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**BENZENE METABOLITE HYDROQUINONE UP-REGULATES
CHONDROMODULIN-I AND INHIBITS TUBE FORMATION IN HUMAN BONE
MARROW ENDOTHELIAL CELLS**

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Running title: HQ modulates ChM-I and endothelial cell tube formation

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ABBREVIATIONS

The abbreviations used are: HQ, hydroquinone; ChM-I, chondromodulin-I; rhChM-I, recombinant human ChM-I; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SRB, sulforhodamine B; HSC, hematopoietic stem cell; TrHBMEC, transformed human bone marrow endothelial cell; HUVEC, human umbilical vein endothelial cell; HCAECs, human coronary artery endothelial cells; NQO1, NAD(P)H:quinone oxidoreductase.

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ABSTRACT

Bone marrow is a major target of benzene toxicity and NAD(P)H:quinone oxidoreductase (NQO1), a protective enzyme against benzene toxicity, is present in human bone marrow endothelial cells which form the hematopoietic stem cell (HSC) vascular niche. In this study, we have employed a transformed human bone marrow endothelial cell (TrHBMEC) line to study the adverse effects induced by the benzene metabolite hydroquinone. Hydroquinone inhibited TrHBMEC tube formation at concentrations which were not overtly toxic as demonstrated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or SRB (Sulforhodamine B) analysis. Hydroquinone was found to up-regulate chondromodulin-I (ChM-I), a protein that promotes chondrocyte growth and inhibits endothelial cell growth and tube formation. Recombinant human ChM-I protein inhibited tube formation in TrHBMEC suggesting that up-regulation of ChM-I may explain the ability of hydroquinone to inhibit TrHBMEC tube formation. To explore this possibility further, anti-ChM-I siRNA was used to deplete ChM-I mRNA and protein. Pretreatment with anti-ChM-I siRNA markedly abrogated hydroquinone induced inhibition of tube formation in TrHBMEC. Overexpression of the protective enzyme NQO1 in TrHBMEC inhibited the up-regulation of ChM-I and abrogated the inhibition of tube formation induced by hydroquinone. In summary, hydroquinone treatment up-regulated ChM-I and inhibited tube formation in TrHBMEC; NQO1 inhibited hydroquinone induced up-regulation of ChM-I in TrHBMEC and protected cells from hydroquinone induced inhibition of tube formation. This study demonstrates that ChM-I up-regulation is one of the underlying mechanisms of inhibition of tube formation and provides a mechanism that may contribute to benzene induced toxicity at the level of bone marrow endothelium.

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INTRODUCTION

Benzene is an occupational and environmental pollutant. Chronic exposure to benzene can induce aplastic anemia, myelodysplastic syndrome and leukemia (Travis *et al.*, 1994). Although questions remain regarding its mechanism of action, it is known that benzene requires metabolism to induce its adverse effects and hydroquinone is considered to be one of the major toxic metabolites derived from benzene (Ross, 1996). Benzene is metabolized to phenolic derivatives mainly in the liver (Ross, 1996) and both catechol and hydroquinone have been shown to persist in bone marrow after benzene exposure (Rickert *et al.*, 1979). Through auto-oxidation or peroxidase mediated oxidation, hydroquinone can be converted to reactive 1,4-benzoquinone capable of reacting with proteins, DNA and lipids (Ross, 2000). NADPH:quinone oxidoreductase 1 (NQO1, DT-diaphorase), a flavin-containing quinone reductase, can protect cells from toxic effects via reduction of quinone substrates to less reactive hydroquinone forms, which are more water soluble and easily conjugated and excreted (Ross, 1997).

The major target of benzene toxicity is bone marrow where the main hematopoietic cell generating system is in close contact with the stromal microenvironment. Bone marrow stroma is composed of a variety of different cell types providing structural and functional support for hematopoiesis. Among these cell types, endothelial cells have a specific biological relevance. Recent advances in stem cell studies have revealed two HSC niches which play an important role in the homeostatic regulation of HSCs: the osteoblastic niche (osteoblasts and hematopoietic cells) and the vascular niche (hematopoietic cells and endothelial cells) (Caplan and Dennis, 2006; Friedenstein *et al.*, 1974; Dexter *et al.*, 1977; Li and Li, 2006). The vascular niche offers an alternative niche for mobilized stem cells which promotes proliferation and further differentiation or maturation and release into the circulatory system (Kopp *et al.*, 2005; Avecilla *et al.*, 2004). Damage to the vascular niche could result in abnormal hematopoiesis. Diseases of myelodepression, aplastic anemia and leukemia can all be characterized by abnormal proliferation of blood cells resulting from abnormal and poorly regulated hematopoiesis. Therefore, in this study,

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hydroquinone induced toxicity in the bone marrow vascular microenvironment was examined by using TrHBMEC as a model system.

Among the endothelial cell functions, capillary tube formation is a prerequisite for the establishment of a continuous vessel lumen and further angiogenesis (Matsumura *et al.*, 1997). Recent advances in hypovascular mesenchymal tissues have unveiled some endothelial cell tube formation inhibitors including ChM-I and its related-gene, tenomodulin (Hiraki and Shukunami, 2005). Interestingly, ChM-I was identified as one of the genes up-regulated by treatment of TrHBMEC with hydroquinone in an Affymetrix microarray study in our lab (Zhou *et al.*, 2008). ChM-I was originally identified as a chondrocyte growth promoting factor and then purified as an angiogenesis inhibitor which inhibits DNA synthesis, cell growth and tube formation in endothelial cells (Hiraki *et al.*, 1991). ChM-I has also been reported to promote apoptosis in human coronary artery endothelial cells (HCAECs) (Yoshioka *et al.*, 2006). ChM-I is initially synthesized in a type II transmembrane glycoprotein precursor form with total of 334 amino acids in human. After C-terminal cleavage by the furin endoprotease, it is secreted from the cell immediately as a mature but shorter 120 amino acid protein (Hiraki *et al.*, 1999;Azizan *et al.*, 2001). ChM-I is expressed at high levels in avascular tissues such as cartilage and cornea, but under normal conditions, ChM-I expression in endothelium is very low (Hiraki and Shukunami, 2005;Hiraki and Shukunami, 2000). In this work, a tube formation assay was first performed to assess the ability of hydroquinone to inhibit TrHBMEC tube formation. Further studies were then performed to investigate whether ChM-I was a mediator of the effects of hydroquinone on tube formation in TrHBMEC. In addition, based on the documented protective role of NQO1 against benzene toxicity, we overexpressed NQO1 in TrHBMEC to investigate whether NQO1 could protect cells from hydroquinone induced up-regulation of ChM-I and inhibition of tube formation.

MATERIALS AND METHODS

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Materials - Hydroquinone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and β -mercaptoethanol were obtained from Sigma Chemical (St. Louis MO). Human ChM-I siRNA sequences were obtained from Dharmacon (Lafayette, CO). RNeasy Kit was obtained from Qiagen (Valencia, CA). Recombinant human ChM-I and rabbit polyclonal antisera against human ChM-I were generated in the Hiraki lab (Hiraki *et al.*, 1999). Mouse anti-NQO1 monoclonal antibody was generated in our lab. Mouse anti- β -Actin antibody was obtained from Sigma. Lipofectamine 2000 transfection reagent was obtained from Invitrogen (Carlsbad, CA). Matrigel was obtained from BD Bioscience (San Jose, CA). Butyl Sepharose 4 Fast Flow was obtained from GE Healthcare (Piscataway, NJ). Amicon Ultra-4 3,000 NMWL centrifugal filter devices were obtained from Millipore (Billerica, MA).

