Chapter 2

Roles of SRF in Endothelial Cells During Hypoxia

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Additional information is available at the end of the chapter

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1. Introduction

Oxygen is a basic need for human life. Maintaining adequate oxygen supply is essential for proper cellular functions. In normal tissue, the oxygen supply usually matches metabolic requirements, and even if there is a brief oxygen shortage, the body can overcome it by an increase in the oxygen extracted from the blood or an increase in local blood flow. In advanced solid tumors, however, due to uncontrolled cell proliferation, the oxygen consumption rate often exceeds the oxygen available around the area, resulting in local hypoxia. The diffusion distance from blood vessels to surrounding tissues is usually no more than 100-200 μm; therefore, the further into the center of the tumors, the lower the oxygen level gets. As measured by Eppendorf probe, pO2 in normal tissue is between 17 and 65 mm Hg, while in wide range of tumors, pO2 can go down to 2 mm or even to zero.

As a result of oxygen deficiency, two things can happen to the suffering cells. Cells can either stop proliferation and die of apoptosis or necrosis, or fight back by taking adaptive processes that lead to increased proliferation, migration and tissue reorganization. While the ultimate fate of the cells varies with tissue type, the severity and duration of hypoxia play critical roles in choosing the direction. In moderate oxygen decline (~ 2-7 mm Hg), the cells in oxygen starvation and the cells carrying oxygen (red blood cells) run towards each other. Cancer cells can move away from their original locations to where oxygen is sufficient, while endothelial cells in the blood vessels can also take an action to move out to form new vessels to bring oxygen towards the center of hypoxia. The former process is known as metastasis, and the latter is angiogenesis. Angiogenesis and metastasis support cancer cells to survive through hypoxic crisis and allow malignant progression. Under severe hypoxic condition (< 1 mm Hg), however, cells are prone to die of apoptosis if glycolytic ATP available, otherwise, die of necrosis.

Hypoxia-induced apoptosis proceeds through the mitochondrial pathway, as the mitochondria are the primary site of oxygen consumption in a cell. Under normoxic conditions, the mitochondria consume about 90% of available oxygen in the generation of ATP through oxidative phosphorylation in order to meet the metabolic needs of the cell [1, 2]. When there is not sufficient oxygen to support this process, mitochondrial damage occurs, which leads to apoptotic cell death.

To live or to die for a cell under hypoxia is all regulated through different expression and activation of transcription factors. A number of transcription factors have been reported to respond to oxygen deficiency, including AP-1 [3], FOS [4], JUN [4], CREB/ATF [5], DEC1 [6], EGR1 [7], ETS1 [8], GADD153 [9], GATA2 [10], MASH2 [11], NF-IL-6 [12], NFκB [13], RTEF-1 [14], SMADs [15], SP1 [16], STAT5 [17], and of course, the most popular ones, HIF [18] and p53 [19].

2. Hypoxia inducible factor

Hypoxia inducible factor (HIF) is the best studied transcription factor in hypoxia. Whenever there is a discussion about hypoxia, HIF is always an inevitable topic. HIF is composed of two subunits, α and β. While HIFβ is constitutively expressed, HIFα functions more like an oxygen sensor, varying in response to oxygen level [20]. HIFα has an extremely short half-life under normoxic conditions due to ubiquitination by von Hippel-Lindau factor (VHL). Hypoxia does not change HIFα expression per se but stabilizes it by inhibiting hydroxylation at prolines 402 and 564 so that VHL can no longer bind to HIFα to cause proteasomal degradation. Instead, it enables HIFα to bind to HIFβ in the nucleus, generating a functional heterodimeric transcription factor that is able to activate genes that contain hypoxia-response elements (5′-RCGTG-3′), such as genes coding for glucose transporters, vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS), and erythropoietin (EPO) [21, 22]. In normal tissue, the expression of such genes is to counteract the detrimental impact of hypoxia and to help cells to survive through oxygen crisis. In cancer, however, this role of HIF is abused to support tumor growth and resistance to chemotherapy. Up to date, there are three members in HIF family. HIF-1α is most ubiquitously expressed, while HIF-2α, which shares 48% identity and similar functions with HIF1α, is more restricted to endothelial cells [23]. HIF-3α is the least characterized but may function as a negative regulator of hypoxia, as its dimer with the β subunit has no transcriptional activity [24].

