HIF-1-mediated production of exosomes during hypoxia is protective in renal tubular cells

Wei Zhang[1,3*], Xiangjun Zhou[2,3*], Qisheng Yao[2], Yutao Liu[3], Hao Zhang[1,#], Zheng Dong[3,4#]

[1]Department of Nephrology, The Third Xiangya Hospital of Central South University, Changsha, China; [2]Department of Urology, Taihe Hospital, Hubei University of Medicine, Hubei, China; [3]Department of Cellular Biology and Anatomy, Medical College of Georgia at Augusta University; [4]Department of Nephrology, The Second Xiangya Hospital of Central South University, Changsha, China

* Wei Zhang and Xiangjun Zhou contributed equally to this work

# Hao Zhang and Zheng Dong contributed equally to this work

[#] Corresponding authors: Zheng Dong, Department of Nephrology, The Second Xiangya Hospital of Central South University, Changsha, China; and Department of Cellular Biology and Anatomy, Medical College of Georgia at Augusta University and Charlie Norwood VA Medical Center, 1459 Laney Walker Blvd, Augusta, GA 30912, USA

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Abstract

Exosomes are nano-sized vesicles produced and secreted by cells to mediate intercellular communication. The production and function of exosomes in kidney tissues and cells remain largely unclear. Hypoxia is a common patho-physiological condition in kidneys. This study was designed to characterize exosome production during hypoxia of rat renal proximal tubular cells (RPTC), investigate the regulation by hypoxia-inducible factor-1 (HIF-1), and determine the effect of the exosomes on ATP-depletion induced tubular cell injury. Hypoxia did not change the average sizes of exosomes secreted by RPTC cells, but it significantly increased exosome production in a time-dependent manner. HIF-1 induction with dimethyloxalyl glycine (DMOG) also promoted exosome secretion, whereas pharmacologic and genetic suppression of HIF-1 abrogated the increase of exosome secretion under hypoxia. The exosomes from hypoxic RPTC cells had inhibitory effects on apoptosis of RPTC cells following ATP-depletion. The protective effects were lost in the exosomes from HIF-1α knockdown cells. It is concluded that hypoxia stimulates exosome production and secretion in renal tubular cells. The exosomes from hypoxic cells are protective against renal tubular cell injury. HIF-1 mediates exosome production during hypoxia and contributes to the cytoprotective effect of the exosomes.

Introduction

Exosomes are small, membrane-bound vesicles of 50-150 nm in diameter that are produced by cells and secreted to extracellular space. The contents of exosomes include a variety of proteins, RNAs, DNAs, and other types of molecules. After secretion, exosomes may traffic to other cells to release their contents and thus act as an important mean of cell-cell communication. The biogenesis, secretion, and cargo contents of exosomes depend on cellular context and are regulated under various patho-physiological conditions(6, 20). Recent research has implicated exosomes in renal patho-physiology (3, 7, 29). However, the role and regulation of exosomes in kidney cells and tissues remain poorly understood.

Hypoxia is an important patho-physiological condition encountered by cells and tissues. For example, hypoxia occurs in ischemic diseases, such as myocardial infarction, stroke, and acute kidney injury. Hypoxia is also known to be a feature of solid tumors that may contribute to tumorigenesis, therapy resistance, and cancer metastasis. In response to hypoxia, cells may activate various signaling pathways and a myriad of transcription factors for cell survival and adaptation, and when hypoxic stress becomes overwhelming, cell death ensues. Hypoxia-inducible factors (HIF) are a family of transcription factors that are responsive to hypoxia and mainly responsible for cellular adaptation (22). As such, HIFs are known to regulate energy utilization, metabolism, oxidative stress, and cell death and survival. Functional or transcriptionally active HIF consists of a heterodimer of α and β subunits. While HIF- β is constitutively expressed, HIF-α is stabilized and expressed under hypoxia. Therefore, HIF activity is largely dependent on the level of HIF-α expression. Under normoxia or normal oxygen (21% O_2)

conditions, HIF-α is hydroxylated at specific proline residues by prolyl hydroxylases, resulting in its association with the E3 ligase VHL and consequent proteosomal degradation. In hypoxia or low oxygen conditions, proline hydroxylation in HIF-α is blocked, leading to HIF-α accumulation, dimerization with HIF- β, and then translocation to the nucleus for hypoxic gene transcription.

In the field of cancer research, exosome secretion was reported to increase under hypoxia and this increase appeared HIF-1α dependent (11). Hypoxic exosomes were further shown to be a mediator that aggravates tumor progression by transmission of particular content that may promote tumor cell survival and invasion (23). In kidney, increased exosome secretion was detected in ureteral obstructed kidney as well as MCT and HK2 cell lines under hypoxic conditions (2). However, the regulatory mechanism and function of exosome secretion in renal tubular cells under hypoxia remain largely unclear. In this study, we characterized exosome production during hypoxia of renal tubular cells, determined the role of HIF-1, and analyzed effect of exosomes on ATP-depletion associated injury.

Materials and Methods

Antibodies and special reagents

Antibodies and reagents were purchased from the following sources: monoclonal anti-CD63 and anti-TSG-101 antibodies from System Biosciences (Palo Alto, CA, USA); anti-Caspase-3, anti-HIF-1α and anti-GAPDH antibodies from Cell Signaling Technologies (Beverly, MA, USA); secondary antibodies for immunoblot analysis from Jackson Immuno Research Laboratories Inc. (West Grove, PA， USA); DMOG from

Sigma (St. Louis, MO, USA); YC-1 from Cayman Chemical (Michigan, USA); exosome-deplete FBS from Gibco (Thermo Fisher Scientific, MA, USA).

Cell culture

The RPTC line was originally obtained from Dr. Ulrich Hopfer (Case Western Reserve University, Cleveland, OH) and cultured in DMEF/F12 media containing 10% FBS for experiments. Wild-type and HIF-1α-null mouse embryonic fibroblasts (MEFs) were obtained from Dr. Gregg Semenza (Johns Hopkins University, Baltimore, MD) and grown in DMEM medium with 15% FBS. HIF-1α knockdown RPTC cells were generated as described in our previous work (19). All the cells were maintained at 37°C in an atmosphere of 5% CO_2. 2% exosome-depleted FBS (instead of normal FBS) was used for cell culture during experimental treatment.

Hypoxic Incubation

Hypoxia treatment was conducted in a hypoxia chamber as described previously (26). Briefly, dishes with cells were transferred into a hypoxia chamber (COY Laboratory Products, Ann Arbor, MI) containing 1% or 0.2%, 2%, 4%, 8% oxygen respectively according to the experiment design. The oxygen tension in the chamber was monitored and maintained by a computerized sensor probe. Humidity was maintained with a humidifier. `All the medium used for hypoxia treatment was pre-incubated in the chamber for over 6 hours to reach a balanced oxygen concentration.

Exosomes isolation and identification

Cell culture medium was collected and subjected to a series of centrifugation (800 × g; 10 min, 2000 × g; 20 min, 10,000 × g; 30 min). The resultant supernatant was finally subjected to ultracentrifugation (Rotor SW28 and SW55, Optima™ L-100 XP, Beckman, USA) at 100,000 × g for 90 min to collect exosomes in pellet, which was lysed for protein analysis, resuspended in phosphate buffered saline (PBS) for quantification or −80°C storage.

Nanoparticle-tracking analysis (NTA)

We completed Nanoparticle Tracking Analysis (NTA) with ZetaView (Particle Metrix Inc, Germany) to analyze the size distribution and concentration of the exosomes preparations as described recently(9, 30). Isolated exosomes were diluted with 1:500 or 1:1000 in particle-free PBS and resuspended before being injected into the sample cell chamber. Size distributions and particle concentrations were assessed with NTA software. Exosome concentration analysis was normalized with the total number of cells from the corresponding dish. To quantify the cell number, the cells in each dish were harvested at the end of treatment and digested into suspension by trypsin for quantification with a TC20 Automated Cell Counter.

Transmission electron microscopy (TEM)

TEM was conducted by Electron Microscopy Core of Augusta University as described previously (10, 30). 3μl of exosomes pellet solution was applied on formvar carbon-coated 200-mesh copper electron microscopy grids, and incubated at room temperature for 5min, and then subjected to standard uranyl acetate staining. The grid was washed

with PBS for three times and allowed to semi-dry at room temperature before observation in transmission electron microscope (Hitachi H7500 TEM, Tokyo, Japan).

