Cell Research (2010) 20:919-934.

www.nature.com/cr

ORIGINAL ARTICLE

Proto-oncogenic H-Ras, K-Ras, and N-Ras are involved in muscle differentiation via phosphatidylinositol 3-kinase

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Oncogenic H-Ras G12V and its variants have been shown to inhibit muscle differentiation. However, the role of proto-oncogenic Ras (c-Ras) in muscle differentiation remains unclear. The active GTP-bound form of Ras has been known to associate with diverse effectors including Raf, phosphatidylinositol 3-kinase (PI3K), Ral-GDS, and other molecules to transmit downstream signals. We hypothesize that c-Ras may stimulate muscle differentiation by selectively activating PI3K, an important mediator for muscle differentiation. In our experiments, inhibition of c-Ras by farnesyltransferase inhibitors and a dominant negative form of H-Ras (Ras S17N) suppressed muscle differentiation. Consistently, individual knockdown of H-Ras, K-Ras, and N-Ras by siRNAs all blocked muscle differentiation. Interestingly, we found that c-Ras preferentially interacts with PI3K rather than its major binding partner c-Raf, during myogenic differentiation, with total c-Ras activity remaining unchanged. PI3K and its downstream myogenic pathway, the Nox2/NF-κB/inducible nitric oxide synthase (iNOS) pathway, were found to be suppressed by inhibition of c-Ras activity during differentiation. Furthermore, expression of a constitutively active form of PI3K completely rescued the differentiation block and reactivated the Nox2/NF-κB/iNOS pathway in c-Ras-inhibited cells. On the basis of our results, we conclude that contrary to oncogenic Ras, proto-oncogenic H-Ras, K-Ras, and N-Ras are directly involved in the promotion of muscle differentiation via PI3K and its downstream signaling pathways. In addition, PI3K pathway activation is associated with a concurrent suppression of the otherwise predominantly activated Raf/Mek/Erk pathway.

***Keywords*:** c-Ras; PI3K; muscle differentiation

Cell Research (2010) **20**:919-934. doi:10.1038/cr.2010.92; published online 6 July 2010

Introduction

Proto-oncogenic Ras protein (c-Ras) transduces signals from cell surface receptors into the cytoplasm via specific effector pathways and regulates diverse cellular processes including cell proliferation, differentiation, and apoptosis [1-4]. In mammals, four distinct Ras proteins including H-Ras, K-Ras4A, K-Ras4B, and N-Ras have been identified. These four Ras proteins are located on the inner surface of the plasma membrane and function as molecular switches. They are tightly regulated by guanine nucleotide exchange factors and GTPase-activating proteins (GAPs) [5-7]. Although they are believed to have similar biological functions, there have also been several reports that the four Ras proteins have unique biological functions and participate in different signaling cascades [8-10]. The main diversity among Ras isoforms originates from the 24 C-terminal amino acids, in which less than 15% of the residues are identical (hence this has been termed the hypervariable region) [6]. The hypervariable region also contains an essential signal for localizing Ras to the inner surface of the plasma mem-

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Received 13 October 2009; revised 1 February 2010; accepted 15 March 2010; published online 6 July 2010

brane. The CAAX box in this region experiences a post-translational modification making it more hydrophobic, which facilitates introduction of Ras proteins into their final locations [11]. Contrary to c-Ras, oncogenic Ras and its variants render the GTPase insensitive to GAP regulation, leading to constitutive activation in the GTP-bound form and resulting in activation of aberrant downstream signaling pathways [2].

Many investigators have reported the role of diverse signaling molecules in muscle differentiation including phosphatidylinositol 3-kinase (PI3K), Akt, Rac, $p70^{S6K}$, phospholipase C-β1 and phospholipase C-γ1, mammalian target of rapamycin and p38 MAPK [12]. Among them, PI3K seems to be a master molecule for muscle differentiation because it regulates all other molecules previously mentioned during muscle differentiation [13]. In addition, PI3K has been identified to activate NADPH oxidase, thus generating H_2O_2 and leading to NF-κB activation, followed by increased expression of inducible nitric oxide synthase (iNOS) and subsequent NO generation during the myogenic process [14, 15]. In contrast, the Raf/Mek/Erk pathway seems to suppress muscle differentiation [16].

Oncogenic H-Ras G12V and its variants have been shown to inhibit myogenic differentiation [17-19], prompting the idea that Ras is a negative regulator of muscle differentiation. However, it was recently shown that association of DA-Raf 1 with GTP-Ras led to decreases in Ras-Raf interaction, consequently reducing Raf/Mek/Erk activity during muscle differentiation [20]. Also, it is well known that active, GTP-bound Ras associates with a wide variety of effectors including Raf, PI3K, Ral-GDS, Rho GTPases, and other molecules to transmit downstream signals controlling distinct cellular events [2, 21]. These ideas prompted us to study the potential roles of c-Ras, instead of oncogenic Ras, in muscle differentiation using H9c2 and C2C12 cell lines. Here, we demonstrate that c-Ras stimulates muscle differentiation via PI3K and its downstream pathway. Also, we demonstrate that H-Ras and K-Ras are directly involved in muscle differentiation, while N-Ras contributes indirectly to muscle differentiation by preventing apoptosis.

Results

Proto-oncogenic c-Ras positively regulates muscle differentiation in H9c2 cells

Using H9c2 cells, we generated stable cell lines overexpressing a dominant negative form of H-Ras (dnRas) containing a serine (S) to asparagine (N) substitution at residue 17 (S17N). This substitution was shown to inhibit the ability of c-Ras to interact with PI3K, c-Raf, and all other downstream effectors. Expression levels of Ras were evaluated in mock transfectants (mock) and two stably transfected clones expressing the dnRas construct (R1 and R2) by immunoblotting. Compared with mock cells, c-Ras expression showed an almost twofold increase in R1 and R2 clones (Figure 1A). To examine the role of c-Ras in muscle differentiation, we induced differentiation by culturing cells in differentiation medium (DM). After 6 days of incubation in DM, we observed morphological transformation (Figure 1B) and changes in the expression levels of muscle-specific markers such as myogenin and myosin heavy chain (MHC) via immunoblotting (Figure 1C). As shown, mock cells cultured in DM showed distinct myotube formation and increased expression of differentiation markers, compared with cells grown in proliferation medium (PM). However, R1 and R2 clones remained undifferentiated in DM. Mock cells treated with two farnesyltransferase inhibitors, $LB42907{\cdot}H_2SO_4$ (LB) and farnesylthiosalicylic acid (FTS), in DM also did not show any signs of muscle differentiation (Figure 1B and 1C). To further verify the results, activity of the muscle creatine kinase (MCK) promoter was monitored by luciferase assay using the MCK luciferase reporter plasmid, MCK-Luc. Inhibition of c-Ras by dnRas and farnesyltransferase inhibitors reduced MCK promoter activity when measured 3 days after induction of differentiation by DM (Figure 1D), further supporting a role for c-Ras in myogenic differentiation.

H-Ras, K-Ras, and N-Ras are essential for muscle differentiation

Three distinct Ras isoforms (H-Ras, K-Ras, and N-Ras) have been identified in mammals. To determine which isoforms are involved in muscle differentiation, we suppressed their expression levels by transfecting isoform-specific siRNAs at 100 nM concentrations. All isoform-specific siRNAs effectively suppressed their respective target genes without affecting expression of other isoforms (Figure 2A). Furthermore, isoform-specific siRNAs completely blocked muscle differentiation (Figure 2B-2D). To demonstrate which form of Ras functions as the major inducer of PI3K for controlling muscle differentiation, we investigated the phosphorylation levels of Akt and Erk. As shown in Figure 2C, we observed that knockdown of each isoform decreases phosphorylation of Akt compared to control. However, levels of Akt phosphorylation were still maintained above levels of cells grown in PM. Contrarily, knockdown of each Ras isoform did not affect phosphorylation of Erk, which was decreased in DM (Figure 2C). Interestingly, siRNAs specific to H-Ras and K-Ras mainly blocked muscle differentiation without affecting cell survival, while siRNA