The most prominent role of HIF during hypoxia is to support angiogenesis through transcriptional activation of VEGF. VEGF belongs to a family that contains VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta-like growth factor. VEGF-A, the first growth factor that was identified to have special effects on endothelial cells, further splits into five isoforms. VEGF is mainly produced by endothelial cells, macrophages, fibroblasts, and smooth muscle cells. It promotes endothelial cell migration, proliferation and survival through its receptors, VEGFR-1 (Flt-1) and/or VEGFR-2 (Flk-1/KDR), which are pre-

dominantly expressed on endothelial cells [25]. In addition, VEGF can also bind to three other transmembrane proteins: VEGFR-3 (Flt-4), which is expressed mainly on lymphatic endothelial cells and only responds to VEGF-C and -D, Neuropilin-1 and Neuropilin-2, which work as co-receptors with VEGFR-2 [26]. Hypoxia-induced VEGF up-regulation is considered to be the major driving force for angiogenesis during tumor progression [27]. Tremendous effort has been made in cancer chemotherapy to inhibit this process and has achieved some significant results, but some expectations have not been met. In addition to VEGF, HIF also regulates several other angiogenic factors such as placenta-like growth factor, platelet-derived growth factor, angiopoietin-1 and -2 [28].

3. p53

Like HIF, p53 is expressed at a low level under normal oxygen conditions and degraded constantly by MDM2 through ubiquitination [29, 30]. Under cellular stress like hypoxia, however, ATM/ATR kinases become active and phosphorylate p53 at its N terminus, which disrupts its interaction with MDM2 and thus, p53 becomes stabilized and moves into the nucleus to activate pro-apoptotic genes [31]. As mentioned above, hypoxia induces apoptosis through mitochondrial damage. The mitochondrial integrity is guarded by Bcl-2 family proteins which include anti-apoptotic members like Bcl-2 and Bcl-X_L, and also pro-apoptotic members, such as Bax and Bak. The balance between these two teams is critical to the fate of a cell. Bcl-2 is an integral membrane protein that targets the outer mitochondrial membrane, and it can form homodimers with each other or heterodimers with Bax. Bax, on the other hand, can do the same. When Bcl-2 predominates, mitochondria stay intact and cells are protected. However, while Bax is in excess, Bax homodimers become dominant, the cells are susceptible to apoptosis. Bax expression is regulated by p53; therefore, p53 activation increases the ratio of Bax to Bacl-2 and reduces the chance of Bcl-2 and Bax association. It has been postulated that 50% reduction in the formation of Bcl-2/Bax complexes can drive the cells toward apoptosis [32]. When Bax inserts into the outer mitochondrial membrane, it opens pores to allow the molecules sequestered in between outer and inner mitochondrial membrane to leak out into the cytosol. One of the released molecules is cytochrome c, which can bind to the apoptotic protease activating factor-1 (APAF-1] and promote it to form an apoptosome. The apoptosome then binds caspase-9 and activates it to cleave two other caspases, caspase-3 and -7. These two caspases orchestrate apoptosis through cleavage of key substrates within the cell, resulting in cell death.

p53 and HIF1α are an odd couple, one supporting cell death and the other supporting cell survival. These two transcription factors can interact with each other directly because HIF1α contains two p53-binding sites within its ODD domain [33]. Unlike HIF, p53 appears to be less sensitive to oxygen level change. Under moderate hypoxic conditions, HIF1α binds to HIF1β to activate genes that promote cell survival, while p53 still remains inactive. Some *in vitro* studies even showed that in such a situation p53 actually promotes HDM2-mediated

HIF1α degradation [34]. Under severe oxygen poverty, however, HIF1α becomes dephosphorylated and may choose to help p53 to induce cell death [35].

4. Hypoxia activates SRF

Although many transcription factors have been studied extensively under hypoxia [36], the reaction of serum response factor (SRF) to oxygen shortage has rarely been discussed.

SRF regulates numerous genes that are involved in cellular responses to mitogenic stimuli as well as cellular stress [37-39]. These genes fall into many diversified categories, including immediate early genes (FOS, EGR1, etc.), cytoskeletal genes (ACTB, CFL1, DES, DSTN, TTN, KRT17, etc.), muscle-related genes (ACTA2, MYH6, MYH11, SM22α, TNNT1, ATP2A1, etc.), growth factors (IGF2, FGF10, FGFR3, TGFB1i1, etc.), extracellular matrix proteins (CCN1, CTGF, etc.), cell adhesion molecules (ITGA1, ITGA5, ITGB1, etc.), intercellular junctional molecules (TJP1, CDH5, CDH11, etc.), neuronal receptors (NR4A1, NR4A2, etc.), and apoptosis regulators (BCL2). This list is still growing. All these genes contain a common DNA sequence, $CC(A/T)_6GG$, so-called CArG box or serum response element (SRE), which SRF recognizes. Some of these genes contain multiple CArG boxes, for example, EGR1 has six and CCN1 has five, and even SRF itself has four SRE sequences [40], indicating a tight regulation by SRF. In addition to the hundreds of genes that SRF directly regulates, a growing number of genes that do not contain SRE have been found to respond to SRF activition [41, 42].