Western blot analysis

Cell lysate and exosomal proteins were extracted with 2% SDS buffer. Protein concentration was quantified with a BCA protein assay kit (Thermo Scientific, USA). Total exosomal protein loading for Western blot was normalized with total cell number of the corresponding dishes as described above. Protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% milk for 1 h at room temperature, then immunoblotted with primary antibody at 4℃ overnight. The blot membrane was then washed 3 times and incubated with horseradish peroxidase-conjugated secondary antibodies. The blot signal was revealed with a chemiluminescence kit (Bio Rad).

Statistical analysis

All values were expressed as mean ± SD. Statistical analysis was conducted using the GraphPad Prism software (San Diego, CA). Comparison between two groups were performed by Student t-test. For multiple comparisons, ANOVA was used. $P<0.05$ was considered reflecting significant differences. Each experiment was conducted independently for at least 3 times.

Results

Isolation and characterization of exosomes produced by RPTC cells

Exosomes were isolated from cultured media of RPTCs by serial ultracentrifugation. by Nanoparticle-tracking analysis (NTA) of the isolated samples indicated that most of the particles had a size of 50-150nm in diameter with a peak at around 100 nm (Figure 1A, 1B). Immunoblot analysis (Figure 1C) further detected CD63 and TSG101, two widely accepted markers of exosomes, in the isolated samples. As expected, the exosome sample from 30 ml cell culture medium showed higher content of CD63 and TSG101 than that from 15 ml medium (Figure 1C). In addition, electron microscopy revealed the presence of exosomes within the expected size range of exosomes (50-150 nm) and bilayer cup-shaped morphology (Figure 1D and E). The results verify our exosome isolation protocol and indicate that renal tubular cells produce and secret typical exosomes.

Enhanced exosome production during hypoxia of RPTC cells

After characterizing isolated exosomes, we examined the impact of hypoxia on exosome secretion in RPTCs. The cells were exposed to normoxia or hypoxia (1% oxygen) for 6, 12, and 24 hours. Western blot analysis (Figure 2A) showed a time-dependent increase of exosomal protein CD63 and TSG101 under both normoxia and hypoxia conditions. This time-dependence was expected, because more exosomes were secreted into culture medium as the incubation time increased. Interestingly, further comparison indicated consistently higher CD63 and TSG101 expression in hypoxia group than normoxia group at all time points. NTA analysis (Figure 2B) also confirmed that more exosomes were produced by hypoxic cells than normoxic cells after normalization with equal cell

numbers, particularly at 12 h (1.5 fold higher than normoxia, $P<0.05$) and 24h (1.9 fold higher than normoxia, $P<0.05$). In addition, we evaluated the sizes of the exosomes produced the cells under hypoxia and normoxia conditions by NTA. The average diameter of the exosomes from normoxic cells was 108.2±4.1nm, which was indifferently from that of hypoxic exosomes (105.6±3.4 nm) (Figure 2C). Having observed the time-dependent increase of exosomes secretion in 1% hypoxia condition, we checked the effect of different hypoxic concentrations on exosomes production. As shown in Figure 2 D and E by Western blot and NTA analyses, 0.2% , 1% and 2% oxygen hypoxic concentrations could significantly increase exosomes secretion as compared with the corresponding control condition. However, moderate hypoxia of 4% and 8% oxygen did not induce exosome production.

Effects of DMOG and YC-1 on exosome production in RPTC cells

HIFs are the major transcription factors that are responsive to hypoxia in mammalian cells(22). Thus, we examined if HIF was involved in the increased exosome production during hypoxia of RPTC cells. We initially tested the effects of DMOG (pharmacological inducer of HIF) and YC-1 (pharmacological inhibitor of HIF) were respectively used with or without hypoxia treatment for 24h. DMOG increased CD63 and TSG101 expression (Figure 3A) as well as exosome production (Figure 3B) in normoxic cells. Moreover, YC-1 reduced exosome production from hypoxic cells. These data suggested the involvement of HIF in the production and secretion of exosomes in hypoxic renal tubular cells.

Hypoxia-induced exosome production is suppressed in HIF-1-deficient cells

To further prove the role of HIF in hypoxia-induced exosome production, we turned to HIF-deficient cells. In kidneys, renal tubular cells mainly express HIF-1, while vascular endothelial cells express HIF-2(21). Therefore, we established HIF-1α-knockdown renal tubular cell lines by stably transfecting HIF-1α shRNAs. Compared to scrambled sequence transfected cells (Scr), HIF-1α shRNA transfected cell lines (KD) had lower HIF-1α expression under both normoxia and hypoxia conditions (Figure 4A). In nomoxia, these cells produced similar amounts of exosomes, but upon hypoxia exposure HIF-1α knock down cells (KD-hy) produced much less exosomes than scrambled sequence-transfected cells (Figure 4B). Consistently, hypoxia-induced expression of exosomal proteins, CD63 and TSG101, was also suppressed in HIF-1α knockdown cells (Figure 4C).

We further compared HIF-1α knockout (KO) and wild-type (WT) mouse embryonic fibroblasts for exosome production. HIF-1α expression was markedly lower in KO cells under both normoxia and hypoxia conditions, verifying HIF-1α knockout in KO cells (Figure 5A). Hypoxia significantly increased exosomes production in WT cells, but this inductive response was largely attenuated in KO cells as shown by exosomal protein and NTA analyses (Figure 5B and 5C).

Cytoprotective effect of the exosomes produced by hypoxic renal tubular cells

We wondered about the function of the exosomes produced by renal tubular cells, especially those induced to produce under hypoxia. Considering that hypoxia triggers cell stress, we hypothesized that the exosomes produced under hypoxia may help the cells coup with the stress. To test this possibility, we observed the effect of the exosomes

isolated from normoxia or hypoxia cells on ATP-depletion associated cell injury in RPTC cells. ATP-depletion was induced by inclusion of azide (inhibitor of Cytochrome Oxidase, Complex IV of mitochondrial respiratory chain) in cell incubation buffer. In this model, apoptosis developed within 1-3 hours after ATP-depleted cells were returned to normal culture medium. Pre-incubation with exosomes derived from normoxic cells (exo-Nc24h) had marginal effects on apoptosis, but the exosomes from hypoxic cells (exo-Hy24h) significantly suppressed apoptosis (Figure 6). The effect of hypoxic exosomes was confirmed by morphological assessment (Figure 6A, 6B) and immunoblot analysis of caspase-3 cleavage or activation (Figure 6C).

Role of HIF-1 in producing cytoprotective exosomes by RPTC cells under hypoxia

Finally, we determined if HIF-1α was involved in the protective effect of exosomes from hypoxic cells. To this end, we collected exosomes from HIF-1α knockdown cells and scrambled sequence-transfected cells to test their effects on apoptosis following azide-induced ATP-depletion. Representative cell morphology is shown in Figure 7A and the percentage of apoptosis is presented in Figure 7B. ATP-depletion induced ~30% apoptosis in RPTC cells, which was reduced to 17% by the exosomes isolated from scrambled sequence-transfected cells under hypoxia (exoScr-Hy group), whereas the exosomes isolated from normoxic, scrambled sequence-transfected cells (exoScr-Nc group) did not have significant effects. Notably, the exosomes isolated from hypoxic, HIF-1α-knockdown cells (exoKD-Hy) did not reduce apoptosis either. The morphological results were confirmed by immunoblot analysis of active/cleaved caspase-

3, a biochemical hallmark of apoptosis (Figure 7C). These results support a critical role of HIF-1 in producing protective exosomes during hypoxia of renal tubular cells.

Discussion

The diagnostic potential and bio-effects of exosomes have drawn tremendous attention in recent years in biomedical research (29). In the current study, we have presented the results about the regulation of exosome production by hypoxia in renal tubular cells. We have further shown evidence that the exosomes from hypoxic cells are cytoprotective for renal tubular cells. Hypoxia is a feature of both acute and chronic kidney diseases in various clinical and experimental conditions (4, 13, 26). Exosomes have recently been implicated in physiological as well as pathophysiological processes in kidney (3, 7, 25). Our data demonstrate that exosome production in renal tubular cell is remarkably increased during hypoxia, which is consistent with previous work in cancer cells (14). Consistently, Borges et al showed the increase of exosomal protein CD63 production in kidney tissues of UUO mice and during hypoxia of renal tubular cells, suggesting the induction of exosomes under these conditions (2). In our study, exosomes were evaluated by a complementary techniques including EM visualization, Nanoparticle-tracking assay, and immunoblot analysis of exosomal proteins CD63 and TSG101.