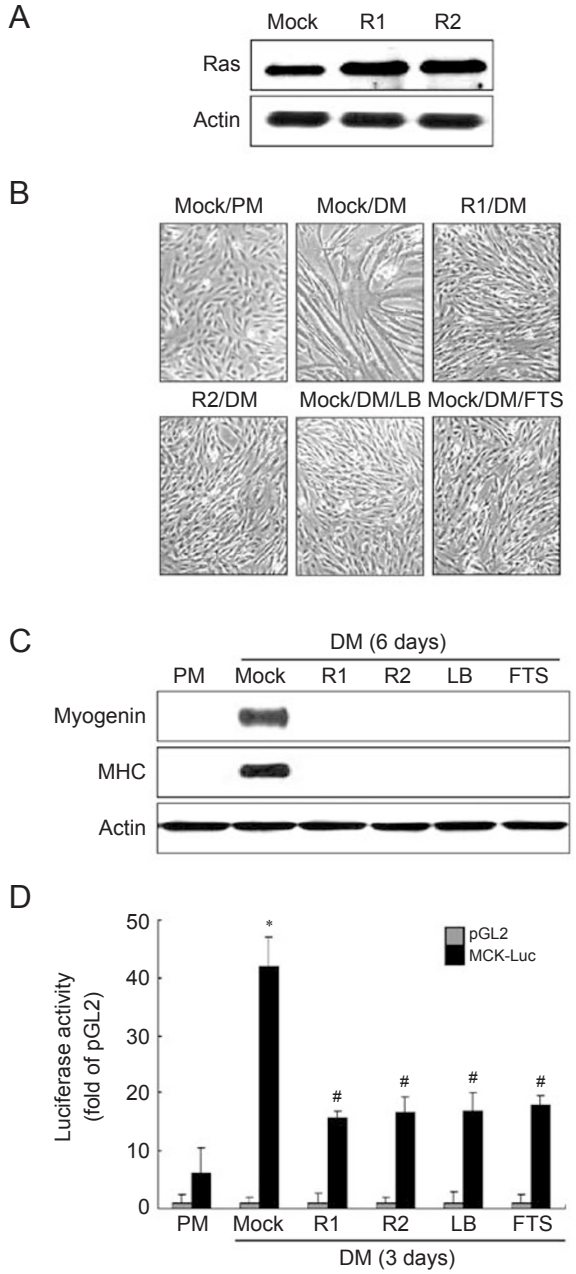


Figure 1 Role of c-Ras in muscle differentiation. **(A)** C-Ras expression as assessed by immunoblotting. H9c2 cells transfected with pcDNA3.0 (mock) and pcDNA3.0-dnRas (R1, R2). **(B-D)** Role of c-Ras in muscle differentiation as assessed by morphological changes, immunoblotting of differentiation markers, and MCK-luciferase assays, respectively. Differentiation was induced by growth in DM for 6 days. Photomicrographs are at ×40 magnification. Mock cells in PM were used as a negative control. PM, proliferation medium; DM, differentiation medium; Mock/PM, mock cells in PM; Mock/DM, mock cells in DM; R1/DM and R2/DM, cells expressing dnRas (R1, R2) in DM; Mock/DM/LB, mock cells treated with 1 μM LB42907·H_2SO_4 (LB) in DM; Mock/DM/FTS, mock cells treated with 15 μM FTS in DM. **P* < 0.05 vs untreated mock cells in PM; #*P* < 0.05 vs mock cells in DM.

specific to N-Ras greatly induced cell death in addition to impairment of cell differentiation (Figure 2B). Increased cell death in N-Ras knockdown cells was due to apoptosis, which was demonstrated by increased processing of caspase-3 and increased expression of Bax compared with cells receiving other siRNA constructs and scrambled controls (Figure 2E).

Selective interaction of c-Ras with PI3K over c-Raf during myogenesis

In previous studies, oncogenic Ras was shown to activate the Raf/Mek/Erk pathway in myogenic cells [22, 23], and thereby block myoblast differentiation [19, 24]. However, proto-oncogenic c-Ras has been shown to activate diverse downstream pathways, including the PI3K and Raf/Mek/Erk pathways [25]. These studies led us to speculate that the ratio of PI3K activation to Raf/Mek/Erk activation may determine differentiative versus proliferative responses to c-Ras activation in myoblasts. Thus, we hypothesized that c-Ras activates PI3K in DM with concomitant downregulation of c-Raf activity. To test this hypothesis, we examined changes in c-Ras association with PI3K p110 subunit (catalytic subunit) and c-Raf proteins via coimmunoprecipitation (Figure 3A, left). In addition to coimmunoprecipitation using anti-pan c-Ras antibody, reverse experiments were also performed (Figure 3B). Both coimmunoprecipitation experiments demonstrated that c-Ras interaction with p110 markedly increased in DM, compared with PM. However, when LB was present in DM, association of c-Ras with p110 decreased almost to basal levels. Association of c-Ras with c-Raf or phospho-c-Raf was dramatically reduced in DM, compared with PM. Reduced levels of association were similar to those observed on inhibition of c-Ras with LB in DM (Figure 3A and 3B). In addition, we confirmed that phosphorylation of c-Raf at Serine 338 is decreased during myogenesis, which is in agreement with previous reports [16]. Immunoblotting also demonstrates that equal amounts of protein were used in immunoprecipitation experiments (Figure 3A, right). These findings indicate that c-Ras effectively recruits and activates PI3K instead of c-Raf during muscle differentiation.

c-Ras stimulates muscle differentiation via PI3K

To further prove that c-Ras is actively involved in muscle differentiation, we next measured c-Ras activity during myogenic differentiation using activated c-Ras affinity precipitation assays. Results showed that activity of c-Ras is significantly increased in cells grown in PM, as compared to serum-free medium controls. Additionally, increases in total c-Ras activity remained constant, regardless of differentiation status (PM vs DM) (Figure

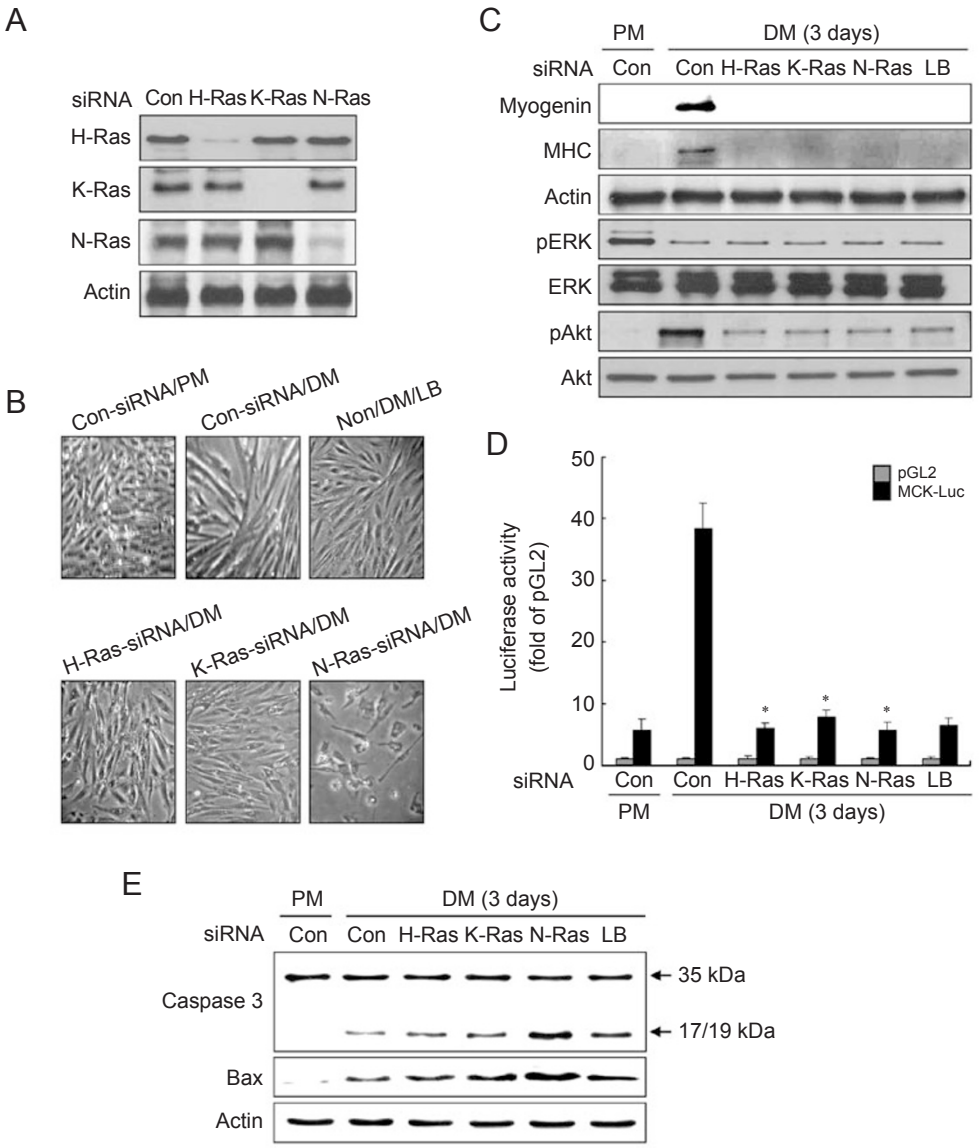


Figure 2 Involvement of H-Ras, K-Ras, and N-Ras in muscle differentiation. **(A)** Expression level of H-Ras, K-Ras, and N-Ras as assessed by immunoblotting. **(B-D)** Knockdown of H-Ras, K-Ras, and N-Ras impairs muscle differentiation as assessed by morphological changes, immunoblotting of differentiation markers, and MCK-luciferase assays, respectively. Differentiation was evaluated after 3 days of growth in DM. Photomicrographs are at ×40 magnification. Scrambled siRNA was used as a negative control for the knockdown experiments. LB in DM was used as a positive control for Ras inactivation. *$P < 0.05$, vs cells transfected with scrambled siRNA in DM (Con). **(E)** Apoptosis in siRNA-treated cells was monitored by immunoblotting of processed caspase-3 and Bax expression.

