Leslie McNaughton

Project Title: Factors and signals regulating expression of the pro-hypertrophic, high molecular weight FGF-2, by human cardiac myofibroblasts.

Student's Name: Leslie McNaughton

Supervisor's Name: Elissavet Kardami

Departmental Affiliation: Institute of Cardiovascular Sciences, St. Boniface Research Centre and Department of Human Anatomy and Cell Sciences

Project Abstract:

Background. Chronic activation of the renin-angiotensin-aldosterone axis causes maladaptive cardiac remodeling leading to heart failure. This is partially due to the conversion of cardiac fibroblasts to an 'activated' hyper-secretory myofibroblast phenotype. Fibroblast growth factor 2 (FGF-2) is a protein implicated in myofibroblast-mediated remodeling. FGF-2 exists as high (Hi-) and low molecular weight (Lo-) isoforms. Hi-FGF-2 stimulates cardiomyocyte hypertrophy and secretion of cytokines associated with heart failure. The main hypothesis under investigation is that human heart-derived myofibroblasts express Hi-FGF-2, which is upregulated by angiotensin II (Ang II), by a mechanism engaging the AT1 (and/or AT2) receptor(s), ERK kinase, and/or matrix metalloprotease, MMP-2, activities. Also under investigation is the effect of an anti-hypertrophic polyphenol, resveratrol, on Hi-FGF-2 expression.

Methods. Primary cultures of human patient heart-derived myofibroblasts were stimulated with Ang II, in the presence or absence of: inhibitors of the AT1 or AT2 receptors, ERK, and MMP-2; or resveratrol. Cell-associated FGF-2, and exported, cell surface-bound FGF-2 levels were examined by western blot analyses, using anti-FGF-2 antibodies.

Results. Human heart myofibroblasts express Hi-FGF-2, which was upregulated by Ang II. This upregulation was reduced by AT1 and AT2 inhibitors, and eliminated by simultaneous AT1 and AT2 blockade. Inhibiting ERK, or MMP-2, or supplementing with resveratrol, eliminated the Ang II-induced Hi-FGF-2 upregulation.

Conclusions. Chronic Ang II elevation in patients may exert some of its deleterious effects via cardiac myofibroblast Hi-FGF-2 upregulation. Both the AT1 and AT2 receptors, as well as the ERK and MMP-2 pathways, are potential targets to reduce Hi-FGF-2 levels. Furthermore, resveratrol, or clinically usable analogs, could be used to decrease myofibroblast Hi-FGF-2.

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Introduction

Heart disease is currently a leading cause of death globally. Although treatments such as those targeting the inhibition of the renin-angiotensin-aldosterone system and beta-adrenergic receptor blockade are currently available, the burden of heart disease remains high. This points to the need for modified or novel treatment strategies. In order to provide novel treatment options, it is important to increase our understanding of both the triggers and molecular mechanisms involved in heart disease.

Cardiac hypertrophy (increased cardiac mass relative to body weight) can occur as a compensatory response in order to sustain cardiac output when stress is placed on the heart. It is classified as adaptive (in the case of exercise-induced hypertrophy, normal post-natal growth, during pregnancy) or maladaptive (pathological). Prolonged hemodynamic stresses such as hypertension, ischemic heart disease, valvular insufficiency and genetic defects can lead to maladaptive cardiac remodeling including hypertrophy and fibrosis. Pathological cardiac hypertrophy is considered a major risk factor in the development of heart failure [1, 2] and is associated significant increase in the risk for sudden death. Identification of neurohumoral agents, cytokines, and growth factor molecules acting as molecular triggers of hypertrophy is important as it can provide therapeutic targets for prevention of hypertrophy. **Fibroblast growth factor 2 (FGF-2) is one such trigger**

FGF-2 is a heparin-binding polypeptide with many biological activities including effects on cell growth and differentiation. It acts by binding to plasma membrane tyrosine kinase receptors (FGFRs) that are present in all cardiac cells including adult cardiomyocytes [3]. A single FGF-2 mRNA gives rise to two different types of isoforms: high molecular weight (Hi; 21-34 kDa) and low molecular weight (Lo;18 kDa). Although the FGF-2 isoforms have different biological activities, most studies to-date have focused on Lo-FGF-2 [4], which is cardioprotective, angiogenic, and mitogenic [3]. Hi-FGF-2 on the other hand is emerging as a strong candidate for triggering pathological cardiac hypertrophy. Studies in the supervisor's laboratory have shown that Hi-FGF-2, made and 'secreted' by cardiac fibroblasts and myofibroblasts, can stimulate cardiomyocyte hypertrophy in a paracrine manner. Recombinant Hi-FGF-2 has been shown to promote myocardial hypertrophy *in vitro* and *in vivo* [5] and unlike Lo-FGF2, Hi-FGF-2 is anti-angiogenic [6], a property which is expected to contribute to an ischemic environment in the hypertrophying heart. Thus increased extracellular Hi-FGF-2 would be expected to act on the myocytes both directly by triggering hypertrophy, and, indirectly, by preventing angiogenesis and creating an ischemic environment, to compound and sustain the hypertrophic response. In addition, experimental models of heart disease (pressure overload hypertrophy in mouse, streptozotocin-induced diabetes and drug-induced cardiotoxicity in the rat) display significant upregulation of cardiac Hi-FGF-2 [7 and unpublished data, Kardami laboratory]. The preferential translation of Hi-FGF-2 over Lo-FGF-2 has been noted under conditions of oxidative stress and heat shock [3].