Our study has further demonstrated an important role of HIF-1 in exosome production during hypoxia of renal tubular cells. In normoxic cells, activation of HIF by DMOG induced exosome production from RPTC cells and, inhibition of HIF by YC-1 blocked exosome production during hypoxia (Figure 4). In addition, hypoxia-induced exosome production was suppressed in HIF-1α-deficient cells (Figure 5). Together, these

results support the role of HIF-1 in mediating exosome biogenesis and secretion in hypoxic renal tubular cells. However, the mechanism whereby HIF-1 regulates exosomes under hypoxia is unclear. In this regard, HIF has been reported to mediate the induction of Rab22 and RAB20 (8, 27), which may be associated with extracellular vesicle formation and secretion (6, 18). In addition, hypoxia may induces ceramide production by the activation of nSMase, another important regulator of exosome secretion(15).

Functionally, exosomes derived from hypoxic cells were demonstrated to facilitate tumor invasion and metastasis in cancer research and they were also reported to be pro-fibrotic during kidney fibrosis by their TGF-beta mRNA content (2, 12, 23). Our current results show that exosomes from hypoxic RPTC cells can protect against renal tubular cell apoptosis following ATP-depletion. The effect, though not complete, suggests a promising, exosome-based approach of cell protection. There are reports about the cytoprotective effects of the exosomes derived from stem or progenitor cells (5, 17), but to our knowledge, the current work is the first to demonstrate the protective effect of renal tubular cell-derived exosomes. Interestingly, our work showed a time-window for RPTC cells to produce cytoprotective exosomes under hypoxia. Specifically, the exosomes collected during 16 and 24 hours of hypoxia were protective, but those collected from over 30 hours of hypoxia were not (data not shown). In addition, we noticed that the cells needed serum (exosomes-depleted FBS in our study) to produce cytoprotective exosomes during hypoxia (data not shown).

How do the exosomes from hypoxic cells protect renal tubular cells? Our current work does not provide a clear answer to this question. Nonetheless, hypoxic stimulation may change the content of exosomes that is believed to be the main mediators affecting

297 recipient cells (1). The content of exosomes may change dynamically in response to time
298 duration of cell culture as well as the cellular context (16). This appears relevant, because
299 in our study RPTC cells started developing apoptosis after >24 hours of hypoxia, a time-
300 point when produced exosomes were no more protective (data not shown). In our
301 previous studies, hypoxia and HIF-1 induced cytoprotective genes or factors in renal
302 tubular cells, including miRNAs (26, 28). In the current study, we have further provided
303 evidence for a role of HIF-1 in the production of cytoprotective exosomes by renal
304 tubular cells under hypoxia (Figure 7).
305 Hypoxic tumor-derived extracellular vesicles negatively regulated NK cell
306 function by a mechanism involving TGF-β and miRNAs transfer(1). Also, exosomes
307 derived from hypoxic oral squamous cell carcinoma (OSCC) cells increased the
308 migration and invasion of OSCC cells by delivering miRNAs in a HIF-1α and HIF-2α-
309 dependent manner (16). Exosomes secreted by human myeloid leukemia cell line K562
310 also enhanced angiogenesis under normoxic and hypoxic conditions. With chronic
311 hypoxia, exosomes could also enhance angiogenesis by targeting factor-inhibiting
312 hypoxia-inducible factor-1 (24). Therefore, HIF-1 and associated miRNAs might be the
313 main effectors in the protection by hypoxic exosomes. However, it is still hard to say the
314 protective effect of hypoxic exosomes completely depends on HIF-1, because numerous
315 transcription factors and cellular signaling pathways are activated during hypoxia that
316 may mediate the protective effect. Further work should identify the molecules in the
317 exosomes from hypoxic renal tubular cells that are responsible for the cytoprotective
318 effect.
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References

1. **Berchem G, Noman MZ, Bosseler M, Paggetti J, Baconnais S, Le Cam E, Nanbakhsh A, Moussay E, Mami-Chouaib F, Janji B, and Chouaib S**. Hypoxic tumor-derived microvesicles negatively regulate NK cell function by a mechanism involving TGF-beta and miR23a transfer. *ONCOIMMUNOLOGY* 5: e1062968, 2016.
2. **Borges FT, Melo SA, Ozdemir BC, Kato N, Revuelta I, Miller CA, Gattone VN, LeBleu VS, and Kalluri R**. TGF-beta1-containing exosomes from injured epithelial cells activate fibroblasts to initiate tissue regenerative responses and fibrosis. *J AM SOC NEPHROL* 24: 385-392, 2013.
3. **Borges FT, Reis LA, and Schor N**. Extracellular vesicles: structure, function, and potential clinical uses in renal diseases. *BRAZ J MED BIOL RES* 46: 824-830, 2013.
4. **Brukamp K, Jim B, Moeller MJ, and Haase VH**. Hypoxia and podocyte-specific Vhlh deletion confer risk of glomerular disease. *Am J Physiol Renal Physiol* 293: F1397-F1407, 2007.
5. **Burger D, Vinas JL, Akbari S, Dehak H, Knoll W, Gutsol A, Carter A, Touyz RM, Allan DS, and Burns KD**. Human endothelial colony-forming cells protect against acute kidney injury: role of exosomes. *AM J PATHOL* 185: 2309-2323, 2015.
6. **Colombo M, Raposo G, and Thery C**. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol* 30: 255-289, 2014.
7. **Erdbrugger U, and Le TH**. Extracellular Vesicles in Renal Diseases: More than Novel Biomarkers? *J AM SOC NEPHROL* 27: 12-26, 2016.
8. **Hackenbeck T, Huber R, Schietke R, Knaup KX, Monti J, Wu X, Klanke B, Frey B, Gaipl U, Wullich B, Ferbus D, Goubin G, Warnecke C, Eckardt KU, and Wiesener MS**. The GTPase RAB20 is a HIF target with mitochondrial localization mediating apoptosis in hypoxia. *Biochim Biophys Acta* 1813: 1-13, 2011.
9. **Helwa I, Cai J, Drewry MD, Zimmerman A, Dinkins MB, Khaled ML, Seremwe M, Dismuke WM, Bieberich E, Stamer WD, Hamrick MW, and Liu Y**. A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. *PLOS ONE* 12: e170628, 2017.
10. **Helwa I, Cai J, Drewry MD, Zimmerman A, Dinkins MB, Khaled ML, Seremwe M, Dismuke WM, Bieberich E, Stamer WD, Hamrick MW, and Liu Y**. A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. *PLOS ONE* 12: e170628, 2017.
11. **Hubbi ME, and Semenza GL**. Regulation of cell proliferation by hypoxia-inducible factors. *Am J Physiol Cell Physiol* 309: C775-C782, 2015.
12. **Khade PK, and Giannakakou P**. RABbing cancer the wrong way. *Proc Natl Acad Sci U S A* 111: 11230-11231, 2014.
13. **Kimura K, Iwano M, Higgins DF, Yamaguchi Y, Nakatani K, Harada K, Kubo A, Akai Y, Rankin EB, Neilson EG, Haase VH, and Saito Y**. Stable expression of HIF-1alpha in tubular epithelial cells promotes interstitial fibrosis. *Am J Physiol Renal Physiol* 295: F1023-F1029, 2008.
14. **King HW, Michael MZ, and Gleadle JM**. Hypoxic enhancement of exosome release by breast cancer cells. *BMC CANCER* 12: 421, 2012.
15. **Kosaka N, Iguchi H, Hagiwara K, Yoshioka Y, Takeshita F, and Ochiya T**. Neutral sphingomyelinase 2 (nSMase2)-dependent exosomal transfer of angiogenic microRNAs regulate cancer cell metastasis. *J BIOL CHEM* 288: 10849-10859, 2013.
16. **Li L, Li C, Wang S, Wang Z, Jiang J, Wang W, Li X, Chen J, Liu K, Li C, and Zhu G**. Exosomes Derived from Hypoxic Oral Squamous Cell Carcinoma Cells Deliver miR-21 to Normoxic Cells to Elicit a Prometastatic Phenotype. *CANCER RES* 76: 1770-1780, 2016.
17. **Lindoso RS, Collino F, Bruno S, Araujo DS, Sant'Anna JF, Tetta C, Provero P, Quesenberry PJ, Vieyra A, Einicker-Lamas M, and Camussi G**. Extracellular vesicles released from mesenchymal stromal cells modulate miRNA in renal tubular cells and inhibit ATP depletion injury. *STEM CELLS DEV* 23: 1809-1819, 2014.
18. **Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, Moita CF, Schauer K, Hume AN, Freitas RP, Goud B, Benaroch P, Hacohen N, Fukuda M, Desnos C, Seabra MC, Darchen F, Amigorena S, Moita LF, and Thery C**. Rab27a and Rab27b control different steps of the exosome secretion pathway. *NAT CELL BIOL* 12: 19-30, 1-13, 2010.