Hypoxia is a form of stress to the cells; therefore, it triggers a response from SRF undoubtedly. As shown in Figure 1, under hypoxic condition, there is not only an increase in the level of SRF expression (Figure 1A), but also an increase in SRF phosphorylation (Figure 1A), which enhances SRF binding activity to SRE (Figure 1B). Moreover, this activation of SRF is independent from either HIF or p53, because neither shut down of HIF with its specific inhibitor Dimethyl Bisphenol A (DBA) (Figure 1C), nor inhibition of p53 with Pifithrin-α (Figure 1D) has impact on hypoxia-induced SRF activation.

5. SRF supports hypoxia-induced angiogenesis

Previously, we have shown that SRF is required for VEGF-induced *in vitro* angiogenesis, and without SRF, VEGF cannot induce endothelial cell proliferation and migration, which are essential for angiogenesis [43]. Our findings were confirmed and extended later by an *in vivo* study on mouse embryonic development, which demonstrated that knockout of SRF in endothelial cells impairs sprouting angiogenesis from arteries to veins [44]. Transcriptional analysis showed that SRF deficiency not only had negative impact on genes responsible for endothelial connection (e.g. VE-cadherin) and adhesion (e.g. integrin α5 and β1), but also suppressed angiogenic factors like VEGF and angiopoietin-1 and -2.

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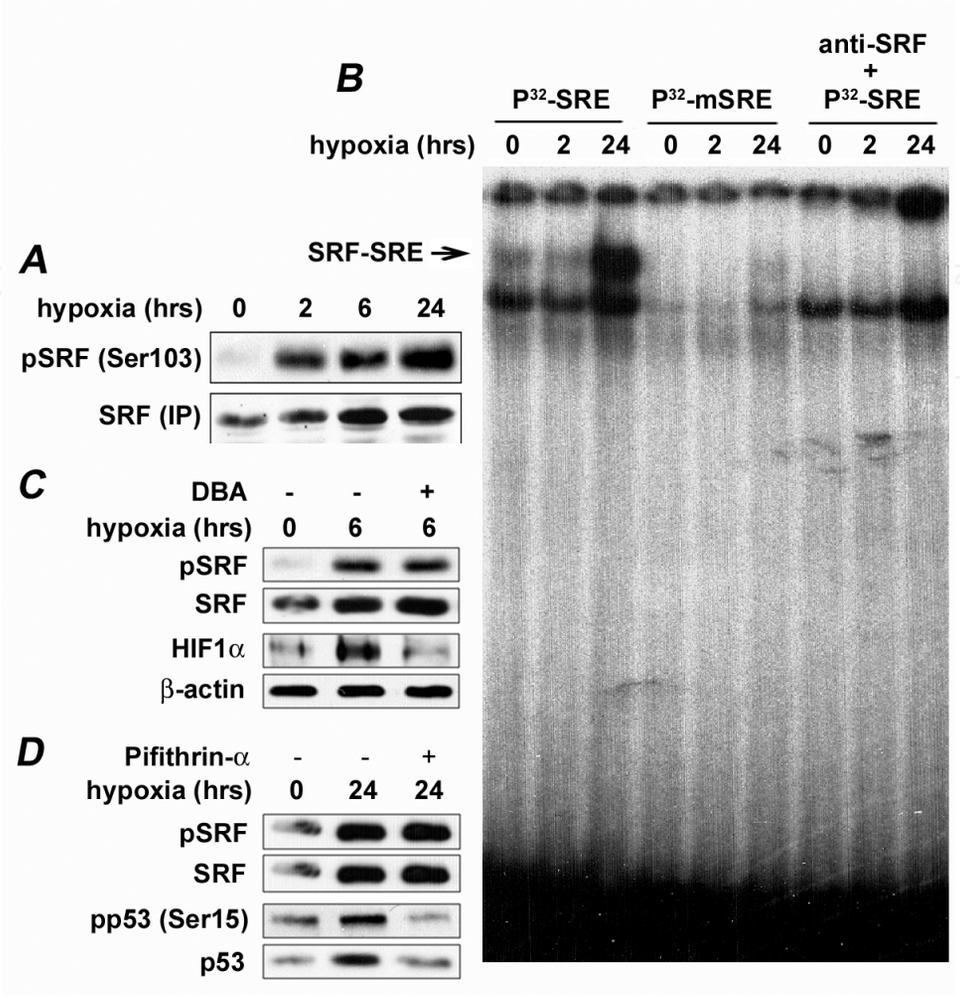

