
NM	R/R	M	Cryoinjury	F	N	N	[53]
NM	R/R	H, I	Occlusion	+/E	ND	N	[22]
NM	R/R	H	Cryoinjury	ND	ND	N	[54]
NM	R/R	I	Reperfusion	+/L-B	ND	N	[55]
AM	D/D°	M	Cryoinjury	ND	ND	N	[56]
AM	D/D°	M	Cryoinjury	ND	ND	N	[57]
AM	D/D°	G	Cryoinjury	ND	ND	N	[58]
AM	D/D°	G	Cryoinjury	ND	ND	N	[59]
AM	D/D	M	Cryoinjury	ND	ND	N	[60]
AM	RB/RB°	V	Normal	ND	ND	N	[61]
AM	RB/RB°	I	Cryoinjury	+/M&S	ND	N	[62]
AM	R/R°	D	Normal	ND	ND	N	[63]
AM	RB/RB°	H	Cryoinjury	+/M&S	ND	N	[64]
AM	RB/RB°	H	Cryoinjury	+/M&S	ND	N	[65]
AM	RB/RB°	I	Cryoinjury	ND	ND	N	[66]
AM	H/H°	ND	Occlusion	ND	ND	N	[1]
AM	RB/RB°	I	Cryoinjury	+/M&S	ND	N	[67]
AM	M/M	V	Normal	ND	+/VEGF	N	[68]
AM	R/R	I	Occlusion	+/L-B	+/VEGF	N	[69]
AM	R/R°	I	Chemical	+/E	ND	N	[70]
AM	R/R°	I	Occlusion	+/E	ND	N	[71]
AM	R/R°	D	Normal	ND	ND	N	[72]
AM	P/P°	H	Occlusion	+/E	ND	N	[73]
AM	R/R	V	Occlusion	ND	ND	N	[74]
F	R/R°	V	Chemical	+	ND	N	[75]
AM	S/S°	I	Occlusion	+/E	ND	N	[76]
AM	R/R	M	Normal	ND	ND	N	[77]

Donor cell: C, myoblast cell line; NM, neonatal myoblast; AM, adult myoblast; F, single muscle fiber.
Species, donor species/host species: M, mouse; R, rat; H, human; D, dog; P, pig; RB, rabbit; HM, hamster; S, sheep; C, cell line; °, autologous transplant.
Tracking: D, dye; H, histology; I, immunostain; T, transgenic; CH, Y chromosome; G, donor specific gene (mdx, or sry); V, viral transfection; Ca, calcium phosphate transfection; E, electroporation; Li, liposome gene delivery; M, metabolic label.
Heart injury: Normal; Cryoinjury, Reperfusion injury, Permanent coronary occlusion, Genetic (i.e., mdx), Chemical, Cardiotoxic agent.
Function improvement/assay: +, improved; L-B, isolated perfused Langendorff with intraventricular balloon; M, closed-chest with intraventricular micromanometer; M-B, micromanometer with balloon; M&S, micromanometer and sonomicrometer; E, echocardiography; F, in vitro force measurements; Ex, exercise regimens; ND, not determined.
Angiogenesis: +, angiogenesis; ND, not determined.
Cell transplant survival intervention: Y, yes; N, no; HS, heat shock.

Table 3
Stem cell and stem cell-derived cardiomyocyte transplantation

Stem cell type	Species D/H	Cell status at transplant	Delivery	Tracking	Heart injury	Function improvement/ assay	Angiogenesis	Refs.
ESC	M/M	CM	CI	E	Genetic	ND	ND	[78]
ESC	M/R	CM	CI	E	Occlusion	+/E	+/BMP2, TGF-beta	[79]
ESC	M/R	CM	CI	Ca	Occlusion	+/E, M, F	ND	[80]
ESC	M/M	CM	CI	Ca	Occlusion	+/M, F	+/VEGF	[81]
BMSC	R/R°	SC, CM	CI	M	Cryoinjury	+/L-B	+	[82]
BMSC	R/R°	SC	CI	D	Normal	ND	ND	[83]
BMSC	H/S	SC	IU	I, PCR	Normal	ND	ND	[84]
BMSC	M/M	SC	CI	T	Reperfusion	ND	+	[85]
BMSC	M/M	SC	CI	T, PCR	Normal, Occlusion	ND	ND	[86][a]
BMSC	R/R	SC	AI	V	Occlusion	ND	ND	[87]
BMSC	H/H°	SC	AI	ND	Occlusion	+/E, TSPECT, Ex	ND	[88]
BMSC	M/M	SC	CI	T, CH	Occlusion	+/M	+	[89]
BMSC	R/R°	S	CI	D	Normal	ND	ND	[72]
BMSC	D/D°	SC	CI	I	Occlusion	+/E	+	[90]
BMSC	M/M	SC	BI	T	Normal	ND	ND	[91]
BMSC	M/M	SC	IU	T	Genetic	ND	ND	[92]
BMSC	H/H°	SC	AI	ND	Occlusion	+/E, TSPECT, Ex, V	ND	[2]
BMSC	M/R	SC	IV	V	Occlusion	ND	+	[93]
BMSC	P/P°	C	CI	M	Occlusion	+/SPECT	+	[94]
BMSC	H/M	SC	CI	V	Normal	ND	ND	[95]
BMSC	M/M	SC	BM T	T	Normal	ND	ND	[96]
BMSC	M/M	SC	BMT	CH	Genetic	ND	ND	[97]
NSC	M/M	SC	BI	T	Normal	ND	ND	[98]
EnSC	M/M	SC	CI	V	Cautery	ND	ND	[99]
HpSC	C/M	SC	CI	V, CH	Normal	ND	ND	[100]
CSC	M	SC	−	T	Normal	ND	ND	[101]
UNK	H	Mobile	HHT	CH	Transplant	ND	+	[102][a]
UNK	H	Mobile	HHT	CH	Transplant	ND	+	[103][a]
UNK	H	Mobile	HHT	CH	Transplant	ND	+	[104]
UNK	H	Mobile	HHT	CH	Transplant	ND	ND	[105]
UNK	H	Mobile	HHT	CH	Transplant	ND	+	[106]
UNK	M	Mobile	SC	I	Occlusion	+/E	+	[107]