Cell culture - TrHBMEC is a transformed microvascular endothelial cell line established from primary bone marrow endothelial cells by Dr. Babette B. Weksler (Weill Medical College, Cornell University) (Schweitzer *et al.*, 1997). Cells were cultured on 0.2% (w/v) gelatin coated cell culture plates in Dulbecco's Modified Eagle Medium (low glucose) supplemented with 5% (v/v) fetal bovine serum, penicillin (100 units/ml), streptomycin (100 units/ml), 3 mM L-glutamine, 10 mM HEPES and 1% (v/v) BME vitamins (Invitrogen, Carlsbad, CA). This medium was used for all experiments. Cells were maintained at 37 °C in 5% CO₂. Cells with passage number ranging from 19-25 were used in this study.

MTT cell proliferation assay – TrHBMEC proliferation following treatment with hydroquinone was measured using the MTT colorimetric assay. In these studies, TrHBMEC were seeded at 2-3 x 10³ cells/well in 96-well plates and allowed to attach for 16 hours. Medium was removed by aspiration and the TrHBMEC were treated with the hydroquinone (1-1000 μ M) in complete medium for 24 hours, after which, the medium was removed and replaced with 200 μ l of fresh medium. After 72 hours the medium was removed and an additional PBS wash was performed to avoid the possibility of a colorimetric reaction between MTT and hydroquinone. MTT (50 μ g) in complete medium (50 μ l) was added to each well for a further 4 hour incubation. Cell viability was determined by measuring the cellular reduction of

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MTT (Mosmann, 1983) to the crystalline formazan product, dissolved by the addition of DMSO (100 μ l). Optical density was determined at 550 nm using a Molecular Devices Thermomax microplate reader. The IC_{50} values were defined as the concentration of hydroquinone that resulted in 50% reduction in cell number compared to the control. These values were determined from percentage of control versus hydroquinone concentration.

Quantitative real time RT-PCR of the ChM-I gene - The relative amount of *ChM-I* mRNA was assessed using TaqMan probe real-time PCR with the ABI 7500 system (Applied Biosystems). Taqman probes for *ChM-I* (Assay ID: Hs00170877_m1) and β -Actin (Part Number: 4333762T) assay were purchased from Applied Biosystems (Foster City, CA), β -Actin was used as the internal control. All of the reactions were run in triplicate or quadruplicate. The fold increase of *ChM-I* mRNA in TrHBMEC after hydroquinone treatment, normalized to an endogenous β -Actin reference and relative to untreated control is given by: $2^{-\Delta\Delta CT}$ (Where: $\Delta\Delta CT = \Delta CT_{\text{hydroquinone treatment}} - \Delta CT_{\text{untreated control}}$, and $\Delta CT = \text{threshold cycles for target ChM-I} - \text{threshold cycles for reference } \beta\text{-Actin}$).

Human ChM-I siRNA transfection - Lipofectamine 2000 transfection reagents were obtained from Invitrogen. Cells (0.5 million) were seeded into 100 mm plates the day before transfection with 15 ml medium containing serum but without antibiotics. On the day of transfection, 600 pmol siRNA was diluted in 1.5 ml culture medium without serum and antibiotics which resulted in a final working siRNA concentration of 33 nM after adding complexes to cells. Lipofectamine 2000 transfection reagent (30 μ l) was added to another 1.5 ml culture medium without serum and antibiotics with gentle mix and incubated for 5 minutes at room temperature. Then the diluted oligomer and lipofectamine 2000 were mixed gently and incubated at room temperature for another 20 minutes to allow for the formation of transfection complexes. The complexes were added onto the cells and mixed gently by rocking the plate back and forth. Cells contain transfection complexes were then incubated under their normal growth conditions for various periods of time until harvest.

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Purification of rhChM-I - Recombinant human ChM-I was prepared as described previously (Hiraki *et al.*, 1999).

Recombinant human NQO1 (rhNQO1) transfection - Fugene HD transfection reagent (Roche Applied Bioscience) and the CMV driven mammalian expression vector, pcDNA3.0 containing human wtNQO1 cDNA (Winski *et al.*, 1998) were used in transfections. Cells were allowed to grow to 80% confluency, Fugene HD transfection reagent, DNA and diluent mix was made following Fugene HD instruction. Cells transfected for at least 18 hours were subjected for further hydroquinone treatment or tube formation assay.

Immunoblot analysis – Mature ChM-I in medium -- TrHBMEC culture media (10 ml for each sample) were collected after treatment. The medium was first loaded to butyl Sepharose column following manufacture's instruction and then eluted with 20 mM Na₂HPO₄ with 40% acetonitrile, collected fractions were concentrated using an Amicon Ultra-4 3,000 NMWL centrifugal filter devices to a similar volume. Samples were then diluted in 5X Laemmli sample buffer to a total volume of 120 µl and heated to 90°C for 5 minutes. Same volumes of samples (25 µl) were then separated by 12% SDS-PAGE. – *Cell lysate proteins* -- TrHBMEC were scraped into PBS and collected by centrifugation. Cell pellets were resuspended in 100 µl RIPA buffer with protease inhibitors added and sonicated on ice for 15 seconds. Sonicates were centrifuged at 13,000 rpm for 5 minutes to remove cellular debris. Following centrifugation, the supernatant was assayed for protein concentrations using the method of Lowry. Protein samples (15 µg to 50 µg) were diluted in 5X Laemmli sample buffer and heated to 90°C for 5 minutes then separated by 12% SDS-PAGE. After SDS-PAGE separation, samples were transferred to polyvinylidene difluoride membranes in 25 mM Tris, 192 mM glycine containing 20% (v/v) methanol at 500 mA. Following transfer, membranes were placed overnight in blocking buffer (10mM Tris-HCl, pH

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8 containing 125 mM NaCl, 0.2% (v/v) Tween-20 and 5% (w/v) non-fat dry milk). Antibodies diluted in blocking buffer were used at the following dilutions and times: Polyclonal antisera against ChM-I 1:5000 for 1 hour at room temperature or 4°C overnight; Anti- β -Actin, 1:10,000 for 0.5 hour at room temperature or 4°C overnight. For all analysis, HRP conjugated goat anti-mouse or anti-rabbit IgG were diluted at 1:5000 in blocking buffer for 0.5 to 1 hour at room temperature. Protein bands were visualized using luminol-based enhanced chemiluminescence as described by the manufacturer (PerkinElmer Life Sciences). Densitometry analysis software was Adobe Photoshop 7.0.

Generation and validation of ChM-I antibody - The polyclonal antibody against human ChM-I precursor and mature protein was generated and validated in the Hiraki lab as described previously (Hiraki *et al.*, 1999). Validation was also extended in the Ross lab. First the recombinant human ChM-I protein was verified using MALDI-TOF MS and then the reactivity of the antibody against the recombinant human ChM-I protein was confirmed. The antibody was also validated using ChM-I protein generated from TNT Quick Coupled Transcription/Translation Systems (cell free protein expression, Promega, WI) using the full length coding region. The antibody was further validated by the detection of decreased formation of ChM-I resulted from anti-ChM-I siRNA treatment.

Tube formation assay - 6-well or 24-well plates were coated with 150 μ l or 30 μ l Growth Factor Reduced matrigel (BD bioscience) per well and incubated at 37°C for 30 minutes. 100,000 or 20,000 TrHBMEC cells (siRNA pretreated cells were cells transfected with anti-ChM-I siRNA for 48 hrs) were seeded onto each well in 5ml or 1 ml media (with or without hydroquinone/ChM-I treatment). Cells were allowed to grow for 16 hours for tube formation observations (10x objective lens, 10x ocular lens, Nikon TMS-F inverted phase contrast microscope). Pictures were taken directly from ocular lens using Nikon Coolpix 990 digital camera. Total lengths of tube-like structures per field were measured using image processing and analysis software (NIH image J version 1.41, available in the public domain via the National Institute of Health, Bethesda, MD). Each experiment was repeated at least 3 times.

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Statistical analysis - Values are presented as the mean \pm SD. Statistical significance was evaluated using the student's *t*-test for comparisons between two mean values. One way ANOVA with Tukey *post hoc* test was used to assess significance between experimental groups. A value of $P < 0.05$ was considered significant.