19. **Peng J, Ramesh G, Sun L, and Dong Z**. Impaired wound healing in hypoxic renal tubular cells: roles of hypoxia-inducible factor-1 and glycogen synthase kinase 3beta/beta-catenin signaling. *J PHARMACOL EXP THER* 340: 176-184, 2012.
20. **Raposo G, and Stoorvogel W**. Extracellular vesicles: exosomes, microvesicles, and friends. *J CELL BIOL* 200: 373-383, 2013.
21. **Rosenberger C, Mandriota S, Jurgensen JS, Wiesener MS, Horstrup JH, Frei U, Ratcliffe PJ, Maxwell PH, Bachmann S, and Eckardt KU**. Expression of hypoxia-inducible factor-1alpha and -2alpha in hypoxic and ischemic rat kidneys. *J AM SOC NEPHROL* 13: 1721-1732, 2002.
22. **Semenza GL**. Hypoxia-inducible factors in physiology and medicine. *CELL* 148: 399-408, 2012.
23. **Steinbichler TB, Dudas J, Riechelmann H, and Skvortsova II**. The Role of Exosomes in Cancer Metastasis. *SEMIN CANCER BIOL* 2017.
24. **Umezu T, Tadokoro H, Azuma K, Yoshizawa S, Ohyashiki K, and Ohyashiki JH**. Exosomal miR-135b shed from hypoxic multiple myeloma cells enhances angiogenesis by targeting factor-inhibiting HIF-1. *BLOOD* 124: 3748-3757, 2014.
25. **van Balkom BW, Pisitkun T, Verhaar MC, and Knepper MA**. Exosomes and the kidney: prospects for diagnosis and therapy of renal diseases. *KIDNEY INT* 80: 1138-1145, 2011.
26. **Wang J, Biju MP, Wang MH, Haase VH, and Dong Z**. Cytoprotective effects of hypoxia against cisplatin-induced tubular cell apoptosis: involvement of mitochondrial inhibition and p53 suppression. *J AM SOC NEPHROL* 17: 1875-1885, 2006.
27. **Wang T, Gilkes DM, Takano N, Xiang L, Luo W, Bishop CJ, Chaturvedi P, Green JJ, and Semenza GL**. Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis. *Proc Natl Acad Sci U S A* 111: E3234-E3242, 2014.
28. **Wei Q, Liu Y, Liu P, Hao J, Liang M, Mi QS, Chen JK, and Dong Z**. MicroRNA-489 Induction by Hypoxia-Inducible Factor-1 Protects against Ischemic Kidney Injury. *J AM SOC NEPHROL* 27: 2784-2796, 2016.
29. **Zhang W, Zhou X, Zhang H, Yao Q, Liu Y, and Dong Z**. Extracellular vesicles in diagnosis and therapy of kidney diseases. *Am J Physiol Renal Physiol* 311: F844-F851, 2016.
30. **Zhou X, Zhang W, Yao Q, Zhang H, Dong G, Zhang M, Liu Y, Chen JK, and Dong Z**. Exosome production and its regulation of EGFR during wound healing in renal tubular cells. *Am J Physiol Renal Physiol* 78-2017, 2017.

Figure Legends

Figure 1 Isolation and characterization of exosomes produced by RPTC cells.

RPTC cells were cultured for 24 hours with exosome-free FBS to collect medium for exosome isolation by serial ultracentrifugation. (A) Size distribution of the isolated particles analyzed by NTA. (B) Representative potential image of the particles during NTA ZetaView quantification. (C) Immunoblot analysis of exosomal proteins CD63 and TSG101 in the exosome preparations from 15 ml and 30 ml cell culture media. (D, E) Examination of isolated exosomes by transmission electron microscopy. Scale bars are 200 nm and 100 nm in (D) and (E), respectively.

Figure 2 Enhanced exosome production during hypoxia of RPTC cells

RPTC cells were cultured in normoxia (21% oxygen) or hypoxia (1% oxygen) for 6, 12 or 24 hours to collect medium for exosome isolation by serial ultracentrifugation. (A) Immunoblot analysis of CD63 and TSG101 in exosomal preparations from normoxic (N6h, N12h, N24h) and hypoxic (H6h, H12h, H24h) RPTC cells. The loading volume of exosome lysis for each condition was normalized with corresponding cell lysate protein. (B) Exosome concentrations analyzed by NTA. Data are means $\pm$ SD; * $P<0.05$ vs nomorxia 12h group, $^{\#}$ $P<0.05$ vs nomorxia 24h group, n=3. (C) Size distribution analysis by NTA for exosomes from nomorxic and hypoxic cells. n=9. (D, E) RPTC cells were cultured in different hypoxic conditions of 0.2%, 1%, 2%, 4% or 8% oxygen for 24 hours, while corresponding control cells were in normoxia (21% oxygen). (D) Immunoblot analysis of CD63 in exosomal preparations from normoxic (N24h) and hypoxia (H24) of different oxygen concentrations. (E) Exosome concentrations from the

corresponding groups as in (D) analyzed by NTA. Data are means + SD; * P＜0.05 vs indicated nomorxia group, n=3.

Figure 3 Effects of DMOG and YC-1 on exosome production in RPTC cells (D) RPTC cells were cultured in normoxia (21% oxygen) with or without 1 mM DMOG, or in hypoxia (1% oxygen) for 24 hours with or without 20 uM YC-1 to collect medium for exosome isolation by serial ultracentrifugation. (A) Immunoblot analysis of CD63 and TSG101 in exosomal preparations and HIF-1α in cell lysate from each conditions. (B) NTA quantification of exosomes. NC: normoxia control; normorxia with DMOG; Hy: hypoxia; Hy+Yc-1: hypoxia with YC-1. Data are means ± SD; n=3; * P＜0.05 vs NC group.

Figure 4 Hypoxia-induced exosome production is suppressed in HIF-1-deficient cells (A) Immunoblot analysis of HIF-1α in scrambled sequence-transfected cells (Scr) and HIF-1α knockdown cells (KD1, KD2) after 6 hours of normoxia or hypoxia incubation. GAPDH was blotted as the internal control. (B) Immunoblot analysis of CD63 and TSG101 in exosome preparations from scrambled sequence-transfected cells (Scr) and HIF-1α knockdown cells (KD) after 24 hours of normoxia or hypoxia incubation. (C) NTA quantification of exosomes from scrambled sequence-transfected cells (Scr) and HIF-1α knockdown cells (KD) after 24 hours of normoxia or hypoxia incubation. Data are means ± SD; n=3; * P＜0.05 vs Scr Normoxia group.

Figure 5 Exosomes production in MEF cells with HIF-1α knockout

(A) Immunoblot analysis of HIF-1α in wild-type MEF cells (WT) and HIF-1α knockout MEF cells (KO) after 6 hours of normoxia or hypoxia incubation. (B) Immunoblot analysis of CD63 and TSG101 in exosome preparations from wide type cells and HIF-1α knockout cells after 24 hours of normoxia or hypoxia incubation. C, NTA test for exosomes quantity from wide type cells and HIF-1α knockout after 24 hours of normoxia or hypoxia incubation. Data are means ± SD (n = 3); * P＜0.05 vs indicated group.

Figure 6 Cytoprotective effect of the exosomes produced by hypoxic RPTC cells

RPTC cells were cultured in normoxia or hypoxia for 16-24 hours to collect medium for exosome isolation. exo-Nc24h: exosomes from cells with 24 hours of normoxia; exo-Hy24h: exosomes from cells with 24 hours of hypoxia. The exosomes were then added to another group of RPTC cells at 5×1011 particles/ml for 12 hours of pre-treatment, followed by ATP-depletion with azide and recovery to show apoptosis. (A) Representative images of cell and nuclear morphologies. After treatment, the cells were stained with Hoechst33342 to record cellular morphology by phase contrast microscopy and nuclear morphology by fluorescence microscopy. The cells were also stained with propidium iodide (PI) to show necrosis. (B) Percentage of apoptosis determined by counting the cells with typical apoptotic morphology. Data are means ± SD (n = 3); * P＜0.05 vs NC group, # P＜0.05 vs Azide (No addition) group. (C) Immunoblot analysis of cleaved caspase-3. β-actin was probed as internal control.

