Ischemic mitral regurgitation (MR) is a common complication of ischemic heart disease that conveys an adverse prognosis after both myocardial infarction (MI) and coronary revascularization (1,2). Extensive work has confirmed the relation of ischemic MR to remodeling and distortion of the ischemic left ventricle (LV) (3–9). As viable myocardial cells are lost, the wall becomes thinner and bulges outward, a process that begins almost immediately and then progresses over weeks to months (10). If the inferior wall of the heart is involved, bulging of this wall also displaces the ischemic left ventricle (LV) (3–9). As viable myocardial cells are lost, the wall becomes thinner and bulges outward, a process that begins almost immediately and then progresses over weeks to months (10). If the inferior wall of the heart is involved, bulging of this wall also displaces the ischemic left ventricle (LV) (3–9).

The study was designed to assess whether post-myocardial infarction (MI) in-scar transplantation of skeletal myoblasts (SMs) could reduce chronic ischemic mitral regurgitation (MR) by decreasing left ventricular (LV) remodeling.

METHODS
An infero-posterior MI was created in 13 sheep, thereby resulting in increasing MR. Two months post-MI, the animals were randomized and in-scar injected with expanded autologous SM (n = 6; mean: 251 x 10⁶ cells) or culture medium only (n = 7). Three-dimensional echocardiography was performed at baseline, before transplantation, and for two months thereafter (sacrifice), with measurements of LV end-diastolic and end-systolic volumes (ESV), ejection fraction (EF), MR stroke volume, and leaflet tethering distance; wall motion score index (WMSi) was assessed by two-dimensional echo.

RESULTS
Measurements were similar between groups at baseline and before transplantation. At sacrifice, transplantation was found to have reduced MR progression (regurgitant volume change: (-1.83 ± 0.32 ml vs. 5.9 ± 0.7 ml in control group, p < 0.0001) and tethering distance (-0.41 ± 0.09 cm vs. 0.44 ± 0.12 cm in control group, p < 0.001), with significant improvement of EF (2.01 ± 0.94% vs. -4.86 ± 2.23%, p = 0.02), WMSi (-0.25 ± 0.11 vs. 0.13 ± 0.03 in controls, p < 0.01) and a trend to a lesser increase in ESV (23.3 ± 3.5 ml vs. 35.4 ± 4.2 ml in control group, p = 0.055).

CONCLUSIONS
Autologous skeletal myoblast transplantation attenuates mild-to-moderate chronic ischemic MR, which otherwise is progressive, by decreasing tethering distance and improving EF and wall motion score, thereby enhancing valve coaptation. These data shed additional light on the mechanism by which skeletal myoblast transplantation may be cardioprotective. (J Am Coll Cardiol 2006;47:2086–93) © 2006 by the American College of Cardiology Foundation.
tethering distance and ischemic MR by locally reshaping the infarcted wall and improving global LV function (Fig. 1). This approach was tested in a chronic ischemic MR sheep model using three-dimensional (3D) and Doppler echocardiography to quantify MR and relate it to 3D changes in valve configuration.

METHODS

All experiments were performed in accordance with the “Guiding Principles in the Care and Use of Animals” of the American Physiological Society.

Chronic ischemic MR. We used the chronic ischemic MR model of Llaneras and Edmunds (27,28), which produces MR only with LV remodeling, eight weeks after ligating the second and third circumflex obtuse marginal coronary arteries. Thirteen sheep (40 to 50 kg), anesthetized with thiopentothal (0.5 ml/kg), intubated and ventilated at 15 ml/kg with 2% isoflurane and oxygen, and given glycopyrrolate (0.4 mg intravenously [IV]) and prophylactic vancomycin (0.5 g IV), underwent sterile left thoracotomy, with procaainamide (15 mg/kg IV) and lidocaine (3 mg/kg IV followed by 2 mg/min) given 10 min before coronary ligation. Sheep received oral amiodarone (400 mg daily) for two days before infarction and oral acebutolol (200 mg) and aspirin (250 mg) the day before infarction. After baseline imaging, the pericardium was opened and the second and third circumflex obtuse marginal branches were ligated to infarct the infero-posterior wall. Imaging was repeated and the thoracotomy closed (Fig. 2A).