RESULTS

Determination of non-overtly toxic concentrations of hydroquinone in TrHBMEC. To assess hydroquinone induced cytotoxicity in TrHBMEC and to generate a dose which did not demonstrate overt toxicity, MTT growth inhibition assays were performed. To avoid the reaction between the residual hydroquinone and MTT, an extra wash with PBS was performed before the addition of MTT. The calculated IC₅₀ of hydroquinone in TrHBMEC was approximately 139 μ M (Figure. 1), which is in agreement with data obtained using trypan blue exclusion as a marker of toxicity (Bironaite *et al.*, 2004). Using a SRB assay to measure cellular protein content, the IC₅₀ value for hydroquinone in TrHBMEC was calculated to be 167 μ M, which correlates well with the MTT assay results since IC₅₀ values obtained using the SRB method are usually higher than those obtained using the MTT method (Vichai and Kirtikara, 2006). Hydroquinone at a dose of 10 μ M was chosen for further studies.

Hydroquinone up-regulated ChM-I mRNA in TrHBMEC. To understand how hydroquinone alters TrHBMEC function, 10 μ M of hydroquinone was used to treat TrHBMEC. ChM-I was identified as one of the genes up-regulated by hydroquinone treatment, from an absent call to a present call in an Affymetrix microarray study (Zhou *et al.*, 2008). To verify ChM-I mRNA up-regulation by hydroquinone treatment, Taqman probe quantitative RT-PCR was performed to evaluate ChM-I mRNA level after hydroquinone treatment in TrHBMEC. As shown in figure 2, hydroquinone treatment successfully up-regulated ChM-I mRNA about 1.6 fold in TrHBMEC compared to untreated control cells. Pretreatment with actinomycin D to inhibit the *de-novo* mRNA synthesis in TrHBMEC blocked the up-regulation of

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ChM-I precursor protein (data not shown) confirming that hydroquinone up-regulated ChM-I at the level of mRNA in TrHBMEC.

Hydroquinone up-regulated ChM-I protein in TrHBMEC. ChM-I is initially synthesized in precursor form, a type II transmembrane glycoprotein (334 amino acids in human), which requires cleavage by the furin endoprotease to secrete the mature 120 amino acid protein (Hiraki *et al.*, 1999;Azizan *et al.*, 2001). The increased expression of ChM-I mRNA in TrHBMEC suggested a potential up-regulation of ChM-I protein. Immuno-blot assays were performed to detect the ChM-I precursor protein in TrHBMEC cell lysates. As shown in figure 3a, hydroquinone treatment for 16 hours up-regulated ChM-I precursor in a dose dependent manner and up-regulation of ChM-I precursor could be observed as early as 6 hr after treatment with 10 μ M hydroquinone treatment (Figure 3b). To ensure that hydroquinone did not block the maturation of ChM-I, the up-regulation of mature ChM-I protein levels in cell culture medium was examined. As shown in figure 3c, the immunoblot results clearly demonstrated that mature ChM-I in medium was also increased by hydroquinone treatment. The increase of mature ChM-I in medium confirmed the secretion of ChM-I from TrHBMEC.

Hydroquinone inhibited TrHBMEC tube formation in matrigel. One important function of ChM-I is to inhibit endothelial cell tube formation (Shukunami and Hiraki, 2007). Since hydroquinone induced up-regulation of ChM-I mRNA and protein were both confirmed, we next determined whether hydroquinone could inhibit TrHBMEC tube formation. A tube formation assay was performed in matrigel coated plates as shown in figure 4C, 4D and 4E. A parallel control study using gelatin coated plate was also performed as shown in figures 4A and 4B. Hydroquinone treatment did not induce obvious morphological change and cell death in cells grown in gelatin coated plates (Figures 4A, 4B). However, hydroquinone treatment inhibited TrHBMEC tube formation in a dose dependent manner on cells grown in matrigel (Figures 4C, 4D and 4E), it almost completely inhibited TrHBMEC tube formation at 10 μ M, a concentration which

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was not overtly toxic as demonstrated by trypan blue exclusion (Bironaite *et al.*, 2004) or MTT (Figure 1) or SRB analysis (data not shown).

Recombinant human ChM-I inhibited TrHBMEC tube formation in matrigel. In order to support the hypothesis that hydroquinone up-regulated ChM-I and inhibited tube formation in TrHBMEC, we examined the effect of direct addition of purified mature human ChM-I protein on the formation of tubes in TrHBMEC. Although ChM-I has been demonstrated to be able to inhibit tube formation in different endothelial cell lines such as HUVEC (Oshima *et al.*, 2004), bovine carotid artery endothelial cells (Hiraki *et al.*, 1999) and HCAEC (Yoshioka *et al.*, 2006), it has not been tested on TrHBMEC. As shown in figure 5, recombinant human mature ChM-I inhibited TrHBMEC tube formation in matrigel in a dose dependent manner.

Pretreatment with anti-ChM-I siRNA in TrHBMEC knocked down ChM-I protein and abrogated the inhibition of tube formation induced by hydroquinone treatment. To define whether the elevation of ChM-I by hydroquinone contributes to hydroquinone induced tube formation inhibition in TrHBMEC, TrHBMEC were first pretreated with anti-ChM-I siRNA to deplete ChM-I mRNA and ChM-I protein. As shown in figure 6a, ChM-I precursor protein was successfully knocked down more than 80% by ChM-I siRNA between 48 hours and 72 hours after siRNA transfection. Importantly, pretreatment with anti-ChM-I siRNA inhibited hydroquinone induced up-regulation of ChM-I (Figure 6b). Cells after 48 hours of anti-ChM-I siRNA treatment were lifted and seeded onto matrigel-coated plates to perform the tube formation assay. Cells treated with either non-targeting control siRNA or anti-ChM-I siRNA maintained tube formation ability when there was no hydroquinone present. However, tube formation was inhibited in TrHBMEC pretreated with non-targeting siRNA in the presence of hydroquinone (10 μ M); whereas inhibition of tube formation was markedly abrogated in TrHBMEC pretreated with anti-ChM-I siRNA and subsequently challenged with hydroquinone (Figure 6c, 6d). These data strongly suggest that ChM-I

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elevated by hydroquinone treatment may be a novel mechanism contributing to hydroquinone induced inhibition of tube formation in TrHBMEC.

Overexpression of NQO1 in TrHBMEC protects cells from hydroquinone induced up-regulation of ChM-I and inhibition of tube formation. NQO1 is known to be a protective enzyme against benzene toxicity. We hypothesized that overexpression of NQO1 could protect TrHBMEC from hydroquinone induced up-regulation of ChM-I and inhibition of tube formation. TrHBMEC were treated with either a control plasmid or plasmid encoding human NQO1. Overexpression of NQO1 was confirmed in TrHBMEC by immuno-blot analysis (Figure 7a). Immuno-blot analysis also demonstrated that cells overexpressing NQO1 inhibited hydroquinone induced up-regulation of ChM-I precursor protein (Figure 7b). Cells transfected with pcDNA3.0 harboring NQO1 (the NQO1 overexpression vector) or control vector were then lifted to perform the tube formation assay in the presence of hydroquinone. Hydroquinone inhibited tube formation in cells transfected with control vector, but the inhibition was abrogated in cells transfected with NQO1 overexpression vector as shown in figure 7c, 7d.

DISCUSSION

Chronic exposure to benzene can result in bone marrow toxicity and is associated with the development of a variety of blood dyscrasias including lymphocytopenia, thrombocytopenia, pancytopenia, and aplastic anemia. Some individuals develop myelodysplastic syndrome that can ultimately progress into acute myelogenous leukemia (Stillman *et al.*, 1997). A recent study further showed that workers exposed to low levels of benzene exhibited a significant decrease in white blood cells and platelets (Lan *et al.*, 2004). These data suggest that a characteristic of the early stages of chronic benzene toxicity could be ineffective hematopoiesis, a process that requires structural and functional support from the bone marrow stroma, including the vascular microenvironment formed by bone marrow endothelial cells. Hydroquinone, a major benzene metabolite, was demonstrated to induce adverse effects in TrHBMEC

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(Figure 1, 4) suggesting that benzene could induce ineffective hematopoiesis through damaging the HSC vascular microenvironment.

