Adenoviral Gene Transfer of FGF-5 to Hibernating Myocardium Improves Function and Stimulates Myocytes to Hypertrophy and Reenter the Cell Cycle

Gen Suzuki, Te-Chung Lee, James A. Fallavollita, John M. Canty, Jr

Abstract—Fibroblast growth factors (FGFs) have diverse actions on the myocardium but the importance of stimulating angiogenesis versus direct effects of FGFs on cardiac myocytes is unclear. We used intracoronary injection of a replication-deficient adenoviral construct overexpressing FGF-5 (AdvFGF-5) to improve flow and function in swine with hibernating myocardium. Two-weeks after AdvFGF-5 (n=8), wall-thickening increased from 2.4±0.04 to 4.7±0.7 mm in hibernating LAD regions (P<0.05) whereas remote wall-thickening was unchanged (6.7±0.4 to 5.8±0.5 mm). This was associated with small increases in resting flow to dysfunctional myocardium, but flow during adenosine was unchanged (LAD 1.45±0.27 versus 1.46±0.23 mL/min per g and remote 4.84±0.23 versus 4.71±0.47 mL/min per g, P=NS). Unexpectedly, animals receiving AdvFGF-5 demonstrated a 29% increase in LV mass over the 2-week period (P<0.05 versus untreated animals with hibernating myocardium and normal shams). Histological analysis confirmed profound myocyte cellular hypertrophy in AdvFGF-5 treated myocardium (19.9±0.32 versus 15.2±0.92 μm in untreated, P<0.001). Myocytes in the proliferative phase of the cell cycle (Ki-67 staining) increased 7-fold after AdvFGF-5 (2.904±405 versus 409±233 per 10⁸ myocyte nuclei in untreated, P<0.05). Myocyte nuclei in the mitotic phase (phosphorylated histone H3 staining) also increased after AdvFGF-5 (127±24 versus 35±13 per 10⁶ myocyte nuclei in untreated, P<0.05). Thus, rather than angiogenesis, stimulation of hypertrophy and reentry of a small number of myocytes into the mitotic phase of the cell cycle are responsible for the effects of AdvFGF-5 on function. Although additional mechanisms may contribute to the improvement in wall-thickening, overexpression of AdvFGF-5 may afford a way to restore function in hibernating myocardium and ameliorate heart failure in chronic ischemic cardiomyopathy. (Circ Res. 2005;96:767-775.)

Key Words: hibernating myocardium ■ growth factors ■ gene therapy

Hibernating myocardium is common in patients with ischemic cardiomyopathy, and myocardial revascularization can improve function and ameliorate symptoms of heart failure. Unfortunately, many patients are not suitable candidates for surgical or percutaneous revascularization and developing nonsurgical approaches to reverse dysfunction and improve perfusion would be desirable. Administration of FGFs as recombinant proteins or overexpression using plasmid and adenoviral vectors elicits multiple effects that could favorably affect flow and function in viable chronically dysfunctional myocardium. Considerable enthusiasm for therapeutic angiogenesis has arisen from promising experimental animal studies using rapidly developing coronary collaterals and ameoreid occluder models.¹⁻⁶ Unfortunately, FGF-mediated improvements in myocardial perfusion are small, and few laboratories have demonstrated objective changes in flow during pharmacological or metabolic stress. In addition, when administered to dogs with chronic well-developed coronary collaterals, FGF did not improve myocardial perfusion.⁵ This raises the possibility that FGFs accelerate collateral growth in animal models yet may not afford improvements when coronary flow reserve is chronically reduced. Such actions could be the basis for the inability to translate experimental findings to clinical studies in patients with chronic coronary disease.⁷

Despite the paucity of data demonstrating increases in coronary flow reserve, many studies show substantial effects of FGFs on myocardial function, raising the possibility that the major therapeutic actions of FGFs may be due to nonangiogenic mechanisms.⁸ For example, FGF has been shown to protect myocytes against irreversible injury¹⁹⁻¹¹ and reversible stunning,¹⁰¹² promote alterations in calcium handling that could improve contractility,¹³ attenuate ischemia-induced myocyte apoptosis, and stimulate myocyte hypertrophy in cell culture.¹⁴ Whereas the relative contribution of each of these mechanisms in models of chronic ischemia in
vivo is unknown, they could improve function independently of changes in myocardial perfusion.

We performed the present study to evaluate the effects of FGF-5, a secreted fibroblast growth factor, in a swine model of chronic collateral-dependent hibernating myocardium where the physiological characteristics of hibernating myocardium, namely reduced resting flow and function with a critical impairment of subendocardial flow reserve, remain unchanged between 3 and 5 months after instrumentation. This contrasts with relatively short-term ameroid instrumented swine models of collateral-dependent myocardium, where transient upregulation of endogenous growth factors and subendocardial infarction near the time of ameroid occlusion could be important in modulating the effects of exogenous growth factors. We overexpressed FGF-5 with an adenovirus that has previously been demonstrated to transfect myocardium after intracoronary administration. A similar approach ameliorated stress-induced dysfunction in a porcine ameroid model where resting function was normal with initially promising results in phase II/III human trials. Our results demonstrated that the salutary effects of AdvFGF-5 on function are dissociated from perfusion and related to stimulating myocyte hypertrophy and inducing a small population of myocytes to reenter the mitotic phase of the cell cycle.

Materials and Methods

Procedures and protocols conformed to institutional guidelines for the care and use of animals in research and are detailed in the expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org. Hibernating myocardium was produced as previously described.15

Serial Physiological Studies

Pigs began studies 4 months after instrumentation, at which time flow, function, and coronary flow reserve are critically reduced. Using a percutaneous approach under propofol sedation, we inserted a 5F multipurpose catheter into the left ventricle using the 6F introducer side port for pressure monitoring and arterial sampling. Regional wall-thickening was assessed with transthoracic echocardiography from a right parasternal approach, and myocardial perfusion was assessed with fluorescent microspheres.20 After an equilibration period, we assessed hemodynamics, flow, and regional wall thickening at rest, during submaximal epinephrine infusion, and after pharmacological vasodilatation with adenosine (0.9 mg/kg per min IV) with phenylephrine infused to prevent adenosine-induced hypotension.

We administered a replication deficient adenovirus containing FGF-5 under control of the CMV promoter (AdvFGF-5, 1×10^10 pfu, n=8) in equally divided doses into the LAD, circumflex, and right coronary arteries over 30 seconds, taking care to avoid reflux into the systemic circulation. First-pass myocardial uptake was enhanced using histamine (25 μg/min IC). As controls, we assessed the effects of a similar dose of nuclear localizing EGFP adenovirus (AdvEGFP, n=2 hibernating and n=2 sham) after intracoronary administration.

An identical physiological study was repeated 2 weeks after AdvFGF-5, after which animals were euthanized under anesthesia. The LV was weighed, sections incubated in TTC to assess infarction, and samples taken for flow and histological analyses.

Apoptosis, Myocyte Hypertrophy, and Cell Cycle Markers

Details are provided in the expanded Materials and Methods. Briefly, we quantified myocyte nuclear density, diameter, and nuclear length to estimate myocyte volume as previously described.22 Myocyte diameter and volume from the AdvFGF-5 group were compared with matched animals with untreated hibernating myocardium (n=10), sham control groups (n=5), and animals receiving AdvEGFP (n=4) to exclude nonspecific effects of the adenovirus. Myocyte apoptosis was assessed using fluorescent TUNEL staining.22

Tissue sections were also incubated with antibodies for the nuclear cell cycle markers Ki-67 (a specific marker for cells that have reentered the growth phase of the cell cycle) and anti-phospho-histone H3 (a marker of mitosis). We quantified positive myocyte nuclei in relation to myocyte nuclear density using both light and fluorescent confocal multifluorescence microscopy (Bio-Rad MRC 1024). Cardiac myocytes were costained with antibodies to tropomin I and nuclei were costained with TO-PRO-3. Similar quantitative results were obtained with each approach (online data supplement).

