**“Regulation of the cardiac fibroblast cell cycle by p44/42 MAPK”**
**5/4/1-7/10-NCD-II**

## PROJECT COMPLETION REPORT

1. Project title **Regulation of the cardiac fibroblast cell cycle by p44/42 MAPK**

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3. Institution: Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum

4. Date of start: 25th March 2011 Duration: 3 years

5. Grant sanctioned

| Year | Grant received | Actual expenditure incurred |
|---|---|---|
| **2011** | **Rs. 18,89,355/-** | **Rs.18,23,384/-** |

Balance of Rs. 65,971/- is being returned to the Council

**6. Objectives of the proposal**

Cardiac fibroblasts play an important role in wound healing following myocardial injury, and in the pathophysiology of myocardial remodeling induced by hypertension, myocardial infarction, and reperfusion injury after ischemia. Central to the involvement of these cells in multiple aspects of cardiac function in the context of myocardial injury is the fact that, unlike cardiac myocytes, they retain their replicative capacity throughout adult life. Surprisingly, however, there is very little information on the kinetics of the cardiac fibroblast cell cycle and the mechanisms that regulate cell cycle progression in these cells. Since p44/42 MAPK is known to be an important modulator of cell proliferation in other cell types, this study focuses on the role of p44/42 MAPK in the regulation of the cardiac fibroblast cell cycle and the less-known mechanisms that link p44/42 MAPK with key components of the cell cycle machinery such as the cyclins, p21CIP1, p27KIP1, Skp2 and FOXO3a.

**The specific objectives included:**

a) Identification of the cell cycle checkpoint(s) regulated by p44/42 MAPK.
b) Evaluation, in p44/42 MAPK-inhibited cardiac fibroblasts, of the expression profile of cell cycle regulatory proteins involved in $G_1$-S transition, such as:
   - cyclins A, D1 and E,
   - the phosphorylation status of the retinoblastoma protein.

c) Delineation of the mechanisms that link p44/42 MAPK, FOXO3a, p21 and p27 in the control of G1-S transition in these cells.

i. To examine whether p44/42 MAPK activation in serum-stimulated cells

- negatively correlates with the expression of p21 and p27
- negatively correlates with FOXO3a levels and its nuclear translocation
- positively correlates with Skp2 levels

ii. Additionally, to uncover potential interaction of p44/42 MAPK with p21, FOXO3a and skp2, expression levels of p21, FOXO3a and Skp2, and nuclear translocation of FOXO3a, would be examined in serum-stimulated, p44/42 MAPK-inhibited cells.

d) Does the increase in p21 expression in p44/42 MAPK-inhibited cells involve its transcriptional up-regulation mediated by FOXO3a and/ or post-transcriptional control involving Skp2?

i. To determine whether FOXO3a is involved in p21 gene regulation,

- Electrophoretic Mobility Shift Assay will be performed to examine nuclear translocation of FOXO3a.
- p21 mRNA levels will be determined in FOXO3a-inhibited, serum-stimulated cells, and
- p21 protein levels will be determined in FOXO3a-inhibited, serum-stimulated cells.

ii. Does Skp2 post-translationally regulate FOXO3a, p21 and p27 levels? Post-translational regulation of FOXO3a, p21 and p27 levels by Skp2 will be determined by analysis of FOXO3a, p21 and p27 levels in Skp2-inhibited, serum-stimulated cells.

e) Probing the possible cross-talk between p44/42 MAPK and other kinases such as PI3K and p38 MAPK that have been implicated in cell cycle regulation in other cell types.

7. **Details of work done**

**Experimental protocols**

**Isolation of cardiac fibroblasts**: The heart tissue from young adult male Sprague Dawley rats is minced into bits and subjected to a series of 10 digestions in dissociation medium containing collagenase type I A, trypsin and pancreatin. Cells are pooled, re-suspended in M199 containing 10% FBS, seeded into two 35 mm cell culture dishes and incubated in a humidified $CO_2$ incubator at $37^0$C in an atmosphere of 95% air and 5% $CO_2$ for 150 minutes. At the end of this period, the supernatant containing unattached cells and debris is discarded, the dishes with the adherent fibroblasts are rinsed with M199 and supplied fresh M199 with 10% FBS.

