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Hypoxia-induced SM22α in A549 cells activates the IGF1R/PI3K/Akt pathway, conferring cellular resistance against chemo- and radiation therapy

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ABSTRACT

Chemo- or radiation-resistance in tumors caused by hypoxia often undermines efficacy of cancer therapy. Thus, therapies that overcome cellular resistance during hypoxia are necessary. SM22α is an actin-binding protein found in smooth muscle, fibroblasts, and some epithelium. We demonstrate that SM22α is induced in A549 non-small cell lung carcinoma cells by hypoxia and its overexpression increased chemo- and radiation-resistance. Hypoxia-mediated induction of SM22α expression is hypoxia-inducible factor-independent. Moreover, SM22α overexpression enhances tumor cell growth and activates the IGF1R/PI3K/Akt pathway via direct interaction with IGF1Rβ. Our results suggest SM22α as a novel regulator of hypoxic survival pathway of A549 NSCLC cells.

Structured summary of protein interactions:
IGFR1 Beta physically interacts with **SM22 alpha** by anti bait coimmunoprecipitation (View Interaction: 1, 2)
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1. Introduction

Cells in rapidly growing tumors quickly outstrip the vascular supply, resulting in a poorly vascularized microenvironment characterized by hypoxia and nutrient starvation [1]. The hypoxic status of various solid tumors has been linked to poor prognosis due to tumor progression toward a more malignant phenotype, with an increased resistance to radiotherapy and chemotherapy treatment [2]. The best characterized hypoxia response pathway is mediated by hypoxia-inducible factor (HIF), a heterodimeric DNA-binding complex composed of two basic helix-loop-helix proteins, namely the constitutive HIF-1β and one of either hypoxia-inducible α-subunits, HIF-1α or HIF-2α [3]. HIF regulates the expression of more than 70 genes, including proapoptotic and antiapoptotic ones, and is considered as a master regulator of the hypoxic response. However, accumulating evidence indicates that HIF-independent pathways also promote cell survival and control angiogenesis under hypoxic conditions by activating phosphoinositotide-3-kinase (PI3K) [4], extracellular signal-regulated kinase 1/2 [5], or p38 mitogen-activated protein kinase [6].

SM22α, which is also called as transgelin, is a shape change-sensitive actin cross-linking/gelling protein found in fibroblasts and smooth muscle [7]. It is a member of the calponin family, which is localized to the cytoskeletal apparatus [8]. SM22α-null mice reveal that SM22α is involved in calcium-independent smooth muscle contraction [9]. Recently, different functions for SM22α are suggested such as a tumor suppressor [10]. Also, our previous studies revealed that SM22α is involved in cellular resistance against anti-cancer drugs or gamma-radiation [11] and in cellular senescence [12].

Here, we present another function of SM22α in A549 non-small cell lung carcinoma (NSCLC) cells. In A549 cells exposed to hypoxic stress SM22α protein levels were elevated, which increased the cell growth and cellular-resistance against anti-cancer drugs and gamma-radiation. Interestingly, hypoxic induction of SM22α in these cells was independent of HIF-1α and activated the insulin-like growth factor receptor (IGFIR)/PI3K/Akt signal pathway via direct interaction between SM22α and IGF1Rβ.

2. Materials and methods

2.1. Cell culture, chemicals, gamma-radiation and transfection

Human NSCLC A549 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in

Abbreviations: EGFR, epidermal growth factor receptor; HIF, hypoxia-inducible factor; IGFIR, type 1 insulin-like growth factor receptor; IgG, immunoglobulin G; MMP-9, metallomatrix protease-9; MMS, methyl methanesulfonate; NSCLC, non-small cell lung carcinoma; PI3K, phosphoinositotide-3-kinase; RT-PCR, reverse transcription-polymerase chain reaction; SA-β-Gal, senescence-associated β-galactosidase; siRNA, silencing RNA.

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RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM glutamine, 10% (v/v) fetal bovine serum (Invitrogen), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen) at 37 °C in a 5% CO_2 humidified incubator. Methyl methanesulfonate (MMS), *cis*-platinum (II)–diamine dichloride (Cisplatin), H_2O_2, and cobalt chloride ($CoCl_2$), were purchased from Sigma–Aldrich (St. Louis, MO, USA). AG1024 was purchased from Calbiochem (La Jolla, CA, USA). Prior to gamma-radiation, cells were seeded into T25 flasks at 1×10^6 cells/flask. On the next day, the cells were exposed to a single dose of 20 Gy (^{60}Co γ-ray source; dose rate, 2 Gy/min) and cultured for the indicated time periods. SM22α-expression vectors and silencing RNA (siRNA) targeting of SM22α were used as described previously [12].