ChM-I is mainly expressed in avascular tissues (Hiraki and Shukunami, 2005; Hiraki and Shukunami, 2000). ChM-I mRNA was reported as an absent call in non-treated TrHBMEC in our microarray study (Zhou *et al.*, 2008) and low expression of ChM-I was confirmed by RT-PCR. With hydroquinone treatment, both ChM-I mRNA and protein were up-regulated in TrHBMEC. A similar up-regulation was observed using HUVEC cells (data not shown). The increased expression of ChM-I in TrHBMEC suggests a novel pathway through which hydroquinone could induce toxicity in TrHBMEC. These findings led us to identify ChM-I as a novel mediator in hydroquinone induced tube formation in TrHBMEC and provide a potential mechanism of how hydroquinone induces adverse effects in a critical bone marrow stromal compartment. The effect of recombinant human ChM-I protein on tube formation inhibition and the abrogation of hydroquinone induced inhibition of tube formation by siRNA knockdown provided strong support for the hypothesis. Mature ChM-I is a multifunctional glycoprotein secreted from cells. It is known to promote chondrocyte growth, inhibit endothelial cell growth, DNA synthesis and tube formation. It has also been reported to be able to suppress T cell responses and synovial cell proliferation (Setoguchi *et al.*, 2004). The eventual destiny of endothelial cells treated with ChM-I could be apoptosis as was observed in HCAECs treated with ChM-I (Yoshioka *et al.*, 2006). The accumulation of ChM-I elevated by hydroquinone could result in adverse effects in the bone marrow endothelial microenvironment critical for the development and differentiation of HSC.

Recent studies have shown that endothelial cell tube formation requires the activation of the Rho GTPases, Cdc42 and Rac1. Cell surface proteolysis mediated through membrane type 1 matrix metalloproteinase is also necessary to create vascular guidance tunnels within the 3D matrix environment (Davis *et al.*, 2007). Whether the above mentioned molecules are involved in ChM-I mediated inhibition of tube formation requires further study.

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Hydroquinone has been demonstrated to up-regulate both message and protein levels of ChM-I, but its exact transcriptional mechanisms of hydroquinone up-regulation of ChM-I expression remains unknown. The ChM-I core promoter region contains an AP-1 binding site (Yanagihara *et al.*, 2000) and hydroquinone has been reported to enhance AP-1 activation in TF-1 erythroleukemia cells (Zheng *et al.*, 2004). Whether hydroquinone can up-regulate ChM-I via AP-1 activation need further investigation. Aoyama *et al.* has reported that methylation status in ChM-I core promoter region determines cell-specific ChM-I expression (Aoyama *et al.*, 2004) and Bollati *et al.* has reported their observations of changes in DNA methylation pattern in subjects exposed to low dose benzene (Bollati *et al.*, 2007). It is also a possibility that hydroquinone up-regulates ChM-I expression via altering methylation status in the ChM-I promoter region.

NQO1's protective role against benzene toxicity has been documented (Ross, 1997; Rothman *et al.*, 1997; Bauer *et al.*, 2003). The NQO1*2 polymorphism is a single nucleotide polymorphism, defined as a C to T change at position 609 of the human NQO1 cDNA, corresponding to a proline to serine change at position 187 of the protein (Ross *et al.*, 1996). The mutant NQO1*2 protein is rapidly degraded by the ubiquitin proteasomal system, resulting in an absence of NQO1 protein in individuals carrying the NQO1*2/*2 genotype (Moran *et al.*, 1999). Epidemiological studies have associated the NQO1*2 genotype with an increased risk of benzene-induced myeloid toxicity and a variety of *de-novo* and therapy induced leukemias (Ross, 2005). Overexpression of NQO1 in TrHBMEC decreased the expression of ChM-I and abrogated the inhibition of tube formation induced by hydroquinone further confirming its protective role against benzene induced toxicity. These studies demonstrate the potential role of NQO1 against hydroquinone induced inhibition of endothelial cell tube formation in bone marrow and suggest individuals carrying the NQO1*2 genotype may be more susceptible to benzene metabolite induced stromal compartment damage.

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In summary, hydroquinone up-regulated ChM-I and inhibited tube formation in TrHBMEC suggesting that elevation of ChM-I by hydroquinone is a novel mechanism underlying hydroquinone induced inhibition of tube formation. In addition, this study provides a potential mechanism of how hydroquinone can induce adverse effects in the bone marrow endothelial microenvironment critical to the development and differentiation of HSC.

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FIGURE LEGENDS

Figure 1. Assessment of hydroquinone induced toxicity in TrHBMEC with MTT assay. TrHBMEC proliferation after hydroquinone treatment was assessed using MTT assay. TrHBMECs were exposed to hydroquinone (5-1000 μ M) for 3 days. Results are expressed as the mean \pm standard deviation of three independent determinations. The calculated IC₅₀ is 139 μ M.

Figure 2. Hydroquinone up-regulated ChM-I mRNA in TrHBMEC. TrHBMEC were treated with 10 μ M hydroquinone for 4 hours. The relative amount of ChM-I mRNA was assessed by TaqMan probe real-time PCR. The fold increase of ChM-I mRNA is given by $2^{-\Delta\Delta CT}$. Values are normalized against endogenous β -Actin. Data represent means \pm SD from 4 experiments. * $p < 0.05$ is considered significant when compared with untreated control.

Figure 3. Hydroquinone up-regulated ChM-I precursor and mature ChM-I protein in TrHBMEC. TrHBMECs were treated with hydroquinone at indicated concentrations and hours. ChM-I precursor protein levels were examined in TrHBMEC sonicates by immunoblot analysis. β -Actin was included as a loading control (3a, 3b). Mature ChM-I protein in culture media were also examined by immunoblot analysis after partial removal of serum proteins and centrifugal concentration. rhChM-I was included as a positive control (3c). Blots represent similar results from three independent experiments. The density of the bands was determined and plotted as the relative fold compared with the untreated control. * $p < 0.05$ is considered significant when compared with untreated control.

Figure 4. Hydroquinone inhibited TrHBMEC tube formation in matrigel. Hydroquinone did not induce obvious morphological change and cell death when TrHBMEC were seeded on gelatin coated plates (A, B) However, hydroquinone inhibited TrHBMEC tube formation in a dose dependent manner

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when cells were seeded on matrigel coated plates, 10 μ M hydroquinone almost completely inhibited TrHBMEC tube formation (C, D, E). Arrows indicate the tube structures. Data represent similar results from three independent experiments. Total tube length per field was measured by image processing and analysis software. * $p < 0.05$ is considered significant when compared with untreated control. ** $p < 0.05$ is considered significant when compared with untreated control and 5 μ M hydroquinone treatment (F).

Figure 5. Recombinant human ChM-I inhibited TrHBMEC tube formation in matrigel.

Recombinant human ChM-I mature protein inhibited TrHBMEC tube formation in a dose dependent manner (A, B, C). Arrows indicate the tube structures. Data represent similar results from three independent experiments. Total tube length per field was measured by image processing and analysis software. * $p < 0.05$ is considered significant when compared with untreated control. ** $p < 0.05$ is considered significant when compared with untreated control and 1 μ g/ml ChM-I treatment (D).

Figure 6. Pretreatment with anti-ChM-I siRNA in TrHBMEC knocked down ChM-I protein and abrogated the inhibition of tube formation induced by hydroquinone treatment.