Statistics

Data are expressed as mean±SE. Differences after treatment with AdvFGF-5 and comparisons between the hibernating and normally perfused remote regions of the same heart were assessed using paired t tests. Details of the piece-wise flow analysis are provided in the online data supplement. Differences among AdvFGF-5–treated animals, age-matched shams, and untreated animals with hibernating myocardium were assessed using a two-way ANOVA and the post-hoc Holm-Sidak test (Sigma Stat 3.0) with P<0.05 considered significant.

Results

Pigs were in good health at the time of study, and TTC staining showed no infarction. Initial physiological studies were performed 123±2 days after instrumentation and repeated 2 weeks after AdvFGF-5 (137±2 days).

Findings before AdvFGF-5 confirmed dysfunctional hibernating myocardium. There were reductions in resting perfusion (LAD 0.98±0.09 versus 1.38±0.15 mL/min per g in remote; P<0.01) with the greatest reduction in the subendocardium (0.79±0.05 versus 1.52±0.16 mL/min per g in remote; P<0.01). Full-thickness flow during epinephrine was attenuated (LAD 1.27±0.09 versus 1.65±0.08 mL/min per g in remote; P<0.01), and subendocardial flow did not increase significantly above resting levels (LAD 0.93±0.09 mL/min per g; P=NS versus rest). Likewise, the increase in full-thickness flow during adenosine was severely attenuated (LAD 1.45±0.27 versus 4.84±0.23 mL/min per g in remote; P<0.001), and subendocardial flow did not increase above the resting value (LAD 0.72±0.17 mL/min per g; P=NS versus rest).

Efficiency of Intracoronary AdvEGFP Gene Transfer in Swine

Figure 1 demonstrates the efficiency of intracoronary gene transfer after pretreatment with histamine to increase endothelial permeability. Fluorescence imaging of EGFP demonstrated a high frequency of cardiac nuclear colocalization. When this was quantified using TO-PRO-3 to assess cardiac nuclei, the EGFP was present in 39±2% of cells. Cytoplasmic staining of EGFP was weaker but still present 2 weeks after AdvEGFP administration. These data confirm a high transfection efficiency after histamine pretreatment with the intracoronary approach that is similar to that reported using LacZ transfection by other laboratories.17
Effects of AdvFGF-5 on Flow and Function in Hibernating Myocardium

Figure 2 summarizes the effects of AdvFGF-5 on flow and function with detailed transmural, circumferential, and relative perfusion analysis provided in the online data supplement. Hemodynamic measurements are summarized in the Table. Two-weeks after AdvFGF-5, LAD wall thickening (ΔWT) increased from 2.4±0.4 to 4.7±0.7 mm (P<0.05 versus initial), whereas wall thickening in normally perfused regions was unchanged. Global LV function and the response to epinephrine were not affected by AdvFGF-5 (online data supplement).

Despite prominent effects on function, there were only small changes in myocardial perfusion when averaged among all samples in the region distal to the stenosis. As summarized in Figure 2, resting perfusion tended to increase in hibernating LAD regions while decreasing in remote regions, but the differences were not significant. There were no significant differences in absolute flow during adenosine vasodilation or epinephrine (online data supplement). When analyzed as paired measurements from LAD pieces pooled from all animals (n=147), there was a small increase in relative resting perfusion (LAD/Remote 0.70±0.02 to 0.75±0.02; P<0.0001) and analysis of the integrated flow deficit showed it to decrease from 18.9±2.9% to 12.5±4% (P<0.05) indicative of a reduction in the resting perfusion deficit size. There was no improvement in relative flow during adenosine (LAD/Remote 0.33±0.02 versus 0.31±0.02), but there was a small reduction in the vasodilated perfusion deficit size (46.7±3.5% versus 43.2±3.5%; P<0.05). Thus, the effects of AdvFGF-5 on flow were primarily restricted to the border regions between normally perfused and hibernating regions.

Effects of AdvFGF-5 on Myocyte Cellular Hypertrophy

Although body weight (96±3 to 104±3 kg) and LV end-diastolic dimension (50±2 to 54±2 mm) increased by only ~8%, echocardiographic estimates of LV mass increased by 29% 2 weeks after AdvFGF-5 (177±19 to 228±15 g; P<0.05). Figure 3 shows that AdvFGF-5 produced myocyte cellular hypertrophy.
Hemodynamic Parameters

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<td>97±6 104±6</td>
<td>124±8† 97±7*</td>
<td>15 433±1395 13 101±1620</td>
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Values are mean±SEM; *P<0.05 Initial vs Final studies; †P<0.05 vs rest.

n indicates no. of pigs; PLVsys, left ventricular systolic pressure; PLVED, left ventricular end diastolic pressure; HR×PLVsys, rate pressure products.

cellular hypertrophy with quantitation summarized in Figure 4. Myocyte diameter was compared with similarly instrumented but untreated animals with hibernating myocardium (controls) and uninstrumented shams of a similar age (AdvFGF-5 134±2 days and untreated hibernating 137±2 days) and body weight (AdvFGF-5 104±3 kg, untreated hibernating 106±4 kg, and normal shams 115±4 kg). The LV mass/body weight ratio in animals receiving AdvFGF-5 was higher than untreated animals (2.5±0.1 versus 2.2±0.1 g/kg; P<0.05) as well as sham controls (1.8±0.2; P<0.05 versus both groups). AdvFGF-5 increased myocyte diameter in parallel with the changes in LV mass (Figure 4). In the LAD region, subendocardial myocyte diameter increased from 16.2±0.40 to 18.6±1.12 μm (P<0.05) in AdvFGF-5–treated animals and both were significantly increased in comparison to sham controls (14.8±0.19 μm; P<0.05). Changes in subepicardial myocyte diameter were even more pronounced (15.2±0.94 to 19.9±0.32 μm after AdvFGF-5, P<0.05, and 13.0±0.83 μm in shams, P<0.05). Changes in myocyte diameter in normally-perfused remote regions were similar to hibernating LAD regions and particularly prominent in the subepicardial layers (20.3±0.7 versus 15.2±0.39 μm in untreated, P<0.05, and 13.7±0.81 μm in shams, P<0.05). Animals receiving AdvEGFP showed no change in echocardiographic LV mass over 2 weeks, and myocyte diameter after AdvEGFP (remote zone myocardium 14.4±1.22 μm) was no different from sham or untreated hibernating groups.

Effects of AdvFGF-5 on Myocyte Nuclear Density, Cell Volume, and Apoptosis

Figure 5 summarizes the effects of AdvFGF-5 on myocyte nuclear density, apoptosis, and calculations of myocyte cellular volume from subendocardial LAD samples. Whereas LAD connective tissue was mildly increased in animals receiving AdvFGF-5 (LAD 6.6±1.1% versus 4.1±0.8% in remote; P<0.05), it was no different in untreated controls (LAD 6.9±0.7% versus 4.5±0.2% in remote; P<0.05). In addition, there were no inflammatory changes in AdvFGF-5–treated pigs.

Due to prominent cellular hypertrophy, AdvFGF-5 treatment produced significant reductions in myocyte nuclear density. In the LAD subendocardium, myocyte nuclear density was 746±26 versus 926±44 nuclei per mm² in untreated (P<0.01) and 1212±36 nuclei per mm² (P<0.01) in shams. Estimates of cell volume using morphometric approaches further confirmed hypertrophy with LAD myocyte cell volume increasing from 11 987±653 to 14 988±901 μm³/nucleus (P<0.05) after AdvFGF-5. The differences in myocyte volume (30%) were proportional to the increase in LV mass (29%) and not indicative of hypertrophy from apoptosis-induced myocyte loss, which, if anything, was lower in animals receiving AdvFGF-5 than untreated hibernating myocardium.