2.2. Hypoxic exposure of lung carcinoma cells

Cells were seeded in 100-mm dishes and grown until confluence. Cells were then subjected to hypoxia (1% O_2) by placing them in an Innova Co-48 hypoxia incubator (New Brunswick Scientific, Edison, NJ, USA). The incubator was evacuated until the desired oxygen partial pressure was reached and then replenished with a gaseous mixture containing 5% CO_2 and 95% N_2. The cells were incubated within the chamber at 37 °C for up to 24 h.

2.3. Western blot and RT PCR analysis

Protein extraction and Western blotting were performed as described previously [13] using primary antibodies specific for human SM22α (Abcam, London, UK), HIF-1α, PI3 K p110, phosphatase and tensin homolog (PTEN), K-Ras (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-Akt (Ser473), Akt, phospho- epidermal growth factor receptor (EGFR [Tyr1068]), EGFR, phospho-IGF1Rβ (Tyr1131)/insulin receptor β (Tyr1146), IGF1Rβ (111A9; Cell Signaling Technology, Danvers, MA, USA), and the corresponding secondary antibodies. RT-PCR analysis of SM22α was performed as described previously [12].

2.4. Measurement of cell death and colony forming assay

Cell death was measured by sub-G_1/G_0 phase analysis of propidium iodide-stained cells using the Epics XL flow cytometer (Beckman Coulter Counter, Fullerton, CA, USA), as described previously [14]. Colony forming assay was performed as described previously [12].

2.5. Senescence-associated β-galactosidase (SA-β-Gal) activity staining

SA-β-Gal activity staining was performed using bromo-4-chloro-3-indolyl-β-D-galactosidase, as described previously [15] and a percentage of positively stained cells were analyzed statistically.

2.6. Immunoprecipitation

Cells were lysed in NP-40 lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 10% glycerol) containing protease inhibi-