Cell transplantation. CELL CULTURES. At the time of infarction, a 10-mg skeletal muscle biopsy was obtained from the vastus lateralis. The biopsy was minced, frozen, and then cultured 14 days before transplantation (25). After enzymatic dissociation, the tissue was centrifuged and the remaining debris eliminated by filtration (100-μm mesh). Fourteen days before transplantation, the cells were plated onto Nunc substrates (10^3/cells/cm^2) and grown in Ham medium supplemented with fetal bovine serum (FBS) (20%, Perbio, Logan, Utah), beta-fibroblast growth factor (5 ng/ml, Sigma, St. Louis, Missouri) and penicillin-streptomycin (10,000 IU/ml and 10 mg/ml, respectively, Gibco, Invitrogen, Cergy Pontoise, France). Cells were expanded twice by trypsinization before reaching confluence, and the culture medium was then shifted to a differentiation medium containing no beta-fibroblast growth factor and 2% FBS.

PREPARATION OF CELLS. Fourteen days after plating, the cells were harvested by trypsinization for implantation.

Figure 1. Mechanistic hypothesis of the efficacy of skeletal myoblast transplantation on chronic ischemic mitral regurgitation (MR). (Left) Baseline configuration of left ventricle (LV) with normal mitral leaflet closure. (Middle) Chronic infero-posterior infarction: loss of viable myocardial cells induce local LV remodeling with outward bulging of the free wall, papillary muscle displacement, and increased leaflet tethering and MR. (Right) Myoblast transplantation reverses local remodeling with decreased tethering distance and improved leaflet coaptation and MR. Ao = aorta; Ant. = anterior; LA = left atrium; MI = myocardial infarction; Post. = posterior.

<table>
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<th>Abbreviations and Acronyms</th>
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<tr>
<td>ESV = end-systolic volume</td>
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<td>FBS = fetal bovine serum</td>
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<tr>
<td>IV  = intravenously</td>
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<tr>
<td>LV  = left ventricle</td>
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<tr>
<td>MHC = myosin heavy chain</td>
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<tr>
<td>MI  = myocardial infarction</td>
</tr>
<tr>
<td>MR  = mitral regurgitation</td>
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<tr>
<td>PM  = papillary muscle</td>
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<tr>
<td>SM  = skeletal myoblast</td>
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<tr>
<td>3D  = three-dimensional</td>
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<td>WMS = wall motion score</td>
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Following one rinse with Ca\(^{2+}\)/Mg\(^{2+}\)-free phosphate-buffered saline (Gibco, Invitrogen), the cells were incubated for 5 min with trypsin-ethylene diamine tetra acetic. Upon dissociation, 10% (final volume) of FBS was added and the cells were centrifuged (1,200 rpm). The cell pellet was washed three times in saline (NaCl 0.9%, Baxter) supplemented with bovine serum albumin (0.5%, Sigma). Cell counting was performed using the Neubauer hemacytometer, and a first assessment of cell viability was done using the trypan blue dye exclusion technique. The cell pellet was then resuspended in saline to a final concentration of 10^8 cells/ml. Aliquots were preserved for further characterization of cell type and viability. Immediately before injection, cells were transferred to 1 ml syringes and warmed to room temperature.

**INTRAMYOCARDIAL INJECTIONS.** Sheep were randomized into two groups receiving culture medium only (n = 7, control group) or cells (n = 6, myoblast group) in a physiologic salt solution with 0.5% bovine serum albumin. Eight weeks after infarction, during a repeat left lateral thoracotomy, 20 to 30 intramyocardial myoblast injections were performed across the bulging infero-posterior infarcted area (mostly underlying the posterior papillary muscle) using a 27-gauge needle. Control sheep received an equivalent volume of culture medium.

**Assessment of results. CELL CHARACTERIZATION.** At each expansion time and at the final harvest, the cells were analyzed by flow cytofluorimetry for the CD56 myogenic marker. The proportion of myogenic cells was evaluated using flow immunofluorescence as previously described (26).