Figure 7 Role of HIF-1 in producing cytoprotective exosomes by RPTC cells under hypoxia

HIF-1-knockdown RPTC cells and scrambled sequence-transfected RPTC cells were cultured in normoxia or hypoxia for 24 hours to collect medium for exosome isolation. exoScr-Nc: exosomes from scrambled sequence-transfected cells with 24 hours of normoxia incubation; exoKD-Hy: exosomes from HIF-1-knockdown cells with 24 hours of hypoxia. The exosomes were then added to another group of RPTC cells at 5×1011 particles/ml for 12 hours of pre-treatment, followed by ATP-depletion with azide and recovery to show apoptosis. (A) Representative images of cell morphology recorded by phase contrast microscopy. (B) Percentage of apoptosis determined by counting the cells with typical apoptotic morphology. Data are means + SD (n = 3); * $P<0.05$ vs NC group, # $P<0.05$ vs Azide (No addition) group. (C) Immunoblot analysis of cleaved caspase-3. β-actin was probed as internal control.

Figure 1

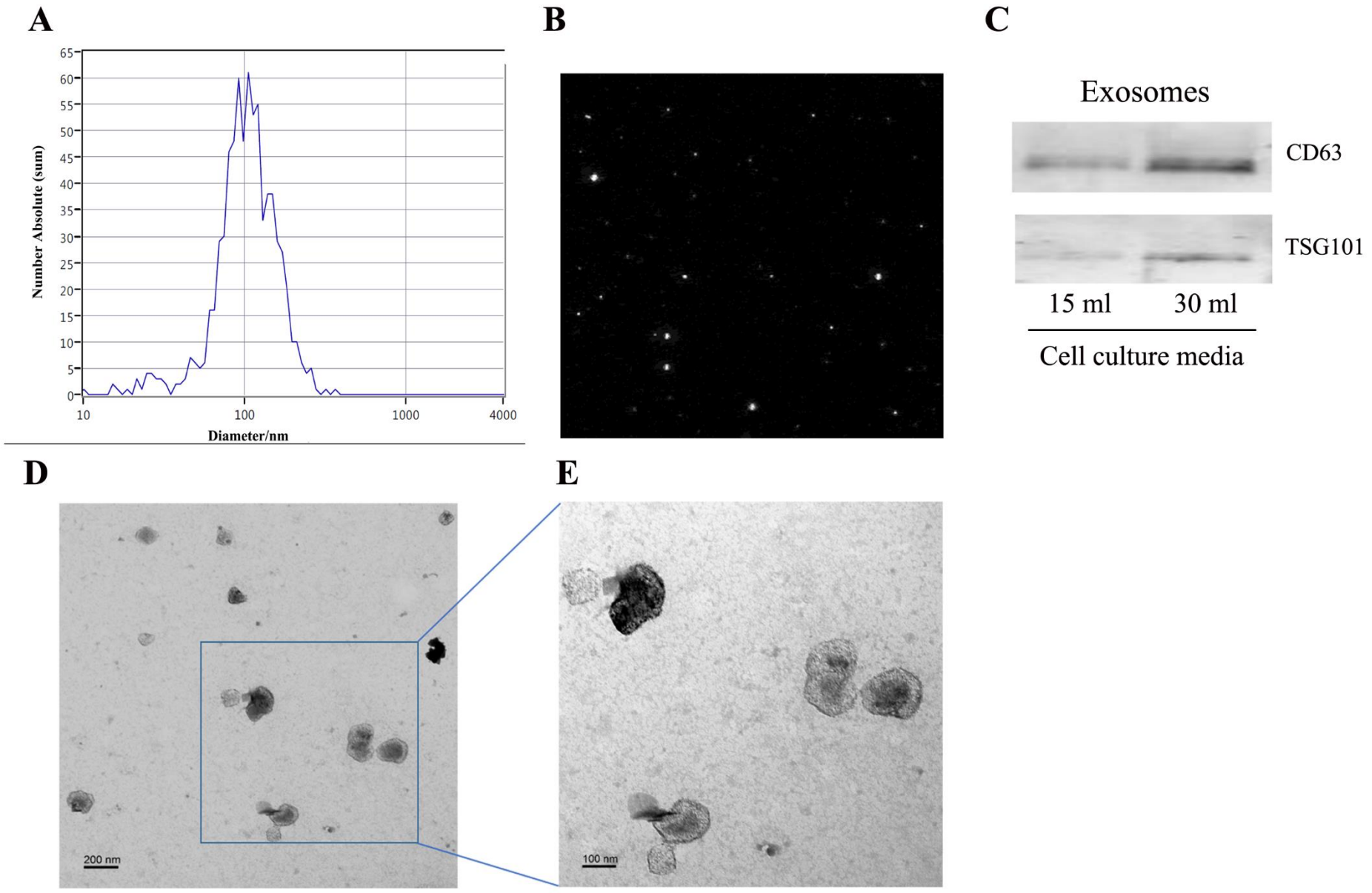
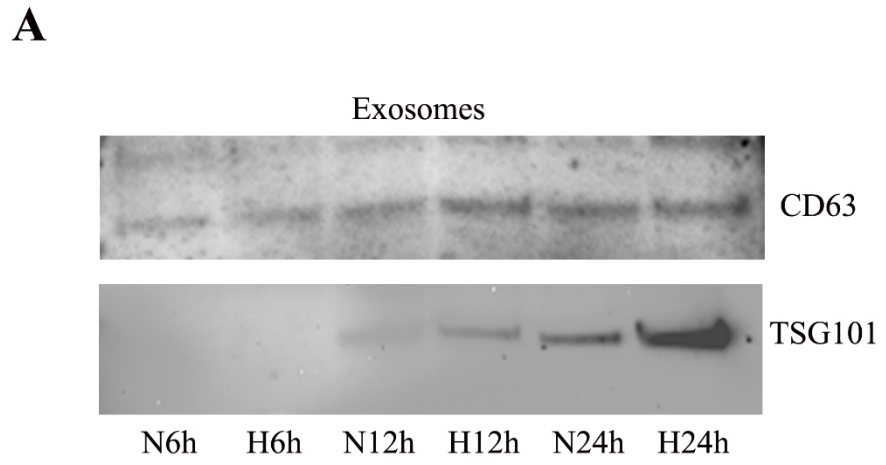
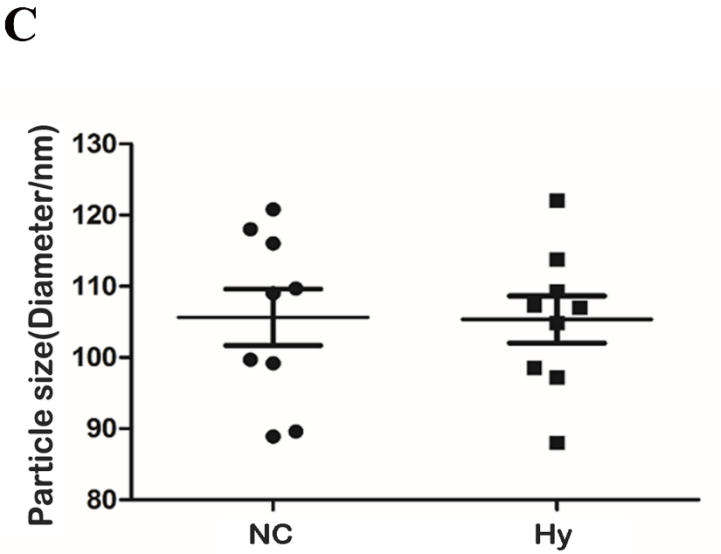
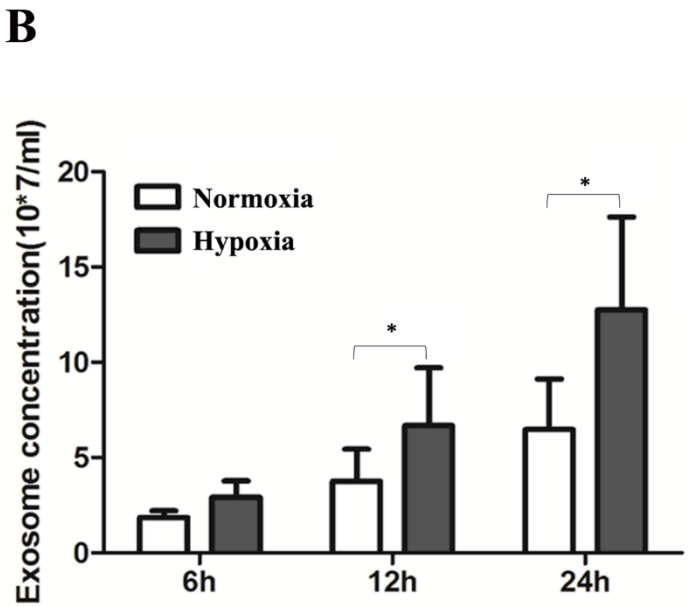
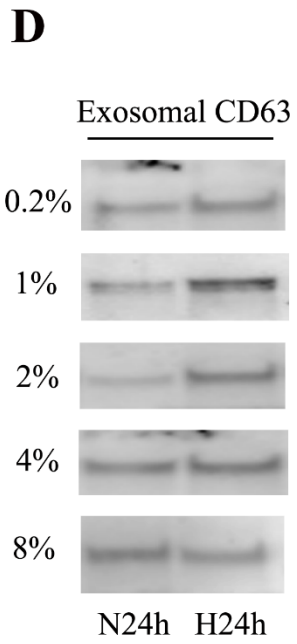