Figure 1. Hypoxia activates SRF in mouse brain endothelial cells (bEND3) regardless HIF and p53 status. A. Cells were cultured in a hypoxic chamber (5% CO_2 : 94% N_2 : 1% O_2) at 37˚C for 2, 6 and 24 hours. Total protein was isolated and immune-precipitated with an antibody against SRF. Total and phosphorylated SRF were detected by Western blot analysis. B. SRF protein activity was analyzed by electrophoretic mobility shift assay with P^{32}-labeled consensus SRE (SRE) and mutant SRE (mSRE) oligos. SRF to SRE binding activity was increased by hypoxic treatment. The lack of binding ability to the mutant SRE probe as well as the super shift with the SRF antibody (anti-SRF) confirmed the binding specificity. C. In the presence of Dimethyl Bisphenol A (DBA), a specific inhibitor for HIF, hypoxic treatment failed to stabilize HIF1α, but did not affect SRF activation. D. Incubation with p53 inhibitor Pifithrin-α suppressed p53 activation by hypoxia but did not affect SRF either.

Here we show that hypoxia-induced angiogenic activity in brain endothelial cells is completely lost when SRF is knocked down by RNA interference (Figure 2), indicating that SRF is essential to hypoxia-induced angiogenesis. On the other hand, when extra copies of SRF gene are introduced into these cells, hypoxia-induced angiogenic activity is enhanced. It has been postulated that hypoxia induces angiogenesis through HIF-VEGF-MAPK/Rho-SRF pathway [45]. From our previous study [43], we know that VEGF does activate SRF through MAPK and Rho pathways. However, this is just one side of a coin. As shown above (Figure 1), the increase of VEGF signaling during hypoxia is due to HIF activation, while hypoxia activates SRF independently from HIF and therefore, independently from VEGF as well. SRF responds to hypoxia directly as other transcription factors like HIF and p53. In addition, SRF also serves

as a downstream regulator in cell proliferation, adhesion and migration, thus any mitogenic factor that aims to stimulate such cellular activities requires SRF involvement.

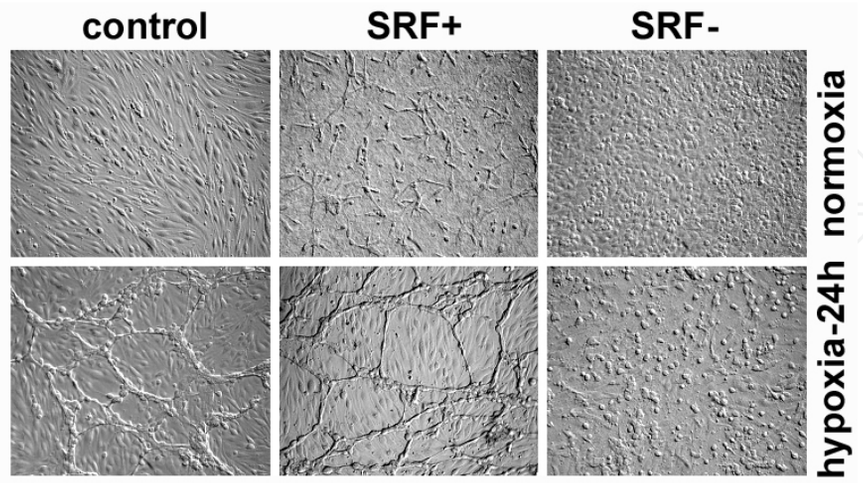

Figure 2. Knockdown of SRF in brain endothelial cells (bEND3) prevents hypoxia-induced angiogenesis. bEND3 cells were cultured in collagen gel matrix under a hypoxic condition. The collagen gel matrix was made of 50% type I collagen in HEPES (pH 8.5) Hanks buffer balanced growth medium. The mixture was solidified in 12-well plates at 37˚C for 20 minutes. Cells were mixed in the liquid gel, plated on top of the solidified gel in the 12-well plates and incubated at 37˚C for additional 20 minutes. More layers of cells were plated in the wells by repeating this step. Eventually, growth medium was added to the top of the solidified gel containing endothelial cells and the plates were incubated at 37˚C for a week. The control cells formed a cobblestone monolayer at the end, while SRF over-expressing cells (SRF+) moved vertically and horizontally within the gel matrix. The cells with SRF knockdown (SRF-), on the other hand, stayed inactively. Under hypoxia, both control and SRF+ cells formed cable-like structure, an indication of angiogenic activity, while the SRF- cells showed sign of death.