TrHBMECs were treated with either scramble siRNA or anti-ChM-I siRNA for the indicated times. ChM-I precursor levels were examined in TrHBMEC sonicates by immunoblot analysis. β -Actin was included as a loading control. Blots demonstrated a successful knock down of ChM-I proteins by anti-ChM-I siRNA treatment (6a). Anti-ChM-I siRNA treatment also efficiently inhibited hydroquinone induced up-regulation of ChM-I (6b). Blots are representative of three independent experiments. The density of the bands was determined and plotted as the relative fold compared with the scrambled siRNA control. * $p < 0.05$ is considered significant when compared with scrambled siRNA control (6a, 6b). TrHBMECs pretreated with either scrambled or anti-ChM-I siRNA for 48 hrs were lifted to perform tube formation assay in the presence or absence of hydroquinone (10 μ M). All cells maintained tube formation abilities in the absence of hydroquinone (6c - A, C). Hydroquinone treatment completely inhibited tube formation in TrHBMECs with scrambled siRNA pretreatment (6c - B) but only partially inhibited tube formation in

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cells pretreated with anti-ChM-I siRNA (6c - D). Arrows indicate the tube structures. Data represent similar results from four independent experiments. Total tube length per field was measured by image processing and analysis software. * $p < 0.05$ (6d).

Figure 7. Overexpression of NQO1 in TrHBMEC protects cells from hydroquinone induced up-regulation of ChM-I and inhibition of tube formation. TrHBMECs were first transfected with either control vector or NQO1 overexpression vector for 18-24 hrs; blot (7a) demonstrated a successful overexpression of NQO1 in TrHBMEC. Cells were then subjected to hydroquinone treatment or the tube formation assay in the presence or absence of 10 μ M hydroquinone. ChM-I precursor protein levels after hydroquinone challenge were examined in TrHBMEC sonicates by immunoblot analysis. Overexpression of NQO1 inhibited hydroquinone induced up-regulation of ChM-I (7b). β -Actin was included as a loading control. Hydroquinone (10 μ M) inhibited tube formation in TrHBMEC transfected with control vector (7c - A, C) but the inhibition was abrogated in TrHBMEC transfected with NQO1 overexpression vector (7c - B, D). Arrows indicate the tube structures. Data represent similar results from three independent experiments. Total tube length per field was measured by image processing and analysis software. * $p < 0.05$ (7d).