Prolonged stress, and the accompanying increases in bioactive molecules (catecholamines, cytokines) result in conversion of the relatively quiescent fibroblasts to activated fibroblasts (myofibroblasts). These cells that play a central role in the remodeling process and induction of hypertrophy and fibrosis by synthesizing collagen and other

extracellular matrix molecules as well as producing bioactive agents such as FGF-2 [8]. It was initially believed that only Lo-FGF-2 was secreted by these cells, while Hi-FGF-2 remained predominantly in the nucleus. However, research in the supervisor's laboratory has demonstrated that cardiac myofibroblasts are major producers of Hi-FGF-2 [9], which becomes exported by the cell by a non-conventional pathway, and acts locally in a paracrine or autocrine fashion. Exported Hi-FGF-2 may either remain tightly bound to the cell surface (and the extracellular matrix) or become liberated from the cell surface into the conditioned media (in culture) or in body fluids.

In cultured rat heart fibroblasts Hi-FGF-2 is upregulated and secreted into the extracellular environment in response to stimulation by angiotensin II (Ang II) [9]. Ang II is a major mediator of the renin-angiotensin-aldosterone system and has been linked to hypertension, renal and heart failure, and is known to promote cardiac hypertrophy [10, 11]. Current strategies for pharmacological management/prevention of heart failure include targeting Ang II production (ACE inhibitors) and/or Ang II plasma membrane receptors (Ang II receptor blockers or ARBs) [10]. In view of the properties of Hi-FGF-2, the relationship between Ang II and Hi-FGF-2 production by cardiac fibroblasts suggests that Hi-FGF-2 is mediating some of the deleterious effects of Ang II. *There is as yet no information as to whether human heart-derived fibroblasts display similar properties to their rat counterparts regarding Hi FGF-2 expression, secretion and upregulation by Ang II.*

Ang II exerts its effects by acting on one of two G-protein linked receptors: AT1 and AT2 [11]. In the heart, these receptors are found in abundance on cardiac fibroblasts and myofibroblasts, and to a lesser extent on cardiomyocytes. Both AT1 and AT2 receptors become upregulated in the heart following cardiac pathologies such as myocardial infarction and dilated cardiomyopathy [12]. The deleterious effects of Ang II (including cardiomyocyte hypertrophy as well as vasoconstriction, aldosterone and vasopressin release, renal sodium reabsorption, increased collagen deposition, and cell proliferation) are mediated by the AT1 receptor. This is demonstrated by the fact that these effects are attenuated by AT1 receptor blockers [10]. On the other hand, the AT2 receptor may function to oppose the actions of the AT1 receptor [11, 12]. In fact, the AT2 receptor is known to be involved in physiological processes such as development and tissue remodeling and the regulation of blood pressure.

Work from supervisor's laboratory indicated that, in rat cardiac myofibroblasts, the AT1 (but not AT2) mediated Hi-FGF-2 up-regulation [9]. Other studies have shown that the Ang II-AT1 signal transduction pathway includes activation of the extracellular signal-regulated protein kinases (ERK), as well extracellular matrix proteases, such matrix metalloproteinase (MMP-2) [13]. The Ang II-AT1 upregulation of MMP-2 activity is responsible for releasing another heparin-bound growth factor (the heparin-bound epidermal growth factor) from the cell surface to the environment, enabling it to then act on cell surface receptors. It is therefore plausible that MMP-2 may also act to liberate cell-bound Hi-FGF-2. *It is not yet known if intermediate signals ERK and MMP-2 influence Hi-FGF-2 up-regulation and/or secretion by cardiac myofibroblasts.*

While the deleterious effects of elevated Ang II are managed through the use of ARBs, there is interest in exploiting naturally occurring compounds that may have anti-hypertrophic properties. Resveratrol is a naturally occurring compound that has been isolated as a

'nutraceutical' from over 70 species of plants. It is also present in a variety of common food products such as grapes, peanuts, and wine [14]. Resveratrol is reported to have several beneficial effects such as promoting increased high-density lipoprotein cholesterol, inhibiting platelet aggregation, improving endothelial function, in addition to anti-inflammatory and antioxidant properties [14, 15, 16]. Resveratrol has also been shown to reduce angiotensin-induced cardiac hypertrophy, and to possess some anti-fibrotic properties [17, 18] by preventing the Ang II-induced ERK activation [19]. *However, there is currently no information as to whether resveratrol can influence the Ang II-induced Hi-FGF-2 up-regulation/release by cardiac fibroblasts.*

In this work we have addressed the following hypotheses: (i) human patient-derived cardiac myofibroblasts express Hi FGF-2 which is upregulated by Ang II, (ii) the Ang II-induced Hi FGF-2 up-regulation by human cardiac myofibroblasts is mediated by the AT1 receptor, the ERK pathway, and MMP-2, and (iii), resveratrol decreases Hi FGF-2 expression and export by cardiac fibroblasts.

Materials and Methods

Cells. Cultures of adult rat heart (ventricle)-derived fibroblasts were obtained at the supervisor's laboratory [9], and kept as a frozen stock at passage P1. Primary cultures of neonatal cardiac fibroblasts were isolated from cardiac ventricles of one-day-old Sprague-Dawley rat pups as published in [20, 21]. The rat-derived (both adult and neonatal) cells were used at passage P2, and grown to near confluence in Ham's Mixture Nutrient F-10 medium (Sigma) containing 10% fetal bovine serum, and 1% penicillin/streptomycin (GIBCO). Twenty-four hours before the various treatments, the cultures were switched to a low serum medium (0.5% serum) supplemented with 10μg/mL each of insulin, transferin, selenium; 20μg/mL ascorbic acid; and 0.2% bovine serum albumin.

Primary cultures of human patient atria-derived fibroblasts were also obtained from frozen stocks available in the supervisor's laboratory in collaboration with Dr. Rakesh Arora. Additional stocks of human patient atria-derived fibroblasts were obtained from Dr. Darren Freed's laboratory. Both stocks were kept frozen at passage P1. They were used at either passage P2 or P3, and grown to near confluence in Lung/Cardiac Fibroblast Basal medium (Cell Applications Inc.), containing 10% fetal bovine serum, and 1% penicillin/streptomycin (GIBCO). As with the rat-derived cells, twenty-four hours before the various treatments, the cultures were switched to a low serum medium (0.5% serum) supplemented with 10μg/mL each of insulin, transferin, selenium; 20μg/mL ascorbic acid; and 0.2% bovine serum albumin.