**Measurement of DNA synthesis**: DNA synthesis was measured in synchronous, sub-confluent cultures of cardiac fibroblasts at passage 2 in terms of [$^3$H]-thymidine incorporation into acid-insoluble fraction.

**Western blot analysis:** Western blot analysis of cell cycle regulatory protein expression (Skp2, p27, p21, Phosho-FOXO3a, Total FOXO3a, and β-actin as loading control) was performed by standard protocols using specific antibodies.

**Skp2, FOXO3a and p42 MAPK knockdown by RNA interference:** Cells from Passage 3 were sub-cultured into 12-well plates at a density of $8x10^4$ cells/ well and incubated for 24 h in a $CO_2$ incubator. After 24 hours, the cells were washed with serum- and antibiotic-free M199 and incubated for ~45 minutes in the transfection medium (*900µl* OPTI-MEM-I */ well).* The siRNA cocktail was then added to each well and incubated for 20 hours in a $CO_2$ incubator. *siRNA cocktail = 98 µl of siRNA (5 pmol of Skp2 or FOXO3a or p42MAPK siRNA from Ambion, USA) + 2µl Lipofectamine (Invitrogen, USA).* After transfection, the cells were put back into M199 with 10% FBS for stabilization for 12 h. At the end of 12 h, proteins were extracted for western blot analysis.

**Real Time PCR**: Sub-confluent cultures of cardiac fibroblasts in M199+10%serum were incubated with/without PD98059 for 6 h and total RNA was isolated using Tri reagent. Following DNase I treatment, 2 µg of total RNA was reverse transcribed to cDNA with random hexamer primers and M-MLV reverse transcriptase. TaqMan quantitative real-time polymerase chain reaction (RT-PCR) analysis was carried out using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, CA) with specific, FAM & VIC-labelled probes. The oligo nucleotide primers and probes for p27 and 18S ribosomal RNA were from Applied Biosystems. PCR reactions were performed, as per the manufacturer's instructions, over a total of 40 cycles. p27 expression was normalized to 18S rRNA.

**Electrophoretic mobility shift assay (EMSA):** EMSA was performed using the LightShift Chemiluminescent EMSA Kit (ThermoScientific, USA). Nuclear extracts were prepared from sub-confluent cardiac fibroblast cultures in M199+10% serum with or without PD98059 for 30 minutes using the ThermoScientific NEPER nuclear extraction kit. Primers for FOXO3a were biotinylated using ThermoScientific 3'-end biotin labeling kit. The nuclear extracts were incubated with the biotinylated probes and components of the LightShift Chemiluminescent kit at 37ºC for 60 minutes, electrophoresed on a 6% non-denaturing gel, transferred to nylon membrane and immobilized by UV cross-linking at 254 nm for 10 minutes. The bands were visualized by enhanced chemiluminescence using streptavidin-conjugated HRP. Specificity of binding was confirmed by competition with excess unlabelled FOXO3a oligonucleotides (200-fold).

**Observations**

a) Western blot analysis showed a significant increase in the levels of the phosphorylated form of p44/42 MAPK following 3 and 6 hours of mitogenic stimulation, pointing to its activation.

b) DNA synthesis, measured in terms of [3H]-thymidine incorporation into acid-insoluble material, was reduced significantly upon MAPK inhibition using PD98059. Further, in PD-treated cells, population-doubling time was strikingly higher, showing that p44/42 MAPK is essential for cardiac fibroblast proliferation.

c) Confirmatory evidence that p44/42 MAPK acts at G1-S was obtained from [3H]-thymidine incorporation kinetics and flow cytometric analysis of MAPK-inhibited cells, which showed

an accumulation of cells at the G0/G1 phase with a corresponding reduction in the number of cells in the S phase.

d) i) A significant decrease in cyclin D1 was observed in serum-stimulated, p44/42 MAPK-inhibited cells.

   ii) A significant decrease in cyclin A was also observed in serum-stimulated, p44/42 MAPK-inhibited cells.

   iii) Cyclin E, however, remained unaffected upon MAPK inhibition, pointing to differential regulation of the cyclins.