**Ultrasonic assessment. ACQUISITIONS.** After general anesthesia and during thoracotomy (for optimal imaging), examinations were performed: 1) at baseline; 2) immediately before injections (8 weeks after infarction, allowing MR to develop); and 3) 2 months after injections. Three-dimensional echo data were acquired with a 5-MHz epicardial transducer (Philips Sonos 5500), scanning through a water bath from the LV apex using a rotating array with its beam aligned through the center of the mitral valve, parallel to the LV long axis (29,30). Onboard 3D software recorded 30 rotated images automatically at 6° increments, with electrocardiographic gating and suspended respiration. Digital images were transferred to a Silicon Graphics workstation (Fig. 2B).

The 3D echo analysis first established a reference frame, the least-squares plane of the mitral annulus (29). Mitral geometry was analyzed within this reference frame from rotated images at mid-systole, the time of closest leaflet-annulus approach. The mitral and aortic annuli were identified as the leaflet hinge points, confirmed by cineloop review. The PMs were traced and their most basal and anterior tips identified by reviewing several adjacent images. The tethering length over which the leaflets and chordae were stretched between the PMs and the relatively fixed...
fibrous portion of the annulus was then measured from posterior PM tip to the medial trigeone of the aortic valve (medial junction of aortic and mitral annuli) (Fig. 2B, right). Tethering length was used because in previous studies it has emerged as the strongest predictor of ischemic MR (6–8,13). These 3D echo measurements have been shown to correlate well with those by sonomicrometer crystal array, both in vitro and in vivo ($r^2 = 0.99$, SEE = 0.7 mm, mean difference = 0.08 ± 0.7 mm [NS vs. 0]) (6,8).

Two-dimensional epicardial echocardiography was performed for wall motion analysis scoring. One parasternal LV long-axis view was recorded, care being taken that the mitral and aortic valves and the apex were simultaneously visualized, with three parasternal short-axis views recorded at the base, PM, and apical levels. Acquisition of digital cineloops and analysis of segmental wall motion and MR stroke volume were performed by a single experienced volunteer blinded to the treatment group.

### 3D LV Volume, Mitral Geometry, and MR Evaluation
Three-dimensional LV volumes were obtained using endocardial border tracings from nine equally spaced rotated views (30). Mitral regurgitation stroke volume was calculated as LV stroke volume minus aortic outflow volume (measured by pulsed Doppler). Regurgitant fraction was calculated as (MR stroke volume)/(forward aortic + MR stroke volumes). Mitral annular area was calculated by projecting the mitral annular hinge points onto the least-squares reference plane. Mitral geometry was analyzed at mid-systole (time of closest leaflet-annulus approach), including the PM–to–annulus tethering distance previously described (6,8,13,15).

### SCORING OF REGIONAL CONTRACTILITY
Using the three short-axis views, the LV was divided into 16 segments according to the American Society of Echocardiography recommendations, and a score was allocated to each segment according to its contractility as (0) normokinetic, (+1) hypokinetic, (+2) akinetic, or (+3) dyskinetic. A global wall motion score (WMS) was derived from the algebraic sum of these values, and the WMS index was then obtained by dividing WMS by the total number of scored segments ($n = 16$) (31).

### Pathologic assessment
Sacrifice was performed after the last ultrasonic evaluation, and the hearts were explanted and rinsed in a saline buffer. Location and extent of MI were visually assessed. The entire portion of the LV containing the MI was fixed in formalin for histology and immunohistochemistry.

### HISTOLOGIC STUDIES
Formalin-fixed paraffin-embedded sections of the whole MI were stained with hematoxylin-safranin for qualitative assessment of scar and grafted cells focusing on fibrosis, fat tissue, inflammation, and myocyte necrosis; and presence, density, organization and differentiation of grafted cells.

### IMMUNOHISTOCHEMICAL STUDIES
On selected paraffin sections, immunohistochemistry for skeletal fast isoform (MY-32 clone monoclonal antibodies (Sigma) and cardiac and skeletal slow isoforms (NOQ7 clone monoclonal antibodies (Sigma) of myosin heavy chain (MHC) was performed as previously described (25).

### Statistical analysis
Echocardiographic measurements were compared among stages and sheep by repeated-measures ANOVA. Significant ANOVAs were explored by two paired $t$ tests (chronic ischemia vs. baseline and vs. sacrifice), with significance at $p < 0.025$ (Bonferroni-corrected). Differences in progression between the two groups were explored by unpaired $t$ test, with significance at $p < 0.05$. All data were reported as means ± SEM. Statistical analysis used StatView 5.0. Reproducibility of echographic measurements was tested by two independent observers and gave a variability of 2.5% of the mean.