Treatments: Cells, cultured in 35 mm or 100 mm dishes, were stimulated with Ang II (Sigma) at 10^{-7} -10^{-5} M in the presence and absence of the AT1 blocker losartan (Merck Frost) at 10^{-5} M, the AT2 blocker PD123319 (Sigma) at 10^{-5} M, the ERK inhibitor U0126 (Calbiochem) at 50μM, MMP2 (matrix-metalloproteinase) inhibitor 1 (Calbiochem) at 50μM, and resveratrol (Calcbiochem) at 50μM dissolved in DMSO. Each experiment included: control group, Ang II-treated group, inhibitor(s) (or resveratrol)-treated group(s), and group(s) treated with Ang II plus inhibitor(s) (or resveratrol); n=3/group. For the Ang II plus inhibitor (or resveratrol at 50μM) groups, all inhibitors were added 1 hour prior to the addition of Ang II. An exception to this is

the experiment performed studying the effects of resveratrol. In addition to the groups listed above, the resveratrol experiment included a group of cells treated with DMSO (vehicle) plus Ang II. This experiment also included two separate Ang II plus resveratrol groups – one in which resveratrol was added one hour prior to the addition of Ang II (pre-treatment group) and one in which resveratrol was added one hour following the addition of Ang II (post-treatment group).

FGF-2 detection: At 48 hours after treatment, culture conditioned media, containing secreted FGF-2, was removed and stored at -70 °C until further analyses. Exported FGF-2 that remained bound to the surface of the cell monolayer was eluted by a brief and gentle wash with 2M NaCl as described below, for select experiments. For detection of cell-associated FGF-2, cells were rinsed three times with cold phosphate buffered saline (PBS), scraped, lysed and used for SDS/PAGE analyses (15% gels) and western blotting for FGF-2 (monoclonal anti-FGF-2 antibody from Upstate Biotechnology, Inc.). Densitometry of western blots were done as in [9]. Probing for β-actin or β-tubulin served as a loading control. Recombinant Hi- and Lo-FGF-2 were included as positive controls [9].

Elution of exported and cell-surface bound FGF-2. Following removal of the conditioned media, cells were washed with 2mL (per 100mm dish) of a high salt (2 M NaCl) solution, with proteolysis inhibitors, for 2 minutes as described in [9]. The 2 M NaCl eluates were diluted to 0.5 M NaCl, supplemented with 100 μL of heparin-sepharose beads (freshly made) and left at room temperature for 2 hours with gentle agitation. Heparin binds FGF-2 and removes it from the solution. Heparin-sepharose beads were precipitated by brief centrifugation, washed with PBS twice, and boiled in 35 μL of 2x SDS/PAGE sample buffer to elute heparin-bound proteins, which were analyzed by gel electrophoresis and western blotting to detect FGF-2 as described above.

Statistical Analysis: Quantitative densitometric analysis of each western blot band was done using a computer program (Quantity One 1-D Analysis Software) connected to a scanner (GS-800 Calibrated Densitometer). All values were expressed as means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test was used for comparing differences among groups using GraphPad InStat 3.0 computer program. Differences among groups were defined as significant ($P<0.05$), very significant ($P<0.01$), or highly significant ($P<0.001$).

Results

Hi-FGF-2 in myofibroblasts, and the effect of Ang II, and its receptor blockers, on Hi -FGF-2 accumulation. This series of experiments was done using adult rat heart-derived, as well as human heart-derived myofibroblasts.

Adult rat heart derived fibroblasts. A first series of studies was conducted using adult rat heart (ventricle)-derived myofibroblasts. These cells are defined as myofibroblasts due to their elevated expression of 'activated' fibroblast markers such as α-smooth muscle actin and EDA-fibronectin, demonstrated in [22]. As shown in Figure 1, non-stimulated cells express both Hi- and Lo-FGF-2, but mostly Hi-FGF-2. Stimulation with Ang II significantly increased relative levels of cell-associated Hi-FGF-2 compared to untreated controls, as published in [9]. Cell-

associated FGF-2 refers to the sum of intracellular FGF-2 plus any FGF-2 that has exited the cell but remained tightly bound to the cell surface. Cells treated with Ang II in the presence of losartan (an inhibitor of the AT1 receptor) had significantly reduced Hi-FGF-2 levels compared to those treated with Ang II alone (Figure 1). Although cells that were treated with Ang II in the presence of PD123319 (an inhibitor of the AT2 receptor) showed a trend towards decreasing the effect of Ang II, data did not reach statistical significance ($P>0.05$). Both the control and treatment groups expressed similar levels of β-tubulin (serving as a loading control). Pilot studies in supervisor's laboratory have shown that rat atria-derived myofibroblasts express Hi-FGF-2 and respond to Ang II in a manner similar to their ventricular counterparts (Jon-Jon Santiago, Ph.D student, unpublished observations).

Human heart (atria)-derived myofibroblasts. These cells have been identified at the supervisor's laboratory as myofibroblasts as they also express activated fibroblast markers in a manner similar to their rat counterparts. Figure 2A shows that these cells express EDA fibronectin, α-smooth muscle actin, procollagen, embryonic smooth muscle myosin, but not muscle markers as desmin or striated muscle myosin. These human atria-derived cells express predominantly Hi-FGF-2, migrating at 22-24 kDa (Figure 2B). Stimulation with Ang II significantly increased human cell-associated Hi-FGF-2 accumulation, compared to untreated cells. Losartan, as well as PD123319, significantly ($P<0.05$) decreased the Ang II induced Hi-FGF-2 up-regulation (Figure 2B). Each blocker individually elicited an approximately 50% decrease in the Ang II-induced Hi-FGF-2 upregulation. In a follow-up series of experiments we tested the combined effect of AT1 and AT2 inhibitors. This portion of the work was done in collaboration with Jon-Jon Santiago (Ph. D student, Institute of Cardiovascular Sciences). Once again, Ang II treated cells showed a significant upregulation of Hi-FGF-2 levels when compared to the untreated cells. Treating fibroblasts with both types of Ang II receptor blockers in combination effectively eliminated the Ang II-induced upregulation of Hi-FGF-2 accumulation: there was no significant differences between Hi-FGF-2 levels in control, untreated cells and those treated with Ang II in the presence of both inhibitors (Figure 2C). It should also be noted that both the control and treatment groups expressed similar levels of β-actin (serving as a loading control).