Cell type: ESC, embryonic stem cell; BMSC, bone marrow stem cell; NSC, neuronal stem cell; EnSC, endothelial stem cell; HpSC, hepatocyte stem cell; CSC, cardiac stem cell; UNK, unknown cell type.
Species, donor species/host species: M, mouse; R, rat; H, human; D, dog; P, pig; HM, hamster; C, cell line; °, autologous transplant.
Cell status at transplant: SC, stem cell; CM, cardiomyocyte; Mobile, mobilized cell population.
Delivery: CI, cardiac injection; IU, intrauterine injection; IV, venous injection; AI, arterial injection; BMT, bone marrow transplant; SCI, subcutaneous injection; BI, blastocyst injection; HHT, human heart transplant; −, not applicable.
Tracking: D, dye; H, histology; I, immunostain; T, transgenic; CH, Y chromosome; G, gene (mdx, or sry); V, viral transfection; Ca, calcium phosphate transfection; E, electroporation; Li, liposome gene delivery; M, metabolic label; PCR, PCR.
Heart injury: Normal, Cryoinjury, Reperfusion injury, Permanent coronary occlusion, Genetic (i.e., mdx).
Function improvement/assay: +, improved; L-B, isolated perfused Langendorff with intraventricular balloon; M, closed-chest intraventricular micromanometer; M-B, micromanometer with intraventricular balloon; M&S, micromanometer and sonomicrometer; E, echocardiography; Ex, exercise regimens; TSPECT, Thallium-201 single photon emission computed tomography; SPECT, [^{99m}Tc]MIBI SPECT; V, ventriculography; F, in vitro force measurements; ND, not determined.
Angiogenesis: +, angiogenesis; ND, not determined.
[a] No cardiomyogenesis observed.

into normal or injured hearts. A number of different stem cells have been examined, including embryonic stem cells (ESC), bone marrow stem cells (BMSC), neuronal stem cells (NSC), endothelial stem cells (EnSC), hepatic stem cells (HpSC), and cardiac stem cells (CSC). In some instances, differentiation was induced in vitro, and consequently stem cell-derived cardiomyocytes were transplanted. In other cases, undifferentiated stem cells were transplanted either directly into the myocardium or alternatively intravenous route. In the latter case, it was assumed that the stem cells were able to 'home' or migrate to the normal and/or injured myocardium prior to differentiation. Several other studies are listed wherein the stem cells were delivered into developing embryos (via blas-

tocyst injection or in utero transplantation); these studies are included as they document the cardiomyogenic potential of several cell types. A series of studies examining female hearts that were transplanted into male recipients are listed; the presence of cardiomyocytes with Y-chromosomes was suggestive of mobilization of an unknown stem cell population. Also cited are studies where analogous transplantation and mobilization experiments yielded negative or markedly different results (studies demarked by **).

3. Characterization of the fate of transplanted cells

The ability to monitor the fate of cells following transplantation into normal or injured hearts is critical. This is particularly important in instances where myogenic stem cells are transplanted, as the ability to both track the donor cells and determine the subsequent level of differentiation is critical. Because we feel that cell tracking and lineage identification is of paramount importance in interpreting the consequence of myocyte and myogenic stem cell transplantation, a relatively large portion of the review is devoted to this topic.

Many studies examining myocyte transfer have relied simply on the presence of histological differences between hearts receiving cell transplants as compared to on-transplant controls. Although this type of analysis is useful as a first approximation of the effect of cellular transplantation, particularly in injured hearts, the preferred approach is to utilize molecular analyses that are able to distinguish donor and host cells. This is readily accomplished using lineage-restricted immune histological analyses in studies wherein non-cardiomyocytes are transplanted. For example, the fate of transplanted skeletal myoblasts was directly monitored by immune histological analyses with antibodies that recognize markers expressed in skeletal but not cardiac myocytes [47]. However, this type of analysis by itself is insufficient to monitor the fate of transplanted cardiomyocytes or stem cells with cardiomyogenic potential, as the successfully integrated (and/or trans-differentiated) cardiomyocytes would be indistinguishable from resident host cardiomyocytes.

A number of approaches have been employed to circumvent this problem. For example, treating donor cells with fluorescent cell-tracking dyes prior to transplantation has been used to monitor their survival in vivo. Because there is typically a high degree of donor cell death following transplantation, caution must be exercised to ensure that the observed signal arises from donor cells rather than from host cells which have acquired dye ‘liberated’ from dead or dying donor cells. Such a scenario could greatly over-estimate donor cell survival, or could mistakenly suggest trans-differentiation events. Conversely, proliferation of the transplanted cells could result in an underestimate of donor cell survival. Metabolic labeling of donor cells has also been employed to track their fate. This typically entails culturing donor cells in the presence of modified nucleotides (as for example, tritiated thymidine or bromodeoxy uridine, see for example Refs. [19,57]), that become incorporated into the cells during DNA replication; the replicated DNA (and consequently the donor cell nuclei) can later be identified in histological sections. This approach also suffers from potential signal dilution if there is significant donor cell proliferation post-transplantation.

Intrinsic genetic differences between donor and host cells have also been employed to monitor cell fate following transplantation. For example, anti-dystrophin immune cytology was used to follow dystrophin-expressing fetal cardiomyocytes or embryonic stem cell-derived cardiomyocytes following transplantation into canine and mouse host animals suffering from Duchenne’s-like muscular dystrophy (the host animals did not express dystrophin, see Refs. [4,78]). Other studies have relied on monitoring for the presence of Y-chromosomes following the transplantation of male donor cardiomyocytes into female recipients, using quantitative PCR amplification of Y-chromosome specific genes to estimate donor cell survival. In situ analyses for the presence of nuclei that contain a Y-chromosome can also be used to track the fate of donor cells. However, given the dramatic quantitative differences that have been reported when using this approach to monitor de novo cardiomyogenic events following the transplantation of human female hearts into male recipients (Table 3), it would appear that the assay is somewhat subjective.

Gene transfer provides an alternative and potentially a superior approach to monitor the fate of myocytes or myogenic stem cells following transplantation. The strategies reported to date include direct reporter gene delivery into donor cells via traditional DNA cell transfection or viral vectors, as well as the use of donor cells derived from transgenic animals carrying lineage restricted or ubiquitously expressed reporter genes (Tables 1–3). In the absence of the ability to clonally expand the transfected donor cells, traditional gene transfer is of limited value due to the relatively low transfection efficiency as well as unstable long-term expression characteristics in transiently transfected cells. However, stable transfection and clonal expansion of myogenic stem cells is certainly a viable approach, as demonstrated by the introduction of expression cassettes suitable for lineage isolation and/or restricted expression in ES cells as well as myogenic bone marrow derived cells (Table 3). Although the use of viral vectors permits very high gene transfer efficiency, reporter gene silencing is problematic. This approach also suffers from potential immunogenic responses against any virally encoded proteins that are expressed in the donor cells.