Reentry of Myocytes Into the Growth Phase of the Cell Cycle After AdvFGF-5

Figure 6 illustrates confocal photomicrographs demonstrating myocyte nuclear Ki-67 staining in an animal treated with...
AdvFGF-5. The frequency of Ki-67 staining, a marker of myocytes in the growth phase of the cell-cycle, was expressed in relation to the number of myocyte nuclei. Myocytes are normally in G0 as confirmed by the low frequency of Ki-67 staining (0.028% of myocytes or 284 ± 69 per 10^6 myocyte nuclei in shams). Untreated animals with hibernating myocardium and animals receiving AdvEGFP had similar low values of Ki-67 staining (447 ± 212 and 213 ± 69 per 10^6 myocyte nuclei, respectively). After AdvFGF-5, Ki-67 positivity increased significantly (LAD 2904 ± 405 and remote 2066 ± 326 per 10^6 myocyte nuclei; both P < 0.05 versus sham and untreated), but the frequency of Ki-67 positive myocyte nuclei after AdvFGF-5 was still less than 1% of myocytes (LAD 0.29 ± 0.04% and remote 0.21 ± 0.03% versus 0.03 ± 0.01% in shams). Increased Ki-67 positivity was not restricted to myocytes but was also detected in fibroblasts, endothelial cells, and smooth muscle cells. Nonmyocyte staining represented 51 ± 7% of all nuclear Ki-67 staining.

Although Ki-67 reflects active cellular hypertrophy it could also indicate myocytes undergoing nuclear division. We therefore quantified phosphohistone H3 staining, a marker of DNA replication (Figure 7). Phosphohistone H3 staining was rare in normal shams (2.4 ± 2.4 per 10^6 myocyte nuclei). It was higher in untreated hibernating myocardium (35 ± 13 per 10^6 myocyte nuclei; P < 0.05) and increased further after AdvFGF-5 (127 ± 24 per 10^6 myocyte nuclei; P < 0.05 versus untreated or shams). Frequencies of myocyte apoptosis were much lower (Figure 4) averaging 4.7 ± 3.2 per 10^6 myocyte nuclei in AdvFGF-5–treated animals. Mitotic myocyte nuclei were also visualized by light microscopy but cytokinesis was not seen. Based on these results, approximately 4% of the Ki-67–positive myocytes (~1 in 10,000) were in the mitotic phase of the cell cycle. The frequency distribution of Ki-67 positive myocyte diameters was similar to unstained cells in untreated hibernating myocardium (15.9 ± 0.3 versus 15.1 ± 0.1 μm). In animals receiving AdvFGF-5.
AdvFGF-5, Ki-67 positive myocyte were smaller (17.4±0.4 versus 18.8±0.1 μm; P<0.05) but a subpopulation of cells smaller than normal (potentially indicative of differentiating stem cells) was not identified (online data supplement).

Discussion
There are several important new findings from our study. First, intracoronary administration of AdvFGF-5 in chronic hibernating myocardium produces only small changes in resting perfusion that are predominantly restricted to regions bordering normal and hypoperfused myocardium. In contrast, AdvFGF-5 induced striking improvements in myocardial function that were limited to dysfunctional LAD regions and disproportionate to increases in perfusion. This was accompanied by increases in LV mass and cellular myocyte hypertrophy throughout the LV. Histological staining demonstrated that AdvFGF-5 induced a population of myocytes to reenter the growth phase of the cell cycle. Collectively, these results support the notion that favorable actions of exogenously administered FGFs are largely independent of functional collateral vessel growth and predominantly related to myocyte remodeling.

Limited Effects of AdvFGF-5 on Flow in Hibernating Myocardium
Our study provides a comprehensive assessment of myocardial perfusion after administration of growth factors that has previously been lacking in the majority of studies evaluating...
angiogenic interventions. AdvFGF-5 elicited small increases in resting perfusion that were largely restricted to the border regions. This small flow change was more difficult to ascertain during vasodilation but the reduction in the perfusion deficit size at rest and after vasodilation was similar (4% to 6% absolute difference in each condition). Importantly, our observations regarding the effects of AdvFGF-5 on the distribution of flow are strikingly similar to observations in many clinical trials of angiogenic growth factors where it has been difficult to measure objective increases in myocardial perfusion despite promising findings based on functional improvement in porcine ameroid models. The most demonstrable change in nuclear perfusion studies has been a reduction in the resting perfusion defect size. Recent studies evaluating intracoronary AdvFGF-4 have also demonstrated small reductions in defect size during adenosine vasodilation that are similar in magnitude to those we observed in hibernating myocardium.

Collectively, the results indicate that mature coronary collaterals supplying chronically dysfunctional hibernating myocardium have a limited ability to increase perfusion after exogenous FGF-5. The fact that this occurs in the setting of resting dysfunction and a critical impairment in flow indicates that factors present in rapidly developing collateral models such as upregulation of other elements of the angiogenic cascade and/or coexisting subendocardial infarction are required to effect an improvement in flow. Thus, the results in pigs with chronic hibernating myocardium are similar to the dog with well-developed chronic collaterals where interventions administered near the point of ameroid occlusion accelerate but were unable to increase collateral flow beyond that attainable with intrinsic angiogenic stimuli. Although speculative, this could be a consequence of intrinsic myocardial adaptations that limit the development of metabolic ischemia during stress in hibernating myocardium.

Effects of AdvFGF-5 on Function in Hibernating Myocardium

In studies where flow and function have been evaluated after growth factor administration, their effect on function has greatly exceeded their effect on perfusion. Consistent with this is the fact that intracoronary AdvFGF-4 improved function in swine with pacing-induced heart failure without altering myocardial perfusion. Whereas findings of hibernating myocardium persisted after AdvFGF-5, there was an improvement in the myocardial flow-function relation. Importantly, the improvement in function occurred regionally and did not reflect altered loading conditions because function in normally perfused remote regions, global function, and systolic and end-diastolic LV pressure remained unchanged after AdvFGF-5. This suggests that AdvFGF-5 may have ameliorated a component of dysfunction that was due to acute myocardial stunning superimposed on chronic hibernating myocardium. The coexistence of stunning is consistent with the observation that reductions in wall thickening in chronic hibernating myocardium exceed the reduction in subendocardial perfusion.

Several additional mechanisms exist through which AdvFGF-5 could affect function disproportionately from perfusion. Previous studies have demonstrated that short-term administration of basic FGFs can protect the heart against reversible and irreversible ischemic injury and produce a “preconditioning like” effect. These actions would also be consistent with the improvement in function restricted to myocardium with limited coronary flow reserve. Although acute administration of FGF can also alter myocyte calcium transients and directly increase contractility in vitro, this mechanism seems less likely because remote zone function, global function, and contractile reserve were not altered after AdvFGF-5. A final mechanism through which AdvFGF-5 could have altered systolic function is through an improvement in myocardial efficiency via activation of NOS. Activation of NOS could produce chronic preconditioning against stunning in hibernating myocardium as well as optimize myocardial efficiency if NO release was chronically downregulated in chronic hibernating myocardium.

AdvFGF-5 Mediated Myocyte Hypertrophy

An alternative explanation for the dissociation between flow and functional effects of AdvFGF-5 could relate to stimulation of myocyte hypertrophy and the resultant regional remodeling. Intracoronary administration of AdvFGF-5 led to significant myocardial hypertrophy with a ~30% increase in LV mass within 2 weeks. Previous studies have established a role for endogenous FGFs in acquired hypertrophy produced by a variety of increased physiological loading conditions, and manipulating exogenous basic FGFs can stimulate myocyte cellular hypertrophy and mitosis in neonatal myocytes in vitro. Our study extends previous work to demonstrate significant FGF-5–dependent myocyte plasticity in vivo. Both cellular and anatomic hypertrophy occurred over a brief time interval without changes in ventricular loading conditions. Although some cellular myocyte hypertrophy was apparent in untreated animals with hibernating myocardium versus sham controls, AdvFGF-5 led to substantially greater increases in LV mass and myocyte diameter.

Although myocyte hypertrophy was global and particularly prominent in the subepicardium, the improvement in function was restricted to the LAD region. The trend toward a reduction in function in the remote zone could be consistent with a hypertrophy mediated reduction in contractility but is more likely due to a resolution of moderate compensatory hyperkinesis as LAD function improved. Because transmural wall thickening is usually strongly related to subendocardial function, the prominent LAD subepicardial hypertrophy after AdvFGF-5 may have allowed hypertrophied myocytes in outer layers to compensate for subendocardial myocytes lost from apoptosis during the development of hibernating myocardium. Additional studies will be needed to determine the relative importance of replacing regional myofibrils via cellular hypertrophy versus other mechanisms.