Figure 1

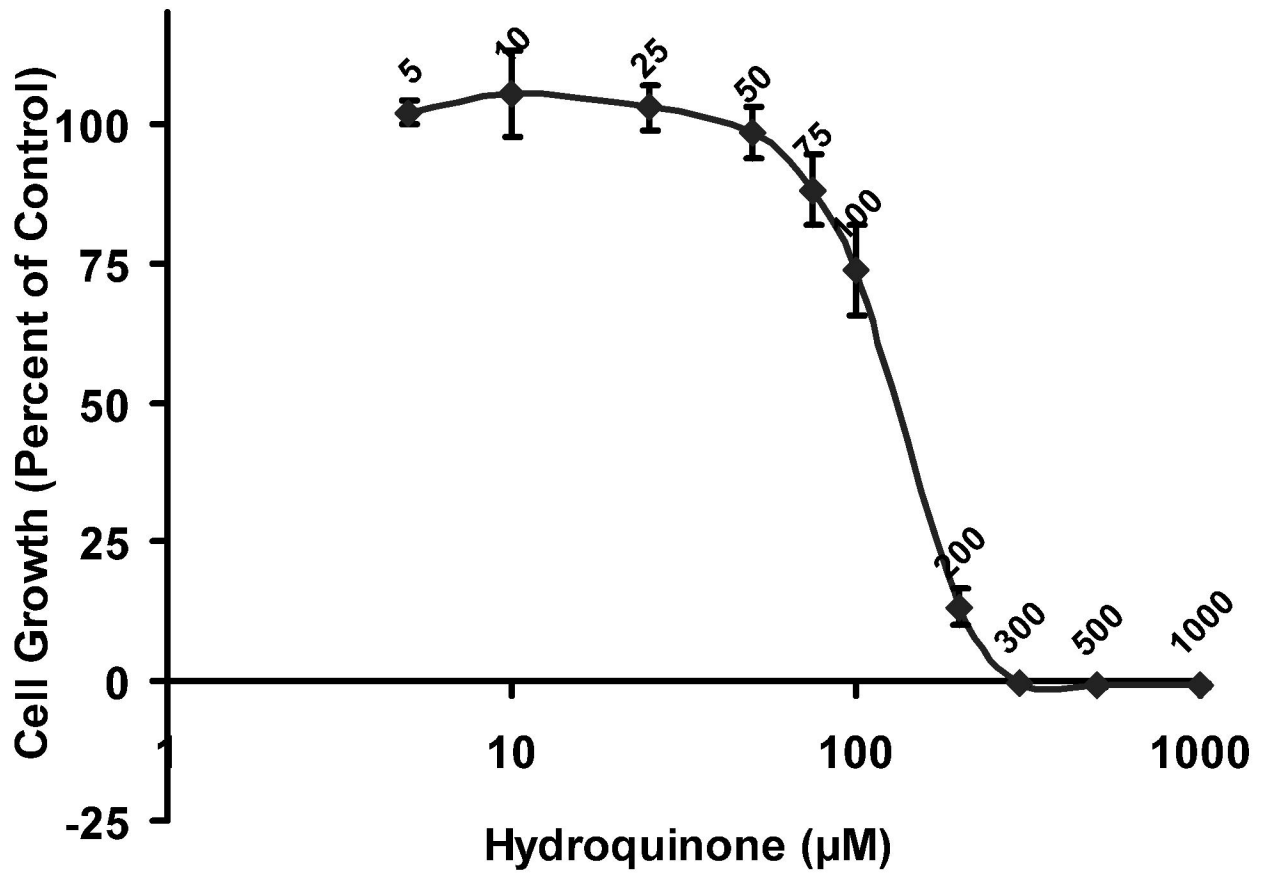


Figure 2

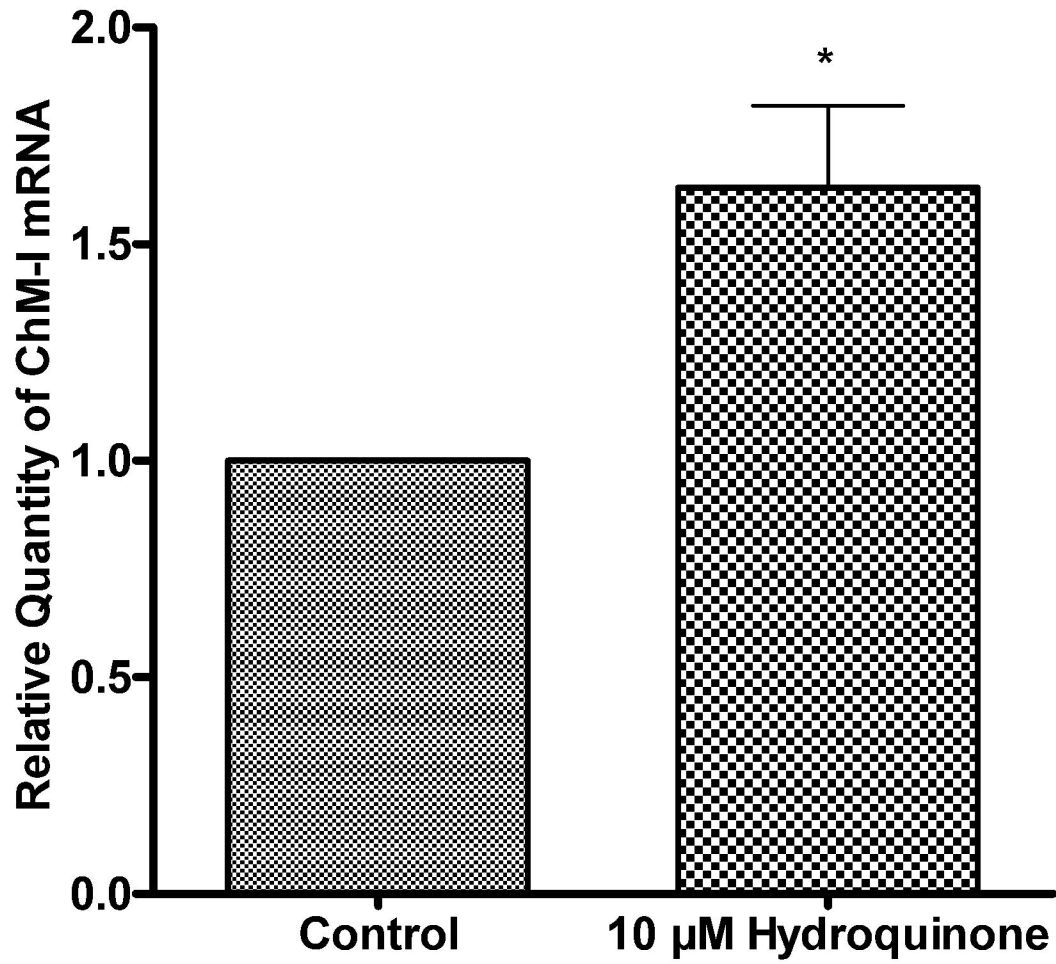


Figure 3a

Hydroquinone 0 μ M 10 μ M 30 μ M

ChM-I
Precursor



β -Actin

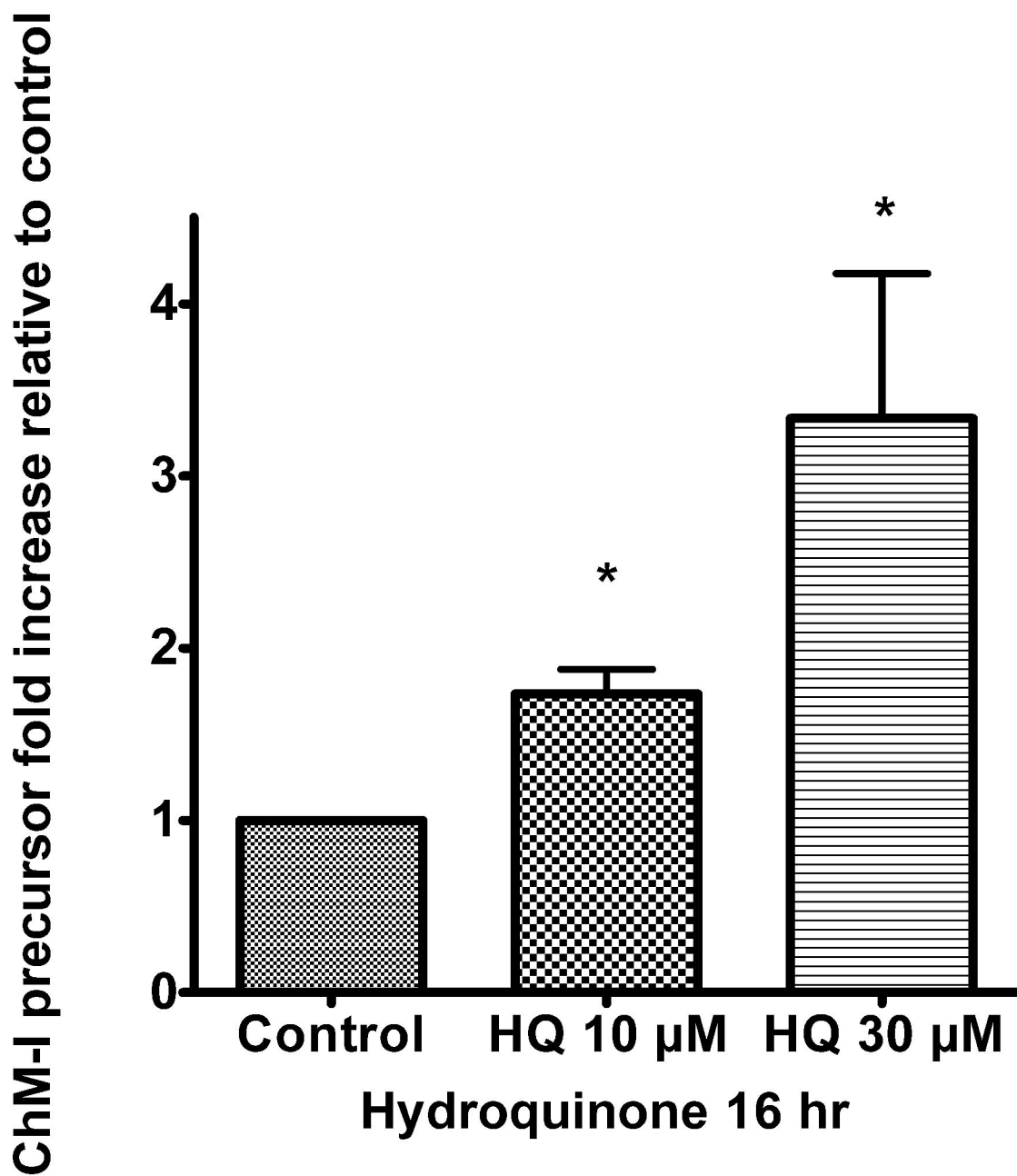
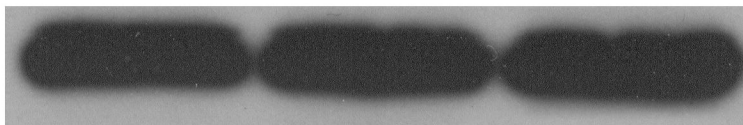
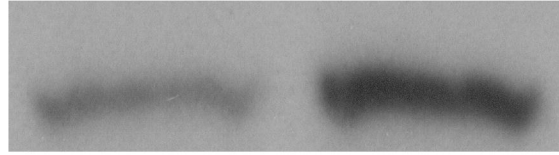