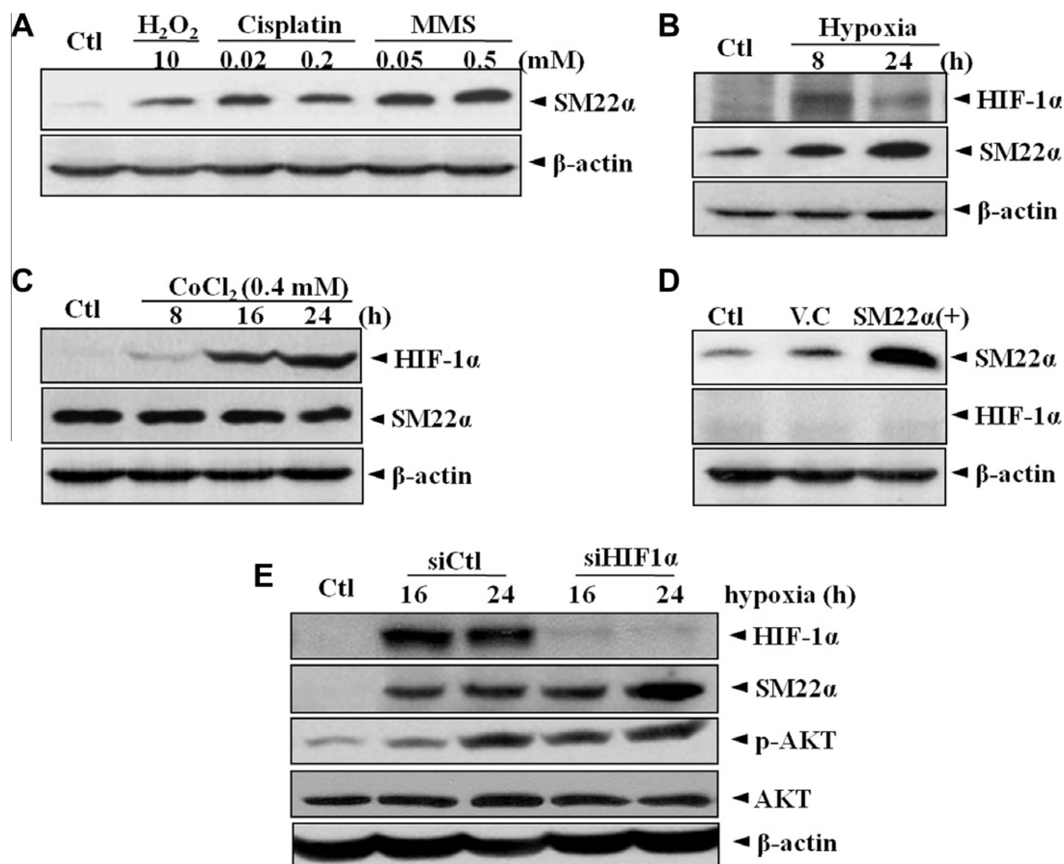

Fig. 1. Hypoxic stress increases SM22α in A549 NSCLC cells. (A) SM22α protein levels were analyzed in A549 cells treated with cytotoxic drugs such as H_2O_2 (10 mM), cisplatin (0.02 or 0.2 mM), or MMS (0.05 or 0.5 mM) for 6 h. (B) SM22α or HIF-1α protein levels were analyzed in A549 cells after exposing to hypoxia (1% O_2) for 8 or 24 h. (C) HIF-1α and SM22α protein levels were analyzed in A549 cells treated with a hypoxia-mimetic drug (0.4 mM $CoCl_2$) for 8, 16, or 24 h. (D) HIF-1α protein levels were analyzed in A549 cells transfected with SM22α expression vector (SM22α (+)) for 48 h. As transfection control, pcDNA3.1 (V.C.) was used. (E) SM22α protein levels were analyzed in A549 cells depleted of HIF-1α by RNA interference (siSM22α) when exposed to hypoxia (1% O_2). Hypoxic activation of AKT by phosphorylation was also showed. Cells in normoxia were compared as control (Ctl). Cells transfected with siRNA control (siCtl) were analyzed in parallel. Protein levels of β-actin were used as an internal control for protein loading.

tors (2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 mM ethylenediaminetetraacetic acid, 130 μM Bestatin, 1 μM leupeptin, 14 μM E-64, 0.3 μM Aprotinin). For immunoprecipitation, cell lysates containing 1 mg protein were incubated with 1 μg anti-IGF1Rβ antibody overnight at 4 °C. The antibody-protein complexes were then incubated with protein A/G plus agarose beads for 2 h at 4 °C, and the beads were pelleted by centrifugation at 2000×g for 2 min. The beads were washed three times with lysis buffer and analyzed by Western blotting using anti-SM22α and anti-IGF1Rβ antibody. For controls of non-specific immunoprecipitation, non-relevant rabbit IgG or anti-EGFR antibody were used in parallel. Cell lysates containing 20 μg proteins were analyzed by Western blotting.

3. Results

3.1. SM22α induction in A549 NSCLC cells under hypoxic condition

Most patients with NSCLC exhibit advanced disease and multi-drug chemotherapy resistance is common in NSCLC [16]. In our previous studies on the chemo-resistance of HepG2 hepatocellular carcinoma cells, we found that SM22α is induced in HepG2 cells by cytotoxic chemicals and the overexpression of SM22α confers chemo- and radiation-resistance [11]. To investigate whether the chemo-resistance of NSCLC cells is also associated with SM22α, its expression in A549 NSCLC cells was analyzed after treatment with cytotoxic chemicals. As shown in Fig. 1A, treatment with a sub-lethal dose of H_2O_2 increased SM22α expression. Cisplatin and MMS, typical cytotoxic agents used in cancer treatment, also elevated SM22α in a dose-dependent manner.

In clinical tumors, hypoxia also induces chemo-resistance [17]. Hypoxia protects A549 cells from drug-induced apoptosis in HIF-dependent or independent manner [18]. Therefore, we examined whether SM22α is also involved in hypoxia-induced cellular resistance of A549 cells. In A549 cells exposed to hypoxia SM22α was accumulated in a time-dependent manner, in which HIF-1α was also increased (Fig. 1B). To evaluate whether HIF regulates the expression of SM22α, A549 cells were treated with cobalt chloride, a chemical inducer of HIF-1α: however, SM22α protein levels were not changed (Fig. 1C). Overexpression of SM22α also did not increase HIF-1α (Fig. 1D), which suggests that hypoxia-inducible SM22α is not regulated by HIF-1α and that SM22α may act as an alternative regulator of survival pathway in hypoxia. To confirm the induction of SM22α irrespective of HIF-1α, HIF-1α-suppressed A549 cells were exposed to hypoxia and SM22α protein levels were analyzed. As shown in Fig. 1E, SM22α was induced by hypoxic stress regardless of HIF-1α. Moreover, SM22α was more elevated when HIF-1α was depleted. In these conditions AKT was also activated with or without HIF-1α, as previously reported [19].