Increased Cell Cycle Markers After AdvFGF-5

A surprising finding was the fact that AdvFGF-5 caused a small population of cardiac myocytes to initiate DNA synthesis. We were able to confirm this using phosphohistone-H3 staining, which has previously been used to label myocyte nuclei in various phases of nuclear division and correlates
with BrdU positivity.\(^3\) Although cytokinesis was not seen, there is considerable difficulty in identifying such short-lived events in histological tissue. Hein et al\(^2\) demonstrated that DNA synthesis increases during hypertrophy and heart failure. Increased Ki-67 was detected without mitosis with DNA replication potentially activated to maintain the DNA content/myocyte volume ratio in hypertrophied myocytes. Regardless of whether cell or nuclear division predominates, the impact of AdvFGF-5 on cellular hypertrophy seems more dominant based on several observations. First, myocytes in the growth phase of the cell cycle (Ki-67) were nearly 20-times higher than those positive for phosphohistone H3. Second, myocyte nuclear density was lower in animals treated with AdvFGF-5. The failure of nuclear density to increase after AdvFGF-5 indicates that cellular hypertrophy exceeded myocyte nuclear hyperplasia making quantitatively important changes in myocyte polyploidy unlikely. Directionally, similar effects were found by manipulating telomerase reverse transcriptase in mice.\(^3\) Finally, we cannot exclude the possibility that some of the Ki-67–positive myocytes reflect stem cell recruitment because the diameters were smaller than nonstained cells in AdvFGF-5–treated hearts and further studies will be required to address this possibility. Collectively, our results support that AdvFGF-5 contributes to functional remodeling through both myocyte cell growth and possibly cell division. This may represent a potentially new approach to reverse the effects of myocyte loss in ischemic cardiomyopathy.

**Methodological Limitations**

The effects of AdvFGF-5 cannot be directly extrapolated to other growth factors because they may have different physiological actions, and further studies are required to evaluate their effect on myocyte hypertrophy.\(^7\) Interestingly, plasmid VEGF has been reported to increase myocyte mitosis leading to nuclear hyperplasia in a porcine ameroid model.\(^3\) We did not colocalize phosphohistone H3 and Ki-67 staining, and it is possible that cellular hypertrophy and nuclear division are occurring in different myocyte populations. It is plausible that AdvFGF-5 is producing hypertrophy in adult myocytes and recruiting a smaller stem cell population responsible for phosphohistone H3 positivity. Although our studies were conducted in “adult” farm-bred swine (more than 100 kg) over 6 months old at the time of study, the animals were otherwise normal, and it is possible that intracoronary gene transfer would be insufficient in aged animals\(^2\) or when coexisting atherosclerosis and endothelial function is present. We injected two-orders of magnitude more viral particles than published clinical trials of intracoronary AdvFGF-4 and one-order higher than previous studies of AdvFGF-5 in swine. Further studies will be required to examine the dose-dependency of the effects we have reported. Finally, we have documented EGFP protein but have not confirmed the presence of FGF-5 protein production 2 weeks after transfection. In a previous study, Giordano\(^4\) confirmed FGF-5 in pigs using a similar approach. Even if the FGF-5 was only transiently expressed, it would not negate the fact that it had lead to substantial increases in LV mass and myocyte hypertrophy in the absence of any alterations in ventricular loading conditions. These were specific to the AdvFGF-5 construct because AdvEGFP did not produce similar effects.

**Clinical Implications**

Our study provides further support for the pleiotropic effects of FGFs on the myocardium, and identifies additional mechanisms to explain the functional improvement after factor interventions. The increases in function and myocyte hypertrophy in hibernating myocardium are more prominent than effects related to angiogenesis, raising the possibility that administration of AdvFGF-5 may be more efficacious as an adjunctive therapy in patients with advanced ischemic cardiomyopathy and heart failure than chronic angina. Further studies evaluating AdvFGF-5 in animal models of heart failure as well as clinical trials will be required to test this possibility.

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**References**


22. Suzuki et al. FGF-5 in Hibernating Myocardium 775
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Abbreviated Title – FGF-5 in hibernating myocardium

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Supplementary Materials and Methods

Procedures and protocols conformed to institutional guidelines for the care and use of animals in research. Hibernating myocardium was produced as previously described. Briefly, pigs were sedated (Telazol; tiletamine 50mg/ml and zolazepam 50 mg/ml)/xylazine (100 mg/ml, 0.022 mg/kg i.m.) intubated and ventilated with a 0.5–2% isoflurane-oxygen mixture. Through a limited pericardiotomy, the proximal LAD was instrumented with a Delrin occluder (1.5 mm). Antibiotics (cefazolin, 25 mg/kg and gentamicin, 3 mg/kg i.m.) were given 1-hour before surgery and repeated after closing the chest. Analgesia included an intercostal nerve block (0.5% Marcaine) and prn intramuscular doses of butorphanol (2.2 mg/kg i.m. q6h) and flenixin (1-2 mg/kg i.m. q.d.).

Serial Physiological Studies

Pigs (n=8) began studies 4-months after instrumentation. We have previously demonstrated that reductions in flow, function and flow reserve remain unchanged after 3-months in this model. Sedation was initiated with Telazol/xylazine and maintained with propofol (5-10 mg/kg/hr i.v.). Under sterile conditions, we inserted a 6-Fr introducer into the left brachial artery. A multipurpose catheter was inserted into the LV apex for microsphere injection. The introducer side port was used to monitor aortic pressure and perform blood withdrawal for microspheres. Animals were heparinized (100 U/kg), and hemodynamics allowed to equilibrate for at least 30-minutes. Regional wall-thickening was assessed with transthoracic echocardiography from a right parasternal approach. All pigs employed for gene transfer study showed anterior dysfunction but dyskinesis was not present under any condition. Systolic wall-thickening (ΔWT=ESWT-EDWT) was measured in LAD and remote regions. Ventricular dimensions and LV mass were calculated using ASE criteria. This was followed by LV
microsphere injection to assess resting perfusion. Subsequently, contractile reserve was evaluated during stimulation with an epinephrine infusion titrated to increase heart rate by approximately 40 beats/min for 10-minutes and wall-thickening and microsphere injection repeated. Finally, pharmacological vasodilation was produced using adenosine (0.9mg/kg/min iv) with phenylephrine infused and titrated to maintain mean blood pressure ~100 mmHg and microsphere flow repeated.

After completing baseline physiological measurements, we administered a replication deficient adenovirus containing FGF-5 under control of the CMV promoter (AdvFGF-5, a gift from Dr. Kirk Hammond, UCSD) produced in clinical grade at the Vector Core Laboratory of the University of Michigan. Previous studies have confirmed first-pass uptake of this construct following intracoronary administration\(^4\). Divided doses (total 2 x 10\(^{12}\) viral particles, 1x10\(^{11}\)PFUs) were injected into the LAD, circumflex and right coronary arteries over 30-seconds taking care to avoid reflux into the systemic circulation. Myocardial uptake was enhanced by an intracoronary infusion of histamine (25μg/min for 3-min) to increase endothelial permeability\(^5\). At the end of the study, catheters were removed and pigs were brought back to the animal facility.

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6 ml/min for 90-seconds. At the end of the study, samples were taken from a midventricular ring, divided into twelve circumferential wedges (Figure 1) with each cut into 3 transmural layers. Dyes were extracted using standard techniques and fluorescence quantified at selected excitation wavelengths.

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**Assessment of myocyte nuclear density, myocyte cell volume and connective tissue**

Myocyte nuclear density was quantified as previously described\(^7,8\). Briefly, tissue samples adjacent to LAD (hibernating) and posterior descending arteries (normal) were fixed (10\% formalin) and paraffin-embedded. We evaluated 300–400 transversely sectioned fields from each sample at a magnification of 600 x using 5-\(\mu\)m sections stained with hematoxylin and eosin. Connective tissue was quantified by point counting of trichrome stained sections, as we
have previously described\textsuperscript{1}. Connective tissue staining was subtracted from the total tissue area to determine the percentage that was represented by myocytes (percent myocyte area). PAS stained sections were used to quantify myocyte diameter and nuclear length (100 transverse myocytes per region) in subendocardial and subepicardial thirds of the LV. Myocyte diameter from theAdvFGF-5 group was compared to matched animals with untreated hibernating myocardium (n=10), untreated sham control groups (n=5).