Figure 3b

Hydroquinone 0 μ M 10 μ M

ChM-I
Precursor



β -Actin

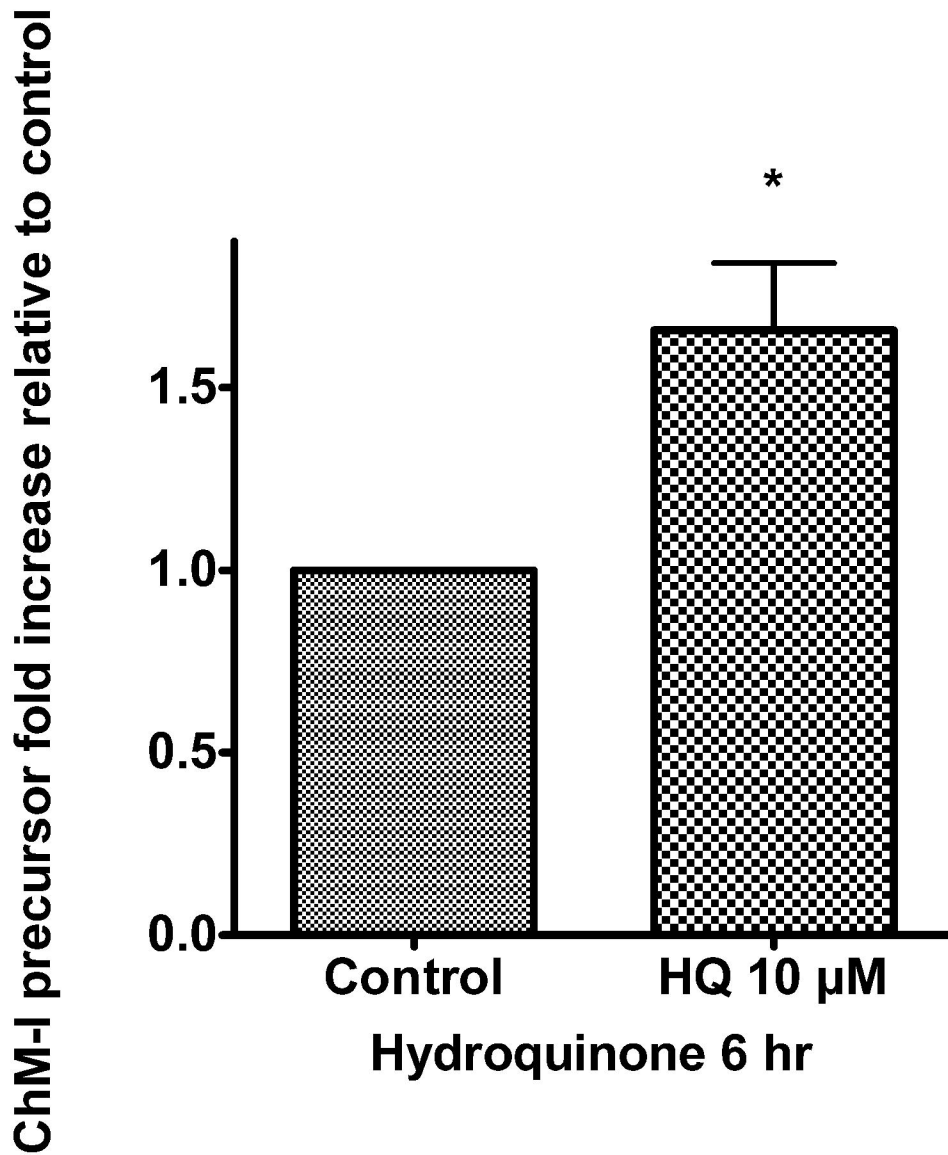
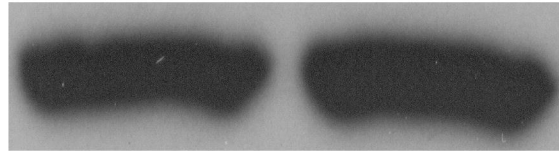


Figure 3c

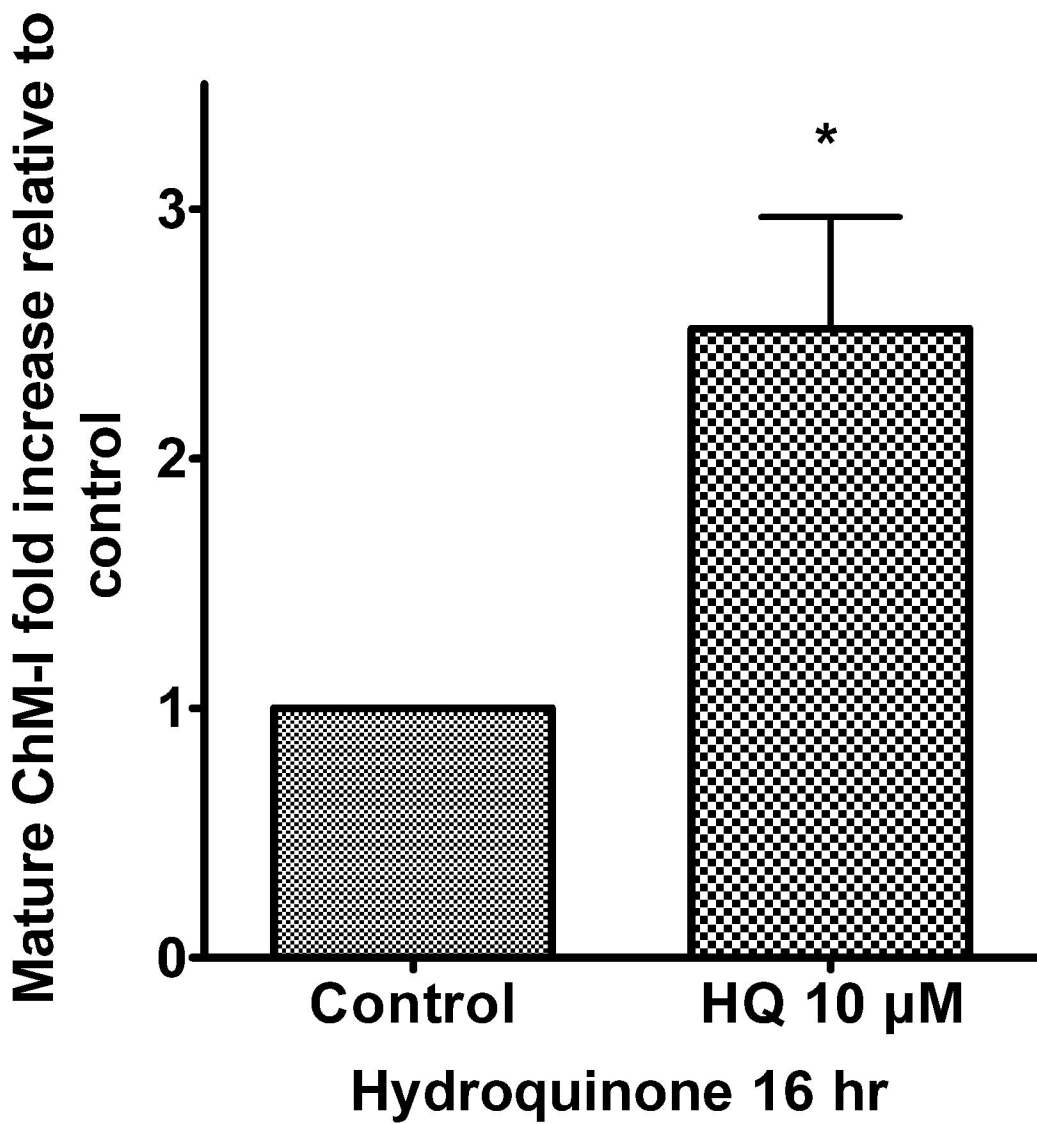
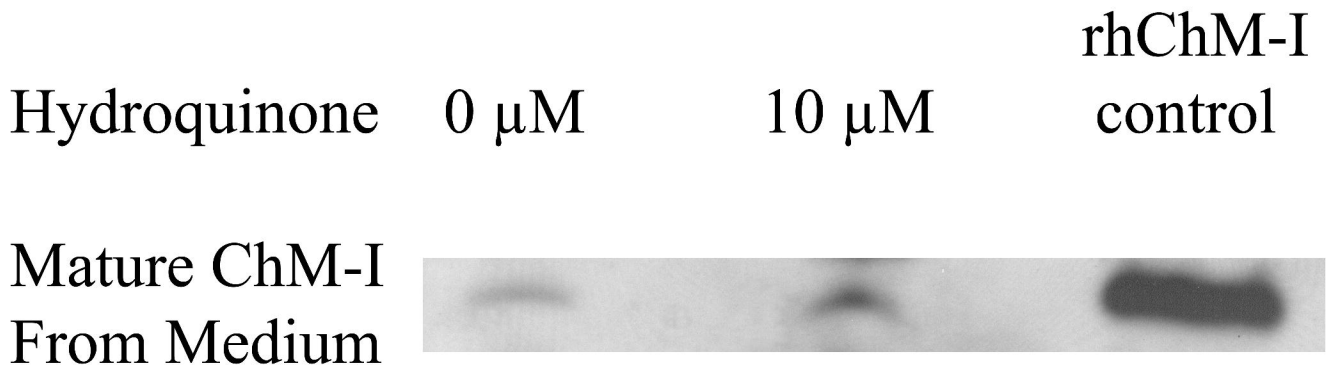


