Supplementary Material and Methods

Western blotting - Primary cortical neurons were lysed briefly in homogenizing buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 5 mM EDTA, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml leupeptin and 4 μg/ml aprotinin). Equal amount of the samples was loaded per lane. For detection of Sp1, and tubulin, blots were incubated with antibodies against Sp1 (1:5000, Upstate Biotechnology, Lake Placid, NY) or tubulin (1:5000, Santa Cruz Biotech., Santa Cruz, CA). An enhanced chemiluminescence kit was used for detection. Western blotting was developed in the linear range used for densitometry. The density of the immunoblots was determined by an image analysis system installed with BIO-ID software (Vilber Lourmat, France).

Reporter Assays - Firefly luciferase and Renilla luciferase activities were measured in a luminometer (Model TD 20/20; Turner Designs, Mountain View, CA) using the reagents provided with the dual luciferase reporter kit (Promega, Madison, WI). The assay was carried out following the manufacturer's recommendations. Transfection efficiency and extract preparations were corrected by normalizing the data to the corresponding Renilla luciferase activity for each construct.

^{35}S-Methionine incorporation - At various time points after OGD treatment, cultured cortical neurons with the density of 5×10^5 cells/cm^2 were washed with PBS and incubated in methionine-free DMEM (Life Technologies-BRL, Rockville, MD), but containing 30 μCi/ml L-^{35}S-methionine (New England Nuclear, Boston, MA) for 1 h.

MTS assay - Primary cortical neurons were plated in 6×10^5 cells/cm^2 in 24-well plates two days before treatment. Following treatment with shRNA transfection for 30 h, neurons were exposed to OGD challenge. Twenty-four hours later, 20 μl of MTS (5 mg/ml) were added to each well, followed by incubation for an additional 30 min. The media was collected and the absorbance measured at 510 nm using a Rainbow Spectra ELISA microplate reader (TECAN). Cell viability was defined relative to corresponding control cells (i.e. relative cell viability = absorbance of treated sample / absorbance of control sample * 100%).

TUNEL staining - Primary cortical neurons were washed twice in PBS before being fixed 1 hour in 4% paraformaldehyde at 4℃, and permeabilized with 0.1% Triton X-

100 in 1% sodium citrate for 5 min at 4℃. Apoptosis was determined by detecting DNA strand breaks with a TUNEL assay, using the *in situ* death detection kit (Roche Diagnostics GmbH, Mannheim, Germany).

Construction of plasmids - The fragments of rat Sp1 promoter (-1600 to -251) was amplified from normal rat genomic DNA using primers Sp1-F(5′-GGACACCCAGAACAGGTAGGTCTG-3′) and Sp1-BglII-R(5′-AGATCTCTCTCTCTGAGGCGTGTA-3′). These PCR product was cloned into plasmid pGL3 vector (Promega, Madison, WI) using the KpnI and BglII restriction sites. The Sp1 5′-UTR (-250 to -1) fragment was amplified from normal rat genomic DNA using primers Sp1-5′UTR-SpeI-F (5′-ACTAGTAGCGAGTCTTGCCATTGG-3′) and Sp1-5′-UTR-EcoRI-R (5′-GGCGCCGGTGGCAGCTGAGGGGCG-3′). The PCR product was cloned into the pRF or pStemRF vector digested by SpeI and EcoRI restriction sites and named as pRsp1F or pStemRsp1F. Bicistronic reporter plasmids pRF and pStemRF were generous gift of J. T. Tseng (National Cheng-Kung University, Taiwan).

Reverse transcription-PCR and real-time PCR analysis - Primary neurons were mixed with 600 µl of TRI reagent (Invitrogen, Carlsbad, CA) to extract cellular mRNA. Single-strand cDNA was synthesized from the cellular mRNA by adding 1 µl of StrataScript™ reverse transcriptase (200 U/µl, Stratagene, La Jolla, CA), 10 µl of buffer (250 mM Tris-HCl, 375 mM KCl, and 15 mM $MgCl_2$), 5 µl of DTT (0.1 M), 1 µl of oligo-dT, and 18 µl of mixed dNTPs (2.5 mM). The mixture was incubated for 50 min at 50°C. The reaction was terminated by heating the mixture to 70°C for 10 min and then icing. Amplification was performed on a thermal cycler (PC800; Astec, Fukuoka, Japan) using DNA polymerase (5 U/µl, Yeastern Biotech). One of the primer sets was added to give a final volume of 20 µl. Reactions were run for the optimal cycles under the following conditions: denaturation at 95°C for 15 sec, annealing at 55°C for 15 sec, and extension at 72°C for 30 sec (repeated for a total of 30 cycles). The primers for the detection of Sp1 cDNA were 5'-CTGCAAGGGTCTGATTCTCTA-3'and 5'-AGCTTGTCCACCTTGAACTA-3'. The primers for the detection of GAPDH cDNA were 5'-TGACAACTTTGGCATCGTGGAAGG-3'and 5'-CAAC GGATACATTGGGGGTAGGAAC-3'. After PCR amplification, an 8.5 µl aliquot of reaction product was analyzed by electrophoresis on ethidium bromide-stained