A
Number Absolute (sum)
65
60
55
50
45
40
35
30
25
20
15
10
5
0
10
100
1000
4000
Diameter/nm
B
C
Exosomes
CD63
TSG101
15 ml
30 ml
Cell culture media
D
200 nm
E
100 nm

Figure 2
(Revised)









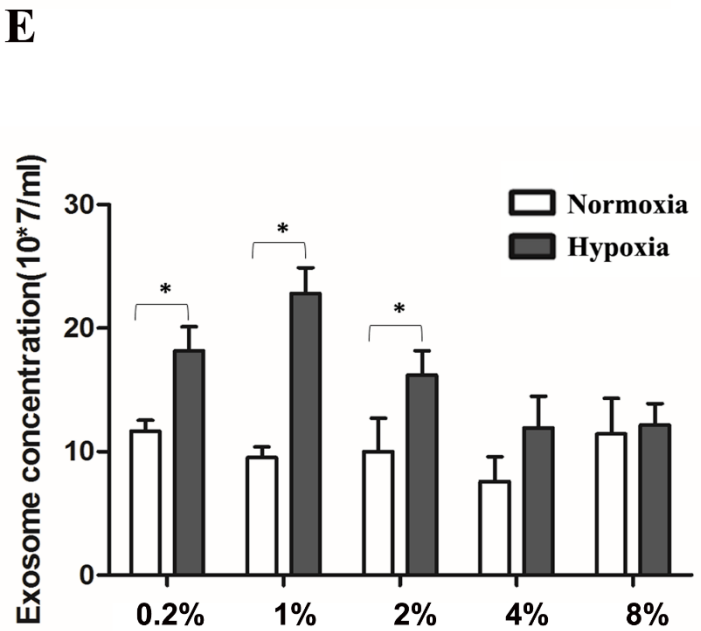
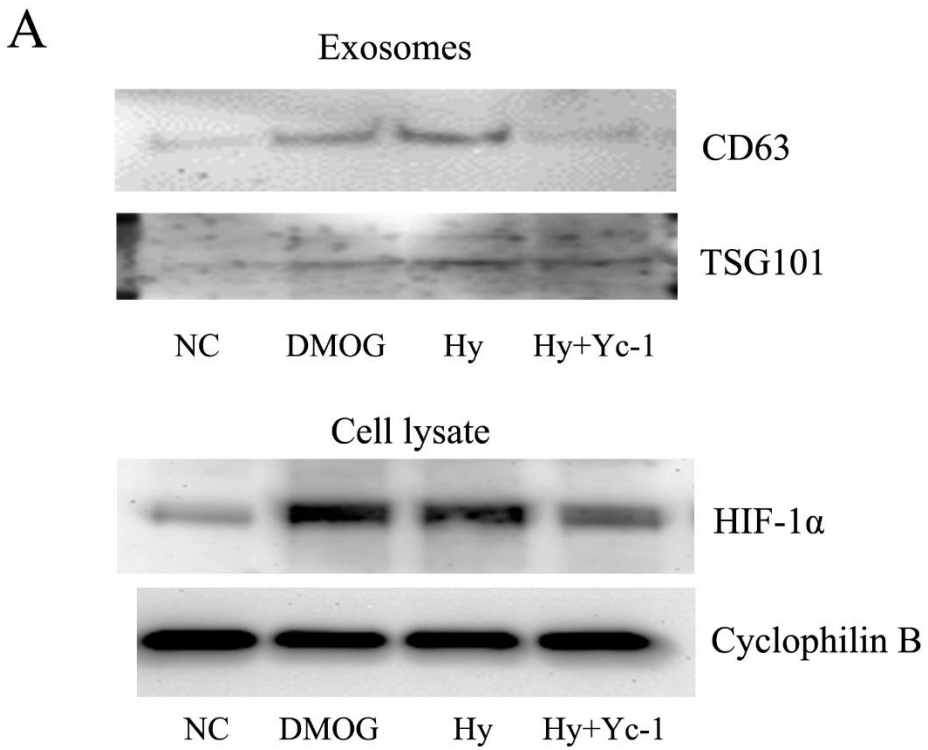


Figure 3


A
Exosomes
CD63
TSG101
NC
DMOG
Hy
Hy+Yc-1
Cell lysate
HIF-1α
Cyclophilin B
NC
DMOG
Hy
Hy+Yc-1

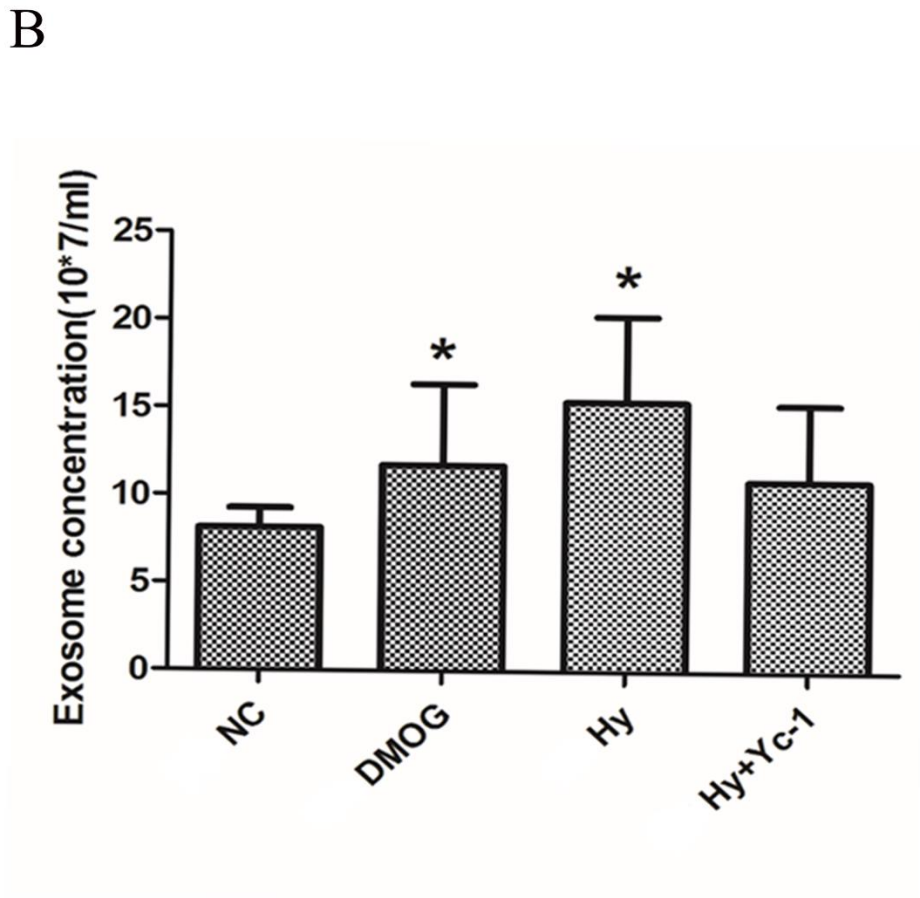

B
Exosome concentration(10*7/ml)
25
20
15
10
5
0
*
*
NC
DMOG
Hy
Hy+Yc-1

Figure 4
(Revised)