Genetically modified animals that carry either lineage restricted or ubiquitously expressed reporter genes offer an alternative strategy with which to monitor donor cell fate following transplantation. This approach has the decided

advantage that expression penetrance of the reporter gene (i.e., percentage of cells expressing as well as the relative level of expression per cell) can be quantitatively assessed in control animals. Moreover, the use of lineage-restricted promoters permits an unambiguous marker for monitoring de novo cardiomyogenic events. An example of the use of fetal donor cardiomyocytes prepared from transgenic mice expressing a nuclear localized β-galactosidase (β-gal) reporter gene is shown in Fig. 1. Dispersed cells prepared from adult transgenic mice indicate that the reporter gene is expressed in cardiomyocytes, but not cardiac fibroblasts (panels A and B). Following transplantation, these cells are readily identified by virtue of their β-gal activity in histological sections of the recipient heart (panels C and D). Reporter genes expressing Enhanced Green Fluorescence Protein (EGFP) have also been used to follow the

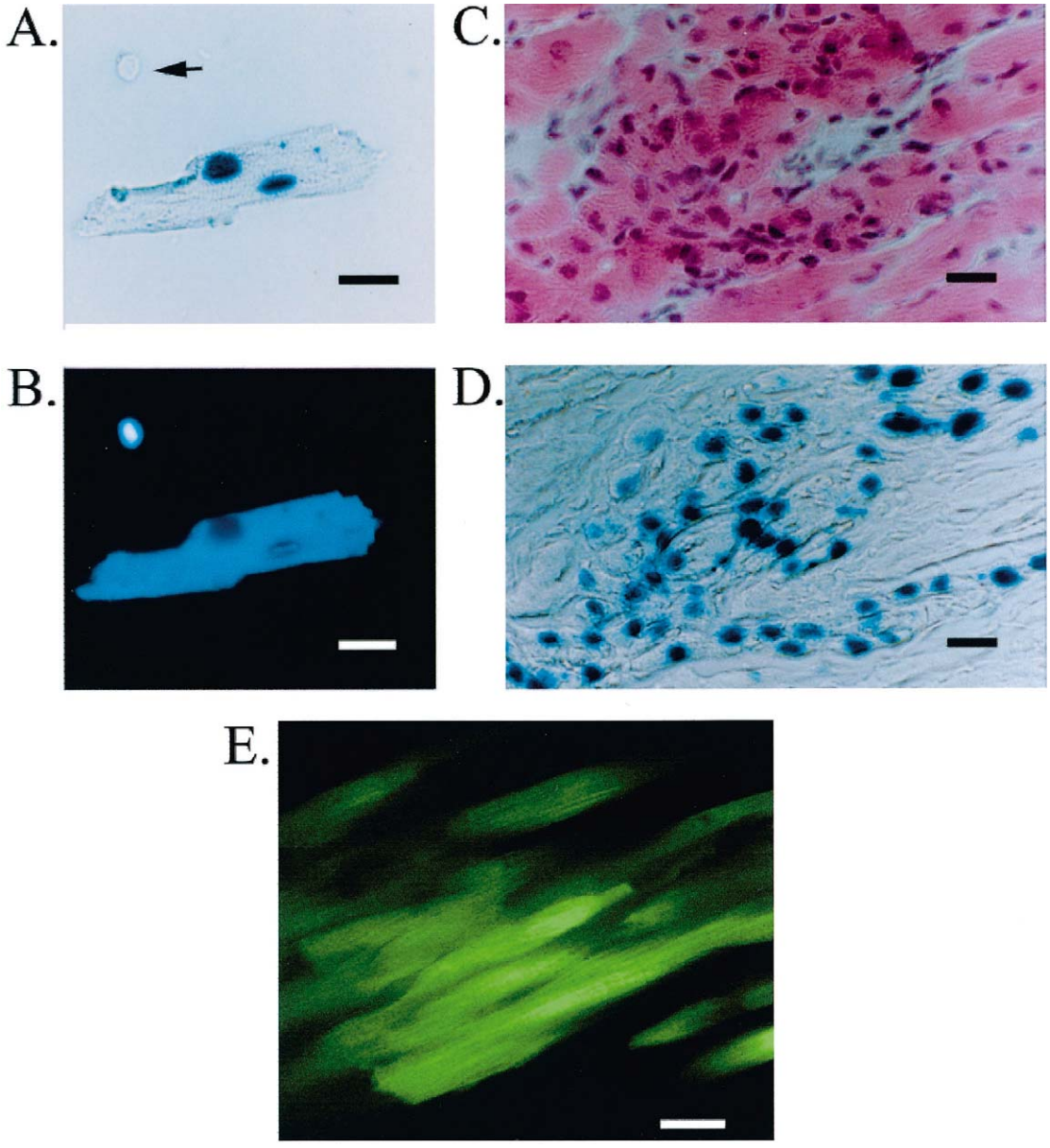

Fig. 1. Tracking of cardiomyocyte grafts in the host myocardium post-transplantation. Panels A and B show the same field of a dispersed cell preparation from an adult mouse heart carrying the nuclear-localized β-gal transgene driven by an α-cardiac MHC promoter (MHC-nLAC). Sample stained with X-gal and Hoechst dye. (A) Brightfield image showing a rod shaped bi-nucleated cardiomyocyte and a cardiac fibroblast (Arrow); (B) fluorescent image of the same field. Note that the β-gal staining is exclusive to cardiomyocyte nuclei and presence of X-gal reaction product quenches the Hoechst staining. Panels C and D show adjacent sections stained with H&E (C) and X-gal (D) from a normal heart transplanted with embryonic day 15 fetal cardiomyocytes carrying the MHC-nLAC transgene. Note the presence of stably grafted donor cells. (E) False-color 2D image of an intracardiac graft of fetal cardiomyocytes expressing EGFP. Image was obtained 35 days post-engraftment during two-photon illumination at a wavelength of 810 nm. Emitted fluorescence was measured in the 500–550-nm range and subsequently encoded in levels of green. Panels A–D are modified from Soonpaa et al. [3]. Magnification bar is 20 μm in all panels.

fate of transplanted myocytes and myogenic stem cells [30,31,89,108–110]. An example of a cluster of EGFP-expressing fetal cardiomyocytes transplanted into a non-transgenic adult mouse heart is shown in Fig. 1, panel E; the use of this EGFP reporter gene is particularly attractive in that gross cell morphology is readily apparent. Moreover, EGFP fluorescence is compatible with a variety of imaging techniques, and as such might be useful to monitor donor cell function in intact hearts (see below). However, care must be exercised with this reporter as dead and dying cardiomyocytes have an auto-fluorescent spectrum that partially overlaps with that of EGFP, and consequently dead or dying host cardiomyocytes can mistakenly be scored as donor cells (M. Rubart, unpublished observation).