agarose gel (1.5 %). Images of the DNA gels after RT-PCR analysis were digitally captured by an image analysis system installed with BIO-ID software (Vilber Lourmat, France). Real-time PCR was carried out with a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). The primers of HIF-1α, GAPDH and NR1 were shown in Table 1. The reaction mixture (total volume 10 μl) contained 1 μl of LightCycler DNA Master Hybridization Probes reaction mix (Roche Diagnostics GmbH, Mannheim, Germany), 3 mM $MgCl_2$, 2 μl of cDNA, 250 nM each of the 2 primers, and 125 nmol/μl each of the dual-labeled fluorescent probes. The primers and the thermal profile were used the same as above. At the end of each annealing step, the fluorescent signal of each reaction was measured at a wavelength of 530 nm with the LightCycler fluorimeter.

RNA interference - To suppress the expression of Sp1, 20 nM Sp1 shRNA (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan) was transfected to cortical neurons with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Thirty hours after transfection, neurons were subjected to OGD for various periods of time. At appropriate times, the cells were lysed and assayed for viability or Western blot analyses as described below.

Immunohistochemistry - Indirect labeling methods were used to determine the levels of Sp1 protein in brain tissues. Briefly, rat brain was fixed in 4% paraformaldehyde in PBS for 16 h at 4°C. Transverse slices of 20 μm thickness were cut and the appropriate slices placed on a microscope slide. After a subsequent wash in PBS, slices were permeabilized and blocked for 60 min in 0.1% Triton X-100/5% goat serum/PBS, and incubated for 12 h at 4°C. Brain slices were incubated with rabbit anti-Sp1 antibodies (1:200, Upstate Biotechnology, Lake Placid, NY), anti-MAb1 antibodies (1:50, Chemicon Europe, Hampshire, UK) and mouse anti-NeuN antibody (1:50, Chemicon, Littleton, CO) overnight. After three washes with PBS, cells were incubated with Alexa 488-conjugated anti-rabbit IgG antibodies (1:200, Molecular Probes, Eugene, OR), Alexa 568-conjugated anti-mouse antibodies (1:200, Molecular Probes, Eugene, OR) and DAPI. The slides were washed three times with PBS and mounted with glycerol. Images were analyzed under a confocal fluorescence microscope (Olympus FluoView^{TM}FV 1000, Melville, NY). Hypoxyprobe (pimonidazole; Chemicon Europe, Hampshire, UK) was intravenously injected into the rat (60 mg/kg) 30 min before MCAO. Animals were sacrificed 45 min after

MCAO and brain tissue was processed for immunohistochemistry.

Supplementary Legends:

Supplementary Videos 1 and 2. - Rats were injected with either saline (Supplementary video 1) or endothelin-1 (ET-1, 240 pmol in 10 µl of saline over 5 minutes; Supplementary video 2) to the left MCA. Video covers a period of approximately 1 minute.