e) We found significant hypophosphorylation of Rb in MAPK-inhibited cells, consistent with impaired G1-S progression.

f) Hypophosphorylation of Rb is brought about by the cyclin-dependent kinase inhibitors (CDKIs), mainly p21 and p27. We observed striking induction of p21 and p27 in MAPK-inhibited cells, which would cause G1-S block.

g) p44/42 MAPK inhibition in mitogen-stimulated cells was found to inhibit Skp2 expression, suggesting that p44/42 MAPK is a positive regulator of Skp2. Inhibition of Skp2 in MAPK-inhibited cells would represent a post-translational mechanism of regulation of CDKIs since Skp2 plays a critical role in the cell cycle by facilitating CDKI degradation.

Subsequently, we focused on the mechanisms underlying the regulation of the cyclin-dependent kinase inhibitors, p27 and p21, by p44/42 MAPK in cardiac fibroblasts.

**Molecular basis of p44/42 MAPK-dependent G1-S transition in cardiac fibroblasts**

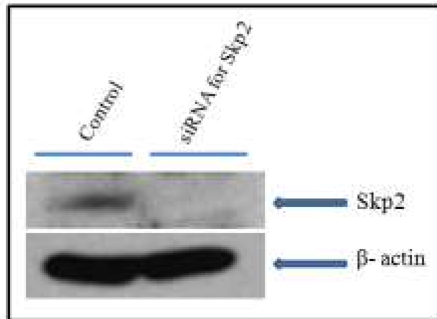
We had proposed to examine the transcriptional and/or post-translational regulation of CDKIs by p44/42 MAPK. As proposed, our approach consisted in uncovering the relationships between p44/42 MAPK, Skp2 (*involved in the post-translation regulation of CDKis*) and FOXO3a *(that regulates CDKIs transcriptionally*) under conditions in which one of these is inhibited to ascertain its role in CDKI expression. *In this regard, a major achievement has been the standardization of the protocol for gene knockdown by RNA interference.* Thanks to the support from the Council for this study, we now have an extremely useful and state-of-the art technique that is important not only in addressing the questions covered under this project but also in cracking several others in the times to come.

**Post-translational regulation of CDKIs by Skp2**

***Skp2 knockdown by RNA interference:*** Cells from Passage 3 were sub-cultured into 12-well plates at a density of $8x10^4$ cells/ well and incubated for 24 h in a $CO_2$ incubator. After 24 hours, the cells were washed with serum- and antibiotic-free M199 and incubated for ~45 minutes in the transfection medium (*900µl* OPTI-MEM-I */ well).*The siRNA cocktail was then added to each well and incubated for 20 hours in a $CO_2$ incubator. *siRNA*

*cocktail = 98 µl of siRNA (5 pmol of control or Skp2 siRNA) + 2µl Lipofectamine.* After transfection, the cells were put back into M199 with 10% FBS for stabilization for 12 h. At the end of 12 h, proteins were extracted for western blot analysis.

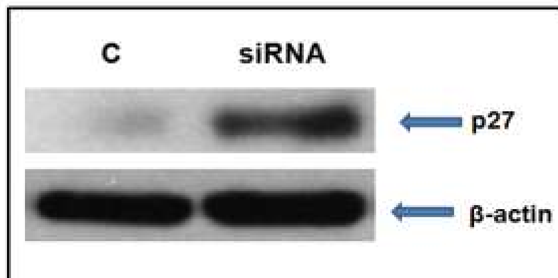
**Validation - Knockdown of Skp2 by RNA interference is evident in the picture below**



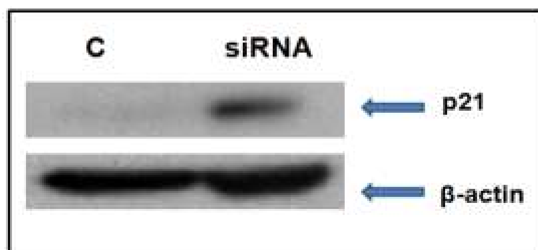

<u>Control</u>: Cells in M199+10% serum
<u>siRNA for Skp2</u>: Cells in M199+10% serum+si RNA for Skp2. Total Skp2 knockdown is shown here.