We used the morphometric measurements to estimate regional variations in myocyte cell volume. Briefly, myocyte volume was determined by 4 independent parameters which include the number of myocyte nuclei per unit area, the average length of myocyte nuclei, the average myocyte diameter, and volume fraction of myocytes in the tissue. Regional variations in Myocyte volume between LAD and remote regions were assessed by use of standard morphological calculations as previously described in detail\textsuperscript{9}.

**Quantitation of Apoptosis with TUNEL**

We quantified apoptosis using the fluorescent TUNEL staining as previously described\textsuperscript{7}. Myocyte apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-labeling (TUNEL, Chemicon Inc) and epifluorescence with an FITC filter. Approximately 300 to 400 fields were examined per sample. The extent of apoptosis was expressed by normalizing the results to the number of myocyte nuclei per mm\textsuperscript{2} in each sample.

**Quantitation of Cell Growth/Cycle Markers-light microscopy**

Immunohistochemistry was used to determine whether AdvFGF-5 promoted myocytes to enter the growth phase of the cell cycle. Adult cardiac myocytes are normally in the quiescent or G\textsubscript{0} phase. The Ki-67 antigen has been used to identify proliferating myocytes although positive staining can reflect adult myocytes developing hypertrophy as well as the potential for cardiac
myocytes to progress to mitosis\textsuperscript{10,11}. Phospho-histone H3 is a nuclear protein that increases in cell nuclei when there is active DNA replication and cells have entered the mitotic phase of cell growth. The number and percent of nuclei labeled with phospho-histone H3 have been closely correlated with the frequency of BrdU uptake in ventricular myocytes\textsuperscript{12}. Thus, nuclear phospho-histone H3 staining is consistent with myocytes having the potential to hypertrophy and/or undergo nuclear division. It is however, not a surrogate marker for ultimate progression of nuclear division to cytokinesis.

Tissue sections (5µm) were incubated with anti-Ki-67 (mouse monoclonal antibody, clone MIB-1, Dako) or anti-phospho-histone H3 rabbit polyclonal antibody (Upstate Biotech). Samples were post-treated with an HRP-conjugated secondary antibody\textsuperscript{10,12}. Positive nuclei were labeled with DAB and initially quantified with light microscopy (600x). We identified myocytes by their morphology on light microscopy and expressed positive Ki-67 nuclear staining relative to myocyte nuclear density in each section (positive myocyte nuclei per million myocyte nuclei). Finally, to determine whether effects of AdvFGF-5 were restricted to cardiac myocytes, we also quantified the frequency of Ki-67 staining in nonmyocyte cells. The specificity of all antibodies was verified by omission of primary antibodies. All of the antibodies have been successfully used in the pig by other laboratories\textsuperscript{13-15}.

**Quantitation of Cell Growth/Cycle Markers-Confocal microscopy**

To further validate the results obtained by light microscopy, additional experiments were performed using the previous antibodies for cell growth/cycle markers with nuclear and myocyte markers that were imaged with confocal microscopy (Bio-Rad MRC 1024). Tissue sections (5µm) were incubated with either anti-Ki-67 (mouse monoclonal antibody, clone MIB-1, Dako) or anti-phospho-histone H3 (rabbit polyclonal antibody, Upstate Biotech). To identify myocytes
we used anti-cardiac TnI antibodies previously demonstrated to react in swine (mouse monoclonal antibody clone 8I-7, Spectral diagnosis, 1:100 or rabbit polyclonal antibody, Santa Cruz, 1:100)\textsuperscript{16}. Nuclei were stained with TO-PRO-3 (Molecular Probes). Samples were post-treated with fluoroisothiocyanate (FITC) conjugated anti-mouse and TRITC conjugated anti-rabbit antibody (Dako)\textsuperscript{10,12}. Multiple fields were digitized and used to quantify the frequency of myocyte Ki-67 staining. Quantitative results using confocal microscopy were compared to the morphometric and light microscopic identification summarized above.

**Efficiency of intracoronary gene transfer assessed using AdvEGFP (n=4)**

We performed additional experiments to quantify the efficiency of adenoviral uptake in vivo as well as exclude an effect of the adenovirus on eliciting myocyte hypertrophy in swine with hibernating myocardium (n=2) and sham controls (n=2). After baseline assessments of flow and function we injected a replication deficient adenovirus overexpressing a nuclear localizing enhanced green fluorescent protein (AdvEGFP). Pigs were studied as outlined above and AdvEGFP (total dose 2 x10\textsuperscript{12} viral particles, Vector Core Facility at the University of Pittsburgh) injected into each of the three major coronary arteries in equally divided doses following pretreatment with intracoronary histamine as outlined for AdvFGF-5.

After two weeks, physiological studies were repeated and tissue was obtained to assess the cellular efficiency of AdvEGFP transfection using direct confocal fluorescent microscopy. Sections were counterstained with phalloidin to identify F-actin (Phalloidin-TRITC, Sigma-Aldrich)\textsuperscript{11}. The effects of AdvEGFP on myocyte diameter in normal remote regions were assessed as outlined below. The frequency of nuclear EGFP in cardiac cells was quantified in 416±16 fields (total area 59±4mm\textsuperscript{2}) for each animal and expressed as a percentage of nuclei in the field which were identified using nuclear staining with TO-PRO-3.
Statistics

Data are expressed as mean ± standard error. Differences after treatment with AdvFGF-5 and comparisons between the hibernating and normally perfused remote regions of the same heart were assessed using paired t-tests. Differences among AdvFGF-5 treated animals, age-matched shams and untreated animals with hibernating myocardium were assessed using a two-way ANOVA and the post-hoc Holm-Sidak test (Sigma Stat 3.0). Differences of p<0.05 were considered significant.

Supplementary Results

Pigs were in good health at the time of study and TTC staining showed no evidence of infarction. Initial physiological studies were performed 123±2 days after instrumentation and repeated 2-weeks after AdvFGF-5 (137±2 days). Over this interval, body mass increased 8% (96±3 kg to 104±3 kg).

Effects of AdvFGF-5 on the Transmural Distribution of Myocardial Perfusion in Myocardial Samples Averaged from Central Core Regions of Hibernating Myocardium

Findings under baseline conditions confirmed hibernating myocardium. There were reductions in resting perfusion (LAD 0.98±0.09 vs. 1.38±0.15 ml/min/g in remote, p<0.01) with the greatest reduction in the subendocardium (0.79±0.05 vs. 1.52±0.16 ml/min/g in remote, p<0.01). Full-thickness flow during epinephrine was attenuated (LAD 1.27±0.09 vs. 1.65±0.08 ml/min/g in remote, p<0.01) and subendocardial flow did not increase significantly above resting levels (LAD 0.93±0.09 ml/min/g, p-ns vs. rest). Likewise, the increase in full-thickness flow during adenosine was severely attenuated (LAD 1.45±0.27 vs. 4.84±0.23 ml/min/g in remote,
p<0.001) and subendocardial flow did not increase above the resting value (LAD 0.72±0.17 ml/min/g, p-ns. vs. rest).

**Effects of AdvFGF-5 on Relative Perfusion in Individual Samples From the Area at Risk**

The effects of AdvFGF-5 on absolute and relative flow (excluding border regions) are summarized in Figure 2. Hemodynamic measurements are summarized in Table 1. Two-weeks after AdvFGF-5, resting perfusion tended to increase in hibernating LAD regions while decreasing in remote regions, but the differences were not significant. There were no significant differences in absolute flow during epinephrine or adenosine stress. When analyzed as paired measurements from individual myocardial pieces (n=147), there was a small increase in relative resting perfusion (LAD/Remote 0.70±0.02 to 0.75±0.02, p<0.0001). In contrast, there was no improvement during epinephrine or adenosine stress (LAD/Remote epinephrine 0.76±0.04 vs. 0.74±0.05 and adenosine 0.33±0.02 vs. 0.31±0.02).