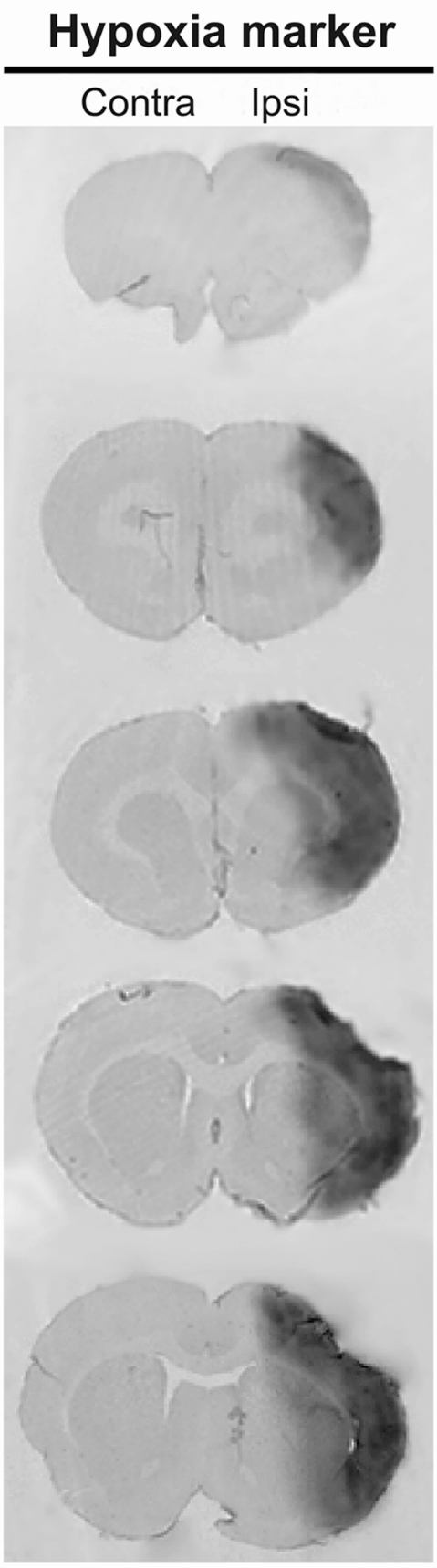
Supplymentery Fig. 1. Immunohistochemistry for the *in vivo* hypoxia marker pimonidazole - The pitroimidazole was injected intravenously as described in Materials and Methods. A strong signal was shown in the rat ipsilateral brain at 0.45 hr of recovery from MCAO. Contra: Contralateral side; Ipsi: Ipsilateral side.

Supplementary Fig. 2. Genes expression modulated by Sp1 - ***A***, Data obtained from the GEO accession no. GSE63. After filtering, Student's *t* test was performed. ***B***, The percentage of genes that contained at least one putative Sp1 binding site. The TF-SEARCH program was used to investigate the potential of Sp1 binding site. ***C***, The 'Core Analysis' function included in IPA (Ingenuity System Inc, USA) was used to interpret the data (32 genes) in the context of biological processes. Significance of the biofunctions was tested by the Fisher Exact test p-value. Top ten Diseases and Disorders significantly altered in core analysis. In brackets, number of genes from the input file implicated in each annotation. Significance at $p < 0.05$.

Supplementary Fig. 3. Sp1 expression in U87 glioma cells and A549 lung cancer cells - The time course of Sp1 expression after OGD treatment in U87 cells (A) or A549 cells (B) was assessed. Total lysates of U87 or A549 cells were obtained at various time points following OGD challenge. Sp1 expression was detected by performing immunoblotting with anti-Sp1 antibodies, using tubulin as an internal control. All experiments were carried out independently in triplicate and were expressed as a percentage of the levels in the naive-controls. Statistical analysis was carried out using the one-way ANOVA with appropriate post hoc tests.

Supplementary Fig. 4. The 5'-flanking region of Sp1 gene was analyzed with Software to predict the IRES-conserved sequence.