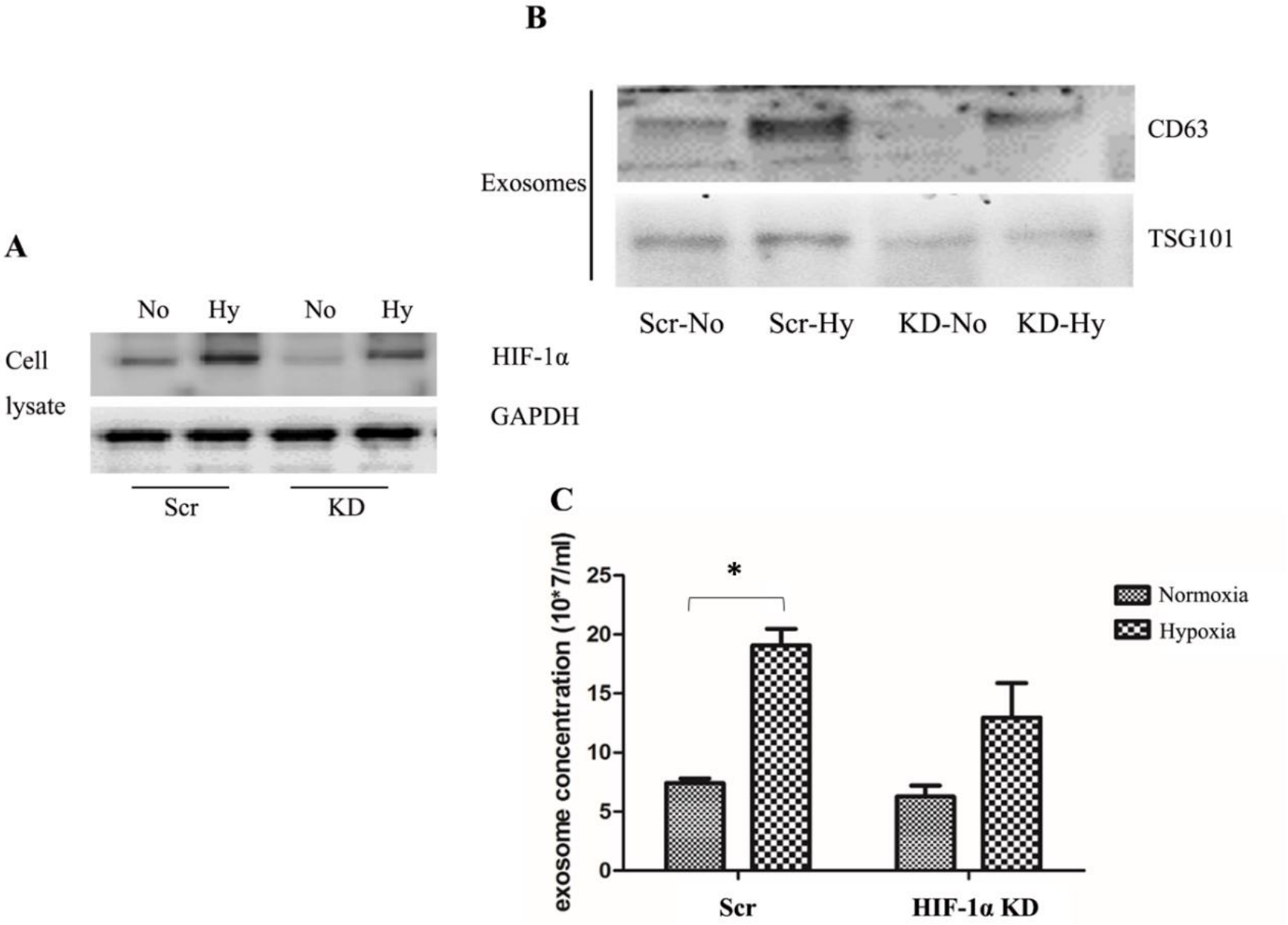


Figure 5
(Revised)