Once a methodology is established to track the donor cells, standard histochemical, immune cytological and ultrastructural analyses can be performed to monitor their fate. In the case of myocyte transplantation, this simply entails determining if the donor cells survive, proliferate, and/or acquire a highly differentiated phenotype. Donor cell survival can be monitored by cell counts post-transplantation. Nuclear localized β-gal reporters (as described above) are particularly well suited for this application as relatively thick heart sections (300+ μm) can be processed and the surviving cell number directly counted using a dissecting microscope. Because of its relatively small size, the entire mouse heart can be rapidly and quantitatively surveyed with this assay. This approach is robust enough to permit quantitation of relatively large grafts (Fig. 2, panel A), and is sensitive enough to detect the presence of a single surviving bi-nucleated cardiomyocyte (Fig. 2, panel B).

Additional information pertaining to the mechanism of cell loss (i.e., apoptosis, necrosis) can be obtained via combinatorial histochemical and/or immune histological analyses [34,35]. Donor cell proliferation can be monitored by a number of direct (i.e., thymidine or BrdU incorporation) or indirect (i.e., PCNA or Ki67 immune cytology) analyses. The merits and demerits of these approaches have recently been reviewed in the context of monitoring adult cardiomyocyte DNA synthesis [111] and accordingly are not discussed here. The level of cardiomyogenic differentiation can also be assessed by traditional histochemical, immune histological and ultrastructural analyses. Once again, β-gal reporter genes are particularly useful as the β-gal/X-GAL reaction product can be directly visualized with standard transmission electron microscopy. Fig. 3 shows an example of transplanted fetal cardiomyocytes expressing a nuclear-localized β-gal reporter. Due to the extensive sample processing, the X-GAL reaction product leaches out of the nucleus, and appears as a peri-nuclear electron dense deposit that is sometimes lodged between the adjacent myofibers. Donor cells can thus be identified without the loss of resolution that can occur with immunological-based analyses. As always, the accuracy of the analyses are directly dependent upon the fidelity of the reagents and/or approaches used to document differentiation.

Following the fate of myogenic stem cells requires

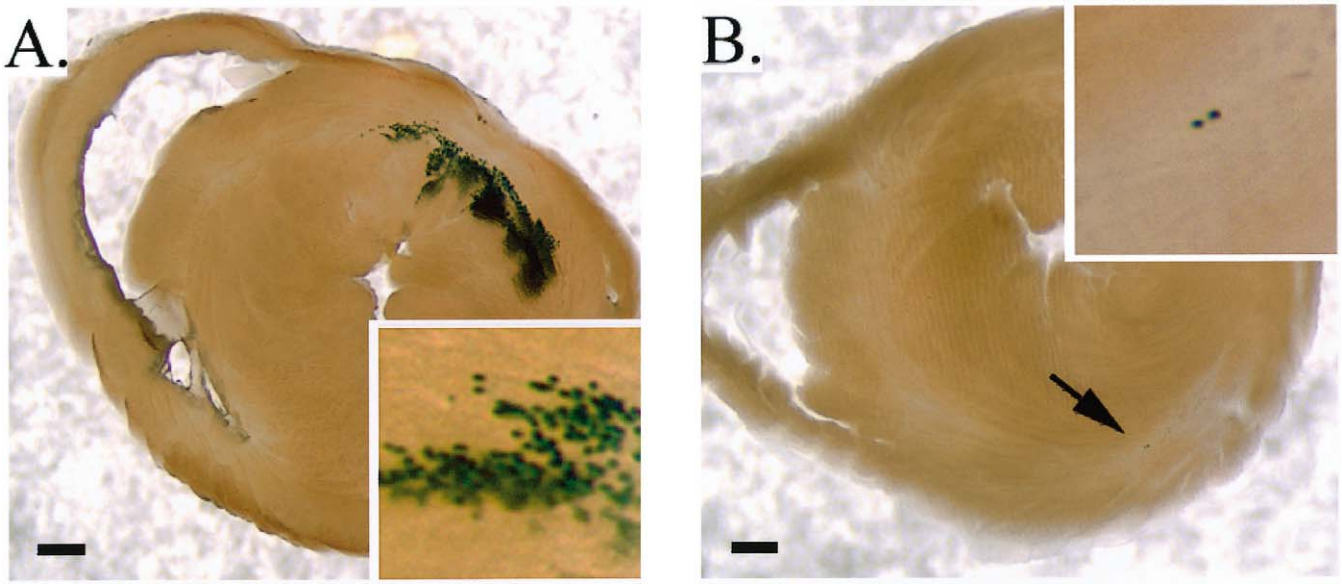

Fig. 2. Quantification of fetal cardiomyocyte graft seeding efficiency. (A) Low power view of a thick heart section (300 μm) from a non-transgenic mouse host transplanted with embryonic day 15 fetal MHC-nLAC cardiomyocytes. The transplanted heart was harvested, fixed, sectioned by vibratome, and stained with X-gal. The grafted cells are identified by virtue of their blue nuclei. Individual cells can be readily visualized and counted under higher magnification (see inset). (B) Photomicrograph illustrating the sensitivity of the MHC-nLAC assay system. A single bi-nucleated cardiomyocyte (arrow) was tracked using this assay system following transplantation. Inset shows higher magnification of the transplanted cell. Magnification bar is 0.5 mm in all panels.