6. SRF protects endothelial cells against hypoxia-induced apoptosis

Several studies indicate that hypoxia-induced apoptosis is solely dependent on the mitochondrial pathway [46-48], which is regulated by Bcl-2 family members [49, 50]. Hypoxia induces an increase in the ratio of the pro-apoptotic protein Bax to the anti-apoptotic protein(s) Bcl-2 and/or Bcl-X_L, thereby increases mitochondrial permeability and enables release of cytochrome c to cytoplasm [51]. Cytochrome c released into the cytoplasm forms complexes with Apaf-1 and triggers a caspase cascade to execute apoptotic cell death [52, 53]. It has been demonstrated in neuronal cells that hypoxia-induced Bax expression and DNA fragmentation are mediated through induction of nitric oxide (NO) [54, 55]. NO in endothelial cells is generated by both the endothelial and inducible isoforms of nitric oxide synthase (eNOS and iNOS) via oxidation of the substrate, L-arginine. Hypoxia can induce both iNOS and eNOS expression because the iNOS gene promoter has the hypoxia response element for HIF1 [56, 57] and the eNOS gene promoter has binding sites for HIF2 [58]. NO has a dual action on the vascular endothelium: at low concentrations (nM), as are present under basal conditions, it protects cells against apoptotic stimuli [59]. When its levels become elevated (μM), as in the case of severe ischemia/hypoxia, NO also initiates apoptosis in both endothelial and non-endothelial cells [60, 61].

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Activation of eNOS, iNOS and SRF is dependent on Rho GTPase-regulated actin dynamics. Actin de-polymerization activates eNOS [62, 63] and iNOS [64, 65] but suppresses SRF, resulting in apoptosis [66, 67]. Conversely, actin polymerization activates SRF but suppresses eNOS and iNOS, supporting cell survival.

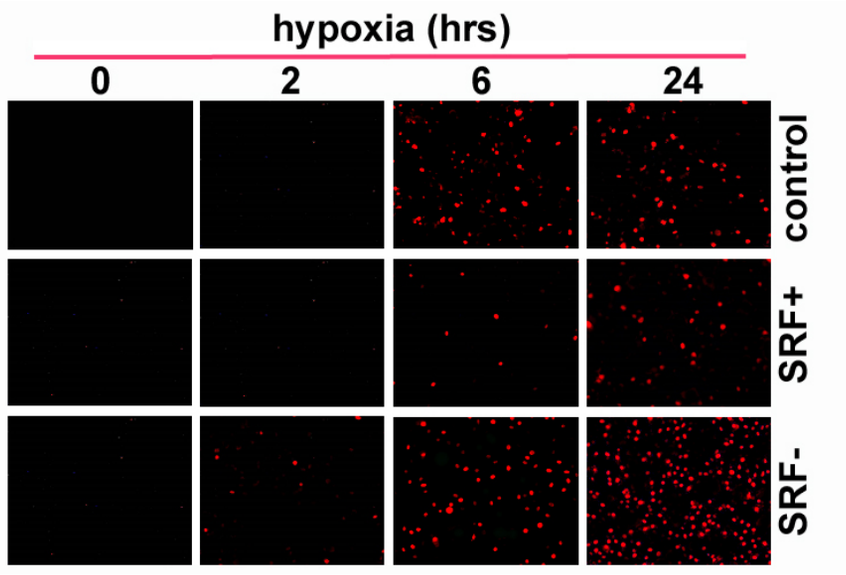

Figure 3. Knockdown of SRF in brain endothelial cells increases hypoxia-induced apoptosis. bEND3 cells were cultured on cover slips under a hypoxic condition for 2, 6 and 24 hours. TUNEL assays were performed to detect apoptosis. Apparently, overexpression of SRF (SRF+) promoted cell survival, while knockdown of SRF (SRF-) made cells more vulnerable to hypoxic damage.