4A). Although c-Ras activity remained high during differentiation in DM, Erk activity decreased to basal levels found in serum-free medium controls (Figure 4B). In contrast, PI3K activity in differentiated cells was approximately twofold higher than that of proliferating cells, which is in accordance with previous reports [26]. Inhibition of c-Ras activity by dnRas (R1 and R2) and farnesyltransferase inhibitors (LB and FTS) significantly reduced PI3K activity, but did not affect Erk activity in DM (Figure 4B). To further verify effects of PI3K, we evaluated differentiation in previously established stable cell lines expressing dominant negative PI3K (Δp85) and constitutively active PI3K (p110*) [26]. Using previously described assays, we found that inhibition of PI3K blocked muscle differentiation, while constitutively active PI3K induced differentiation (Figure 4C-4E). To examine effects of PI3K on c-Ras activity, we measured active c-Ras while treating cells with a PI3K inhibitor

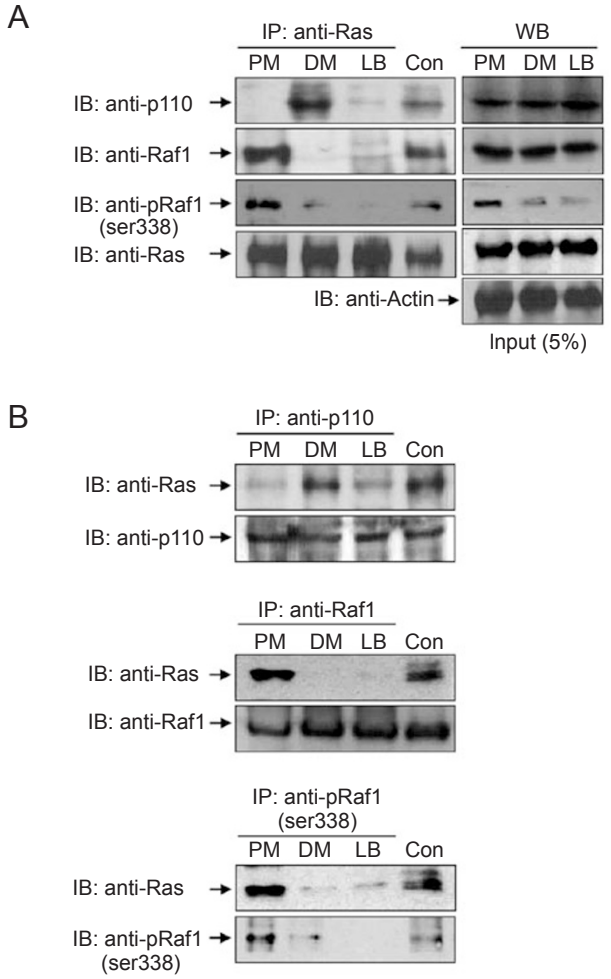


Figure 3 Interaction of c-Ras with PI3K and c-Raf. **(A** and **B)** Co-immunoprecipitation experiments in H9c2 cells cultured with or without LB for 3 days in PM or DM. Lysates were immunoprecipitated (IP) using anti-c-Ras **(A**, left**)**, or anti-PI3K p110 subunit (anti-p110), anti-c-Raf, and anti-phospo-c-Raf (Ser338) antibodies **(B)**. IPs were analyzed by immunoblotting (IB) with indicated antibodies. Total protein lysates (50 μg) were loaded as a non-immunoprecipitated antibody controls for each protein (Con). Expression of p110, Raf1, and Ras was measured using 5% of each IP input by immunoblotting under PM, DM, and LB treatment conditions **(A**, right**)**.

LY294002 (LY) at concentrations of 1, 3, and 5 μM during myogenic differentiation. As expected, c-Ras activity was maintained (Figure 4F). Using cells whose PI3K activity was blocked by either Δp85 or LY294002, as well as cells whose activity was enhanced by p110*, we also found the maintained c-Ras activity (Figure 4G). Additionally, to confirm the same amounts of PI3K enzyme input that was used in PI3K activity assay, a portion of the immunoprecipitated enzyme was subjected to immunoblotting, and was probed with the anti-p85 antibody (p85). In Δp85 cell lines, although Δp85 was transfected, the amount of p85 did not increase. It is because Δp85 inhibits the PI3K activity by competing with wild-type p85. These results demonstrate that c-Ras is upstream of PI3K, not vice versa.

Constitutively active PI3K completely substitutes for c-Ras during muscle differentiation

Our findings suggest that PI3K may act as a master mediator downstream of c-Ras to promote muscle differentiation. Therefore, we tested the effect of constitutively active PI3K on muscle cell differentiation. Using H9c2 cells, we generated a cell line stably expressing both constitutively active PI3K and dnRas (p110*+dnRas), as well as a cell line expressing only constitutively active PI3K (p110*). Expression levels of p110 in both p110* and p110*+dnRas cell lines were approximately twofold higher than in mock cells. Levels of c-Ras in p110*+dnRas cells was almost twofold higher compared to p110* and control cells (Figure 5A). Compared with mock cells, p110* cells differentiated at a much faster rate, as previously shown [26]. The p110* cells fully differentiate into multinucleated myotubes after 3 days in DM, whereas mock cells take 6 days. More interestingly, inhibition of c-Ras activity by dnRas or FTS did not block myogenic differentiation in p110* cells (Figure 5B). Since myogenin is an early myogenic marker, its expression reaches maximum levels by 3 days post-differentiation. Accordingly, lack of differences in myogenin expression was observed between mock cells and p110* cells. MHC, a late myogenic differentiation marker, was markedly increased in p110* and p110*+dnRas cells, compared with mock cells cultured in DM (Figure 5C). MCK is also a late myogenic marker. Luciferase assays performed on cells after 3 days in DM demonstrated an increased activity of the MCK promoter in p110* cells compared to mock cells, regardless of c-Ras inhibition (Figure 5D). While c-Ras activity was maintained, Erk activity decreased in DM, compared to cells in PM, and decreased Erk phosphorylation did not change in p110* cell lines with or without c-Ras inhibition. In contrast, PI3K activity was higher in p110* cells compared to mock cells, even when c-Ras was inhibited by dnRas or FTS (Figure 5E). These data show that constitutively active PI3K can substitute for c-Ras in muscle differentiation.

c-Ras regulates downstream signaling molecules of PI3K

We previously found that H_2O_2 originating from Nox2 stimulates muscle differentiation via the NF-κB activation/iNOS expression pathway, and that PI3K is upstream of the Nox2/NF-κB/iNOS pathway [15, 27, 28]. As a