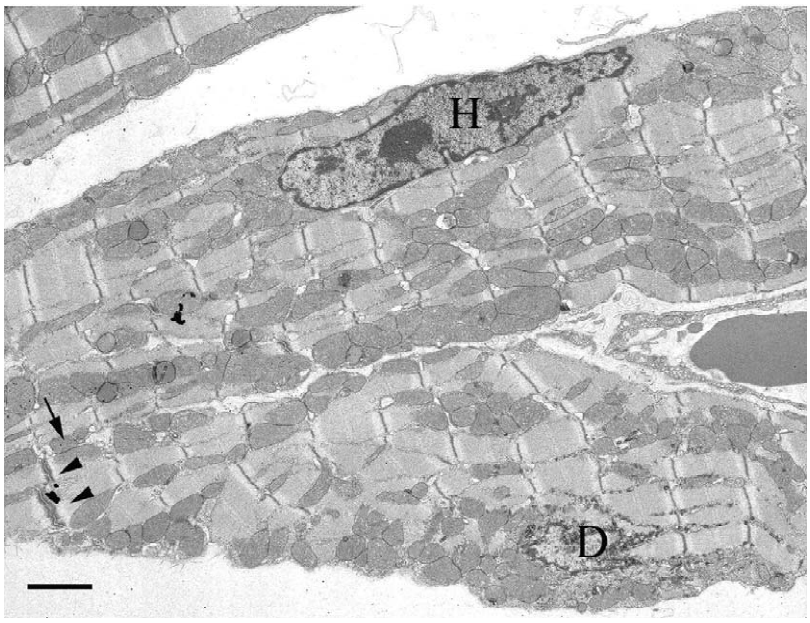

Fig. 3. Ultrastructural analysis of transplanted MHC-nLAC fetal cardiomyocytes. D, donor cell nucleus, identified by presence of perinuclear, electron-dense, X-gal reaction product. H, host cell nucleus, identified by lack of X-gal reaction product. Arrowheads demark fascia adherens/desmosomes between donor and host cells. Arrow demarks putative gap junctions between donor and host cells. Magnification bar is 1 μm.

additional considerations. The use of ubiquitous reporter genes provides a robust approach to monitor donor cell survival and/or homing to injured regions of the heart, but is dependent upon secondary analyses (as described above) to monitor the degree, if any, of myogenic differentiation [85,89,91,98,100]. While the use of lineage-restricted reporter genes provides an unambiguous way to monitor stem cell differentiation into a specific lineage, in the absence of myogenic differentiation additional analyses are required to confirm the presence of the donor cell [86]. Perhaps the ideal situation is combinatorial assays wherein compatible ubiquitous and lineage-restricted reporters are both employed in the donor cell.

4. Mechanisms by which cellular transplantation can impact heart function

A number of the studies cited in Tables 1–3 indicate that myocyte or myogenic stem cell transplantation into injured hearts resulted in improved cardiac function as compared to non-transplanted controls. In most instances it was not clear if the effect on cardiac function resulted as a direct consequence of the transplanted cells participating in a functional syncytium with the host myocardium, or alternatively if the presence of exogenous cells imparted an indirect yet beneficial effect on the heart. This point is underscored by the observation that in some cases injured hearts transplanted with cell types that a priori would not possess the potential to contribute to cardiac work cycles, as for example skeletal myocytes (Table 2), fibroblasts [18,67,112], constituents of the vascular system [113,114], and extravascular smooth muscle [18,112,115–117] nonetheless exhibited some functional improvement as compared to hearts which did not receive cells. Furthermore, improved function was also observed in several cardiomyocyte transplantation studies wherein the number of seeded cells as assessed by histochemical analysis was insufficient to impart a direct functional effect [37].

It is relatively straightforward to understand how cellular transplantation can improve cardiac function if a significant number of donor cells directly participate in a functional syncytium with the host myocardium. However, it is also important to consider potential mechanisms via which transplanted donor cells could benefit cardiac function without directly contributing to systolic contraction. For example, an angiogenic response was observed in many studies following cell transplantation (Tables 1–3). An increase in vascular supply could result in the sparing of the chronically ischemic, functionally overloaded myocardial tissue bordering the infarcted region. This notion is directly supported by the observation that intravenous injection of human angioblasts into rats with myocardial infarcts resulted in enhanced blood vessel formation with a concomitant salvage of the at risk myocardial tissue [118]. Sparing of at risk myocardial tissue, resulting in preserved left ventricular function, is also observed clinically following coronary artery bypass grafting/angioplasty [119]. Similarly, clinical interventions to enhance vascular supply can promote the recovery of hibernating myocardium [120]. An analogous effect from donor cell-induced angiogenesis could contribute to the impact on cardiac function.

In addition to enhanced angiogenesis, myocyte or

myogenic stem cell transplantation may also alter the normal sequela of post MI scar formation and, consequently, ventricular remodeling. Indeed, a consistent observation following skeletal myoblast transplantation in a number of studies was a pronounced attenuation of post MI ventricular dilation (Table 2). According to LaPlace's Law, retention of a more 'normal' pump size and shape should greatly aid cardiac function. Kloner and colleagues have also suggested that by simply thickening the left ventricular wall, cell transplantation can reduce the degree of systolic dyskinesis, which over time could also impart a functional effect [37]. Given these collective experimental and clinical precedents, it is quite reasonable to conclude that the improvement in cardiac function observed in some of the studies cited above are quite independent of donor cell contractile activity.

A number of experimental read-outs have been employed to quantitate the effect of myocyte or myogenic stem cell transplantation. As indicated above, traditional morphometric analyses have clearly documented a reduction in the severity of ventricular remodeling post-injury. A number of direct functional analyses have also been employed to monitor the effect of cell transplantation into injured hearts (Tables 1–3). Many of the studies in rodents comprised ex vivo pressure–volume studies of Langendorff-perfused heart preparations with inflatable balloons inserted into the left ventricle. In some cases, improved exercise tolerance was observed in animals that also showed functional improvement with subsequent ex vivo analyses. Closed chest pressure–volumes studies using intra-ventricular transducers have also been performed. Echocardiographic analyses have been used to monitor function in both rodents and larger animals following cell transplantation. Additionally, ultrasonic crystals have been used to monitor regional wall motion across the infarcted region of the heart of larger animals following cell transplantation. Although these studies have documented that global improvement of cardiac function can occur following cell transplantation, in most instances the assays employed were unable to identify the underlying mechanism (that is, distinguishing between direct contraction of the donor cells versus a beneficial effect imparted upon the surviving host myocardium). Understanding the mechanistic basis for improved cardiac function is of critical importance when attempting to effect modifications aimed at enhancing the intervention.