Effects of inhibiting ERK and MMP-2 on the Ang II-induced upregulation of Hi-FGF-2. The effect of Ang II on cell-associated Hi-FGF-2 accumulation in the presence (and absence) of inhibitors of the ERK pathway, or MMP-2 activity, was tested using human heart (atria)-derived fibroblasts. As expected, and as seen in Figure 3A, Ang II significantly increased cell-associated Hi-FGF-2 compared to the untreated groups. However, treating cells with the ERK activity inhibitor U0126 abolished the Ang II effect on Hi-FGF-2 up-regulation. In a similar manner, use of the MMP-2 inhibitor effectively eliminated the Ang II cell-associated Hi-FGF-2 up-regulation. Both types of inhibitors seemed to even down-regulate control Hi-FGF-2 levels, although this was only a trend and not statistically significant. All groups expressed similar levels of β-actin (loading control).

Exported FGF-2 that remains tightly bound to the cell surface can be eluted with a 2M salt wash of the cell monolayer. Using this approach we determined the effect of U0126 on the Ang II-induced up-regulation of exported, cell surface-bound Hi-FGF-2. Figure 3B shows a set

of pilot data: the Ang II-induced increase in cell surface-bound Hi-FGF-2 was reduced by the ERK inhibitor.

Effects of resveratrol on the Ang II-induced upregulation of Hi-FGF-2. This series of experiments was done using neonatal rat heart-derived, as well as human heart-derived myofibroblasts.

Neonatal rat heart derived fibroblasts. These cells have similar features as their adult counterparts regarding FGF-2 expression and regulation [8, 23]. As expected, Ang II significantly increased relative levels of cell-associated Hi-FGF-2 compared to untreated controls. Resveratrol was added to the cells one hour prior or one hour after Ang II addition (pre- and post- treatment, respectively). The Ang II induced Hi-FGF-2 upregulation was significantly reduced by resveratrol, in both pre-treated and post-treated cells (Figure 4A). In fact both pre- and post-treatment with resveratrol brought levels of cell-associated Hi-FGF-2 down to those of the non-Ang II-treated group. Both the control and treatment groups expressed similar levels of β-tubulin (loading control).

Human heart (atria)-derived fibroblasts. Pilot studies using human cells indicated that resveratrol (which needed to be dissolved in DMSO in a stock solution) reduced the Ang II-induced cell-associated Hi-FGF-2 up-regulation, Figure 4B. DMSO itself had no effect on Hi-FGF-2 levels. In a subsequent experiment the effect of resveratrol on the Ang II-induced cell surface-associated Hi-FGF-2 upregulation was investigated. Pilot data, shown in Figure 4C, indicate that resveratrol reduced cell-surface associated, exported Hi-FGF-2.

Discussion

Main and novel findings of this work are:

1. Human heart (atria)-derived myofibroblasts express Hi-FGF-2 as a predominant FGF-2 isoform.
2. Ang II up-regulates expression and release of Hi-FGF-2 by human heart myofibroblasts and the mechanism is mediated by both AT1 and AT2 receptors.
3. The ERK pathway, as well as MMP-2 activity, are mediating the Ang II-induced Hi-FGF-2 upregulation
4. Resveratrol prevents the Ang II-induced Hi-FGF-2 upregulation

Angiotensin II upregulates Hi-FGF2 in human cardiac myofibroblasts. Ang II has been linked to the development of pathological cardiac remodeling, including maladaptive cardiomyocyte hypertrophy and fibrosis. It exerts effect at the vascular level, such as vasoconstriction, and can also affect cardiac cells, myocytes and non-myocytes, directly, promoting fibrosis and hypertrophy [10]. Ang II is known to stimulate FGF-2 gene expression and protein upregulation [24], although, traditionally, not much attention has been paid as to the FGF-2 isoforms involved. Yet, it is only the Hi-FGF-2 isoform (rodent or human) that can induce cardiac hypertrophy in experimental models [5]. Data presented here demonstrate, for the first time, that Ang II does indeed upregulate Hi-FGF-2 in human heart myofibroblasts, implying that Hi-FGF-2 may mediate at least some of the deleterious effects of Ang II in the human heart.

Previous studies [9] and data shown here, have documented that in rat models Ang II clearly upregulates Hi-FGF-2 in both neonatal and adult heart (ventricle)- derived cardiac myofibroblasts. In addition, rat atria-derived myofibroblasts displayed similar Hi-FGF-2 regulation as their ventricular counterparts (unpublished observations). It is reasonable to conclude that the ability of Ang II to upregulate cardiac myofibroblast Hi-FGF-2 is not species-specific, stage-specific, or cardiac compartment- specific. This would imply that the mechanism mediating Hi FGF-2 upregulation is likely to be similar between rat-heart and human heart derived myofibroblasts.

Blockade of both AT1 and AT2 are required to fully prevent Ang II-induced Hi-FGF-2 upregulation in human heart (atria)-derived myofibroblasts.