3.2. Protective effects of SM22α against anti-cancer drugs and gamma-radiation in A549 cells

SM22α-overexpressing A549 cells (Fig. 2A) were challenged with various cytotoxic agents and cell death was measured (Fig. 2B). A sub-lethal dose of cisplatin (240 μM, 24 h) and MMS (1 mM, 24 h) in control cells (V.C.) caused severe cell death (48% by cisplatin and 85% by MMS); however, SM22α-overexpression resulted in reduction of cell death to approximately 20%. Cell death mediated by gamma-radiation was also diminished by SM22α-overexpression (50% in control cells but 9% in SM22α-overexpressing cells). These results indicate that induction of SM22α by hypoxia may be closely involved in cellular resistance of hypoxic cells against cytotoxic agents. Reversely, siRNA-mediated knockdown of SM22α sensitized the cells to a variety of damaging agents (Fig 2C). When control cells (siCtl) were treated with cisplatin

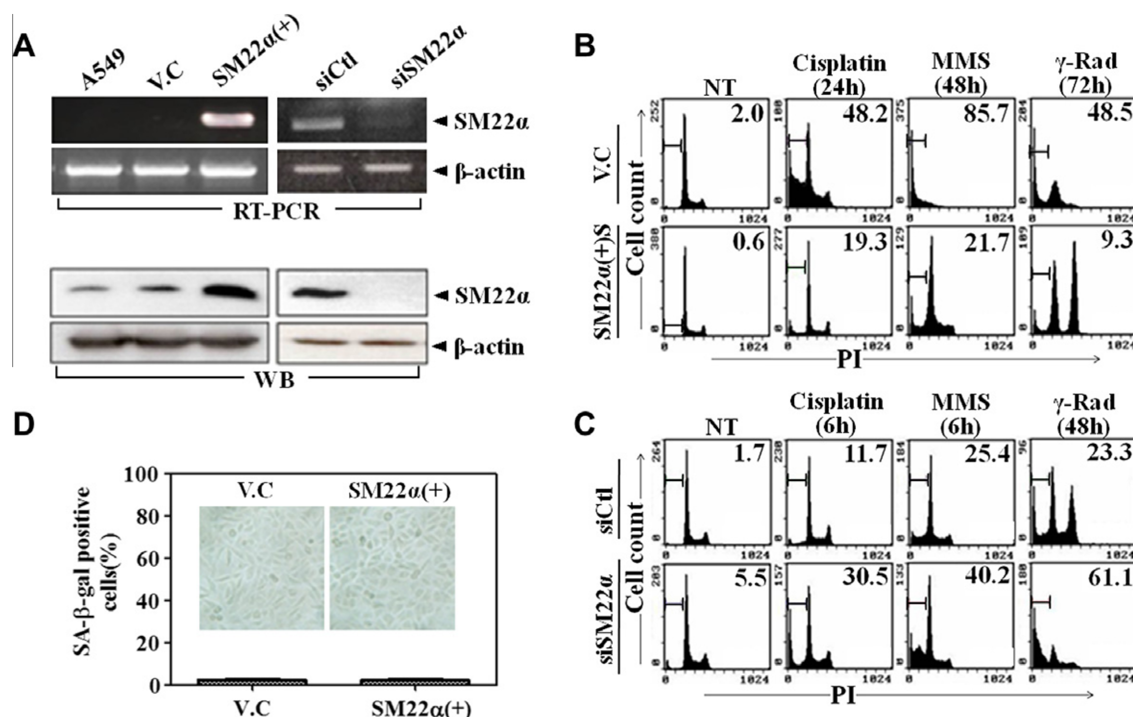

Fig. 2. SM22α is associated with the cellular resistance of A549 cells. (A) SM22α- overexpressing or suppressing A549 cells were prepared by transfection with SM22α-expressing vector, SM22α(+), or SM22α-targeted siRNA (siSM22α) for 48 h. For controls, cells transfected with empty pcDNA3.1 vector (V.C.) or siRNA control (siCtl) were analyzed in parallel. (B) SM22α-overexpressing A549 cells were exposed to cytotoxic reagents (240 μM cisplatin for 24 h, 1 mM MMS for 48 h) and then cell death was analyzed by flow cytometric analysis of propidium iodide-stained cells. Cell death induced by gamma-irradiation (20 Gy) was also analyzed at 72 h after irradiation. The percentage of cell death is indicated within the plot. (C) SM22α-depleted A549 cells were exposed to cytotoxic reagents (240 μM cisplatin for 6 h, 1 mM MMS for 6 h) and then cell death was analyzed. Effect of gamma-irradiation (20 Gy) was also analyzed 48 h after irradiation. (D) SA-β-Gal activity staining was performed on SM22α-overexpressing cells to evaluate cellular senescence.

(240 μM, 6 h) or MMS (1 mM, 6 h) percentage of cell death was 11.7% or 25.4%. However, when SM22α was depleted cell death was elevated approximately 2- or 3-fold (30.5% or 40.2%).