Accordingly, an imaging-based assay was recently developed with the goal of determining if myocytes or myogenic stem cells can participate in a functional syncytium with the host myocardium following transplantation [31,109,110]. The system had two major requirements. The first was the ability to image some aspect of cardiac function at the cellular level within an intact heart. This was accomplished by using two-photon laser scanning microscopy to monitor the changes in fluorescence intensity of calcium sensitive dyes in response to remote electrical stimulation (see Ref. [110] for detailed characterization of the system). An example is shown in Fig. 4. Panel A shows a two-dimensional image from a control heart which was perfused with the calcium sensitive dye Rhod-2. The heart was also perfused with cytochalasin D to facilitate excitation-contraction uncoupling. The preparation was paced at a remote site, and the fluorescence signal from three cardiomyocytes at a depth of approximately 100 μm from the epicardial surface was acquired in line scan mode. The position of the line scan is indicated by the horizontal white line in panel A, and integrated traces generated from the line scan data are shown in panel B. As can be seen, this assay can be used to record cytosolic calcium transients at the single cell level (as approximated by changes in Rhod-2 fluorescence) within intact hearts.

The second system requirement was the ability to distinguish host cardiomyocytes from the transplanted cells. In a proof of concept study, fetal donor cardiomyocytes from EGFP-expressing transgenic mice were transplanted into non-transgenic hosts, and subjected to Rhod-2 fluorescence imaging as described above. The optical paths of the imaging system were modified such that Rhod-2 fluorescence (560–650 nm) and EGFP fluorescence (500–550 nm) could be sampled simultaneously [31,109]. Using this approach, calcium transients were simultaneously recorded from donor cardiomyocytes (as identified by EGFP fluorescence) and juxtaposed host cardiomyocytes (which lacked EGFP fluorescence). The study demonstrated that donor and host cardiomyocytes are electrically coupled to one another, strongly supporting the notion that transplanted cardiomyocytes can participate in a functional syncytium with the host myocardium. This approach may be useful to monitor the capacity of myogenic (i.e., skeletal myoblasts) and cardiomyogenic stem cells to functionally integrate with the host myocardium following transplantation, provided that a transgene suitable for imaging is utilized.

5. Strategies to enhance cellular transplantation

Quantitative PCR studies revealed that the preponderance of donor cardiomyocytes die following transplantation into the heart, with typically less than 5% of the cells successfully seeding the myocardium [38]. Comparable seeding efficiencies have been obtained by morphological analysis of transplanted tissue [19], as well as by direct counts of donor cells carrying the nuclear-localized β-gal reporter described above (Soonpaa, Pasumarthi, Rubart and Field, unpublished results). Similarly, with two exceptions, transplantation and/or homing of myogenic adult stem cells have resulted in at best only modest accumulation of de novo muscle cells (see Table 3). A high rate of myogenic differentiation was reported in one study when 'lin−/kit+' hematopoietic stem cells were directly in-