**The Effects of AdvFGF-5 on the Circumferential Distribution of Myocardial Perfusion and the Estimated Perfusion Deficit Size**

We next evaluated the circumferential distribution of perfusion and the relative flow deficit. Figure 3 summarizes absolute and relative full-thickness perfusion measurements averaged in each of the 12 regions. Under resting conditions, both absolute and relative perfusion increased in 3 of 12 circumferential segments reducing the integrated flow deficit from 18.9±2.9% to 12.5±2.4% (p<0.05). During adenosine, significant increases in perfusion were restricted to a single segment with a small reduction in the vasodilated perfusion deficit size from 46.7±3.5% to 43.2±3.5% (p<0.05). Perfusion distributions during epinephrine were more variable with no significant differences in segmental, absolute or relative perfusion and no
reduction in the perfusion deficit size (16.6±3.0% vs. 18.0±4.0%, p-ns). Thus, AdvFGF-5 produced heterogeneous effects on myocardial perfusion that were more prominent in regions bordering normal and hibernating myocardium leading to small but significant reductions in the relative perfusion deficit size at rest and following vasodilation.

**The Distribution of Myocyte Diameters in Ki-67 Positive Cells After AdvFGF-5**

Figure 4 summarizes data which are pooled from all regions of the heart based on the global nature of increased Ki-67 and myocyte hypertrophy observed after intracoronary AdvFGF-5. There was a broad distribution of myocyte diameters in both Ki-67 positive myocytes and unstained myocytes. In untreated hearts, Ki-67 positive cells were similar in diameter to those that were not stained (average diameter: 15.1 ± 0.1 µm in unstained vs. 15.9 ± 0.3 µm in Ki-67 positive). In contrast, in AdvFGF-5 treated animals, Ki-67 positive cells were significantly smaller than those that were unstained (average diameter: 18.8 ± 0.1 µm in total vs. 17.4 ± 0.4 µm in Ki-67 positive). We did not observe a bimodal distribution that would suggest mobilization of resident cardiac stem cells as a mechanism for the increased Ki-67 staining after AdvFGF-5.

**Correlation between Ki-67 and phospho-histone H3 positive Cells with Light and Confocal Fluorescence Microscopy**

We confirmed the correlation between light (morphometrically identified myocytes) and confocal microscopy (fluorescent counterstained myocytes) in a subgroup of animals with paired results plotted in Figure 5. Sections were prepared from the same tissue block but the samples used were not adjacent slices. There was close correlation between Ki-67 positive myocytes using confocal or light microscopy (y=1.2523x-3.78, R²=0.96). The correlation between light and confocal microscopy with phospho-histone H3 staining was also good although there was
more variability due to the limited number of points sampled ($y=0.89x+1.91$, $R^2=0.41$). These data support the notion that the quantitation of myocyte cell cycle markers using morphometric criteria and light microscopy provide quantitatively similar results to those with multiwavelength confocal microscopy where myocytes are identified by immunostaining.
Supplementary Figures

Figure 1. Analysis of circumferential flow distributions – Samples from posterior (A) through septal (L) myocardium are shown on TTC-stained ring (left). Full-thickness absolute flow distributions are summarized in middle graphs. Hibernating LAD regions were easily delineated by analyzing flow during adenosine vasodilation. Regions of intermediate flow between normal and hibernating regions were considered borders (shaded regions) reflecting an admixture of normal and hypoperfused tissue. Once defined from vasodilated flow, the central samples in normal and hypoperfused regions were combined to obtain weighted averages. Individual pieces were also expressed as fractions of average full-thickness values in normal regions (right graphs). The area below the dotted line indicates the relative flow deficit with the unshaded area representing changes before (initial) and after (final) AdvFGF-5. See text for further description.

Figure 2. Average flow excluding border regions – Initially, resting LAD flow was reduced in comparison to remote normally perfused myocardium. LAD subendocardial flow reserve was critically impaired and unable to increase in response to epinephrine or adenosine stress. Average values were no different two-weeks after AdvFGF-5. Relative flow changes in individual pieces (n=147) showed small increases at rest but no change during epinephrine or adenosine. Thus, the effects of AdvFGF-5 on perfusion to central regions of hibernating myocardium were limited to small changes in relative perfusion with no change in absolute blood flow in any transmural layer.

Figure 3. Circumferential distribution of perfusion in full-thickness samples including border regions. Absolute and relative flow measurements in each of 12 circumferential regions were averaged to assess whether the perfusion deficit size changed reflecting an improvement in
at the perfusion boundary. When border regions were included, we found significant increases in absolute and relative resting flow in the anterolateral (C,D,E) but not septal (J, K) samples leading to a reduction in the relative flow deficit from 18.9±2.9 to 12.5±2.4%. During adenosine, flow increases were only significant in region C after AdvFGF-5 and the relative flow deficit decreased from 46.7±3.5 to 43.2±3.5%. There was no improvement during epinephrine.

**Figure 4 – Frequency distribution of myocyte diameter in Ki-67 positive myocytes from animals treated with AdvFGF-5 vs. untreated controls with hibernating myocardium.** Data are pooled from all regions of the heart based on the global nature of increased Ki-67 and myocyte hypertrophy observed after intracoronary AdvFGF-5. There was a broad distribution of sizes in both Ki-67 positive myocytes and unstained myocytes. In untreated hearts, Ki-67 positive cells were similar in diameter to those that were not stained. In contrast, in AdvFGF-5 treated animals, Ki-67 positive cells were significantly smaller than those that were unstained. The distribution of myocyte size was unimodal in each circumstance with no discernible increase in the frequency of small myocytes potentially indicative of stem cell recruitment as a cause of Ki-67 staining in this model.

**Figure 5 – Correlation between Ki-67 and phospho-histone H3 positive cells with light versus confocal fluorescence microscopy.** The dashed lines represent the ideal identity relation (i.e. a one to one relation between the two measurement techniques). There was good correlation between Ki-67 positive myocytes with confocal (y) vs. light microscopy (y=1.25x-3.78, R²=0.96, n=14). While there were fewer data points for comparison due to the low rates of phospho-histone H3 staining, there was also a nearly one to one correlation between light and confocal microscopy was more moderated than Ki-67 group (y=0.89x+1.91, R²=0.41, n=10).
Thus, quantitation by light microscopy and morphometric identification of myocytes provided similar results to confocal immunofluorescence.

References


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<th>PLVEDP (mmHg)</th>
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<th>Heart Rate (bpm)</th>
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Values are mean±SEM; * p<0.05 Initial vs. Final studies; †p<0.05 vs. rest.

n - number of pigs; PLVsys – left ventricular systolic pressure; PLVEDP – left ventricular end diastolic pressure; HR x PLVsys – rate pressure products.
Table 2. Echocardiographic Measurements.

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* p<0.05 vs. Initial; † p<0.05 vs. Rest
Figure 2

Absolute Flow (ml/min/g)

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Relative Flow

- LAD
  - Initial
  - AdvFGF-5
- Remote
  - Initial
  - AdvFGF-5

* p<.05 vs. Initial
ns

n=147
Figure 3
Figure 4

**FGF-5 Treated**

- • Unstained Myocyte (n=3,374; 18.8 ± 0.1 µm)
- • Ki-67 Myocyte (n=764; 17.4 ± 0.4 µm)

**Untreated**

- • Unstained Myocyte (n=2,490; 15.1 ± 0.1 µm)
- • Ki-67 Myocyte (n=151; 15.9 ± 0.3 µm)
Ki-67 Staining

$y = 1.25x - 3.8$

$R^2 = 0.96$

$n = 14$

Phospho-Histone H3 Staining

$y = 0.90x + 1.9$

$R^2 = 0.41$

$n = 10$

Figure 5
Online Supplement

Adenoviral Gene Transfer of FGF-5 to Hibernating Myocardium Improves Function and Stimulates Myocytes to Hypertrophy and Reenter the Cell Cycle

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James A. Fallavollita

and

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From the VA WNY Health Care System and the Departments of Medicine and Physiology&Biophysics and the Center for Research in Cardiovascular Medicine at the University at Buffalo.

Supported by the VA, AHA, NHLBI, Albert and Elizabeth Rekate Fund and the Oishei Foundation.