### RESULTS
Myocardial infarction was created in 13 animals: 7 controls and 6 given myoblasts. All animals remained available for echocardiography at two months after infarction (pretreatment) and at time of sacrifice (4 months post-MI). Param-
eters were similar between groups at baseline and at 2 months (pretreatment) (Table 1, columns 1 and 2).

**Cell cultures.** Cultures yielded to 251 ± 42 million cells (170 to 150 million) with 60 ± 2.7% (50% to 70%) myogenic cells.

**3D ultrasonic functional assessment and evaluation of MR.** PROGRESSION OF ISCHEMIC MR. At the time of acute infarction, LV dilation was limited and, as at baseline, the leaflets closed at the annular level with only trace MR. At two months post-MI, LV volume was comparable between groups (LV volume: 30.4 ± 5.6 ml in treatment group vs. 31.4 ± 3.5 ml in control group, p = 0.32; and 28.1 ± 5.9 ml in treatment group vs. 28.56 ± 2.09 ml in control group for end-systolic volume, p = 0.31). With bulging of the affected wall, the leaflets were apically tented with mild-to-moderate MR (regurgitant fraction: 27.4 ± 4.8% vs. 30 ± 5% in the treatment and control groups, respectively; p = 0.51). Papillary muscle tip displacement away from the annulus was comparable between groups (2.9 ± 0.06 cm vs. 3.07 ± 0.05 cm, p = 0.87) (Table 1, Fig. 3).

REVERSAL OF ISCHEMIC MR. Two months after myoblast treatment (i.e., 4 months post-infarction), there was a reduction in ischemic MR (5.8 ± 0.59 ml/beat before treatment vs. 4.0 ± 0.51 ml/beat 2 months after treatment, p < 0.01) and in MR progression (regurgitant volume: −1.83 ± 0.32 ml/beat vs. 5.9 ± 0.7 ml/beat in the treatment and control groups, respectively, p < 0.0001) (Figs. 3 and 4A), with a reduction of tethering distance (2.9 ± 0.06 cm pretreatment vs. 2.51 ± 0.04 cm 2 months after treatment in the treated group, p < 0.01) and in its progression (−0.41 ± 0.09 cm vs. 0.44 ± 0.12 cm in the treatment and control groups, respectively, p < 0.01) (Fig. 4B). This paralleled a significant improvement of the left ventricular ejection fraction (+2.01 ± 0.94% in treatment group vs. −4.86 ± 2.23% in control group, p = 0.02) (Fig. 5A) and WMS index (−0.25 ± 0.11 in treatment group vs. 0.13 ± 0.03 in control group, p < 0.01) (Fig. 5B). The benefits of myoblast treatment were also suggested by a trend (almost significant) to a lesser increase in global ESV (23.3 ± 3.5 ml in the treatment group vs. 35.4 ± 4.2 ml in control group, p = 0.055). Mitral annular area increased eight weeks post-MI in parallel with development of MR, but its progression did not decrease significantly after treatment (Table 1).

Pathologic assessment. Scar sizes were similar between the two groups and always extended to the posterolateral LV wall and the posteromedial papillary muscle. In control subjects, scars were always transmural, with fibrous tissue intermingled with fat tissue (Fig. 6A). In the majority (5 of 6) of transplanted animals, large areas of grafted cells were

![Figure 3](image_url). Mid-systolic apical two-dimensional echo images. (Left) Mild bulging of the infarcted infero-posterior wall with apical leaflet tenting relative to the annulus (dashes) in the direction of tethering (upward arrows). Mild-to-moderate MR in the more globular remodeled LV is seen below. (Right) Cell transplantation restores a more normal LV shape with less bulging of the infero-posterior wall (upward arrows) and decreases tethering distance with improved coaptation and no MR (bottom panel) despite important LV dilation. Abbreviations as in Figure 1.
found within the scars (Fig. 6B). These cells displayed typical histologic features of well-differentiated skeletal myotubes (Fig. 7A) and often had a tendency to organize in parallel (Fig. 7B). In all transplanted animals, antibodies directed against the fast MHC isoform identified the presence of skeletal muscle structures surrounded by fibrotic and residual cardiac tissue, which was not labeled (Fig. 7C). Grafted myocytes maintained their skeletal myocyte differentiation and were all negative for the slow MHC isoform (Fig. 7D).