Treatments currently available for the pharmacological management and prevention of heart failure function by blocking the renin-angiotensin-aldosterone system. This blockade is achieved by a variety of mechanisms, and includes targeting Ang II converting enzyme inhibitors (ACE inhibitors), direct renin inhibitors, and Ang II plasma membrane receptor blockers (ARBs). All ARBs that are currently available function by preventing binding of Ang II to its AT1 receptor, and may even function to activate the AT2 receptor [10]. Our data showed that blockade of AT1 through Losartan in human cells did indeed attenuate the Ang II-induced upregulation, as it did in rat heart fibroblasts. Interestingly, however, the AT2 receptor inhibitor PD123319 was also effective in decreasing the Ang II effect in human cells, and in fact, blockade of both AT1 and AT2 receptors was required to return Hi-FGF-2 to normal, pre-Ang II stimulation, levels. In the rat model, AT2 inhibition did show a trend towards decreasing the effect of Ang II on Hi-FGF-2 levels, although data did not reach significance. At this point, therefore, it would be prudent to repeat the rat myofibroblast work with a higher sample number, and by also employing combined used of AT1 and AT2 blockers to ascertain whether AT1 alone or both receptors are involved in Hi FGF-2 upregulation in the rat (thus no definite conclusion can be drawn).

While AT1 has clearly been shown to mediate the deleterious effects of Ang II, and certainly Hi FGF-2 upregulation could be considered as such, AT2 is thought to exert overall beneficial effects [11, 12]. This would contrast with its ability for Hi FGF-2 up-regulation documented here, and needs to be investigated further. Additional AT2 inhibitors may need to be tested in case PD123319 has non-specific effects. Selective depletion of AT1 and/or AT2 receptors via silencing RNA technology may also help to address this issue. At any rate, a requirement for both AT1 and AT2 receptor blockade to reverse the Ang II-induced Hi FGF-2 upregulation has implications at the clinical setting. It is possible that blocking AT2, or both AT1 and AT1, at least for a time, may prove beneficial to some patients.

The Ang II-induced up-regulation of Hi-FGF-2 is dependent on ERK and MMP-2 activation. The ERK pathway is well known to be required for the activation of FGF-2 gene and protein expression by many triggers, including FGF-2 itself, in an auto-stimulation loop [24]. Thus the ability of the potent ERK pathway inhibitor U0126 to eliminate the Ang II-induced Hi-FGF-2 upregulation is in general agreement with previous work, although previous studies have not differentiated between FGF-2 isoforms, and have not examined human heart fibroblasts. In addition our data showed that the ERK pathway was also required to achieve increases in

exported, cell-associated Hi-FGF-2, which can then go on to exert paracrine/autocrine activities. This is likely to reflect the overall increase in Hi-FGF-2 gene and protein expression but we cannot exclude the possibility that the ERK pathway also contributes specifically to increased Hi-FGF-2 export, or that it prevents Hi-FGF-2 turnover (degradation). These topics require further investigation.

MMP-2 activity was also found to be required for the Ang II induced Hi-FGF-2 upregulation by human cardiac myofibroblasts. The Ang II/AT1 pathway is known to enhance MMP-2 activity [25]. The increased MMP-2 activity allows release of another heparin-bound growth factor (the heparin-bound epidermal growth factor) from the cell surface to the environment, enabling it to then bind to and activate its cognate cell surface receptors [13]. It is therefore plausible that MMP-2 may also act to liberate human myofibroblast cell-surface-bound Hi-FGF-2 which would then be available to activate its own FGFR receptors in an autocrine manner. This would contribute to further ERK activation, and further stimulation of FGF-2 expression. Thus we suggest that a potential mode of action of MMP-2 is to allow FGF-2 auto-induction. The ability of MMP-2 to 'liberate' FGF-2 from extracellular matrix (lens capsule) which can then bind to FGFR of endothelial cells and promote their survival has been established [26].

Resveratrol prevents the Ang II-induced Hi FGF-2 upregulation. There is increasing evidence that the natural polyphenol resveratrol exhibits multiple beneficial cardiovascular effects, acting as an anti-oxidant, reducing inflammation, hyper-coagulative state, dyslipidemia, obesity, hypertriglycemia, hyperinsulinemia, ischemia and reperfusion injury, sympathetic tone, and endothelial dysfunction [16]. Resveratrol has additionally been shown to reduce cardiac hypertrophy [17, 20]. Resveratrol has anti-aging properties that may contribute to prevention of heart failure. Previous studies have focused primarily on the direct anti-hypertrophic effect of resveratrol on cardiomyocytes. Data presented in this report indicated that resveratrol may exert some of its beneficial effects in the heart by preventing the Ang II-induced Hi-FGF-2 up-regulation in myofibroblasts. Resveratrol diminished Hi-FGF-2 levels in both rat heart-derived and human-heart-derived cells; it also reduced relative levels of exported, cell-associated Hi-FGF-2, suggesting that it can decrease auto- and paracrine-activities by Hi- FGF-2.

Resveratrol has been reported to inhibit both ERK and MMP-2 activities in fibrosarcoma cells [27], and thus it may also do so in cardiac myofibroblasts; this would explain its ability to prevent the Ang II-induced Hi-FGF-2 upregulation. It is of interest that resveratrol was able to reduce Ang II-induced Hi-FGF-2 upregulation irrespectively of whether it was added before or after Ang II stimulation. In the post-treatment scenario, Ang II has had enough time (one hour) to initiate early downstream signal transduction, but this evidently is not sufficient to elicit Hi FGF-2 upregulation, as it is was still prevented by resveratrol. It is possible that a sustained activation of late signals downstream of Ang II are needed for Hi FGF-2 protein upregulation and this is prevented by resveratrol.

Since resveratrol appears to successfully inhibit the Ang II up-regulation of both cell associated and exported Hi-FGF2, it follows that this molecule (or others like it) may be considered as a potential therapy aimed at reducing Hi-FGF-2 levels and consequently preventing or reducing cardiac hypertrophy. Furthermore, resveratrol may be able to successfully

reduce Hi-FGF-2 levels if given either as a preventative therapy, or as a treatment in a background of prolonged Ang II elevation and the accompanying cardiac pathologies. Nevertheless, and despite it multiple beneficial effects in experimental models, use of resveratrol has not yet reached the clinical stage, perhaps because of its short half-life causing it to be metabolized fast by the body [16]. It may be that resveratrol analogs with improved bioavailability may need to be developed for effective clinical applications.