Representative picture from one of three experiments.

**<u>Skp2 knockdown by RNA interference induces p27 & p21:</u>** As shown in the earlier experiments, exposure of cardiac fibroblasts to 10% serum results in p44/42 MAPK activation and Skp2 induction. To ascertain whether Skp2 has a negative regulatory effect on CDKIs under these conditions, which would facilitate G1-S transition, the expression of CDKIs was determined following Skp2 knockdown. Remarkably, Skp2 silencing in these cells resulted in the expression of both p27 and p21, as shown below.



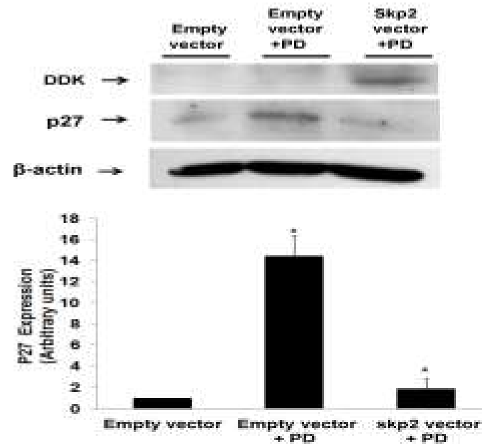

<u>C</u>: Cells in M199+10% serum that do not express p27.
<u>siRNA</u>: Cells in M199+10% serum+ siRNA for Skp2. p27 induction upon Skp2 knockdown is seen here.
Representative picture from one of three experiments that yielded similar results.




<u>C</u>: Cells in M199+10% serum that do not express p21.
<u>siRNA</u>: Cells in M199+10% serum+ siRNA for Skp2. p21 induction upon Skp2 knockdown is seen here.
Representative picture from one of three experiments that yielded similar results.

**Forced expression of Skp2 in ERK1/2-inhibited cells abrogates p27Kip1 protein expression.** As Skp2 knockdown restored p27Kip1 protein levels in serum-stimulated cells, we checked whether forced expression of Skp2 protein in ERK1/2-inhibited cardiac fibroblasts (that do not express Skp2) would attenuate p27Kip1 protein expression. Strong constitutive expression of Skp2 was achieved by transfecting ERK1/2-inhibited cells with Skp2 ORF clone in a ready-to-transfect pCMV-6 expression vector. Skp2 expression was confirmed using antibody against the COOH-terminal DDK Tag of the vector. Forced expression of Skp2 in these cells prevented the up-regulation of p27Kip1 protein), affirming thecentrality of Skp2 in p27Kip1 regulation by ERK1/2 in these cells.

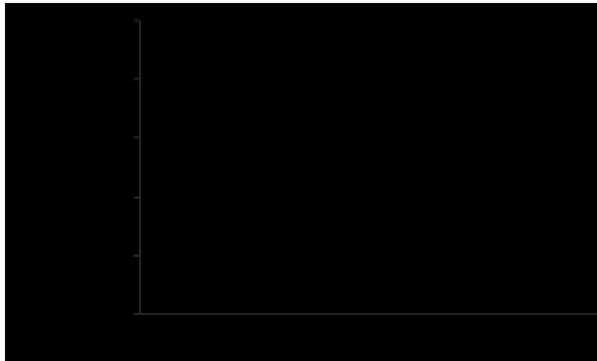
Figure 6: Forced expression of Skp2 in ERK-inhibited cells attenuated p27Kip1 protein expression




***Together, the findings show that p44/42 MAPK up-regulates Skp2 that in turn may inhibit p27 and p21 by promoting their <u>post-translational degradation</u> to facilitate cell cycle progression.***

**Additionally, does p44/42 MAPK regulate p27 transcription in cardiac fibroblasts?** We also checked if, in addition to post-translational regulation of CDKI via Skp2, p27 is subject to transcriptional regulation as well. Sub-confluent cultures of cardiac fibroblasts in M199+10%serum were incubated with/without PD98059 for 6 h and total RNA was isolated using Tri reagent according to the manufacturer's instructions. Following DNase I treatment, 2 µg of total RNA was reverse transcribed to cDNA with random hexamer primers and M-MLV reverse transcriptase. TaqMan quantitative real-time polymerase chain reaction (RT-PCR) analysis was carried out using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, CA) with specific, FAM-labelled probes. The