Chemo-resistance of HepG2 cells is associated with cellular senescence induced by SM22α overexpression [11,12]. SM22α overexpression severely inhibited cell growth and activated the $p16^{INK4a}$/pRB senescence signaling pathway. Moreover, the expression of several metallothionein isoforms, such as MT1G, increased dramatically. In A549 cells, however, SM22α overexpression did not induce cellular senescence, which was validated by SA-β-Gal staining (Fig. 2D). In addition, the expression of metallothionein isoforms did not change (Supplementary Fig. 1). Although SM22α overexpression induces chemo-resistance in HepG2 and A549 cells, its regulatory mechanisms in HepG2 and A549 cells are different.

3.3. SM22α overexpression in A549 cells activates the PI3K/Akt pathway

In contrast to HepG2 cells, SM22α overexpression in A549 cells increased cell growth (Fig. 3A). The colony forming ability of SM22α-overexpressing A549 cells was higher than control cells by approximately twofold. SM22α-knockdown resulted in the opposite effect (Fig. 3B).

The PI3K/Akt pathway is a key regulator of cancerous growth and induction of hypoxia-related proteins [20]. It is also associated with radiation-resistance. Activation of this pathway can occur as a result of stimulation of receptor tyrosine kinases, such as EGFR or vascular endothelial growth factor receptor. Moreover, mutation or overexpression of PI3K or Akt itself, which are frequently found in NSCLC, can also lead to activation of the PI3K/Akt pathway. Because SM22α was associated with chemo- and radiation-resistance and its overexpression stimulated cell growth in A549 cells, we examined whether PI3K/Akt pathway is activated directly by SM22α. Overexpression of SM22α led to accumulation of phosphorylated Akt and SM22α-knockdown resulted in the opposite effect (Fig. 3C). However, EGFR, a typical receptor tyrosine kinase responsible for Akt activation, was not changed. In addition, the levels of PI3K-p110 and K-ras, which are all upstream regulators of Akt, were not influenced. PTEN, an inhibitor of the PI3K pathway, was also unchanged, indicating that Akt in these cells is modulated by other signaling molecules. Expression of components of the PI3K/Akt pathway and SM22α were also examined in A549 cells exposed to hypoxic conditions (Fig. 3D). In hypoxic A549 cells, SM22α and phosphorylated Akt were elevated, while phosphorylated EGFR and PTEN remained unchanged as in SM22α-overexpressing cells.

3.4. SM22α overexpression in A549 cells activates the IGF1R/PI3K/Akt pathway by direct interaction with IGF1Rβ

IGF1R is another upstream regulator of Akt and an important receptor tyrosine kinase in NSCLC [21]. Hypoxic A549 cells, in which SM22α was highly increased, showed dramatically elevated phosphorylation of IGF1Rβ (Fig. 4A). Hypoxic conditions lead to the