Abbreviated Title – FGF-5 in hibernating myocardium

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We used the morphometric measurements to estimate regional variations in myocyte cell volume. Briefly, myocyte volume was determined by 4 independent parameters which include the number of myocyte nuclei per unit area, the average length of myocyte nuclei, the average myocyte diameter, and volume fraction of myocytes in the tissue. Regional variations in Myocyte volume between LAD and remote regions were assessed by use of standard morphological calculations as previously described in detail\textsuperscript{9}.

**Quantitation of Apoptosis with TUNEL**

We quantified apoptosis using the fluorescent TUNEL staining as previously described\textsuperscript{7}. Myocyte apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-labeling (TUNEL, Chemicon Inc) and epifluorescence with an FITC filter. Approximately 300 to 400 fields were examined per sample. The extent of apoptosis was expressed by normalizing the results to the number of myocyte nuclei per mm\textsuperscript{2} in each sample.

**Quantitation of Cell Growth/Cycle Markers-light microscopy**

Immunohistochemistry was used to determine whether AdvFGF-5 promoted myocytes to enter the growth phase of the cell cycle. Adult cardiac myocytes are normally in the quiescent or G\textsubscript{0} phase. The Ki-67 antigen has been used to identify proliferating myocytes although positive staining can reflect adult myocytes developing hypertrophy as well as the potential for cardiac
myocytes to progress to mitosis\textsuperscript{10,11}. Phospho-histone H3 is a nuclear protein that increases in cell nuclei when there is active DNA replication and cells have entered the mitotic phase of cell growth. The number and percent of nuclei labeled with phospho-histone H3 have been closely correlated with the frequency of BrdU uptake in ventricular myocytes\textsuperscript{12}. Thus, nuclear phospho-histone H3 staining is consistent with myocytes having the potential to hypertrophy and/or undergo nuclear division. It is however, not a surrogate marker for ultimate progression of nuclear division to cytokinesis.

Tissue sections (5µm) were incubated with anti-Ki-67 (mouse monoclonal antibody, clone MIB-1, Dako) or anti-phospho-histone H3 rabbit polyclonal antibody (Upstate Biotech). Samples were post-treated with an HRP-conjugated secondary antibody\textsuperscript{10,12}. Positive nuclei were labeled with DAB and initially quantified with light microscopy (600x). We identified myocytes by their morphology on light microscopy and expressed positive Ki-67 nuclear staining relative to myocyte nuclear density in each section (positive myocyte nuclei per million myocyte nuclei). Finally, to determine whether effects of AdvFGF-5 were restricted to cardiac myocytes, we also quantified the frequency of Ki-67 staining in nonmyocyte cells. The specificity of all antibodies was verified by omission of primary antibodies. All of the antibodies have been successfully used in the pig by other laboratories\textsuperscript{13-15}.

**Quantitation of Cell Growth/Cycle Markers-Confocal microscopy**

To further validate the results obtained by light microscopy, additional experiments were performed using the previous antibodies for cell growth/cycle markers with nuclear and myocyte markers that were imaged with confocal microscopy (Bio-Rad MRC 1024). Tissue sections (5µm) were incubated with either anti-Ki-67 (mouse monoclonal antibody, clone MIB-1, Dako) or anti-phospho-histone H3 (rabbit polyclonal antibody, Upstate Biotech). To identify myocytes
we used anti-cardiac TnI antibodies previously demonstrated to react in swine (mouse monoclonal antibody clone 8I-7, Spectral diagnosis, 1:100 or rabbit polyclonal antibody, Santa Cruz, 1:100)\textsuperscript{16}. Nuclei were stained with TO-PRO-3 (Molecular Probes). Samples were post-treated with fluoroisothiocyanate (FITC) conjugated anti-mouse and TRITC conjugated anti-rabbit antibody (Dako)\textsuperscript{10,12}. Multiple fields were digitized and used to quantify the frequency of myocyte Ki-67 staining. Quantitative results using confocal microscopy were compared to the morphometric and light microscopic identification summarized above.

**Efficiency of intracoronary gene transfer assessed using AdvEGFP (n=4)**

We performed additional experiments to quantify the efficiency of adenoviral uptake in vivo as well as exclude an effect of the adenovirus on eliciting myocyte hypertrophy in swine with hibernating myocardium (n=2) and sham controls (n=2). After baseline assessments of flow and function we injected a replication deficient adenovirus overexpressing a nuclear localizing enhanced green fluorescent protein (AdvEGFP). Pigs were studied as outlined above and AdvEGFP (total dose 2 x10\textsuperscript{12} viral particles, Vector Core Facility at the University of Pittsburgh) injected into each of the three major coronary arteries in equally divided doses following pretreatment with intracoronary histamine as outlined for AdvFGF-5.

After two weeks, physiological studies were repeated and tissue was obtained to assess the cellular efficiency of AdvEGFP transfection using direct confocal fluorescent microscopy. Sections were counterstained with phalloidin to identify F-actin (Phalloidin-TRITC, Sigma-Aldrich)\textsuperscript{11}. The effects of AdvEGFP on myocyte diameter in normal remote regions were assessed as outlined below. The frequency of nuclear EGFP in cardiac cells was quantified in 416±16 fields (total area 59±4mm\textsuperscript{2}) for each animal and expressed as a percentage of nuclei in the field which were identified using nuclear staining with TO-PRO-3.
**Statistics**

Data are expressed as mean ± standard error. Differences after treatment with AdvFGF-5 and comparisons between the hibernating and normally perfused remote regions of the same heart were assessed using paired t-tests. Differences among AdvFGF-5 treated animals, age-matched shams and untreated animals with hibernating myocardium were assessed using a two-way ANOVA and the post-hoc Holm-Sidak test (Sigma Stat 3.0). Differences of p<0.05 were considered significant.

**Supplementary Results**

Pigs were in good health at the time of study and TTC staining showed no evidence of infarction. Initial physiological studies were performed 123±2 days after instrumentation and repeated 2-weeks after AdvFGF-5 (137±2 days). Over this interval, body mass increased 8% (96±3 kg to 104±3 kg).

**Effects of AdvFGF-5 on the Transmural Distribution of Myocardial Perfusion in Myocardial Samples Averaged from Central Core Regions of Hibernating Myocardium**

Findings under baseline conditions confirmed hibernating myocardium. There were reductions in resting perfusion (LAD 0.98±0.09 vs. 1.38±0.15 ml/min/g in remote, p<0.01) with the greatest reduction in the subendocardium (0.79±0.05 vs. 1.52±0.16 ml/min/g in remote, p<0.01). Full-thickness flow during epinephrine was attenuated (LAD 1.27±0.09 vs. 1.65±0.08 ml/min/g in remote, p<0.01) and subendocardial flow did not increase significantly above resting levels (LAD 0.93±0.09 ml/min/g, p-ns vs. rest). Likewise, the increase in full-thickness flow during adenosine was severely attenuated (LAD 1.45±0.27 vs. 4.84±0.23 ml/min/g in remote,
p<0.001) and subendocardial flow did not increase above the resting value (LAD 0.72±0.17 ml/min/g, p-ns. vs. rest).

**Effects of AdvFGF-5 on Relative Perfusion in Individual Samples From the Area at Risk**

The effects of AdvFGF-5 on absolute and relative flow (excluding border regions) are summarized in Figure 2. Hemodynamic measurements are summarized in Table 1. Two-weeks after AdvFGF-5, resting perfusion tended to increase in hibernating LAD regions while decreasing in remote regions, but the differences were not significant. There were no significant differences in absolute flow during epinephrine or adenosine stress. When analyzed as paired measurements from individual myocardial pieces (n=147), there was a small increase in relative resting perfusion (LAD/Remote 0.70±0.02 to 0.75±0.02, p<0.0001). In contrast, there was no improvement during epinephrine or adenosine stress (LAD/Remote epinephrine 0.76±0.04 vs. 0.74±0.05 and adenosine 0.33±0.02 vs. 0.31±0.02).