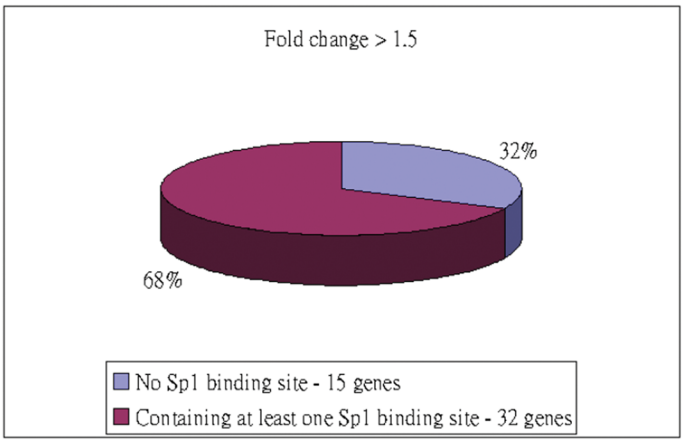
Sup. Fig.1



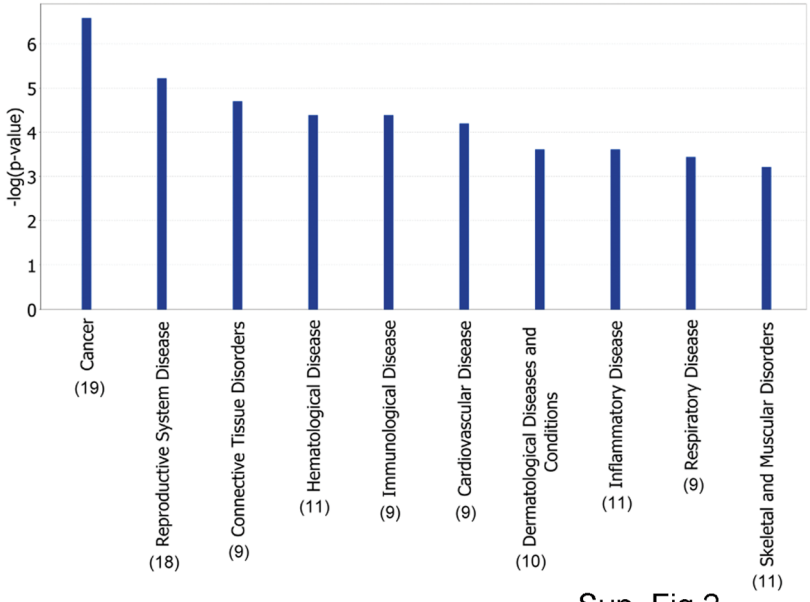
A.

	Gene Symbol	Accession	*P*-value	Fold change
1	BC026782	NM_001025575	2.35E-03	1.50
2	Slbp	NM_009193	2.59E-02	1.50
3	Hspd1	NM_010477	8.48E-04	1.50
4	Nqo1	NM_008706	3.54E-03	1.51
5	Litaf	NM_019980	5.64E-03	1.54
6	Ch25h	NM_009890	3.32E-02	1.54
7	Map2k3	NM_008928	1.48E-02	1.56
8	Hist1h3a	NM_013548	2.23E-02	1.56
9	Cort	NM_007745	4.61E-03	1.56
10	Dnajb3	NM_008299	1.74E-02	1.57
11	Tnfaip8	NM_134131	2.07E-02	1.59
12	Hspa5	NM_022310	1.29E-02	1.59
13	Hmgcr	NM_008255	1.30E-02	1.62
14	Btg1	NM_007569	3.51E-03	1.62
15	Ptn	NM_008973	4.11E-02	1.62
16	Iigp2	NM_019440	3.78E-03	1.62
17	Wwtr1	NM_133784	4.14E-03	1.63
18	Lrrc6	NM_019457	1.15E-02	1.64
19	Sstr2	NM_001042606	4.67E-03	1.64
20	Piga	NM_011081	1.15E-02	1.64
21	Ldlr	NM_010700	2.32E-03	1.66
22	Golim4	NM_175193	6.90E-03	1.67
23	Spsb1	NM_029035	3.01E-02	1.69
24	Slc30a1	NM_009579	1.81E-02	1.75
25	Mest	NM_008590	1.19E-04	1.76
26	Pgm1	NM_025700	2.86E-02	1.79
27	Ndel1	NM_023668	7.80E-03	1.79
28	Fam107b	NM_025626	3.74E-03	1.85
29	Amotl2	NM_019764	1.54E-02	1.86
30	Klf10	NM_013692	1.14E-02	1.88
31	Hsph1	NM_013559	2.23E-03	1.88
32	Thbd	NM_009378	1.00E-02	1.92
33	Serpinh1	NM_001111043	1.19E-02	2.02
34	Gjb2	NM_008125	1.48E-03	2.16
35	Dusp1	NM_013642	3.37E-03	2.20
36	Map3k1	NM_011945	8.25E-04	2.21
37	Phf13	NM_172705	9.49E-03	2.21
38	Ier3	NM_133662	7.01E-04	2.29
39	Jun	NM_010591	8.62E-04	2.38
40	Procr	NM_011171	9.99E-03	2.61
41	Hba-a1	NM_008218	4.70E-03	2.67
42	Nfkbiz	NM_030612	7.25E-05	3.17
43	Socs3	NM_007707	6.79E-03	3.35
44	Nr4a1	NM_010444	1.18E-02	3.44
45	Egr1	NM_007913	1.11E-03	3.68
46	Gadd45g	NM_011817	1.03E-04	3.97
47	Tnfaip6	NM_009398	2.31E-03	4.71

B.