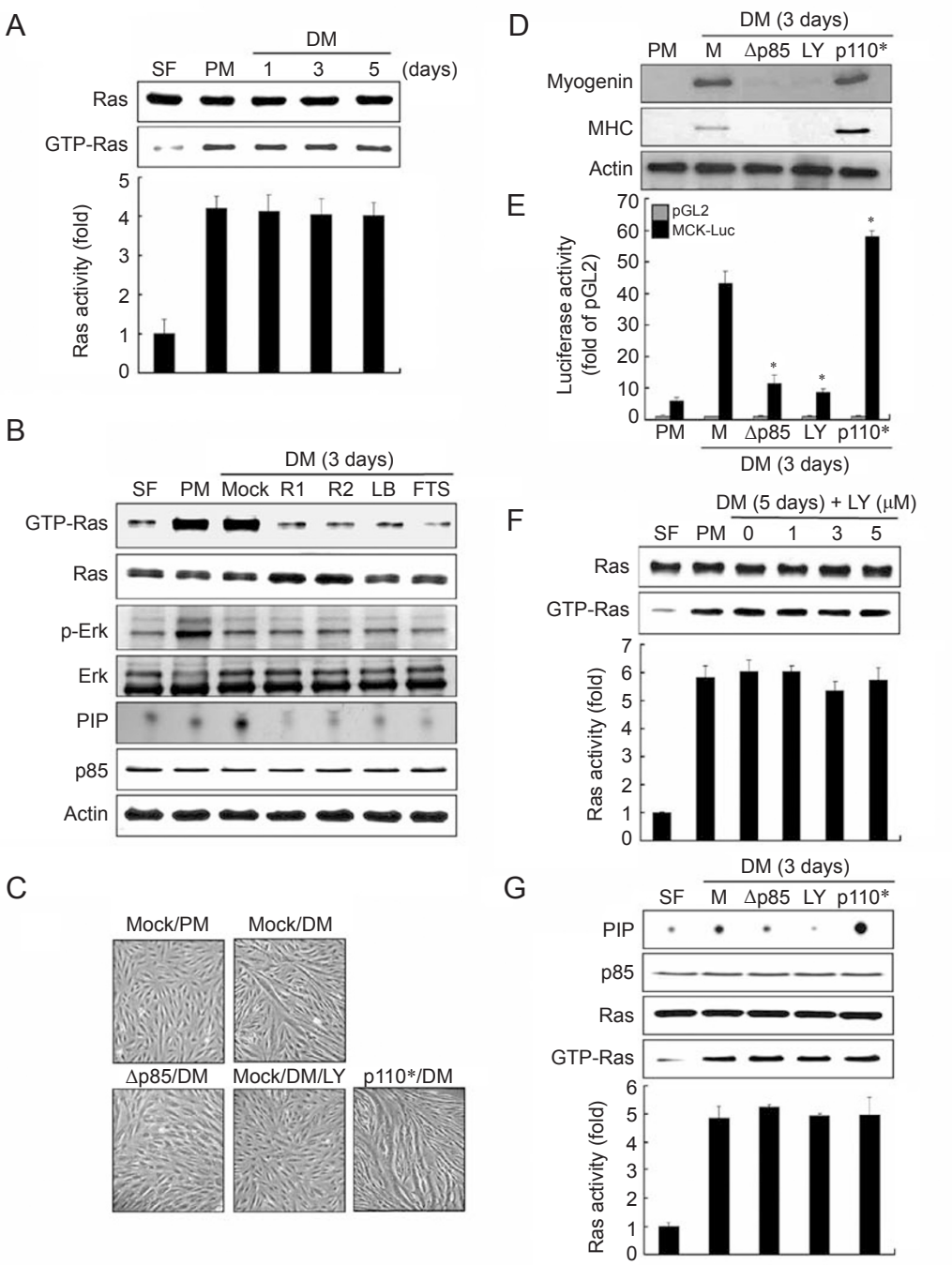


Figure 4 Constant activation of c-Ras and regulation of PI3K activity by c-Ras during muscle differentiation. **(A)** c-Ras activity during muscle differentiation was assessed by activated Ras affinity assays. Serum-free medium (SF) served as a negative control. Densitometric analysis of GTP-Ras is shown as fold over SF condition ±SE. Equal assay loading was ensured by immunoblotting of c-Ras from assay inputs. **(B)** Effect of c-Ras inhibition on PI3K activity was assessed by activated Ras affinity assays, immunoblotting, and TLC. Mock cells incubated in SF for 12 h or PM for 3 days were used as negative and positive controls, respectively. Protein levels of c-Ras, Erk, and actin were used as loading controls in immunoblots. **(C**-**E)** PI3K positively regulates muscle differentiation as assessed by morphological changes, immunoblotting of differentiation markers, and MCK-luciferase assays, respectively. Photomicrographs are at ×40 magnification. **(F** and **G)** No effect of PI3K on c-Ras activity was observed, as assessed by activated Ras affinity assays, immunoblotting and TLC. In all experiments, differentiation was induced by growth in DM for indicated periods of time. M, mock cells in DM; Δp85, cells transfected with pcDNA3.0-Δp85 (Δp85) in DM; LY, mock cells treated with 5 μM LY294002 in DM; p110*, cells transfected with pCMV-p110 (p110*) in DM; p85, a portion of the immunoprecipitated enzyme using anti-phosphotyrosine antibody was immunoblotted and probed with an anti-p85 antibody. *$P < 0.05$ vs untreated mock cells in DM.

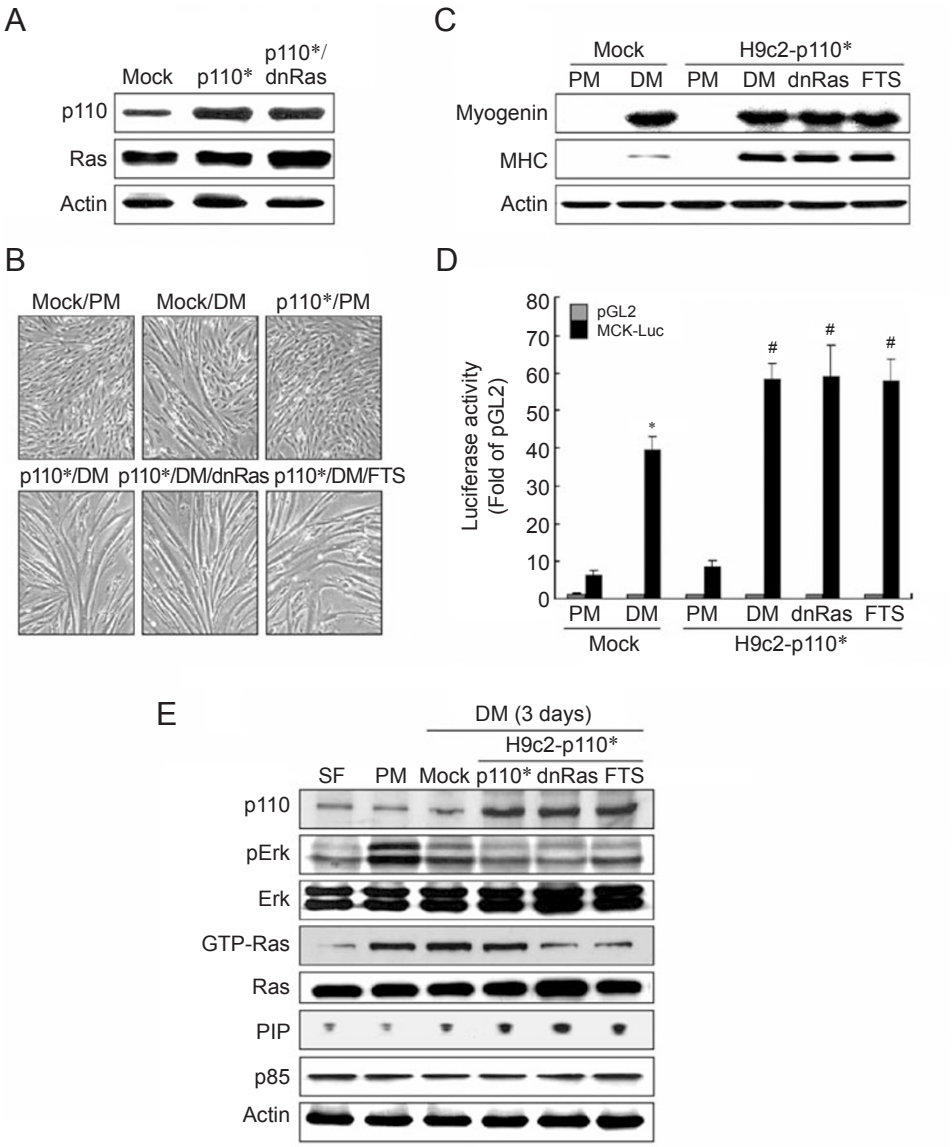
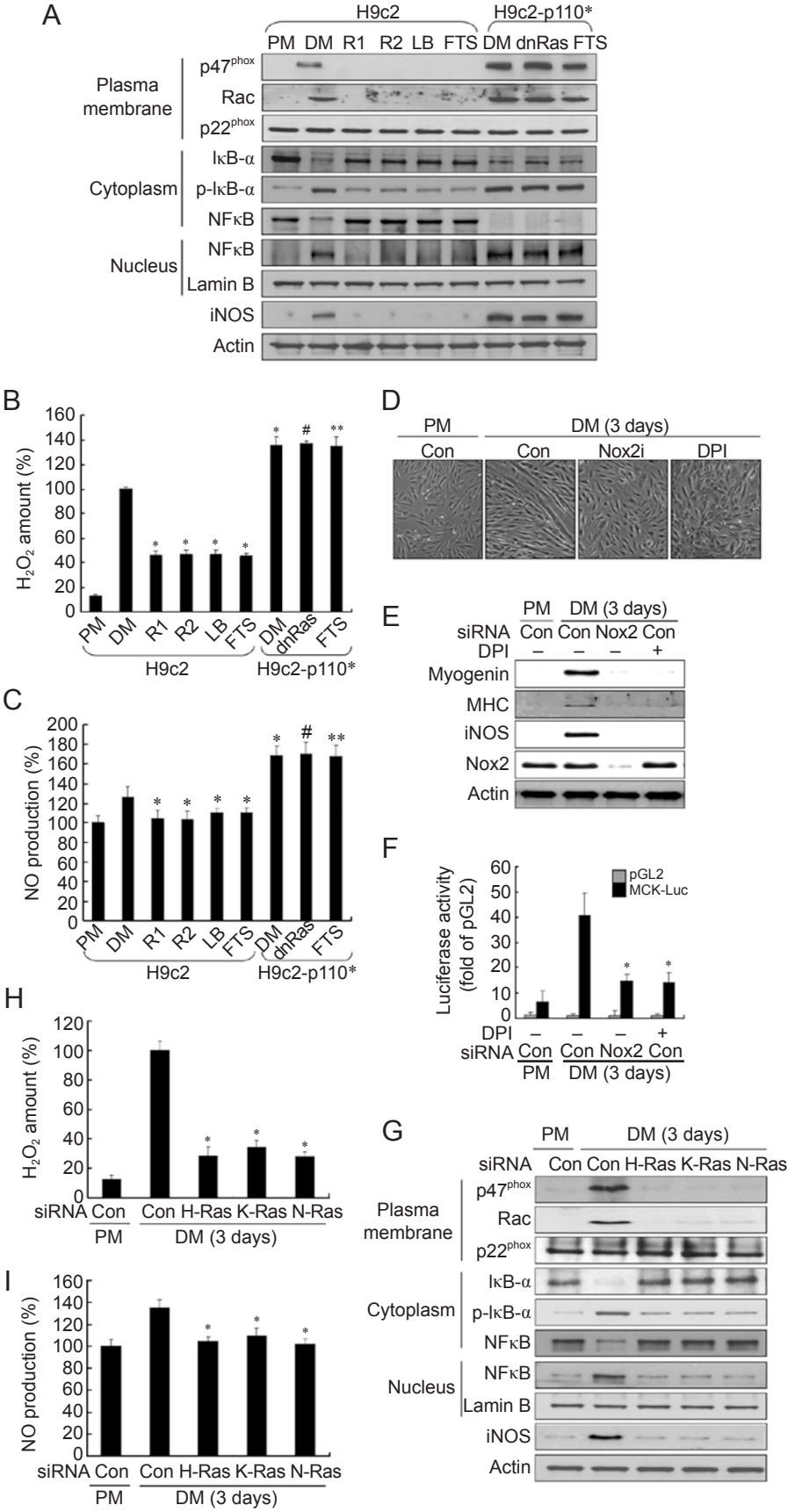