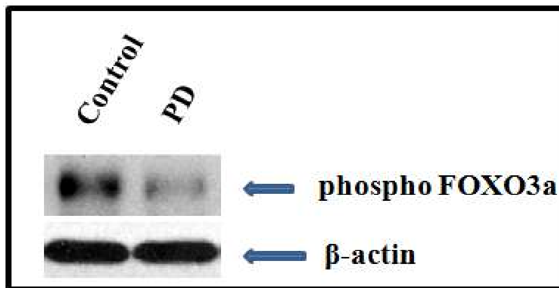
oligo nucleotide primers and probes for p27 and 18S ribosomal RNA were from Applied Biosystems. PCR reactions were performed, as per the manufacturer's instructions, over a total of 40 cycles. 27 expression was normalized to 18S rRNA. As shown below, inhibition of p44/42 MAPK using PD98059 significantly enhances transcription of p27**.**

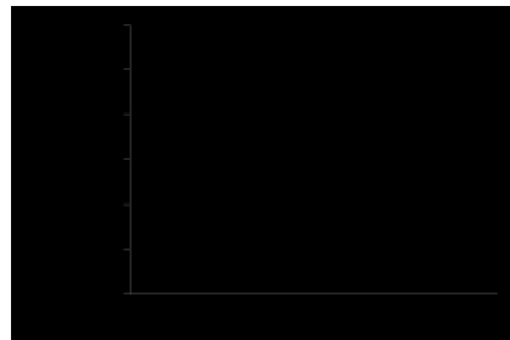


**Control: Cells+10% serum**
**PD-6hrs: Cells+10% serum+PD 98059 for 6 hours**

**A significant increase in p27 mRNA levels upon MAPK inhibition with PD98059**

**How does p44/42 MAPK transcriptionally regulate CDKIs In cardiac fibroblasts?**
Having shown that p27 is also transcriptionally regulated, we focused on FOXO3a, which is a major transcription factor for CDKI. The phosphorylation status of FOXO3a determines its activity level, with the phosphorylated form being retained in the cytosol and hence transcriptionally inactive. We examined whether p44/42 MAPK regulates FOXO3a in cardiac fibroblasts. Cells stimulated with 10% serum for 3 h in the presence (PD3 in the picture below) or absence (C3 in the picture below) of p44/42 MAPK inhibitor were compared for phospho FOXO3a levels. A significant reduction in the inactive phospho FOXO3a in MAPK-inhibited cells, shown below, provided preliminary evidence of its activation upon p44/42 MAPK inhibition.

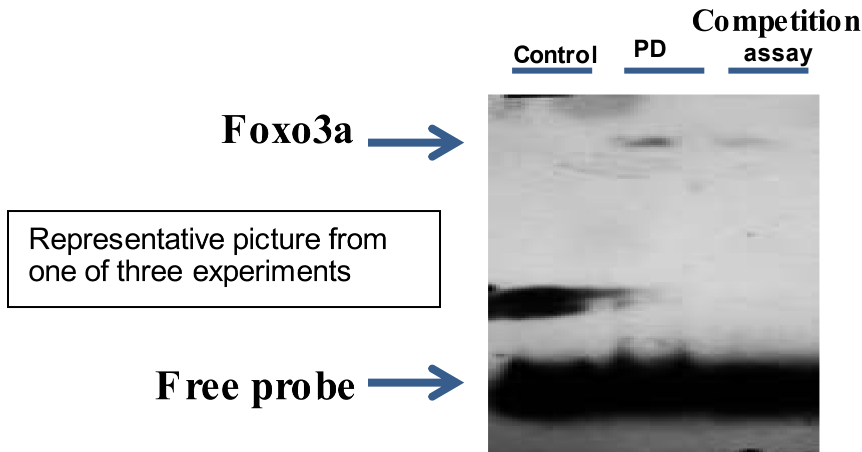


PD3 vs C3, $p < 0.01$, n=3

**Electrophoretic mobility shift assay (EMSA):** To confirm activation of FOXO3a, we performed EMSA, using the LightShift Chemiluminescent EMSA Kit. Nuclear extracts were prepared from sub-confluent cardiac fibroblast cultures in M199+10% serum with or without PD for using the NEPER nuclear extraction kit. Primers for FOXO3a were biotinylated using ThermoScientific 3'-end biotin labeling kit. The nuclear extracts were incubated with the biotinylated probes and components of the LightShift Chemiluminescent kit at 37ºC for 60 minutes, electrophoresed on a 6% non-denaturing gel, transferred to nylon membrane and immobilized by UV cross-linking at 254 nm for 10 minutes. The bands were visualized by enhanced chemiluminescence