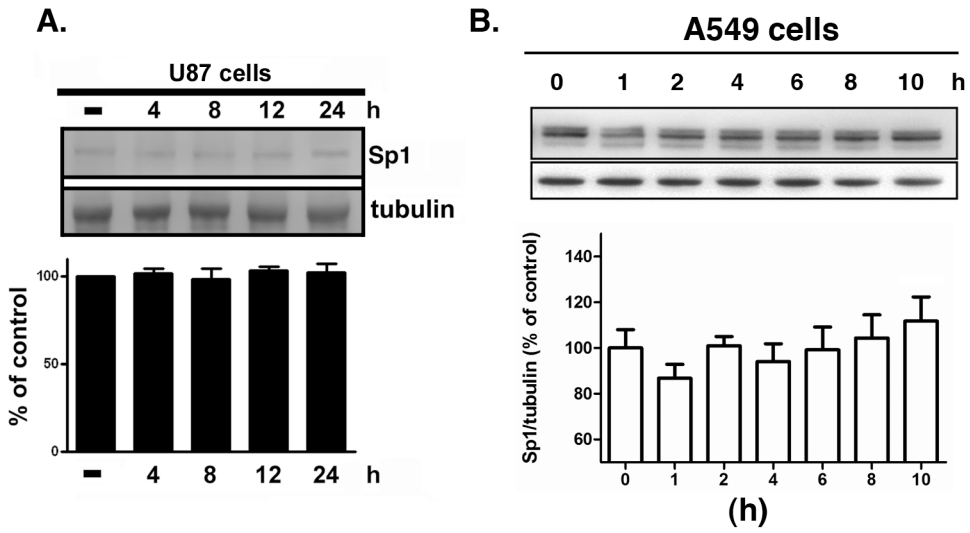


C.



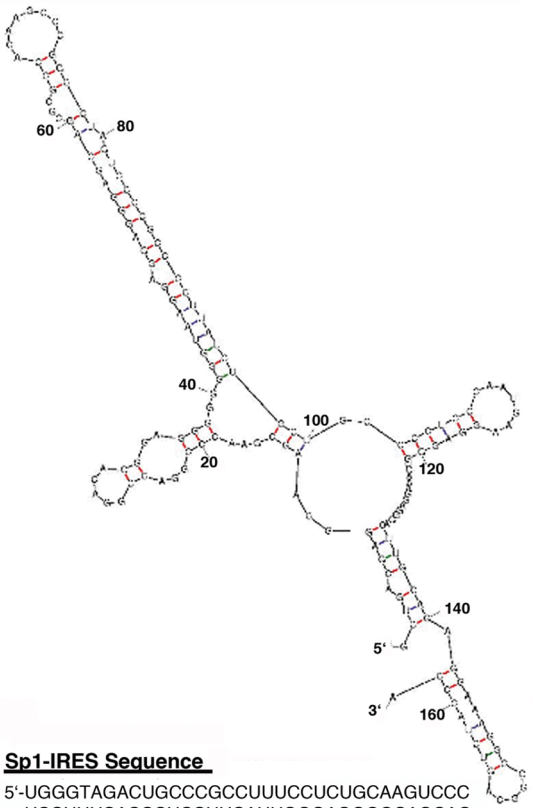
Sup. Fig.2

Sup. Fig. 3


A.
U87 cells
-
4
8
12
24
h
Sp1
tubulin
% of control
100
50
0
B.
A549 cells
0
1
2
4
6
8
10
h
Sp1/tubulin (% of control)
140
120
100
80
60
(h)

Sup. Fig.4

(a).



(b).

Sp1-IRES Sequence

5'-UGGGTAGACUGCCCGCCUUUCCUCUGCAAGUCCC
UCCUUUCACCCUCCUUCAUUGGGAGGGGCAGCAG
AUAAGGGGCGGGGACUAGGCCGGGCUUGUGGCGC
GCUGCUCCCUCCUCCUUACCCCCCCCUCCCUGUCC
GGUCCGGGUUCGCUUGCCUCGUCAGC-3'