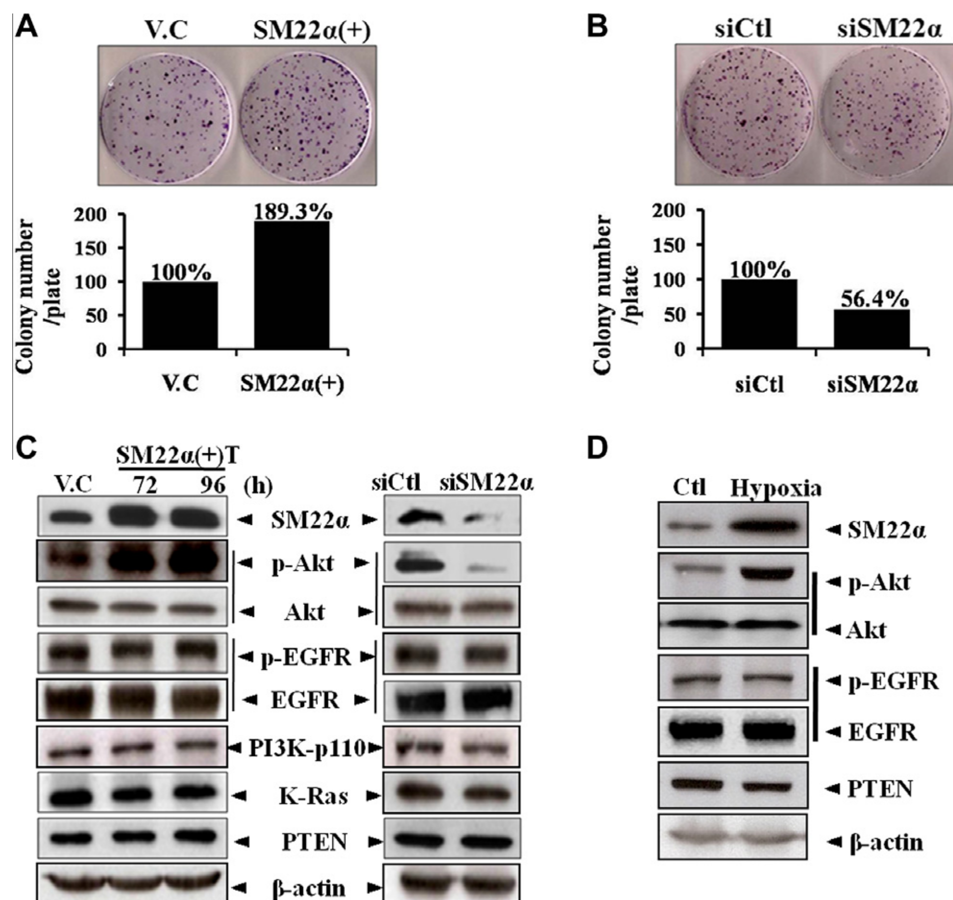

Fig. 3. SM22α overexpression enhances the growth of A549 cells, which is mediated by Akt activation. (A) Colony-forming assays were performed for A549 cells transfected with SM22α-expressing or control vector. Clonogenic survival of tested cells was expressed as a percentage relative to transfection control. (B) For A549 cells transfected with siRNA-targeted against SM22α, colony-forming assay was also performed. (C) Cells transfected with control or SM22α-expressing vectors were harvested at 72 or 96 h post-transfection and Western blot analysis was performed on indicated proteins. A549 cells transfected with siRNA against SM22α were also analyzed at 48 h post-transfection. (D) SM22α and phosphor-AKT protein levels were analyzed in A549 cells exposed to hypoxic conditions (1% O_2) for 24 h.

regulation of numerous cellular events. To examine the direct relationship between increased SM22α and phosphorylation of IGF1Rβ and Akt, their phosphorylations were analyzed in SM22α-overexpressing A549 cells. Fig. 4B shows that IGF1Rβ phosphorylation was increased only by SM22α-overexpression. Moreover, Akt activation in SM22α-overexpressing A549 cells was inhibited completely by AG1024, a specific inhibitor of IGF1R kinase activity (Fig. 4C), which suggest that Akt activation by SM22α is mediated by IGF1R auto-phosphorylation.

Additional evidence of activation of IGF1R by SM22α was obtained using a point mutant of SM22α. While creating the SM22α-overexpression vector, we obtained a point mutant of SM22α gene in which the native A residue at nucleotide position 266 was changed to G (Supplementary Fig. 2). This mutation altered the Lys residue (codon: AAG) at amino acid position 89 to Glu (codon: GAG). Growth of A549 cells transfected with this SM22α-mutant ($SM22\alpha^{L89E}$) was indistinguishable from that of non-transfected cells. In addition, cellular resistance against cytotoxic chemicals or radiation was not observed in these cells (Supplementary Fig. 3). $SM22\alpha^{L89E}$ also did not activate Akt or IGF1Rβ phosphorylation (Fig. 4D). Based on these results, we concluded that SM22α is important for IGF1Rβ activation and Lys89 of SM22α protein is critical for this activation.

In general, IGF1R is activated by specific ligands (e.g., IGF-I, IGF-II) in the extracellular space. However, the activation mode of IGF1R by SM22α, a cytoskeletal protein that interacts with actin, may be different from that of specific ligands. One of possible activation mode is direct interaction between SM22α and the intracellular domain of IGF1R, which induces auto-phosphorylation of IGF1R. Co-immunoprecipitation of SM22α from A549 cell lysates using an anti-IGF1Rβ antibody confirmed our hypothesis. As shown in Fig 5, SM22α was co-immunoprecipitated with IGF1Rβ in SM22α-overexpressing cells and IGF1Rβ was phosphorylated (Fig. 5A). However, $SM22\alpha^{L89E}$ was not co-immunoprecipitated with IGF1Rβ despite of its overexpression (Fig. 5B). Finally, we confirmed that hypoxia-induced SM22α was coimmunoprecipitated with IGF1Rβ, which was phosphorylated (Fig. 5C), revealing that SM22α is directly involved in hypoxia-induced IGF1R activation.

Although the underlying activation mechanisms remain to be identified in future studies, our findings clearly demonstrate that SM22α overexpressed in A549 cells induces activation of the IGF1Rβ/PI3K/Akt pathway via direct interaction with IGF1Rβ, leading to cellular resistance against apoptotic cell death caused by hypoxic stress or cytotoxic agents. This study provides the first demonstration of hypoxic regulation of SM22α and also suggests that SM22α is a novel regulator that positively modulates the IGF1Rβ/PI3K/Akt pathway in A549 cells.