Figure 5 Constitutively active PI3K restores muscle differentiation in c-Ras-inhibited cells. **(A)** Expression of p110 and c-Ras, as assessed by immunoblotting in H9c2 cells expressing constitutively active PI3K (p110*). **(B-D)** Effect of constitutive activation of PI3K on myogenesis as assessed by morphological changes, immunoblotting of differentiation markers, and MCK-luciferase assays, respectively. Photomicrographs are at ×40 magnification. **(E)** Activity of Erk, c-Ras, and PI3K was measured by immunoblotting, activated Ras affinity assays, and TLC, respectively. In all assays, differentiation was induced by growth of cells in DM for 3 days, with or without c-Ras inhibition (dnRas or FTS). Phosphotyrosine was immunoprecipitated from cell extracts and performed PI3K assay (PIP). A portion of the immunoprecipitated enzyme was subjected to immunoblotting and probed with an anti-p85 antibody (p85). *$P < 0.05$ vs untreated mock cells in PM; #$P < 0.05$ vs mock cells in DM.

result of increased iNOS expression, nitric oxide (NO) production was enhanced during muscle differentiation. Nitric oxide is known to play an essential role in cell signaling [29]. Therefore, we investigated whether c-Ras regulates the Nox2/NF-κB/iNOS pathway, and whether constitutive activation of PI3K can completely substitute for c-Ras activity in regulation of this signaling pathway during muscle differentiation. Activity of the Nox2/NF-κB pathway and expression of iNOS were determined at 3 days post-differentiation. Suppression of c-Ras activity by dnRas and farnesyltransferase inhibitors all inhibited activities of Nox2 and NF-κB, resulting in decreased expression of iNOS. However, effects of c-Ras inhibition on this signaling pathway were completely abolished in


A
H9c2
H9c2-p110*
PM DM R1 R2 LB FTS DM dnRas FTS
Plasma membrane
p47phox
Rac
p22phox
Cytoplasm
IκB-α
p-IκB-α
NFκB
Nucleus
NFκB
Lamin B
iNOS
Actin
B
H2O2 amount (%)
PM DM R1 R2 LB FTS DM dnRas FTS
H9c2
H9c2-p110*
C
NO production (%)
PM DM R1 R2 LB FTS DM dnRas FTS
H9c2
H9c2-p110*
D
PM
DM (3 days)
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IκB-α
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p110* cells (Figure 6A). Also, generation of H_2O_2 and NO was significantly suppressed by c-Ras inhibition in mock cells, but completely restored in p110* cells (Figure 6B and 6C). To demonstrate that Nox2 directly participates in muscle differentiation, we inhibited Nox2 by using diphenyleniodonium (DPI), an inhibitor of Nox, as well as a specific siRNA to Nox2. Treatment with DPI and knockdown of Nox2 suppressed muscle differentiation, indicating that Nox2 directly stimulates muscle differentiation (Figure 6D-6F). Furthermore, individual knockdown of H-Ras, K-Ras, and N-Ras decreased levels of H_2O_2 and NO, prevented translocation of Rac and $p47^{phox}$ to the plasma membrane, prevented translocation of NF-κB to the nucleus, and decreased expression of iNOS (Figure 6G-6I). Hence, all three isoforms of c-Ras regulate the PI3K/Nox2/NF-κB/iNOS pathway in muscle differentiation and constitutively active PI3K can substitute for c-Ras.

H_2O_2 contributes to the muscle differentiation as a signal messenger molecule

To determine whether H_2O_2 generated during muscle differentiation stimulates muscle differentiation downstream of PI3K, we treated differentiating cells with 4 mM Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid, a superoxide scavenger) and 0.2 mM deforaxamin (DFOM, iron chelator) for 3 days and investigated the effects on differentiation (Figure 7A-7C). Treatment with antioxidants completely blocked muscle differentiation in mock cells, as well as p110* cells, indicating that H_2O_2 is an essential signaling molecule downstream of PI3K.

c-Ras stimulates muscle differentiation via PI3K in C2C12 myoblasts as well

To validate our findings in H9c2 cells, we examined effects of c-Ras inhibition on muscle differentiation in C2C12 cells, and whether constitutive activation of PI3K can rescue differentiation block induced by c-Ras inhibition. As expected, identical results to those obtained in H9c2 cells were observed in C2C12 cells. Stable cell lines expressing constitutively active PI3K (C2C12-p110*), dnRas (R1 and R2), or both (p110*+dnRas) were established using C2C12 cells. Levels of p110 and c-Ras expression were increased approximately twofold in respective stable cell lines (Figure 8A). Suppression of c-Ras activity inhibited both morphological transformation and expression of differentiation markers in C2C12 cells as in H9c2 cells (Figure 8B and 8C). Activities of c-Ras, Erk, and PI3K in C2C12 and C2C12-p110* cells, with or without c-Ras inhibition, were in accordance with results obtained from H9c2 cells (Figure 8C). Additionally, inhibition of PI3K by Δp85 blocked muscle differentiation, while constitutive activation of PI3K by p110* induced differentiation (Figure 8D and 8E). As with H9c2 cells, PI3K inhibition or activation did not affect overall c-Ras activity (Figure 8F). Lastly, we performed individual knockdown experiments of H-Ras, K-Ras, and N-Ras in C2C12 myoblasts with identical results observed in H9c2 cells (Figure 8G-8I). Our results indicate that c-Ras regulates muscle differentiation via PI3K activation.

Discussion

Here, we demonstrate for the first time that c-Ras positively regulates muscle differentiation by activating the PI3K myogenic Nox2/NF-κB/iNOS pathway. Constitutive activation of PI3K completely restored muscle differentiation after suppression of c-Ras activity, supporting the idea that PI3K is the sole molecule downstream of c-Ras responsible for muscle differentiation. However, our results do not refute previous findings that oncogenic Ras blocks muscle differentiation. Rather, our results suggest that constitutive and strong activation of oncogenic Ras may transmit aberrant signals downstream to block muscle differentiation, while physiological levels of c-Ras activity are essential for PI3K activation and muscle differentiation.