In conclusion. Taken together this work points to a potential signal transduction pathway resulting in Hi-FGF-2 up-regulation by human cardiac myofibroblasts. In this pathway, Ang II activates its receptors (AT1, AT2), followed by downstream activation of the ERK pathway and MMP-2 activity. The ERK pathway stimulates FGF-2 gene expression and increased protein accumulation and secretion; MMP-2 contributes to the 'liberation' of extracellular, cell-bound Hi-FGF-2, that can act in a positive feed-back loop to activate its own gene expression (via ERK). Secreted Hi FGF-2 can also activate other genes associated with a pro-fibrotic response (unpublished observations). Resveratrol, likely by blocking ERK and MMP-2 activation, prevents Hi-FGF-2 up-regulation.

Hi-FGF-2 has been shown to stimulate maladaptive cardiac hypertrophy, a major risk factor in the development of heart failure. It is very likely that Ang II, which is elevated during chronic heart disease, exerts some of its deleterious effect via cardiac myofibroblast Hi-FGF-2 up-regulation. By successfully blocking Hi FGF-2 up-regulation by myofibroblasts one might prevent Ang II- associated maladaptive cardiac changes. Although some available therapies are currently aimed at blocking this pathway (AT1 receptor blockers described earlier), the burden of heart disease remains high, and a subset of patients do not respond to the current therapies. There is a need for enhanced or novel treatment strategies. Based on the results of this study, both the AT1 and AT2 receptors, as well as the ERK and MMP-2 pathways, are potential targets to reduce Hi-FGF-2 levels. Furthermore, resveratrol, or analog compounds if they become available, may interfere with the Ang II up-regulation of Hi-FGF-2.

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Student's signature

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Figure 1: Adult rat heart (ventricle)- derived myofibroblasts express Hi-FGF-2 which is upregulated by Ang II via the AT1 receptor. Shown below is representative western blots and corresponding cumulative data showing relative Hi-FGF-2 levels(cell-associated) in control and Ang II-treated cells (± Losartan, ± PD123319). Brackets indicate comparisons between groups, where ***, **, * correspond to P<0.001, 0.01, 0.05, respectively (n=3).

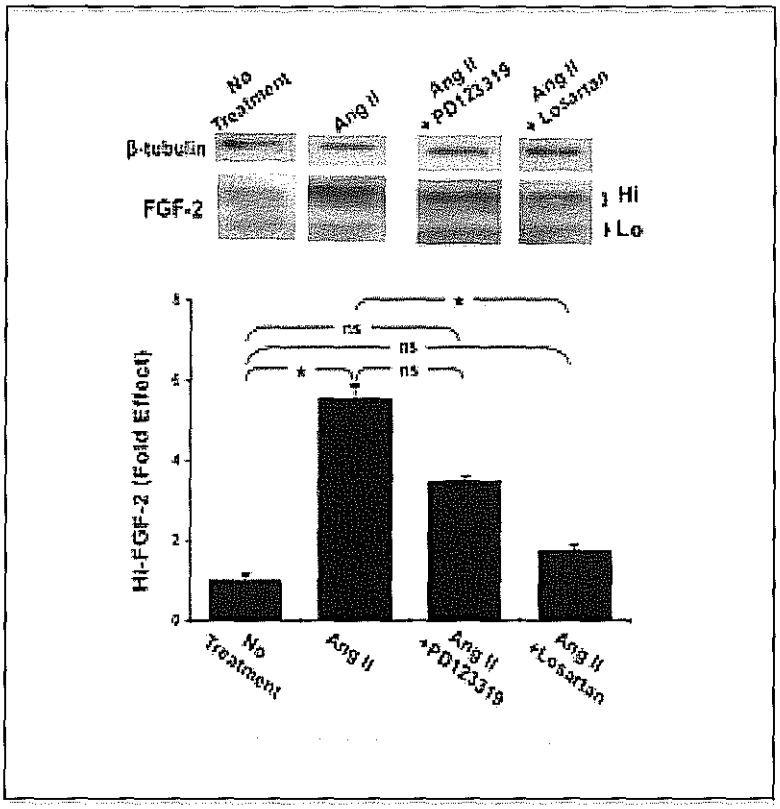

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Figure 2: Human heart (atria)- derived myofibroblasts express Hi-FGF-2 which is upregulated by Ang II via both AT1 and AT2 receptors. (A) Western blot showing that cells derived from human atrial tissue (n=3) express myofibroblasts markers such as EDA-fibronectin, smooth muscle embryonic (Smemb) mysin, procollagen, vimentin and alpha-smooth muscle actin (a-SMA). They do not express striated muscle myosin, or desmin, represening cardiac myocyte markers. Data courtesy of Jon Jon Santiago (Supervisor's Laboratory). (B) Representative western blots and corresponding cumulative data showing relative Hi-FGF-2 levels(cell-associated) in control and Ang II-treated cells (± Losartan, ± PD123319). (C) Representative western blots and corresponding cumulative data showing relative Hi-FGF-2 levels in control and Ang II-treated cells (± combined ARBs), as indicated. Brackets indicate comparisons between groups, where ***, **, * correspond tp P<0.001, 0.01, 0.05, respectively (n=3).