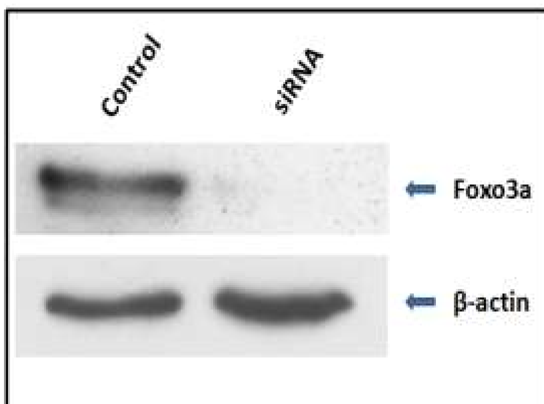
using streptavidin-conjugated HRP. Specificity of binding was confirmed by competition with excess unlabelled FOXO3a oligonucleotides (200-fold). Mobility shift, indicating nuclear translocation and DNA binding of FOXO3a, is evident in the following picture.




Representative picture from one of three experiments

**Activation of FOXO3a is coupled to regulation of p27.** We asked if activation of FOXO3a, as revealed by the experiments described above, is coupled to regulation of p27. To ascertain this, the effect of FOXO3a knockdown by RNA interference on PD 98059-induced p27 expression was evaluated. We had two groups of cells in M199+10% serum, both of which were treated with the p44/42 MAPK inhibitor (PD98059) to induce p27 expression but one had FOXO3a silenced. As shown below, PD-treated cells expressed p27, which was abolished in FOXO3a-silenced cells, showing that FOXO3a is an obligate requirement for p27 expression.

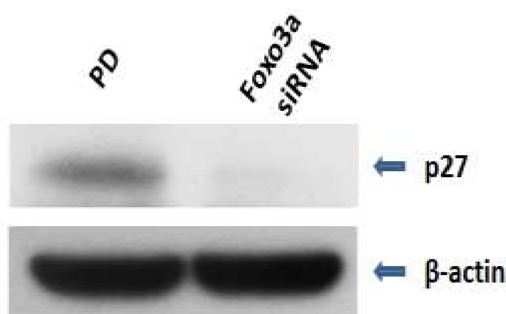
**Validation - Foxo3a expression is dramatically inhibited by siRNA**




**Control: Cells in M199+10% serum+MAPK inhibitor**
**siRNA: Cells in M199+10% serum+MAPK inhibitor+siRNA for FOXO3a. Total inhibition of FOXO3a is evident here.**

**The experiment was repeated three times. Results were comparable, with total inhibition of FOXO3a by siRNA.**

**FOXO3a knockdown inhibits p27 protein expression in PD-treated cells**

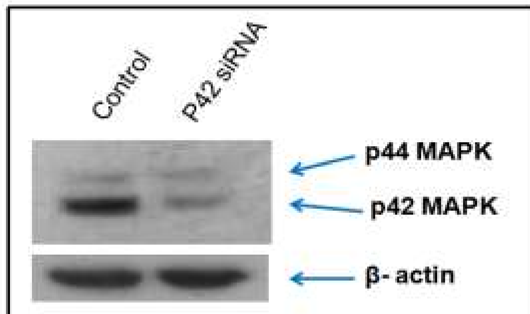



**PD: Cells in M199+10% serum+MAPK inhibitor PD98059**
**FOXO3a siRNA: Cells in M199+10% serum+PD98059+ siRNA for FOXO3a. Complete inhibition of p27 expression is evident here.**