In our isoform-specific knockdown experiments, we showed that H-Ras and K-Ras are directly involved in muscle differentiation, while N-Ras maintains cell survival by possessing anti-apoptotic functions during differentiation. Many reports support the notion that these

Figure 6 Substitution of constitutively active PI3K for c-Ras in activation of the Nox2/NF-κB/iNOS pathway. **(A)** Activity and expression of the Nox2/NF-κB/iNOS pathway were assessed by immunoblotting after manipulation of c-Ras (R1, R2, LB, and FTS) and PI3K (p110*) during muscle differentiation. Actin and lamin B were used as loading controls. Cells in PM or DM were used as negative or positive controls. **(B** and **C)** H_2O_2 and NO production levels were measured under various conditions. **(D-F)** Nox2 involvement in muscle differentiation was assessed by morphological changes, immunoblotting for differentiation markers, and MCK-luciferase assays, respectively. DPI, an inhibitor of Nox, and specific siRNA to Nox2 were used in these experiments. Photomicrographs are at ×40 magnification. **(G-I)** Knockdown of Ras isoforms impairs the Nox2/NF-κB/iNOS pathway as assessed by immunoblotting, and decreases H_2O_2 and NO production. In all experiments, muscle differentiation was induced by growth in DM for 3 days. *P < 0.05 vs untreated mock cells in DM or cells transfected with scrambled siRNA in DM (Con); #P < 0.05 vs R1 cells in DM; **P < 0.05 vs FTS-treated mock cells in DM.

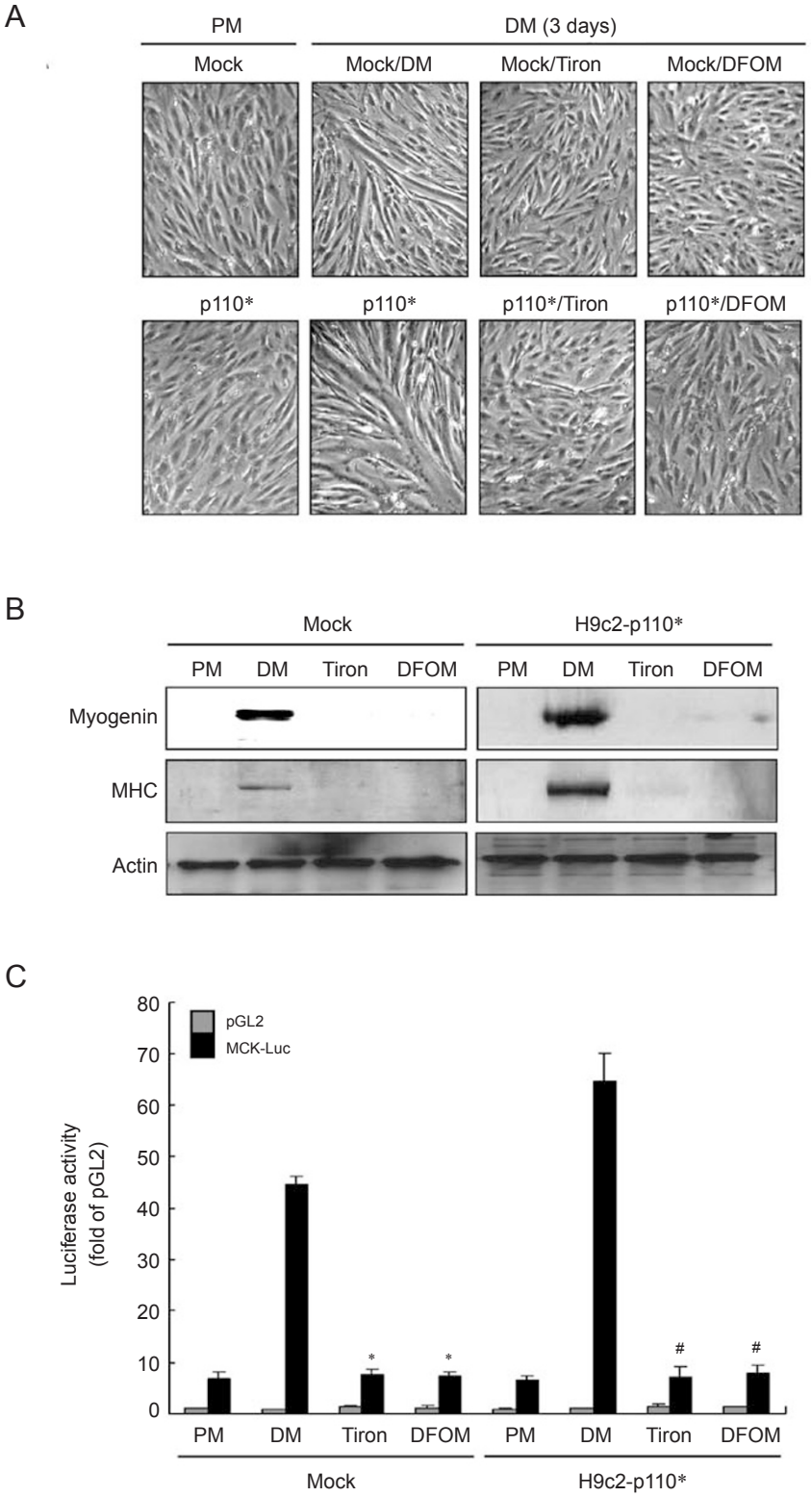

Figure 7 H_2O_2 generated during muscle differentiation is an important signaling molecule. **(A**-**C)** Effect of antioxidants on muscle differentiation was assessed by morphological changes, immunoblotting for differentiation markers, and MCK-luciferase assays, respectively. Photomicrographs are at ×40 magnification. Mock and constitutively active PI3K (p110*) expressing cells were grown in DM and treated with or without 4 mM Tiron and 0.2 mM DFOM for 3 days. *P < 0.05 vs untreated mock cells in DM; #P < 0.05 vs untreated p110* cells in DM.