**The Effects of AdvFGF-5 on the Circumferential Distribution of Myocardial Perfusion and the Estimated Perfusion Deficit Size**

We next evaluated the circumferential distribution of perfusion and the relative flow deficit. Figure 3 summarizes absolute and relative full-thickness perfusion measurements averaged in each of the 12 regions. Under resting conditions, both absolute and relative perfusion increased in 3 of 12 circumferential segments reducing the integrated flow deficit from 18.9±2.9% to 12.5±2.4% (p<0.05). During adenosine, significant increases in perfusion were restricted to a single segment with a small reduction in the vasodilated perfusion deficit size from 46.7±3.5% to 43.2±3.5% (p<0.05). Perfusion distributions during epinephrine were more variable with no significant differences in segmental, absolute or relative perfusion and no
reduction in the perfusion deficit size (16.6±3.0% vs. 18.0±4.0%, p-ns). Thus, AdvFGF-5 produced heterogeneous effects on myocardial perfusion that were more prominent in regions bordering normal and hibernating myocardium leading to small but significant reductions in the relative perfusion deficit size at rest and following vasodilation.

**The Distribution of Myocyte Diameters in Ki-67 Positive Cells After AdvFGF-5**

Figure 4 summarizes data which are pooled from all regions of the heart based on the global nature of increased Ki-67 and myocyte hypertrophy observed after intracoronary AdvFGF-5. There was a broad distribution of myocyte diameters in both Ki-67 positive myocytes and unstained myocytes. In untreated hearts, Ki-67 positive cells were similar in diameter to those that were not stained (average diameter: 15.1 ± 0.1 µm in unstained vs. 15.9 ± 0.3 µm in Ki-67 positive). In contrast, in AdvFGF-5 treated animals, Ki-67 positive cells were significantly smaller than those that were unstained (average diameter: 18.8 ± 0.1 µm in total vs. 17.4 ± 0.4 µm in Ki-67 positive). We did not observe a bimodal distribution that would suggest mobilization of resident cardiac stem cells as a mechanism for the increased Ki-67 staining after AdvFGF-5.

**Correlation between Ki-67 and phospho-histone H3 positive Cells with Light and Confocal Fluorescence Microscopy**

We confirmed the correlation between light (morphometrically identified myocytes) and confocal microscopy (fluorescent counterstained myocytes) in a subgroup of animals with paired results plotted in Figure 5. Sections were prepared from the same tissue block but the samples used were not adjacent slices. There was close correlation between Ki-67 positive myocytes using confocal or light microscopy (y=1.2523x-3.78, R²=0.96). The correlation between light and confocal microscopy with phospho-histone H3 staining was also good although there was
more variability due to the limited number of points sampled (y=0.89x+1.91, R²=0.41). These data support the notion that the quantitation of myocyte cell cycle markers using morphometric criteria and light microscopy provide quantitatively similar results to those with multiwavelength confocal microscopy where myocytes are identified by immunostaining.
Supplementary Figures

Figure 1. Analysis of circumferential flow distributions – Samples from posterior (A) through septal (L) myocardium are shown on TTC-stained ring (left). Full-thickness absolute flow distributions are summarized in middle graphs. Hibernating LAD regions were easily delineated by analyzing flow during adenosine vasodilation. Regions of intermediate flow between normal and hibernating regions were considered borders (shaded regions) reflecting an admixture of normal and hypoperfused tissue. Once defined from vasodilated flow, the central samples in normal and hypoperfused regions were combined to obtain weighted averages. Individual pieces were also expressed as fractions of average full-thickness values in normal regions (right graphs). The area below the dotted line indicates the relative flow deficit with the unshaded area representing changes before (initial) and after (final) AdvFGF-5. See text for further description.

Figure 2. Average flow excluding border regions – Initially, resting LAD flow was reduced in comparison to remote normally perfused myocardium. LAD subendocardial flow reserve was critically impaired and unable to increase in response to epinephrine or adenosine stress. Average values were no different two-weeks after AdvFGF-5. Relative flow changes in individual pieces (n=147) showed small increases at rest but no change during epinephrine or adenosine. Thus, the effects of AdvFGF-5 on perfusion to central regions of hibernating myocardium were limited to small changes in relative perfusion with no change in absolute blood flow in any transmural layer.

Figure 3. Circumferential distribution of perfusion in full-thickness samples including border regions. Absolute and relative flow measurements in each of 12 circumferential regions were averaged to assess whether the perfusion deficit size changed reflecting an improvement in
at the perfusion boundary. When border regions were included, we found significant increases in absolute and relative resting flow in the anterolateral (C,D,E) but not septal (J, K) samples leading to a reduction in the relative flow deficit from 18.9±2.9 to 12.5±2.4%. During adenosine, flow increases were only significant in region C after AdvFGF-5 and the relative flow deficit decreased from 46.7±3.5 to 43.2±3.5%. There was no improvement during epinephrine.

**Figure 4 – Frequency distribution of myocyte diameter in Ki-67 positive myocytes from animals treated with AdvFGF-5 vs. untreated controls with hibernating myocardium.** Data are pooled from all regions of the heart based on the global nature of increased Ki-67 and myocyte hypertrophy observed after intracoronary AdvFGF-5. There was a broad distribution of sizes in both Ki-67 positive myocytes and unstained myocytes. In untreated hearts, Ki-67 positive cells were similar in diameter to those that were not stained. In contrast, in AdvFGF-5 treated animals, Ki-67 positive cells were significantly smaller than those that were unstained. The distribution of myocyte size was unimodal in each circumstance with no discernible increase in the frequency of small myocytes potentially indicative of stem cell recruitment as a cause of Ki-67 staining in this model.

**Figure 5 – Correlation between Ki-67 and phospho-histone H3 positive cells with light versus confocal fluorescence microscopy.** The dashed lines represent the ideal identity relation (i.e. a one to one relation between the two measurement techniques). There was good correlation between Ki-67 positive myocytes with confocal (y) vs. light microscopy (y=1.25x-3.78, R²=0.96, n=14). While there were fewer data points for comparison due to the low rates of phospho-histone H3 staining, there was also a nearly one to one correlation between light and confocal microscopy was more moderated than Ki-67 group (y=0.89x+1.91, R²=0.41, n=10).
Thus, quantitation by light microscopy and morphometric identification of myocytes provided similar results to confocal immunofluorescence.

References


Table 1. Hemodynamic Parameters

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<th>n</th>
<th>PLVsys (mmHg)</th>
<th>PLVEDP (mmHg)</th>
<th>Mean Aortic (mmHg)</th>
<th>Heart Rate (bpm)</th>
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Values are mean±SEM; *p<0.05 Initial vs. Final studies; †p<0.05 vs. rest.

n - number of pigs; PLVsys – left ventricular systolic pressure; PLVEDP – left ventricular end diastolic pressure; HR x PLVsys – rate pressure products
Table 2. Echocardiographic Measurements.

<table>
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<th>LAD ΔWT (mm)</th>
<th>Remote ΔWT (mm)</th>
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<th>LVESD (mm)</th>
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* p<0.05 vs. Initial; † p<0.05 vs. Rest
Figure 2

Transformed to Fractional Flow Reserve

**Absolute Flow (ml/min/g)**

- **LAD**
- **Remote**

**Relative Flow**

**Rest**

- Epinephrine
- Adenosine

**Adenosine**

- Initial
- AdvFGF-5

* p<.05 vs. Initial

**n=147**

ns
Figure 3

Rest

Epinephrine

Adenosine

**Absolute Flow (ml/min/g)**

**Relative Flow**

**Relative Flow Deficit**

- Absolute Flow (ml/min/g)
- Relative Flow
- Relative Flow Deficit

* *p<0.05 vs. Initial
Figure 4

FGF-5 Treated

- Unstained Myocyte (n=3,374; 18.8 ± 0.1 µm)
- Ki-67 Myocyte (n=764; 17.4 ± 0.4 µm)

Untreated

- Unstained Myocyte (n=2,490; 15.1 ± 0.1 µm)
- Ki-67 Myocyte (n=151; 15.9 ± 0.3 µm)
**Ki-67 Staining**

- $y = 1.25x - 3.8$
- $R^2 = 0.96$
- $n = 14$

**Phospho-Histone H3 Staining**

- $y = 0.90x + 1.9$
- $R^2 = 0.41$
- $n = 10$

Figure 5