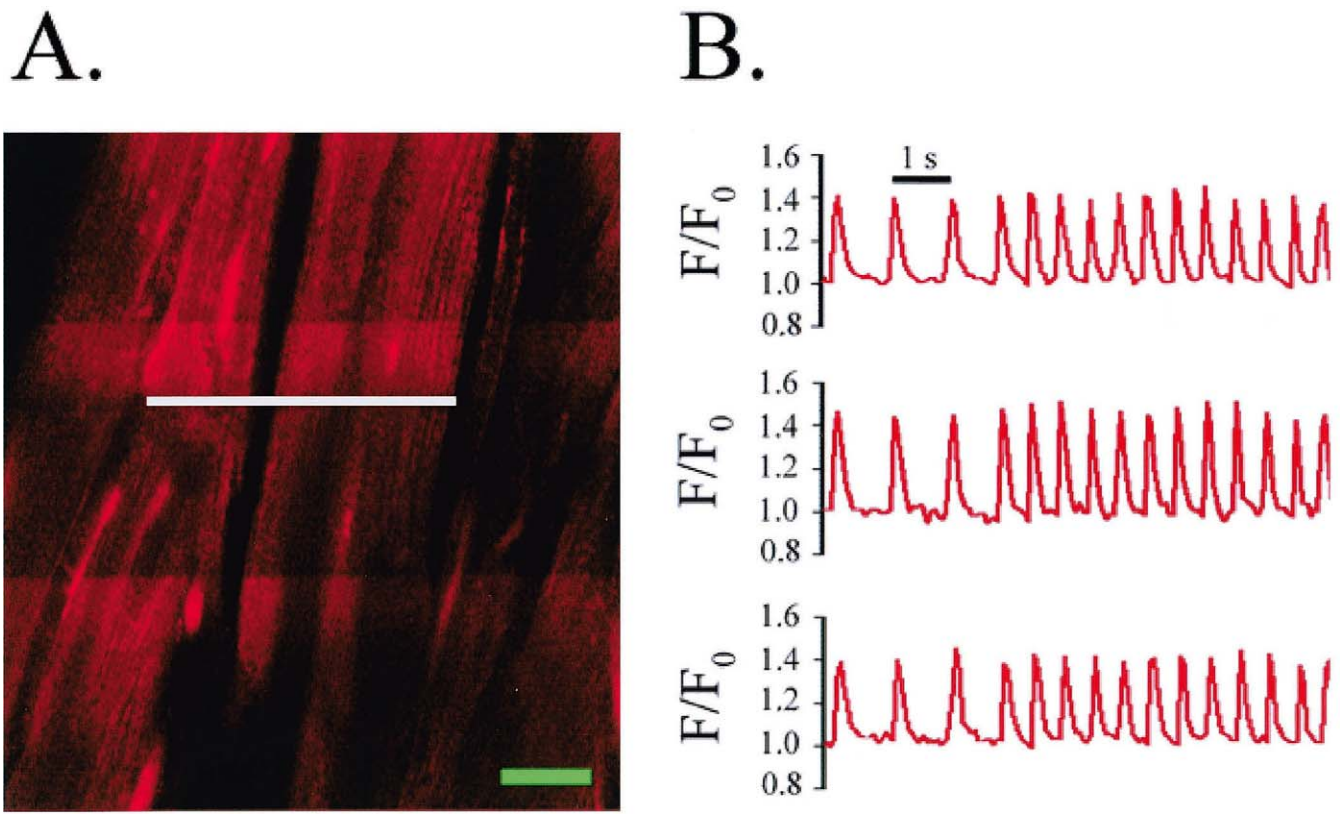

Fig. 4. Imaging of single myocyte calcium transients in the anterior left ventricle of a rhod-2 loaded mouse heart. (A) Full-frame false color image during electrical stimulation at 2 Hz. Image was acquired during two-photon excitation at 810 nm and emission was measured in the 560–650-nm range. Intensity of Ca^{2+}-bound rhod-2 was encoded off-line in shades of red. Electrical stimuli resulted in simultaneous and approximately uniform increases in rhod-2 fluorescence along the entire length of the image. The increase in fluorescence of the myocytes is due to stimulus-evoked increases in cytosolic calcium concentration. Magnification bar (green) is 20 μm. (B) Time course of normalized rhod-2 fluorescence changes in three juxtaposed myocytes during stimulation at 1 and 2 Hz. Rhod-2 fluorescence was acquired in line scan mode. The position of the line scan is indicated by the white horizontal line in panel A. Signal intensity, F, was averaged along the scan line for each myocyte, normalized to the diastolic fluorescence, F_o, and plotted as a function of time.

jected into hearts with coronary artery ligations [89]. In contrast, preliminary studies using an analogous system failed to identify any cardiomyogenic events [86]. The other exception, as discussed above, was the relatively high level of cardiomyocyte chimerization in female hearts transplanted into male recipients that was observed in one of five studies using similar analytical techniques.

Collectively these data suggest that interventions aimed at enhancing donor cell survival, homing, and/or post-transplantation proliferation may be required to achieve high levels of de novo cardiomyocyte seeding. As can be seen in Tables 1–3, some efforts to enhance myocyte and myogenic stem cell seeding have already been initiated. These include relatively descriptive efforts wherein the effects of donor cell age as well as the timing of cell transplantation post-injury were determined. Other studies have attempted to directly enhance cell survival. These include efforts to inhibit apoptosis (by treating cells with apoptosis inhibitors prior to transplantation), to enhance angiogenesis (by the co-delivery of angiogenic factors with the donor cells), and to enhance retention of donor cells (by incorporating them into three dimensional scaffolds or by providing supplementary matrix). In a very clever variation of this approach, Murry and colleagues subjected donor cells to heat shock prior to transplantation in an effort to activate the endogenous protective HSP70 gene [35]. Although this intervention resulted in a transient decrease in the level of donor cell apoptosis following transplantation, it was not clear if this translated to a permanent increase in donor cell seeding. Finally, several studies have demonstrated that co-delivery of growth factors and ES cells resulted in enhanced cardiomyogenic seeding (Table 3).

The availability of transgenic mouse models expressing cardioprotective or cardioproliferative gene products provide a potentially useful experimental system to identify pathways with which to enhance myocyte and myogenic stem cell transplantation. For example, expression of a number of different transgenes has resulted in a marked reduction in reperfusion injury following transient coronary artery occlusion [121]. If reperfusion injury is responsible for a major component of myocyte death post-transplantation, the use of donor myocytes or myogenic stem cells from these transgenic mice should

markedly enhance de novo cardiomyocyte seeding. Donor cells from transgenic mice expressing pro-survival genes should also be more efficient at engraftment [122]. A number of transgenic mouse models that exhibit enhanced cardiomyocyte proliferation have also been described [123,124]; transplantation of myocytes or myogenic stem cells from these animals should result in enhanced proliferation of de novo cardiomyocytes with a concomitant increase in graft size.

In a variation of this theme, genetically modified ES cells were recently used to identify gene combinations that permitted proliferative expansion of cardiomyocytes following myogenic differentiation [125]. The experiment utilized a selection scheme that resulted in the generation of pure cardiomyocyte cultures [78]. Undifferentiated ES cells were transfected with a transgene encoding resistance to neomycin under the regulation of the cardiac specific α-myosin heavy chain promoter. The transgene also carried sequences encoding hygromycin resistance under the transcriptional regulation of the PGK promoter (MHC-neo^r/PGK-hygro^r transgene). Stably transfected, undifferentiated ES cells were selected based on hygromycin resistance. Differentiation was then induced, and cardiomyocytes were selected based on G418 resistance. Low rates of ES-derived cardiomyocyte proliferation were observed in these cultures, as evidenced by the relatively small cardiomyocyte colony size and the absence of tritiated thymidine incorporation (Fig. 5, panels A and C, respectively).

In other cultures, transgenes encoding anti-apoptotic activity (specifically, dominant negative p53 and p193 transgenes) as well as pro-proliferative activity (the E1a oncoprotein) were co-transfected with the neomycin/hygromycin selection cassettes. Markedly enhanced car-