Moderate hypoxia induces cell adaptation but not apoptosis. However, when SRF is insufficient (SRF-), cells become vulnerable to cellular stress and even a brief oxygen shortage can trigger apoptotic cell death (Figure 3). On the other hand, forced overexpression of SRF (SRF +) in these cells can make them more resistant to hypoxic damage and able to survive through even more harsh oxygen crisis. The advantage of SRF over HIF is its broad involvement in the molecular regulation of the cell machinery. Once SRF is activated, it not only promotes cell survival through up-regulation of growth factors and cytoskeletal components, but also protects mitochondrial integrity through up-regulation of anti-apoptotic proteins like Bcl-2 [68]. In another word, SRF supports cell survival at multiple levels. Up-regulation of growth factors stimulates cell proliferation and migration, which require adequate supplies of cytoskeletal proteins, because without cytoskeleton to provide the platform, cells cannot proliferate or migrate, and SRF makes sure these molecules available at the time of need. Finally, SRF also makes sure mitochondria intact so that they can provide the energy that cell proliferation and migration need. Mitochondrial integrity depends on the balance between pro-apoptotic and anti-apoptotic proteins, typically, BAX versus Bcl-2. Severe hypoxia activates p53, which drives up-regulation of BAX, pushing cells toward apoptosis, as BAX gene contains four binding sites for p53. On the other hand, hypoxia also activates SRF (as shown above), which drives up-regulation of Bcl-2, pushing cells toward survival, as Bcl-2 gene contains two SREs in its promoter [68]. The BAX and Bcl-2 fight turns into a wrestle between

p53 and SRF. As shown in Figure 4, manipulation of SRF expression can change BAX/Bcl-2 ratio, and ultimately, change the fate of the cells under hypoxia.

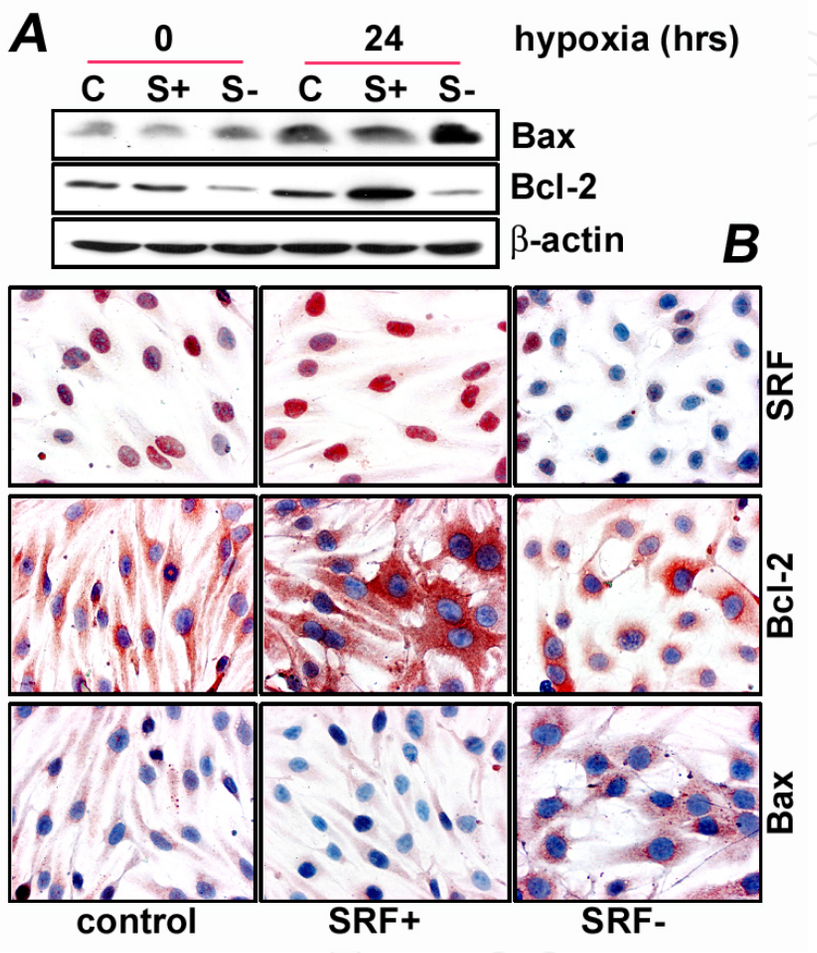

Figure 4. SRF promotes Bcl-2 but suppresses Bax. A. Western blot analysis showed an increase of Bax and a decrease of Bcl-2 in bEND3 cells due to SRF deficiency. B. Immunocytochemistry showed a similar effect.