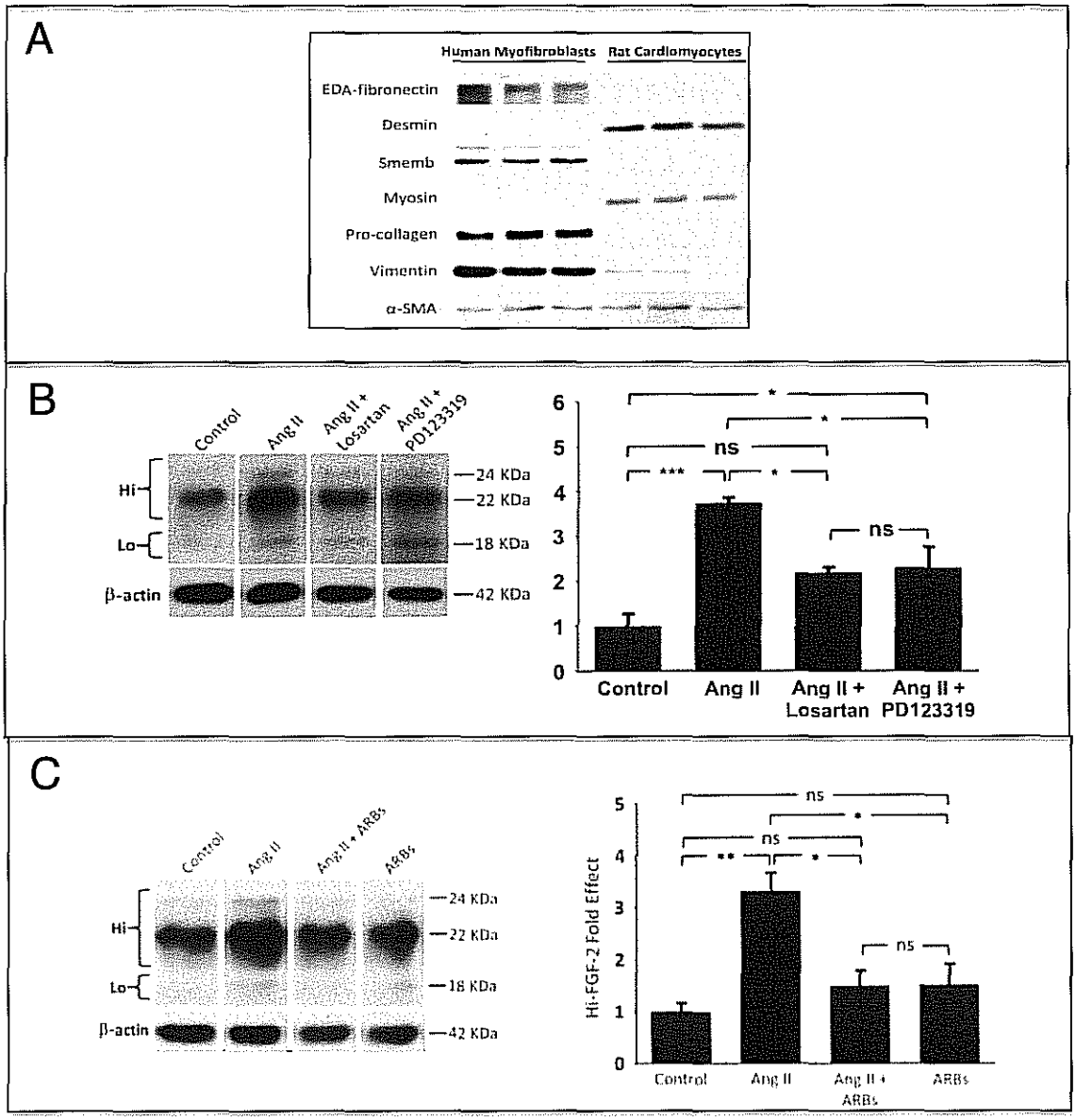

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Figure 3: The Ang II-induced upregulation of Hi-FGF-2 in human heart (atria)-derived myofibroblasts is reduced by inhibitors of the ERK kinase pathway and of MMP-2 activity. (A) Representative western blots and corresponding cumulative data showing relative Hi-FGF-2 levels(cell-associated) in control and Ang II-treated cells (± the ERK inhibitor U0126, ± the MMP-2 activity inhibitor MMP-2-i). (B) Representative western blots showing exported, cell-surface bound Hi-FGF-2 in control and Ang II-treated cell (± U0126). Brackets indicate comparisons between groups, where ***, **, * correspond to P<0.001, 0.01, 0.05, respectively (n=3).