4. Discussion

SM22α was originally isolated and characterized from chicken gizzard smooth muscle [22] and has been established as a transformation- and cell shape-sensitive actin cross-linking/gelling protein [7]. Human SM22α cDNA was cloned during a study that identified genes involved in replicative senescence, and was suggested as a marker for senescence [23]. Thus, initial studies on SM22α had focused on its functions in muscle and senescent cells. Recent studies have revealed novel functions for SM22α in cells other than smooth muscle or fibroblasts. SM22α has been identified as the first ARA54-associated negative modulator for androgen receptor in prostate cells [24]. The mitochondria-associated apoptosis pathway was activated after transfection of SM22α into LNCaP cells and interaction between SM22α and p53 was confirmed by co-immunoprecipitation in these cells [25]. SM22α has been also suggested

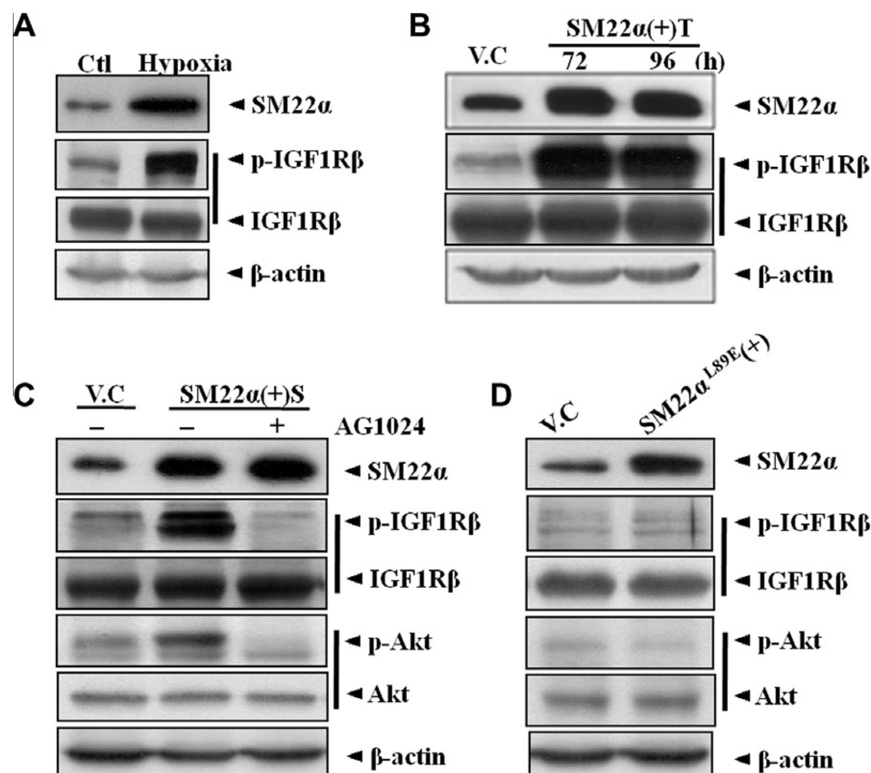

Fig. 4. SM22α-overexpression in A549 cells activates the IGF1Rβ/Akt pathway. (A) A549 cells exposed to hypoxic conditions (1% O_2, 24 h) were analyzed by Western blot of phospho-IGF1Rβ and IGF1Rβ. (B) Phospho-IGF1Rβ and IGF1Rβ protein levels were also analyzed in SM22α-overexpressing A549 cells at 72 h or 96 h post-transfection. (C) Stable transfectants of SM22α (SM22α(+)S) were treated with AG1024 at 10 μM for 24 h, and then analyzed on the status of IGF1Rβ and Akt phosphorylation. (D) An SM22α mutant-expressing ($SM22\alpha^{mut}$) or control vector was transfected into A549 cells for 72 h and the cells were analyzed by Western blot for phosphorylated IGF1Rβ and Akt.