**Representative picture from one of three experiments.**

**<u>RNA interference–mediated inhibition of p44/42 MAPK</u>:** As the regulatory role of p44/42 MAPK in G1-S transition in cardiac fibroblasts exposed to mitogenic stimulation is the focus of this study, we considered it important to reinforce our conclusions based on the use of the specific pharmacological inhibitor of p44/42 MAPK, PD98059, by repeating a couple of key experiments exploiting RNA interference-based p44/42 MAPK inhibition, although this was not proposed earlier. <u>The following pictures demonstrate: a) the efficacy of knockdown, and b) the effect of knockdown on DNA synthesis and p27 expression in mitogen-stimulated cardiac fibroblasts. The observations corroborate the observations with PD98050.</u>

**Validation - Expression profile of p44/42 MAPK after siRNA-mediated knockdown**



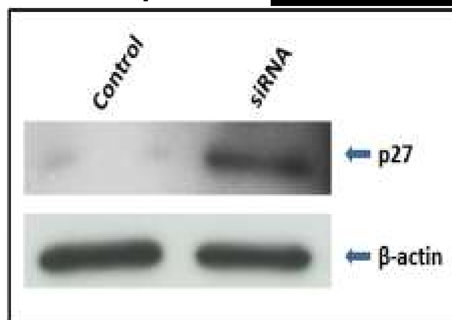

**<u>Control</u>: Cells+10%serum**
**<u>p42 siRNA</u>: Cells+10%serum+siRNA for p42 MAPK**

**The expression of <u>p42 MAPK protein</u> is significantly inhibited by siRNA treatment. Representative picture from one of 3 experiments that yielded similar results.**

**siRNA-mediated knockdown of p42 MAPK significantly decreases DNA synthesis**

p42 siRNA vs control, $p < 0.0001$, n=3

**p42 MAP** **pression**




**<u>Control</u>: Cells+10%serum**
**siRNA: Cells+10%serum+siRNA for p42 MAPK. Inhibition of p42 MAPK by siRNA induces p27.**

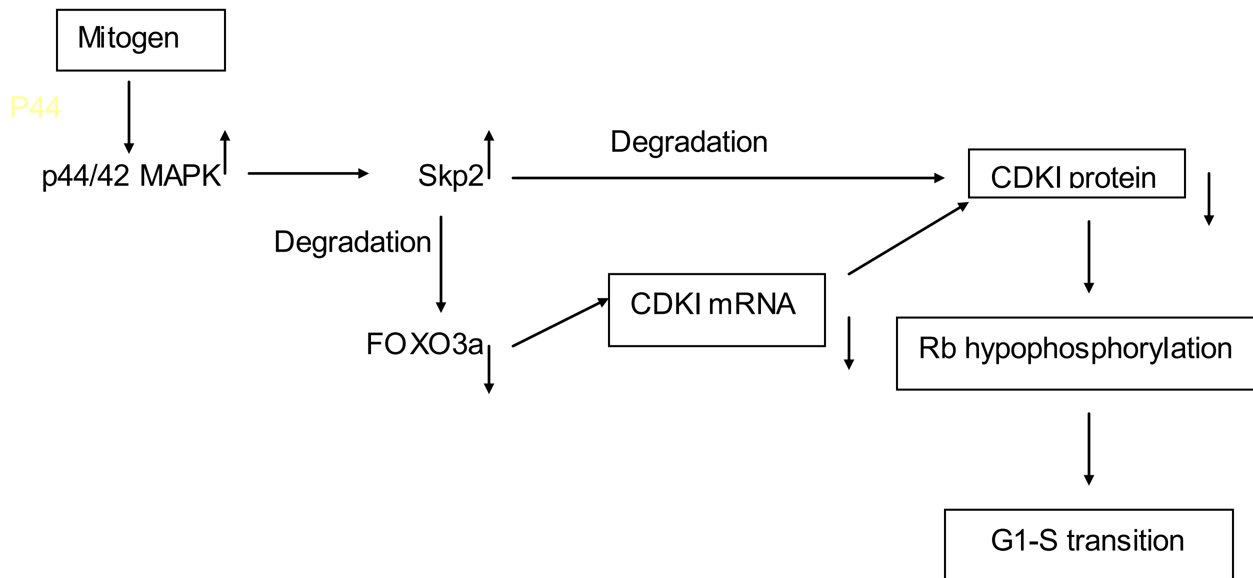
**Representative picture from one of three experiments that gave similar results.**