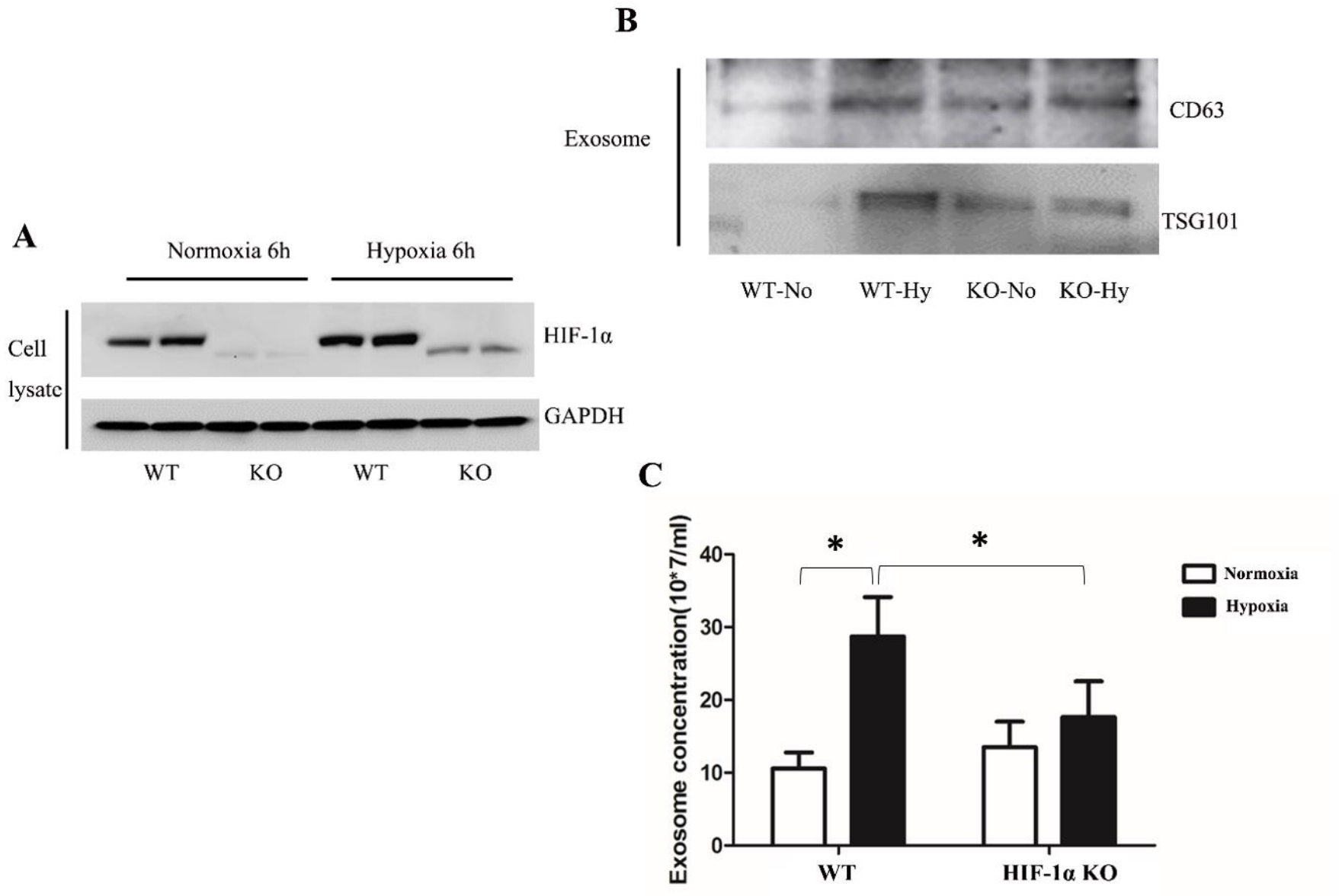


Figure 6

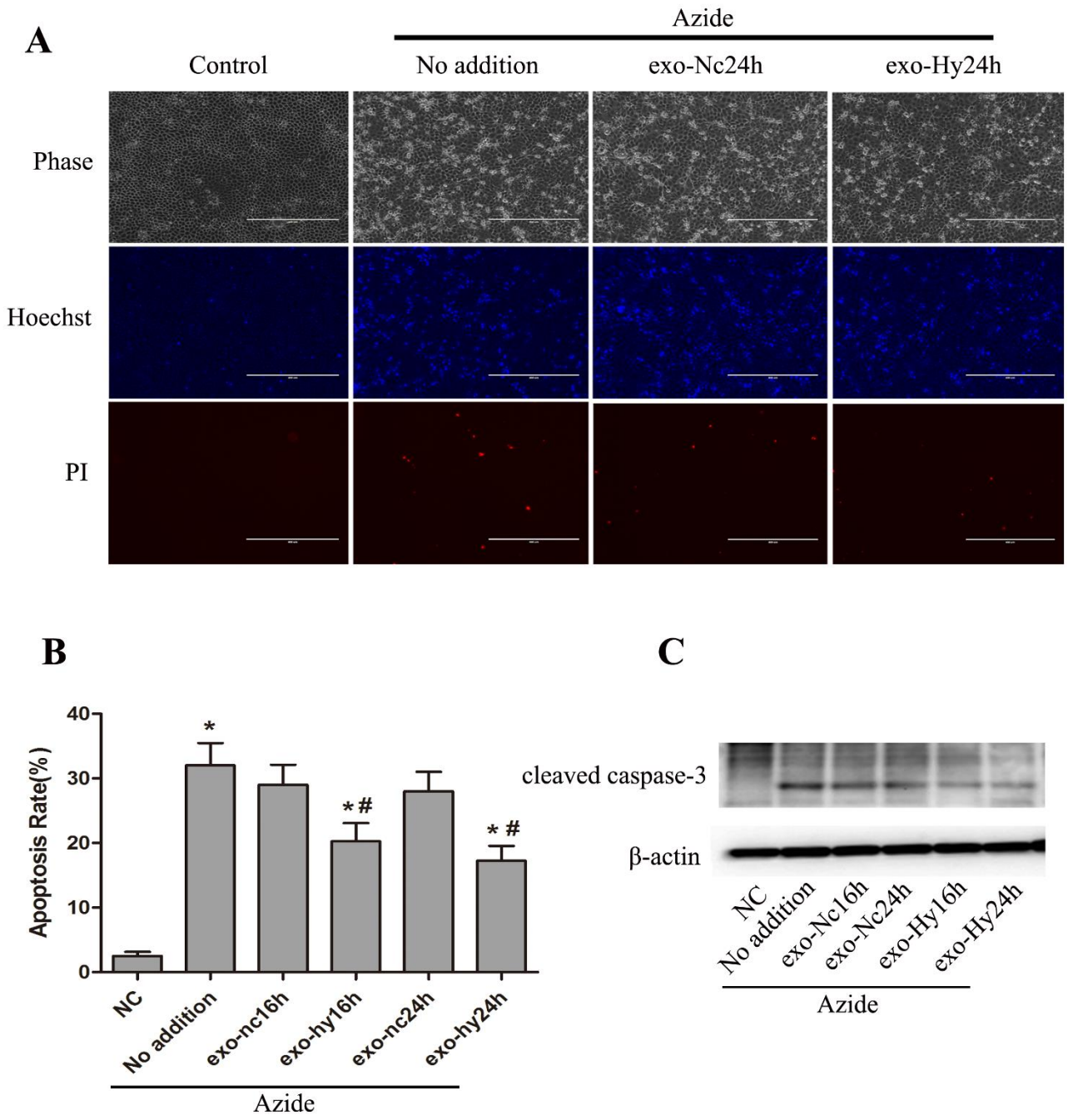
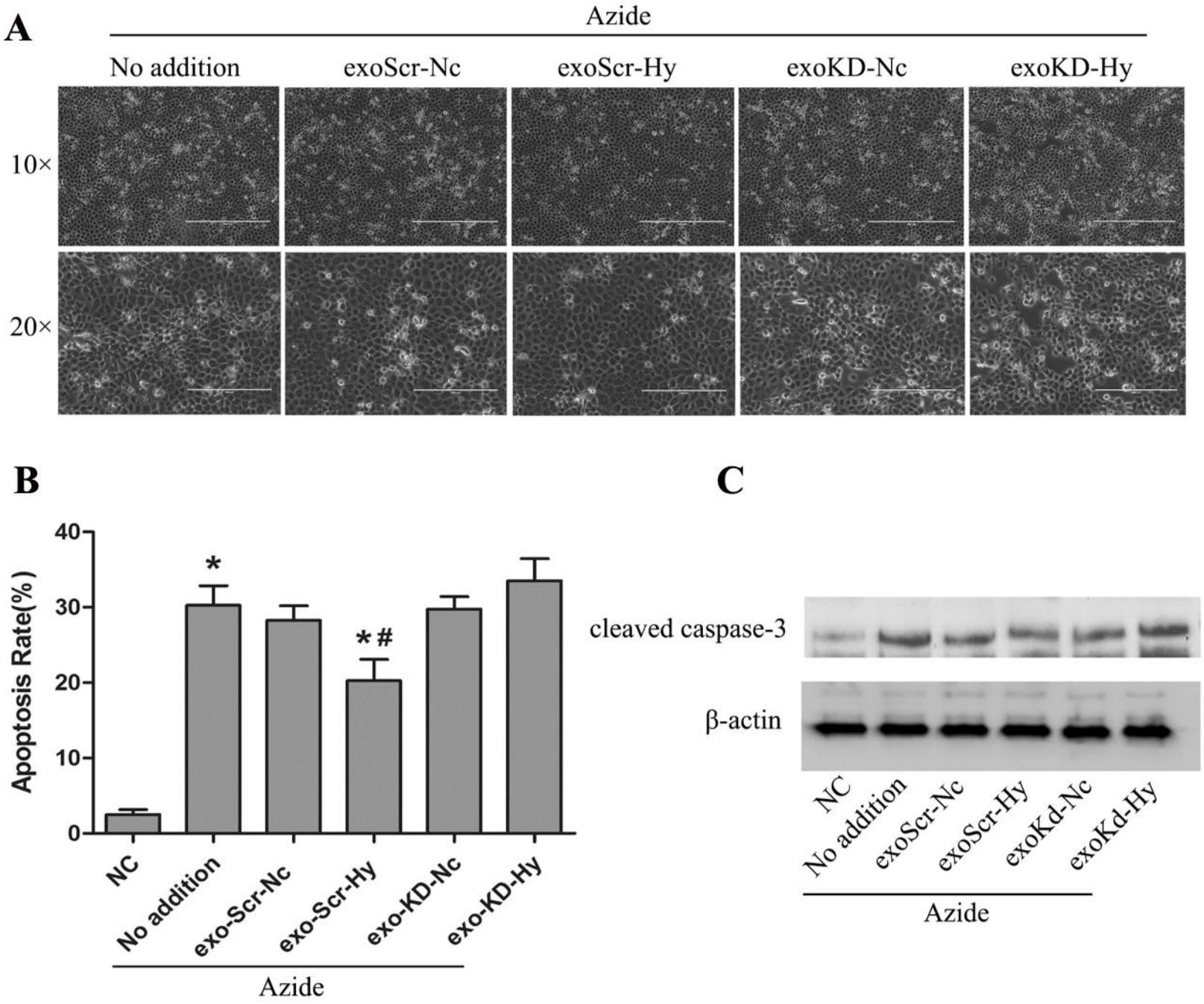

A
Azide
Control
No addition
exo-Nc24h
exo-Hy24h
Phase
Hoechst
PI
B
Apoptosis Rate(%)
40
30
20
10
0
*
*#
*#
NC
No addition
exo-nc16h
exo-hy16h
exo-nc24h
exo-hy24h
Azide
C
cleaved caspase-3
β-actin
NC
No addition
exo-Nc16h
exo-Nc24h
exo-Hy16h
exo-Hy24h
Azide

Figure 7


A
Azide
No addition
exoScr-Nc
exoScr-Hy
exoKD-Nc
exoKD-Hy
10×
20×
B
Apoptosis Rate(%)
40
30
20
10
0
*
*#
NC
No addition
exo-Scr-Nc
exo-Scr-Hy
exo-KD-Nc
exo-KD-Hy
Azide
C
cleaved caspase-3
β-actin
NC
No addition
exoScr-Nc
exoScr-Hy
exoKd-Nc
exoKd-Hy
Azide