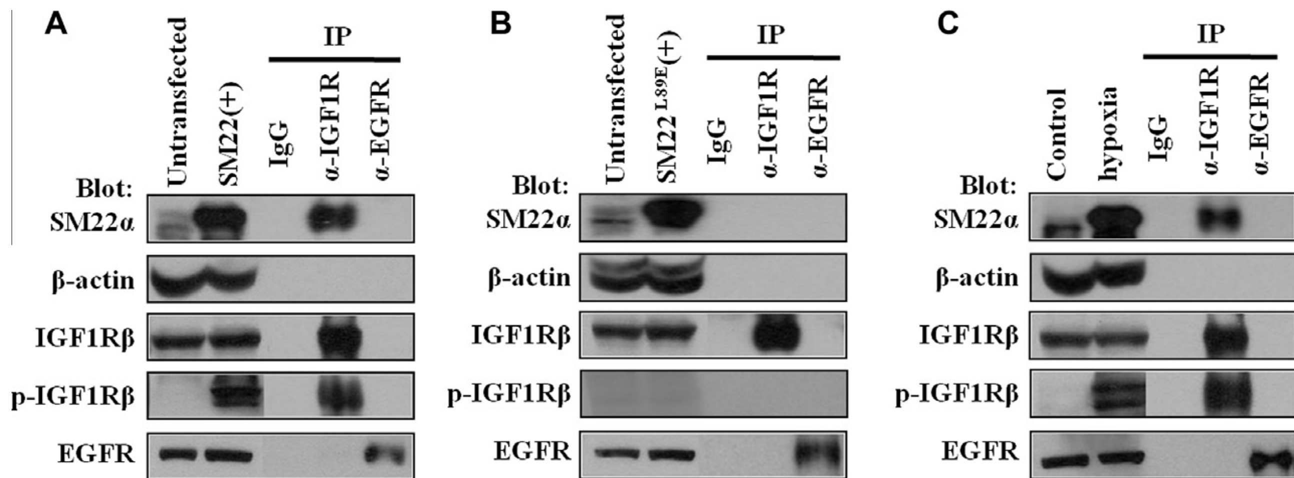

Fig. 5. SM22α in hypoxic A549 cells co-immunoprecipitates with IGF1Rβ. Immunoprecipitates with anti-IGF1Rβ antibody from (A) SM22α-overexpressing cells, (B) SM22α^{mut}- expressing cells, or (C) hypoxic A549 cells were analyzed by Western blot with anti-SM22α or anti-IGF-1Rβ antibodies. Immunoprecipitates with anti-EGFR or rabbit IgG were used as non-specific controls for immunoprecipitation. Cells un-transfected or in normoxia were used as controls for Western blot. 20 μg of total cell lysate was loaded in each lane and analyzed.

as a tumor suppressor. Proteomic analysis revealed that its expression is lost in prostate, breast, colon, and lung cancer [10,26], which is consistent with SM22α-mediated suppression of MMP-9 [27]. In recent studies, we demonstrated SM22α as an inducer of cellular resistance to cytotoxic drugs or radiation. SM22α-overexpressing HepG2 cells became resistant to apoptotic cell death caused by cytotoxic agents, in which metallothionein isoforms, especially MT1G, were significantly induced [11]. These cells were in a state of growth arrest and showed elevated p16^{INK4a} followed by pRB activation, which promotes cellular senescence caused by treatment with a subclinical dose of gamma-radiation or doxorubicin [12]. SM22α overexpression in HepG2 cells also inhibited the activation of IGF1Rβ/Akt and extracellular signal-regulated kinase, thereby suppressing cell proliferation [11].

Here, we examined the functions of SM22α associated with chemo- or radiation-resistance in NSCLC cells, where multi-drug chemotherapy resistance is an obstacle to cancer treatment [17]. Similar to HepG2 cells, cytotoxic drugs induce SM22α in A549 NSCLC cells, in which basal SM22α expression is very low. SM22α-overexpressing A549 cells exhibit dramatic resistance to anticancer drugs and gamma-radiation. More importantly, we have demonstrated that hypoxic conditions, which can be a direct cause of therapeutic resistance [17], also elevate SM22α expression in a HIF-1α-independent manner. However, the mechanism of how SM22α induces chemo-resistance in A549 cells seems to be different from that in HepG2 cells. SM22α-overexpression in HepG2 cells induces growth arrest and inhibits IGF1Rβ/Akt activation. On the contrary, in A549 NSCLC cells, SM22α overexpression enhances cell growth and IGF1Rβ auto-phosphorylation, which is mediated by direct interaction of SM22α with IGF1Rβ. Schnizer et al. reported that hypoxia protects A549 cells from drug-induced apoptosis [18]. They also suggested that cell protection mechanisms under hypoxic conditions are HIF-dependent or -independent. Here, we suggest that SM22α is an important factor in HIF-independent hypoxic response of A549 cells and is a novel regulator of IGF1Rβ activation.

Patients with NSCLC exhibit advanced disease and their resistance to chemo- or radiotherapy makes cancer treatment less effective. In addition, patient's responses to these cancer therapies vary, which makes predictive biomarkers for therapeutic response be an important facet of personalized medicine. In this aspect, it might be an important factor for decision of cancer therapy whether SM22α is induced by chemical or radiation treatment. Also, if a medical treatment can be developed that can modulate SM22α expression during hypoxia or aggressive lung cancer, the efficiency of cancer therapy will be improved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.12.036.

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