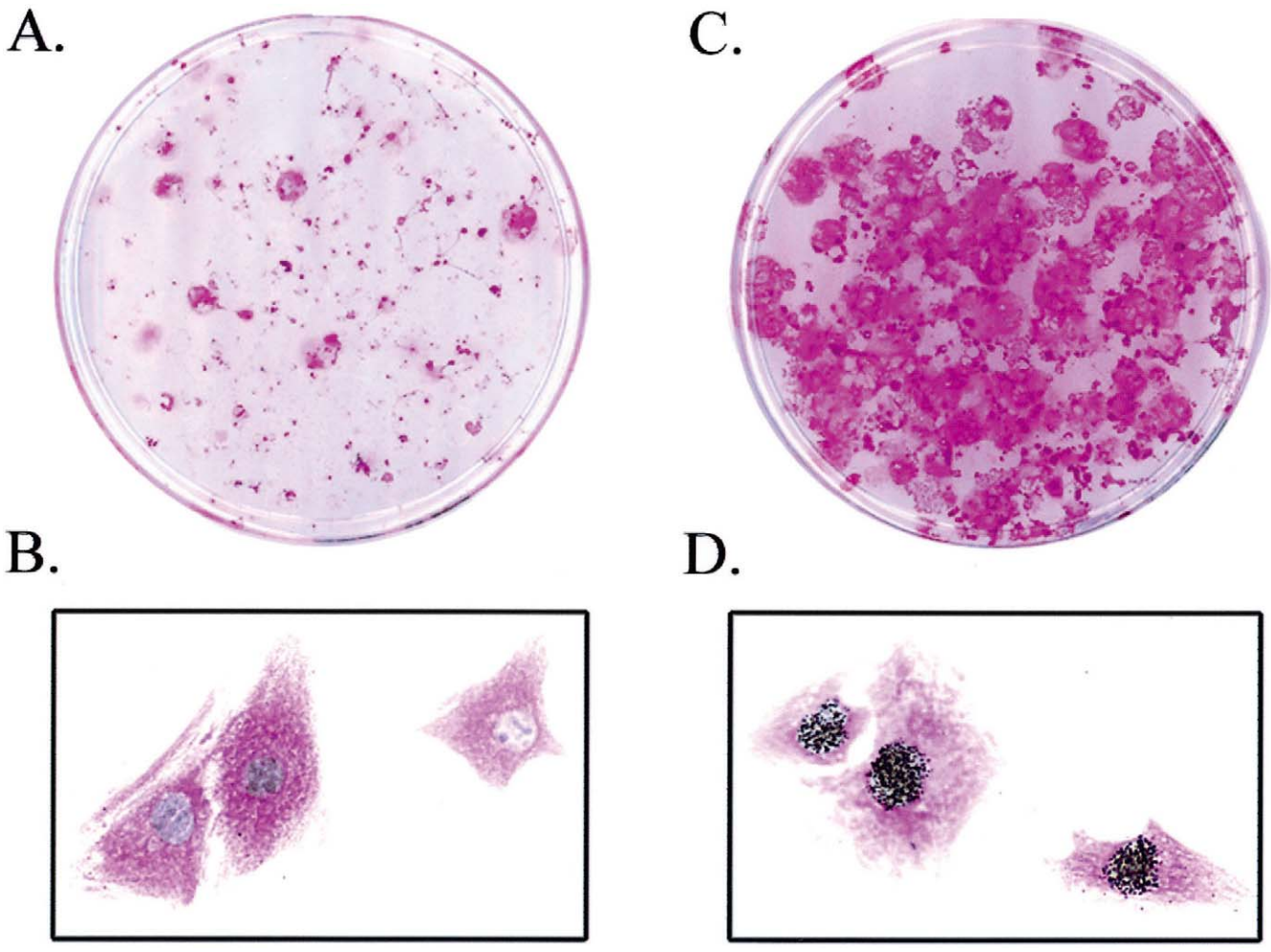

Fig. 5. An ES cell-derived cardiomyocyte colony growth assay. (A,B) Photomicrographs illustrating the yield of ES cell-derived cardiomyocytes in cultures transfected with an MHC-neo^r/PGK-hygro^r transgene alone (A) or in combination with E1A, dominant negative p53 and p193 transgenes (B). ES cell-derived cardiomyocyte colony growth was directly visualized via periodic acid-Schiff (PAS) staining. (C,D) Photomicrographs illustrating dispersed cells from cultures transfected with MHC-neo^r/PGK-hygro^r transgene alone (C) or in combination with E1A, dominant negative p53 and p193 transgenes (D). Dispersed cells were processed for PAS staining followed by tritiated thymidine autoradiography (indicative of cells undergoing DNA synthesis). Note the marked increase in ES cell-derived cardiomyocyte yield when transfected with E1A, dominant negative p53 and p193 (B) as well as increased tritiated-thymidine uptake (D) in comparison to controls (A,C).

diomyocyte proliferation was observed in these cultures, as evidenced by the marked increase in cardiomyocyte colony size and very high rates of tritiated thymidine incorporation (Fig. 5, panels B and D, respectively). These studies demonstrated that combinatorial blockade of p53 and p193 activity (via the expression of the corresponding dominant negative transgenes) was able to abrogate cell cycle-induced apoptosis, thereby resulting in markedly enhanced proliferation of the ES-derived cardiomyocytes. More importantly, the study demonstrated that cell cycle activity can be readily modulated in in vitro generated cardiomyocytes. Although it remains to be seen if these manipulations will ultimately translate to enhanced seeding following transplantation of stem cell-derived cardiomyocytes, preliminary results obtained with fetal cardiomyocytes expressing the same transgenes bode extremely well for this approach.

6. Conclusions

It is clear from the studies reviewed here that myocytes and myogenic stem cells can be stably transplanted into the normal or injured heart, and furthermore that this intervention can have a positive impact on cardiac function. Although improved global cardiac function is the goal of all cell transplantation therapies, it appears that in many cases cited above the mechanistic basis that gives rise to functional improvement is unclear. In this regard the positive correlation between increased angiogenesis and increased function is quite striking. If the preponderance of effected myocardial function resulting from cellular transplantation is mediated via enhanced angiogenesis, there are likely to be better and perhaps safer ways to accomplish this. Similarly, if the main benefit from cell transplantation is to limit dyskinetic wall motion and reduce post-infarction remodeling, then comparative analyses with passive restraint interventions are warranted [126].

In contrast, if the efficiency of myocyte or myogenic stem cell transplantation (or, alternatively, stem cell mobilization) is optimized to the point that there is a substantial reconstitution of the lost muscle mass with nascent functional cardiomyocytes, the intervention should be much more efficacious than simple salvage of at-risk myocardium resulting from cell transplantation-induced angiogenesis. Consequently issues pertaining to the accuracy of tracking of donor cells as well as the assessment of their function post-transplantation are of critical importance. Indeed, this issue underlies our group's concerns (and arguably, our preoccupation) for developing unambiguous assays to track donor cell fate and function. Recent observations of high rates of donor cardiomyocyte coupling following fetal cardiomyocyte transplantation in normal hearts [31,109] bode well for the notion of transplantation- or mobilization-mediated functional reconstitution, provided that stem cell-derived cardiomyocytes can be transplanted or mobilized at a density sufficient for global functional impact. Certainly, a similar set of concerns and caveats are relevant for interventions aimed at inducing cell cycle activity in surviving cardiomyocytes [123].

With respect to the ultimate clinical utility of myocyte and myogenic stem cell transplantation, it is important to recognize that we are still very early in the game. The field of cardiomyogenic stem cell mobilization is even newer. The fact that the potential utility of stem cell-based therapies is becoming highly recognized is underscored by the marked increase in the cell transplantation literature that has occurred over the last 2 years. With more individuals studying the problem, and more importantly with the increase in the intellectual critical mass that will concomitantly be applied to the system, it is likely that new advances will rapidly be made. With luck, the utility of these interventions for the treatment of heart failure will be validated.

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