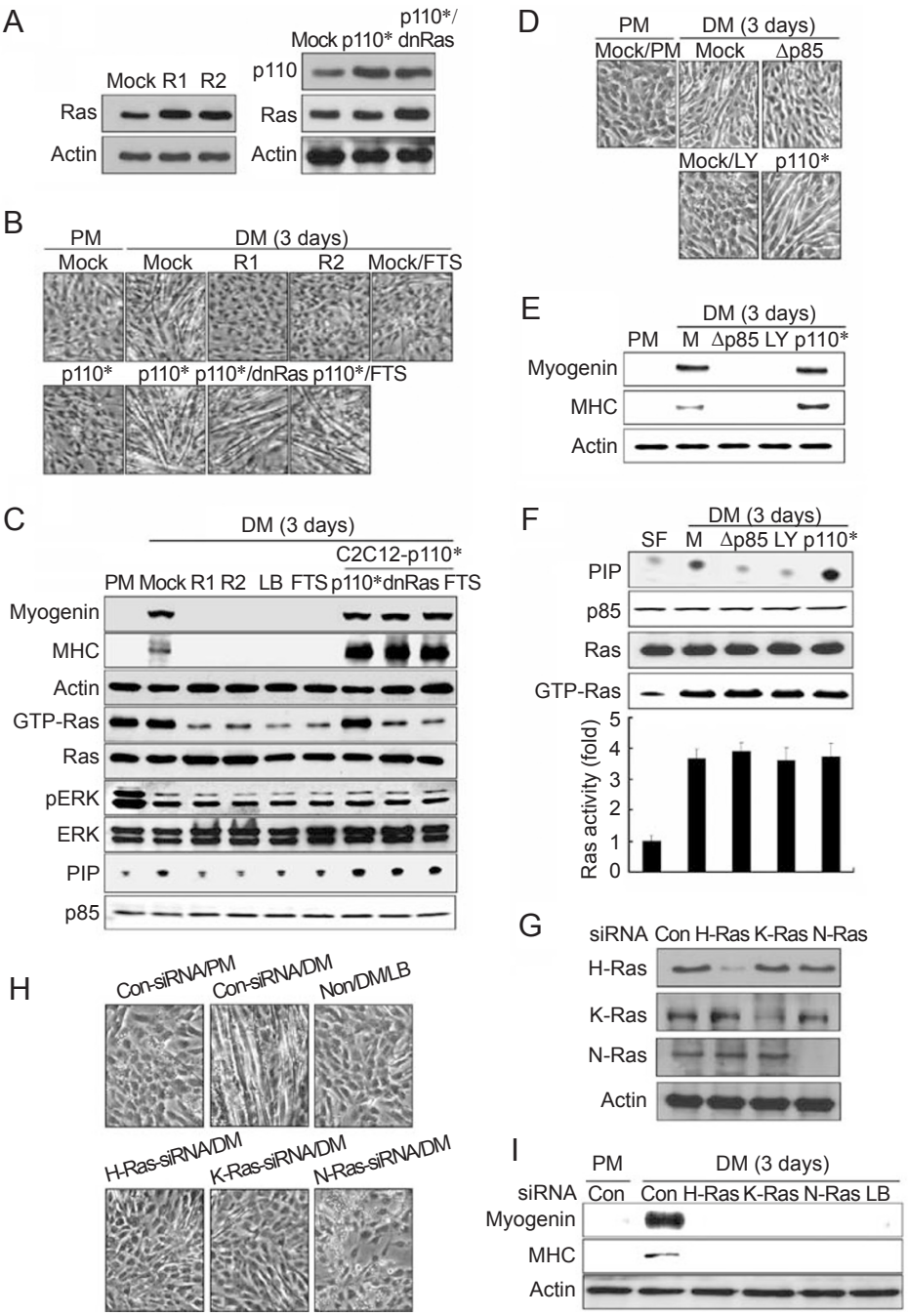

Figure 8 c-Ras regulates differentiation of C2C12 myoblasts via the PI3K pathway. **(A)** Expression of p110 and c-Ras was assessed by immunoblotting after manipulation of c-Ras (R1, R2, dnRas) and PI3K (p110*). **(B)** Muscle differentiation was assessed via morphological changes in c-Ras inhibited, but PI3K activated C2C12 cells. Differentiation was assessed after growing in DM for 3 days. R1 and R2, C2C12 clonal cells expressing dnRas; Mock/FTS, mock cells treated with FTS; p110*/dnRas, C2C12 cells expressing both p110* and dnRas; p110*/FTS, C2C12-p110* cells treated with FTS. **(C)** Constitutively active PI3K (p110*) completely rescues c-Ras inhibition (R1, R2, LB, and FTS) in myogenic differentiation as assessed by immunoblotting, activated Ras affinity assays, and TLC. **(D** and **E)** PI3K manipulation (LY, p110*, and Δp85) modulates muscle differentiation in C2C12 cells as assessed by morphological changes and immunoblotting of differentiation markers. **(F)** No effect of PI3K on c-Ras activity in C2C12 cells as assessed by TLC, activated Ras affinity assays, and immunoblots. **(G)** Expression levels of H-Ras, K-Ras, and N-Ras assessed by immunoblotting in siRNA-transfected C2C12 cells. **(H** and **I)** Impaired differentiation in H-Ras, K-Ras, and N-Ras knockdown cells as assessed by morphological changes and immunoblotting of differentiation markers, respectively. All morphology experiment photomicrographs are at ×40 magnification. In the PI3K assay, a portion of the immunoprecipitated enzyme using anti-phosphotyrosine antibody was subjected to immunoblotting and probed with an anti-p85 antibody (p85).

three isoforms play specialized cellular roles [30, 31]. Since activity of the MCK promoter in K-Ras knockdown cells was a little higher (twofold) than in H-Ras knockdown cells, we believe H-Ras influences activation of the MCK promoter more strongly than K-Ras in later stages of differentiation. However, we also think that compensatory effects from the remaining H-Ras in K-Ras knockdown cells may be insufficient to induce muscle differentiation. Consistent with our results, it was reported that constitutively activated proto-oncogene H-Ras affects MHC promoter activity via the Ras/PI3K/Akt signaling pathway in later stages of differentiation of muscle cells [32]. Direct evidence of K-Ras involvement in muscle differentiation has yet to be reported. However, it was identified that K-Ras functions as a major regulator of Akt activation in erythroid differentiation[33]. Furthermore, K-Ras is required in development of mouse embryos [34, 35], indicating that K-Ras performs specific functions that cannot be carried out by either H-Ras or N-Ras during embryogenesis. Our observations also indicate that knockdown of N-Ras during differentiation induces significantly higher levels of apoptosis. Consistently, N-Ras can protect cells against apoptosis by increasing the ratio of anti-apoptotic molecules Bcl-2 or Bcl-xL to pro-apoptotic molecules Bad or Bax via changes to basal Akt activity and pBad levels through PI3K [36, 37]. Therefore, we think that N-Ras plays a distinct role in balancing cell survival and death via the N-Ras/PI3K/Akt pathway throughout muscle differentiation. In conclusion, we believe all c-Ras isoforms stimulate muscle differentiation via their own unique mechanisms, and are not compensatory for one another.

It is well known that activities of the Raf/Mek/Erk pathway decline with progression of muscle differentiation, and constitutive activation of the Raf/Mek/Erk pathway blocks muscle differentiation [38-40]. If c-Ras is activated during muscle differentiation as we showed, puzzling questions arise regarding how c-Raf activity is suppressed. Previously, an elegant study showed that activated Akt by PI3K switches off the Raf/Mek/Erk pathway by phosphorylating c-Raf, thereby promoting muscle differentiation [38]. Another report showed that DA-Raf 1 can directly associate with active c-Ras, thus precluding Raf/Mek/Erk pathway activation, leading to positive regulation of myogenic differentiation [20]. As a third mechanism of action, we show here that activated c-Ras preferentially interacts with PI3K p110 subunit rather than c-Raf, which could explain the suppression of the Raf/Mek/Erk pathway during muscle differentiation. This preferential interaction may be controlled by a specific scaffold protein reacting to muscle differentiation stimulation. The scaffold protein could operate selectively on activated c-Ras bound to the PI3K p110 subunit, but not bound to c-Raf. Similarly, it was reported that Dapr (differentiation-associated protein), a novel protein, acted as a scaffold protein for Akt during muscle differentiation [41]. These reports along with our data indicate that cells may have devised multiple mechanisms to activate PI3K with concurrent Raf inactivation downstream of c-Ras during myogenic differentiation. Considering that cells have to exit the proliferative stage controlled by the Raf/Mek/Erk pathway prior to undergoing differentiation, the multiple checkpoints controlling Raf activity seem to be reasonable.

Relationships between c-Ras and PI3K have been studied extensively [42-44]. C-Ras and PI3K have been known to interact for many years [45]. However, it is still controversial as to which molecule is upstream of the other. Some groups reported that c-Ras regulates PI3K activity [45-47], whereas others describe PI3K as being upstream of c-Ras [48, 49]. In our experiments, inhibition of c-Ras by dnRas or farnesyltransferase inhibitors suppressed PI3K activity and subsequently its downstream Nox2/NF-κB/iNOS pathway. Conversely, activation or inactivation of PI3K did not affect c-Ras activity. Constitutive activation of PI3K completely rescued muscle differentiation and activation of the Nox2/NF-κB/iNOS pathway, both of which were otherwise suppressed by inhibition of c-Ras activity. Therefore, we conclude that c-Ras is upstream of PI3K, and that PI3K may be the master mediator of c-Ras in muscle differentiation.

In summary, we demonstrated that all c-Ras isforms are pivotal throughout muscle differentiation. In addition, PI3K seems to be the sole master molecule downstream of c-Ras in myogenic differentiation. We also demonstrated that activated c-Ras can preferentially bind to PI3K, but does not bind to c-Raf and does not stimulate the Raf/Mek/Erk pathway, leading to exiting from the cell cycle during muscle differentiation. In future, it will be valuable to identify any possible scaffold proteins that might be involved in selective activation of PI3K with concurrent suppression of c-Raf during myogenic differentiation.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), DMEM/F-12, donor calf serum, horse serum, fetal bovine serum (FBS), and G418 were purchased from GIBCO-BRL (Grand Island, NY, USA). Farnesylthiosalicylic acid (FTS, farnesyltransferase inhibitor) and LY294002 (PI3K inhibitor) were acquired from Calbiochem (San Diego, CA, USA). LB42907·H_2SO_4, another farnesyltransferase inhibitor, was kindly provided from LG Biochem (Daejeon, Korea). [γ-^{32}P] ATP was obtained from Amersham

Biosciences (Little Chalfont, UK). Anti-phosphotyrosine agarose beads, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), DPI, Tiron, and DFOM were purchased from Sigma Chemical (St. Louis, MO, USA). Antibodies specific to MHC, myogenin, actin, H-Ras, K-Ras, N-Ras, caspase-3, Bax, $p47^{phox}$, $p22^{phox}$, IκB-α, p-IκBα, iNOS, Erk, p-Erk, Akt, PI3K p110, p85, Raf-1 (c-Raf), p-Raf-1 (Ser338), lamin B, goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, donkey anti-goat IgG-HRP, and proteins A/G conjugated to agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The phospho-Akt (phospho-Ser473) antibody was obtained from Assay Designs Inc (Ann Arbor, MI, USA). Monoclonal pan-Ras antibody and Raf-1 Ras-binding domain (Raf-1 RBD) agarose beads were purchased from Upstate Biotechnology (Lake Placid, NY, USA).

Cell culture, cDNA constructs, and stable transfection

H9c2 rat cardiac and C2C12 mouse skeletal myoblasts were grown in DMEM/F-12 containing 10% (v/v) donor calf serum and DMEM supplemented with 10% FBS (v/v) (PM), respectively. Cells were induced to differentiate by placing them in DMEM/F-12 containing 1% (v/v) horse serum or 1% (v/v) FBS (DM). To inhibit c-Ras or PI3K activity, 15 μM FTS, 1 μM LB42907·H_2SO_4 (LB), or 5 μM LY294002 (LY) was applied. The pcDNA3.0 constructs containing dominant negative H-Ras (dnRas, H-RasS17N), dominant negative PI3K (Δp85), and the pCMV construct containing constitutively active PI3K (p110*) were described previously [50-52]. Transfection was carried out using GenePORTER according to the manufacturer's instructions (Genetherapy Systems). Stable trasfection was achieved by selecting the transfected cells using 300 μg/ml G418. For establishment of stable cell lines expressing both p110* and dnRas, pcDNA3.0-p110* from pCMV-p110* was constructed and cotransfection of pcDNA3.0-p110* was performed with or without pcDNA3.0-dnRas.