***Active ERK½ is a negative regulator of p21$^{Cip1}$ expression in mitogen-stimulated cardiac fibroblasts***

We also examined whether FOXO3a exerts dual control over p27$^{Kip1}$ and p21 $^{Cip1}$ that regulate G1-S transition but are differentially regulated in different cellular contexts. Western blot analysis showed that inhibition of ERK½ using PD98059 promotes induction of p21$^{Cip1}$ protein in serum-stimulated cells, indicating the regulatory role of ERK½ in p21 expression in cardiac fibroblasts. Real time PCR analysis demonstrated a 3-fold increase in p21$^{Cip1}$ mRNA in serum-stimulated, ERK-inhibited cells, suggesting transcriptional regulation of the p21$^{Cip1}$ gene by ERK½. Importantly, FOXO3a knockdown by RNA interference in ERK½-inhibited cells attenuated p21$^{Cip1}$ mRNA and protein expression, showing that FOXO3a transcriptionally regulates p21$^{Cip1}$ in these cells.

**Conclusions**

The study has been hugely and incredibly rewarding. The findings from this study indicate that the following sequence of events defines the transcriptional and post-translational mechanisms underlying cell cycle progression in cardiac fibroblasts. We present robust data for the first time that p44/42 MAPK and its downstream targets, Skp2 and FOXO3a, are critical players regulating G1-S transition in cardiac fibroblasts exposed to mitogenic stimulation. Taken together, the data show that FOXO3a is a master switch for G1-S transition in cardiac fibroblasts because of its dual control over p27$^{Kip1}$ and p21$^{Cip1}$. FOXO3a may be a potential target for therapeutic manipulation to control cardiac fibroblast hyperplasia and its sequelae in a setting of myocardial injury.





**Abstract**

Cardiac fibroblast hyperplasia associated with augmented matrix deposition profoundly impacts tissue remodeling in the diseased myocardium. However, mechanisms controlling cell cycle progression in cardiac fibroblasts remain unexplored. This study probed ERK1/2-mediated p27$^{Kip1}$ regulation in cardiac fibroblasts. Mitogenic stimulation of quiescent cultures of adult rat cardiac fibroblasts promoted ERK1/2 activation. Reduced DNA synthesis, increased population doubling time, flow cytometric analysis of

cell cycle progression, reduced levels of cyclins D and A, $p27^{Kip1}$ induction, and retinoblastoma protein hypophosphorylation in ERK1/2-inhibited cells indicated ERK1/2-dependence of G1-S transition in cardiac fibroblasts. ERK1/2-active cells expressed Skp2, which facilitates $p27^{Kip1}$ degradation. Skp2 knockdown by RNA interference induced $p27^{Kip1}$ expression in ERK1/2-active cells while its forced expression in ERK1/2-inhibited cells down-regulated $p27^{Kip1}$. Further, transcriptional up-regulation of $p27^{Kip1}$ mRNA in ERK1/2-inhibited cells correlated with activation of FOXO3a transcription factor whose knockdown attenuated $p27^{Kip1}$ mRNA and protein expression in ERK1/2-inhibited cells. Together, the study provides evidence for the first time that, in cardiac fibroblasts, activated ERK1/2 regulates $p27^{Kip1}$ expression transcriptionally and post-translationally via FOXO3a- and Skp2-dependent mechanisms. FOXO3a, which was also found to transcriptionally regulate $p21^{Cip1}$, may be a potential target for therapeutic manipulation to control cardiac fibroblast hyperplasia and its sequelae in a setting of myocardial injury.

8. **Any publications**:

a) S Pramod, K Shivakumar. Mechanisms in cardiac fibroblast growth: An obligate role for Skp2 and Foxo3a in ERK½ MAPK-dependent regulation of $p27^{Kip1}$.
**American Journal of Physiology - Heart and Circ Physiol 306** H844 (2014)

K SHIVAKUMAR
Signature

Date: 12th May 2014