**DISCUSSION**

Ischemic MR is principally caused by LV remodeling with outward PM displacement and increased tethering (4–16). The current study assessed whether chronic ischemic MR can be reduced using autologous skeletal muscle transplantation within the infarcted area at a chronic stage (20–23).

Skeletal myoblasts are precursor cells located under the basal lamina of the adult skeletal muscle and committed to multiply after injury (32). They possess a high potential for division in culture and further multiplication after intramyocardial grafting (33).

Our hypothesis was that myoblast transplantation can reduce ischemic MR through a decrease in localized LV wall deformation and bulging. In the chronic post-MI setting, such a procedure has been effective experimentally in improving global and regional LV systolic function and limiting LV remodeling (25,26). In the current study, myoblast transplantation successfully decreased chronic mild-to-moderate ischemic MR (that was otherwise progressive) by significantly decreasing leaflet tethering distance, consistent with infarct zone reshaping, and repositioning the PM closer to the annulus. This can be understood based on the known dependence of ischemic MR on localized LV remodeling and the central and predominant role of tethering as the final common pathway in inducing ischemic MR, independent of global LV size and function (6,10,11,13). The decrease in tethering length is consistent with the engraftment of myotubes within the infarcted wall and the potential cell-mediated changes in extracellular matrix (34). This mechanistic hypothesis actually fits with previous experiments in which small displacements of the PM tip created large, nonlinear changes in MR (6,8,9). The mild reduction in abnormal wall motion score may reflect limitation of remodeling and outward infarct bulging by new cell growth. This limitation of LV remodeling could explain the significantly lower EF deterioration in the cell group, and the lower ESV increases (25,26). It is beyond the scope of this study to determine whether myoblast transplantation may also provide benefits other than limitation of remodeling, which might include contraction of engrafted myotubes fused with host cardiomyocytes or paracrine mobilization of cardiac stem cells (35,36).

**Study limitations and future directions.** The scope of this study is sharply focused on the problem of inferoposterior MI, a common cause of ischemic MR. In case of global LV dysfunction, the major determinant of MR remains the displacement of the PMs, which are located in the posterior portion of the LV. Therefore, a similar approach could in principle achieve a beneficial effect by injecting skeletal myoblasts around both PM regions (6). Finally, these positive results could justify further studies using percutaneous techniques of myoblast delivery, particularly through the coronary veins (37).

**Figure 5.** Effects of myoblast transplantation on left ventricular ejection fraction function (A) and wall motion score (WMS) (B). MI = myocardial infarction.
Conclusions. In the chronic post-infarction setting, autologous skeletal myoblast transplantation attenuates mild-to-moderate chronic ischemic MR, which is otherwise progressive, by decreasing tethering distance over which the leaflets are stretched and improving ejection fraction and wall motion score, thereby enhancing valve coaptation. Given the limitations of existing methods for repairing ischemic MR in patients (38), it is conceivable that such a procedure may be considered as a complement to standard surgical therapy in the future.

Figure 6. Histology of the subpapillary muscle region. (A) Chronic infarction (after four months) in a control animal characterized by dense fibrous tissue (H&E stain, bar = 600 μm). (B) Scar area in a grafted sheep at two months after transplantation with identification of a group of well-differentiated grafted cells (black arrow) replacing fat and fibrous tissues (H&E stain, bar = 600 μm).

Figure 7. Organized skeletal muscle cells. Treated area in a grafted sheep at two months after transplantation. (A) (×40) High density of grafted cells on traversal section and surrounded by fibrous tissue (H&E stain; bar = 100 μm). (B) (×300) Skeletal myotubes (SM) organized in parallel with multiple peripheral nuclei (*) and abundant myofilaments (H&E stain; bar = 150 μm). (C) Section of an engrafted scar area at two months after transplantation. Immunostaining for the fast isoform of the beta myosin heavy chain (My 32; bar = 200 μm). (D) To a serial section, immunostaining for the slow isoform of beta myosin heavy chain (NOQ7; bar = 200 μm).
REFERENCES