RNA interference

The small interfering RNAs (siRNAs) specific to K-Ras (K-Ras-siRNA) and N-Ras (N-Ras-siRNA) for mouse and rat, as well as mouse H-Ras (H-Ras-siRNA) and a scrambled siRNA, were all purchased from Sigma. For knockdown of rat H-Ras, pools of four siRNA duplexes (Smartpools, Dharmacon) were used [53]. The siRNA target sequences were as follows: rat K-Ras-siRNA sense sequence, 5′-GUG UAU UUG CCA UAA AUA AdTdT-3′; antisense, 5′-UUA UUU AUG GCA AAU ACA CdTdT-3′; rat N-Ras-siRNA sense sequence, 5′-GGU GAA AGA CUC UGA UGA UdTdT-3′; antisense, 5′-AUC AUC AGA GUC UUU CAC CdTdT-3′. Mouse H-Ras-siRNA sense sequence, 5′-GUU GCA UCA CAG UAA AUU AdTdT-3′; antisense, 5′-UAA UUU ACU GUG AUG CAA CdTdT-3′; mouse K-Ras-siRNA sense sequence, 5′-GAA ACC UGU CUC UUG GAU AdTdT-3′; antisense, 5′-UAU CCA AGA GAC AGG UUU CdTdT-3′; mouse N-Ras-siRNA sense sequence, 5′-GAG AUA CGC CAG UAC CGA AdTdT-3′; antisense, 5′-UUC GGU ACU GGC GUA UCU CdTdT-3′. For knockdown of rat Nox2, we purchased Nox2-siRNA from Sigma. The siRNA target sequences were as follows: rat Nox2-siRNA sense sequence, 5′-GUC AUC ACA CUG UGU CUU AdTdT-3′; antisense, 5′-UAA GAC ACA GUG UGA UGA CdTdT-3′. The scrambled siRNA was used as a negative control for the knockdown experiments. The efficiency of siRNAs of all the isoforms was monitored via immunoblotting. The siRNAs (0.5 μg/ml) were transfected into cells using Lipofectamine 2000 Transfection Reagent (Invitrogen, San Diego, CA, USA) for 48 h.

Luciferase assay

H9c2 cells were transfected with 0.5 μg amounts of the pGL2 vector, or MCK-Luc construct, coupled with the internal control plasmid, pSV-β-gal (Promega, Madison, WI, USA). Cells were harvested in 150 μl of luciferase assay lysis buffer [27]. Luciferase and β-gal activities (data not shown) were measured with a microplate reader (Model 680 Microplate Reader, Bio-Rad Laboratories Inc Hercules, CA, USA) using 20 μl of each cell lysate, with luciferase activity being normalized to β-gal activity.

Immunoprecipitation

Cells were washed twice with cold PBS, lysed in cold lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 1 mM NaF), and lysates were cleared by centrifugation at 14 000 r.p.m. for 15 min. Cell lysates (2 mg) were incubated with anti-pan-Ras, anti-PI3K p110 subunit, anti-Raf-1 (c-Raf), or anti-phospho-Raf-1 (Ser338) antibody (2 μg) for 2 h at 4 °C. Proteins A/G conjugated to agarose beads were added for 2 h. Beads were washed three times with washing buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF). The immunopellets were resuspended in SDS-PAGE sample buffer under reducing or non-reducing conditions and subjected to electrophoresis.

Activated Ras affinity precipitation assay

Activated Ras affinity precipitation assays were performed according to the manufacturer's protocol. Briefly, cell lysates (500 μg) were incubated with 5 μg of Raf-1 RBD agarose beads for 30 min at 4 °C. After extensive washing of the agarose beads three times with washing buffer (25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM Na_3VO_4, 10% glycerol, 10 μg/ml leupeptin, 10 μg /ml aprotinin, and 25 mM NaF), the activated Ras (GTP-Ras) bound to Raf-1 RBD argarose beads was released by addition of SDS-PAGE sample buffer. The amount of activated Ras was determined by immunoblotting with a monoclonal pan-Ras antibody.

Thin-layer chromatography (TLC) assay for PI3K

Activity of PI3K was assessed using a previously described PI3K assay [12]. PI3K activity was assayed in phosphotyrosine immunoprecipitates by conversion of PI to phosphatidylinositol-phosphate (PIP). Cells were solubilized by incubation for 30 min in 1 ml of PI3K assay lysis buffer. Anti-phosphotyrosine antibody-conjugated agarose beads (20 μl) were incubated with supernatants containing 500 μg of protein for 1 h at 4 °C. After washing, the immunoprecipitates were resuspended in 100 μl of kinase assay buffer (20 mM Tris-HCl (pH 7.6), 75 mM NaCl, 10 mM $MgCl_2$, 200 μg/ml PI, and 10 μCi [γ-^{32}P] ATP), and incubated for 20 min at room temperature with constant shaking. The reaction was stopped by addition of 100 μl of 1 N HCl and 200 μl of $CHCl_3$-methanol (1:1). The samples were centrifuged, and the lower organic phase was harvested and applied to a silica gel TLC plate (Merck, Whitehouse Station, NJ, USA) coated with 1% potassium oxalate. Thin-layer chromatography plates were developed in $CHCl_3$-CH_3OH-H_2O-NH_4OH (60:47:11.3:2), dried, and visualized

by autoradiography. Additionally, a part of the immunoprecipitated enzyme was subjected to immunoblotting and probed with the anti-p85 antibody to confirm that an equal amount of enzyme was used for the assay (p85, lower panel of each PI3K assay).

Assays for Nox2 and NF-κB activity

Nox2 activity was analyzed as previously described [15]. Nox2 activity was determined indirectly by measuring the translocated amounts of $p47^{phox}$ and Rac proteins forming the active NADPH oxidase to the plasma membrane via immunoblotting [15, 54]. NF-κB activity was assayed by immunoblotting of IκB-α, phospho-IκB-α, and NF-κB protein amounts in nuclear and cytosolic extracts by preparing as previously described [15].

Detection of intracellular H_2O_2

Intracellular H_2O_2 was measured as previously described [55]. Briefly, H_2O_2 was measured using DCF-DA (Molecular Probe) dye. The cells were incubated in DM for 24 h and treated with and without c-Ras inhibitors. Then, the cells were loaded with 10 μM DCF-DA at 37 °C for 30 min, and resuspended in 1 ml of PBS. Fluorescence was measured by a flow cytometer (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ, USA). The mean DCF fluorescence intensity was measured with excitation at 488 nm and emission at 525 nm.

Assay for NO production

The activity of iNOS was determined by assaying nitrites in the conditioned media of cultured cells. To measure nitrite (NO^{2-} or NO^{3-}), 100 μl of culture supernatant was collected, mixed with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride, 2.5% H_3PO_4) and incubated for 10 min at room temperature. Nitrite concentration was determined by measuring absorbance at 540 nm in a 96-well spectrophotometer (Model 680 Microplate Reader, Bio-Rad Laboratories Inc). $NaNO_2$ was used for external calibration. This value was determined for each experiment, and subtracted from the value obtained for each cell sample. Nitrite concentrations were determined from a linear standard curve constructed with known concentrations of sodium nitrite.

Immunoblotting

The immunoprecipitates or total lysates were separated by SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. The membranes were washed twice with TBST before blocking the nonspecific binding with TBS/5% skim milk/1% FCS. The membranes were incubated with the indicated primary antibodies in TBS/2% skim milk/0.7% FCS for 2 h at room temperature. The membranes were washed five times and detected by horseradish peroxidase-conjugated antibodies for 1.5 h at room temperature and viewed using the enhanced chemiluminescence method (Amersham, Braunschweig, Germany). For quantification, band intensity was measured by Bio-Rad imaging densitometric scan (Quantity One, software version 4.6.2).

Statistical analysis

Results were expressed as mean±SE. Error bars represent the mean±SE of three independent experiments performed in triplicate. The difference between two mean values was analyzed using the Student's *t*-test. The difference was considered statistically significant when $P < 0.05$.

Acknowledgments

We are grateful to Dr Julian Downward (Signal Transduction Laboratory, Cancer Research UK, London Research Institute, London, UK) and Dr Masato Kasuga (Research Institute, International Medical Center of Japan, Tokyo, Japan) for providing us with expression vectors for constitutively active form of PI3K (p110*) and dominant negative form of PI3K (Δp85), respectively. We also thank Dr KY Lee (Chungbuk National University, Chungbuk, Korea) for providing us with the MCK-luc vectors. This work was supported by the Korea Science & Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (NO. 20090091346) to SS Kim.

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