The impact of SRF on mitochondrial integrity during hypoxia is not only reflected at the molecular level, but it can also be visualized at the subcellular level. As shown in Figure 5, incubation of brain endothelial cells under a hypoxic condition induces mitochondrial leakage, as reflected by the color change of a fluorescent dye. The longer the hypoxic exposure goes, the fewer intact mitochondria exist. However, forced overexpression of SRF in these cells can reverse the effect of hypoxia, protecting mitochondria against hypoxic damage. Conversely, knockdown of SRF can lower the threshold of mitochondrial tolerance to oxygen deprivation, so that a short hypoxic exposure can cause a massive mitochondrial leakage.

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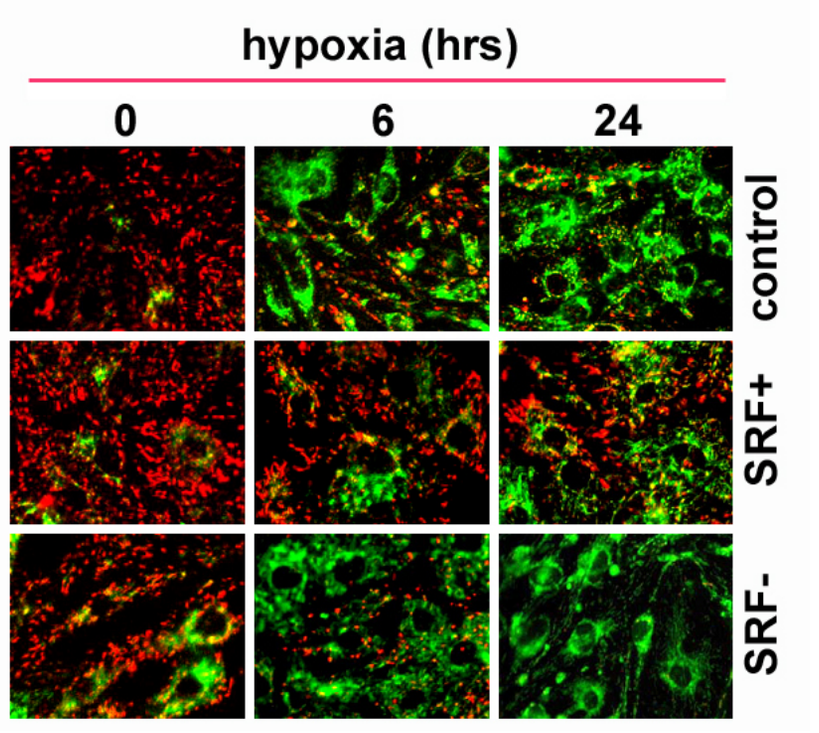

Figure 5. Knockdown of SRF in brain endothelial cells increases mitochondrial permeability during hypoxia. bEND3 cells were cultured on cover slips under a hypoxic condition and stained with a cationic dye. The dye fluoresces differently in healthy vs. apoptotic cells. In healthy cells, the dye accumulates and aggregates in the mitochondria, giving off a bright red fluorescence. While in apoptotic cells, the dye cannot aggregate in the mitochondria due to the altered mitochondrial transmembrane potential, and thus it remains in the cytoplasm in its monomer form, fluorescing green.

Mitochondrial permeability is reflected by the opposite movement of BAX and cytochrome c. Normally, BAX remains in the cytoplasm at a low level, while cytochrome c hides in between the inner and outer membranes of the mitochondria. When cells suffer from an oxygen shortage, BAX jumps, moving toward mitochondria. The insertion of BAX into the outer mitochondrial membrane opens pores to let cytochrome c leak out. Cytoplasmic cytochrome

c binds to Apaf-1 and triggers caspase cascade, leading to apoptotic cell death. During this event, the level of SRF is a determining factor for the fate of the cell. As illustrated in Figure 6, as oxygen crisis prolongs the opposite movement of BAX and cytochrome c increases, and cells prone to die. Manipulation of SRF level can either facilitate this process or reverse it, depending on what we desire.