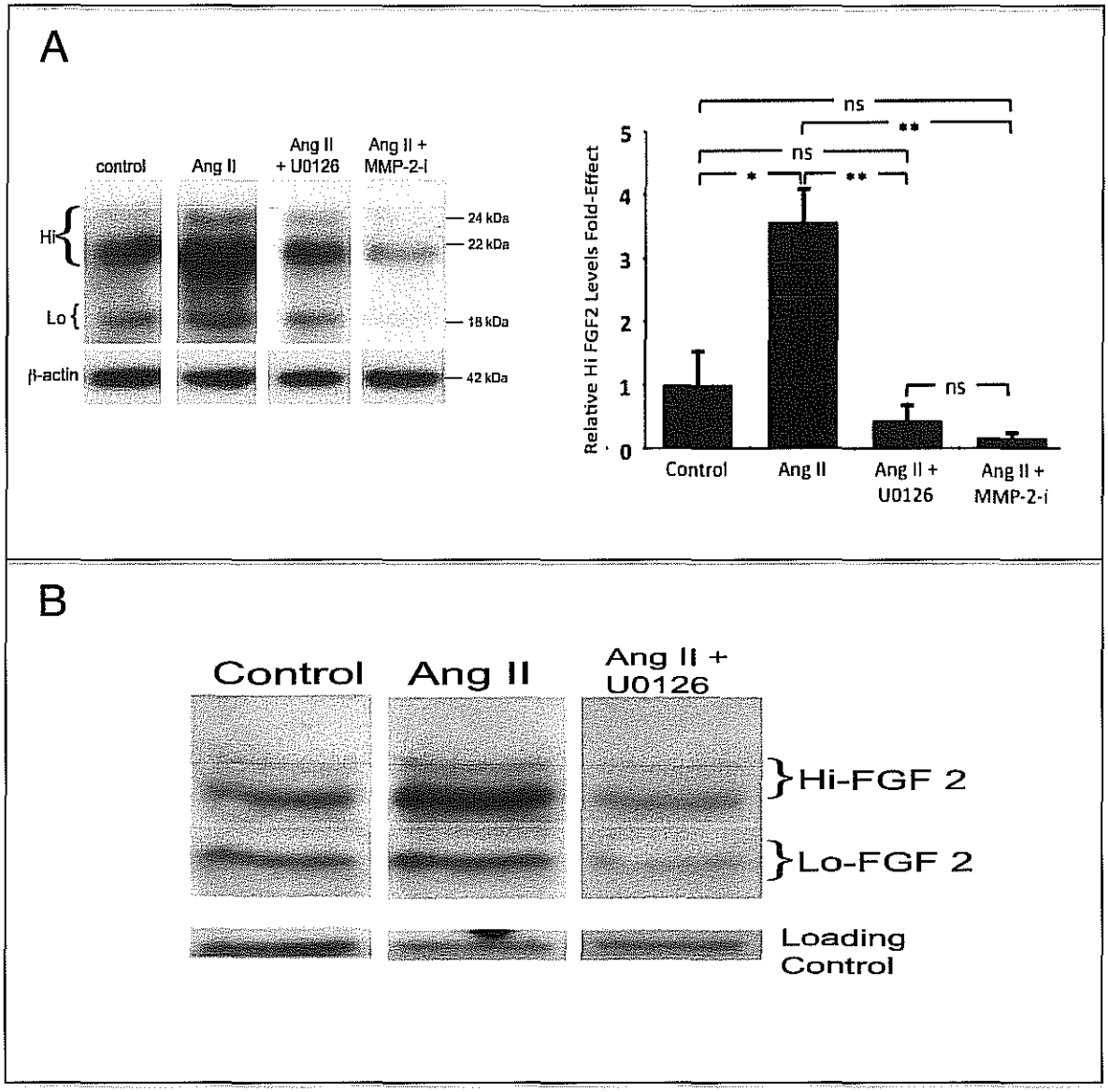

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Figure 4: The Ang II-induced up-regulation of Hi-FGF-2 in neonatal rat and human heart (atria)- derived myofibroblasts is attenuated by resveratrol . (A) Representative western blots and corresponding cumulative data showing relative Hi-FGF-2 levels(cell-associated) in control and Ang II-treated cells (± resveratrol pre-treatment, ± resveratrol post-treatment) using neonatal rat myofibroblasts. (B) Representative western blots showing relative Hi-FGF-2 levels (cell associated) in control and Ang II-treated cells (± resveratrol) using human atrial-derived myofibroblasts. (C) Representative western blots showing relative Hi-FGF-2 levels (exported and cell-surface bound) in control and Ang II-treated human cells (± resveratrol). Brackets indicate comparisons between groups, where ***, **, * correspond to P<0.001, 0.01, 0.05, respectively (n=3).

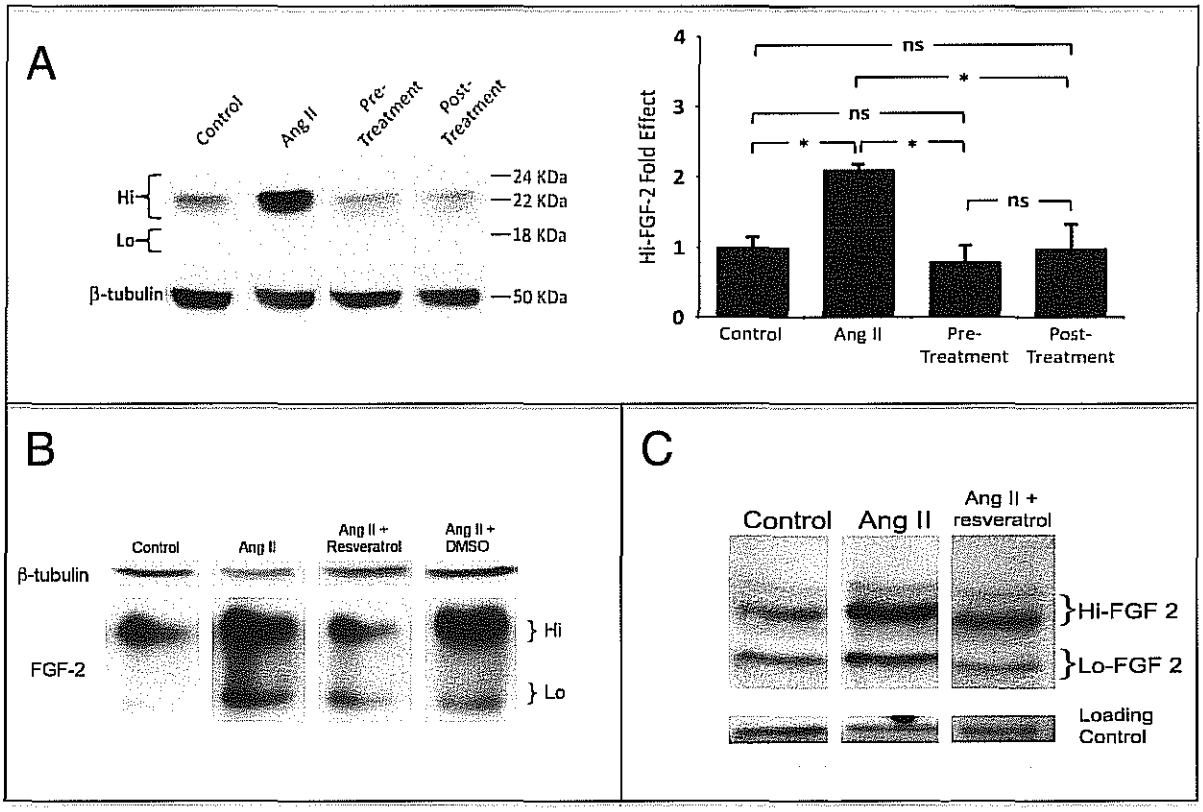

Figure 5: Potential translational applications of this work.