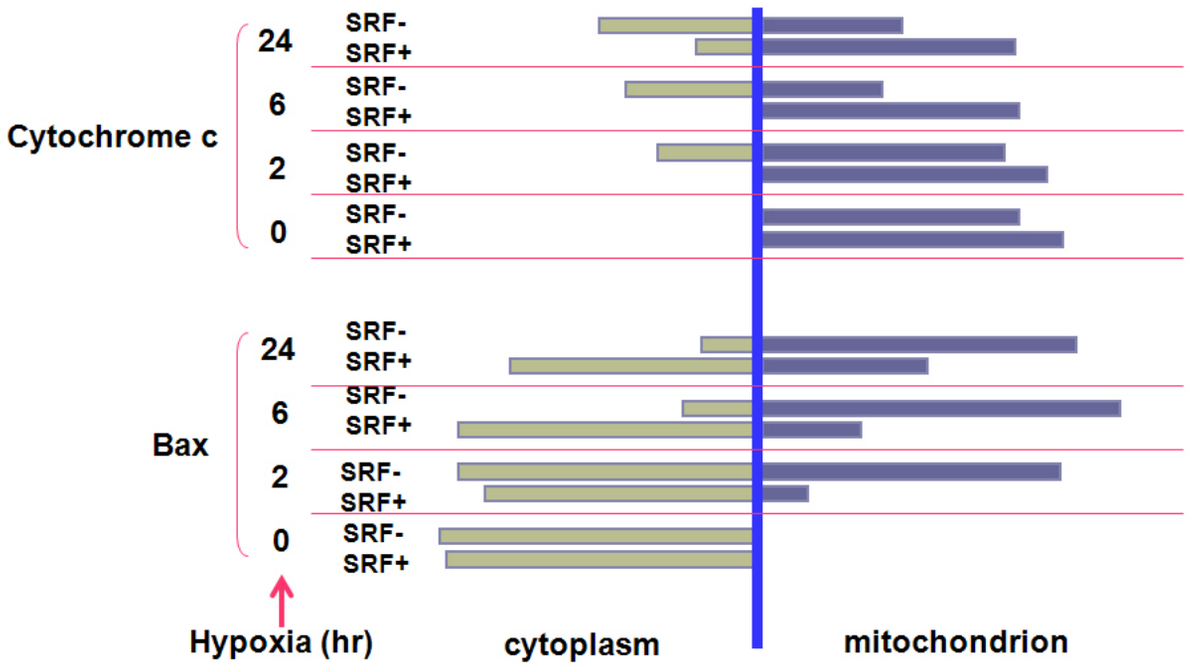

Figure 6. SRF protects mitochondrial integrity. As oxygen deprivation extends, more and Bax binds to mitochondria and opens up channels to allow cytochrome c to escape from mitochondria into cytoplasm, where it forms complexes with Apaf-1 and triggers caspase cascade. With overexpression of SRF, cells can reverse Bcl-2/Bax ratio decrease caused by hypoxia and prevent cytochrome c leakage, while lack of SRF accelerates mitochondrial breakdown.

7. Conclusions

Due to unregulated proliferation of malignant cells, oxygen deficiency is common in tumor development. Cancer cells have learned a few tricks to survive through oxygen crisis, and one of them is to stimulate endothelial cells to build new vessels extending oxygen toward the hypoxic area. However, depending on the severity of hypoxia, endothelial cells may follow the cue to support tumor growth by engaging in angiogenesis, or commit a suicide by engaging in apoptosis and leave the tumor cells to die. It is our interest to guide the endothelial cells to choose the second path. The best known players in the battle against hypoxia are HIF and p53. In general, HIF up-regulates angiogenic factors to promote angiogenesis, while p53 up-regulates pro-apoptotic genes to induce apoptosis. However, the relationship between HIF and p53 is not always a bull-and-bear fight; sometimes they can also join forces to become friends. HIF can bind to MDM2 to stabilize p53 and thereby to promote apoptosis [69]. It has

been reported that HIF-deficient embryonic stem cells resist to hypoxia-induced p53 activation and apoptosis [70]. A similar observation was also reported in neuronal cells where HIF helps p53 to endorse cell death [71]. For this reason, treatments targeting HIF do not always achieve inhibition of tumor angiogenesis.

Unlike HIF, SRF promotes cell survival through multi-level and fundamental regulations. Level 1 – growth factors: as discussed above, SRF is not only activated by growth factors, but also turns around to stimulate growth factor expression. This positive feedback reinforces the signal for cell survival. Level 2 – cytoskeletal components: no matter it is for cancer cells to move away from their primary location to look for new places with better oxygen and nutrient supply, or for cancer cells to allure endothelial cells with chemicals to form new vessels to bring oxygen and nutrients to the tumors, cytoskeletal regeneration and rearrangement are essential requirements. The molecules involved in these processes are tightly controlled by SRF. As shown in our previous study [43], without SRF, even the most potent angiogenic factor VEGF cannot stimulate angiogenesis. Level 3 – anti-apoptosis: hypoxia induces apoptosis through disrupting mitochondrial outer membrane, while mitochondrial integrity is guarded by Bcl-2, which is controlled by SRF. Therefore, SRF should be a better candidate for cancer gene therapy, and a treatment targeting SRF, instead of HIF, should achieve better results.

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