Figure 4

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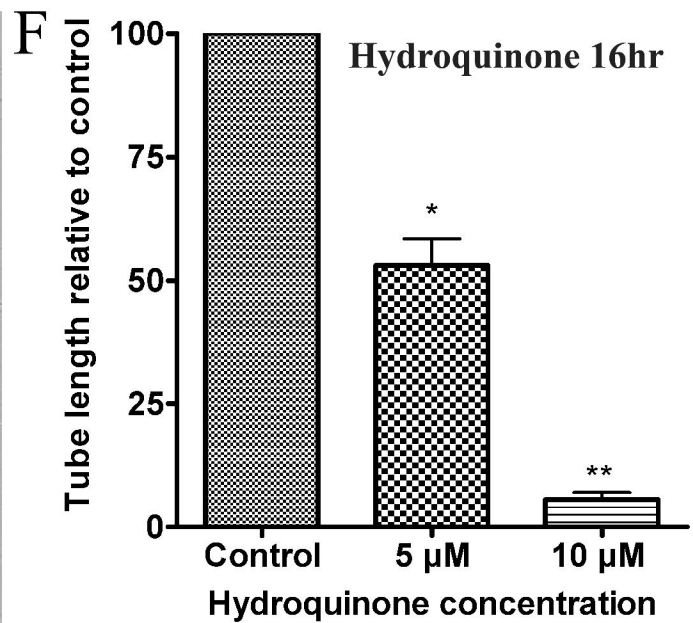
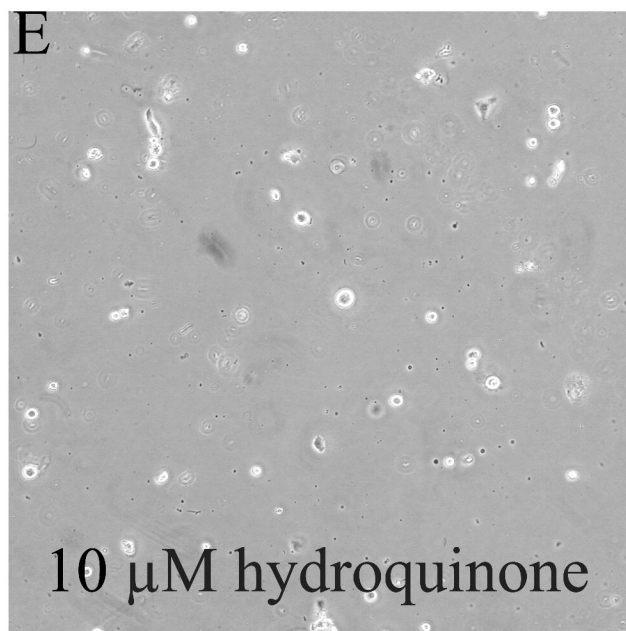
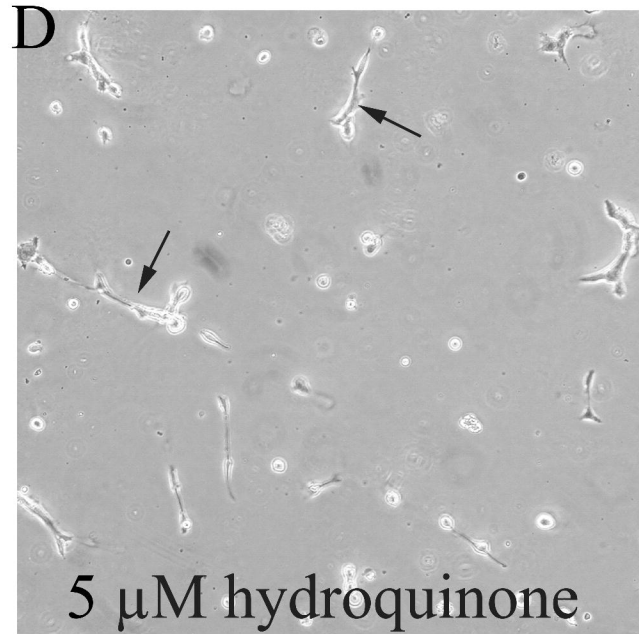
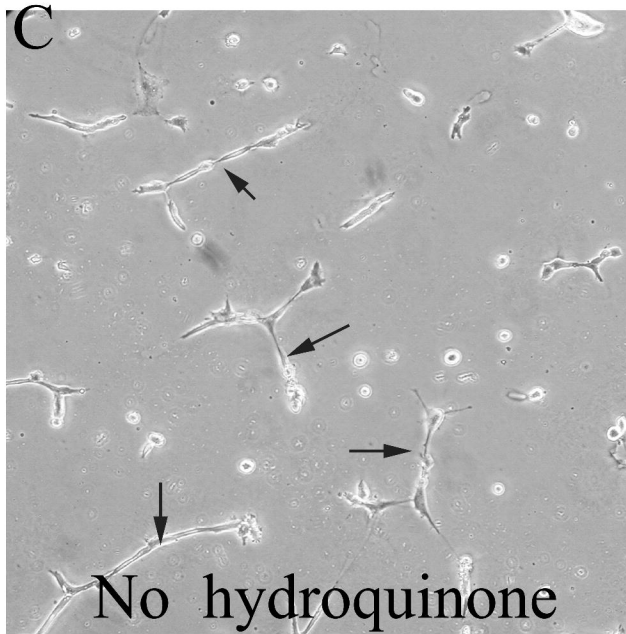
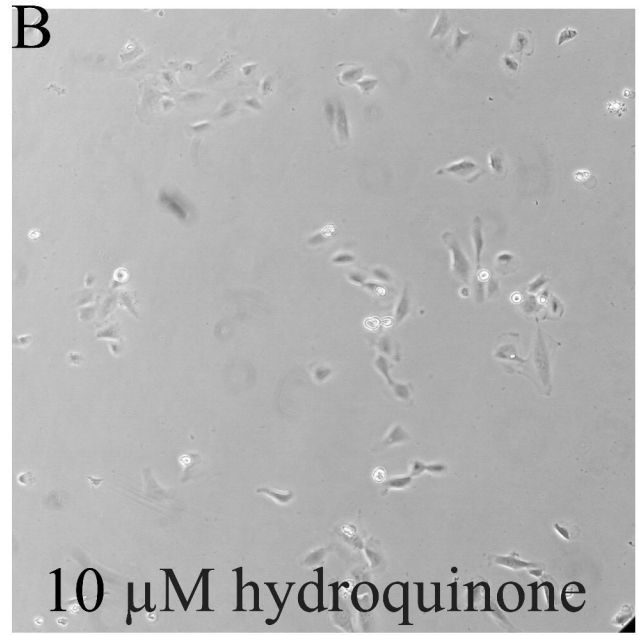
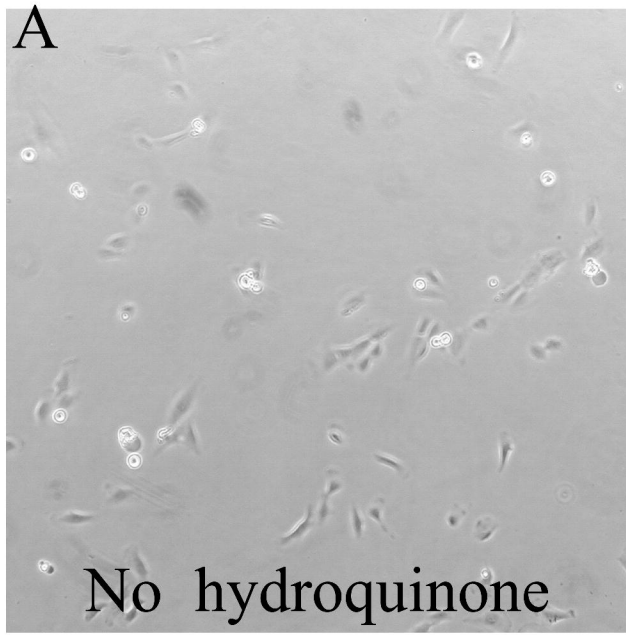


Figure 5

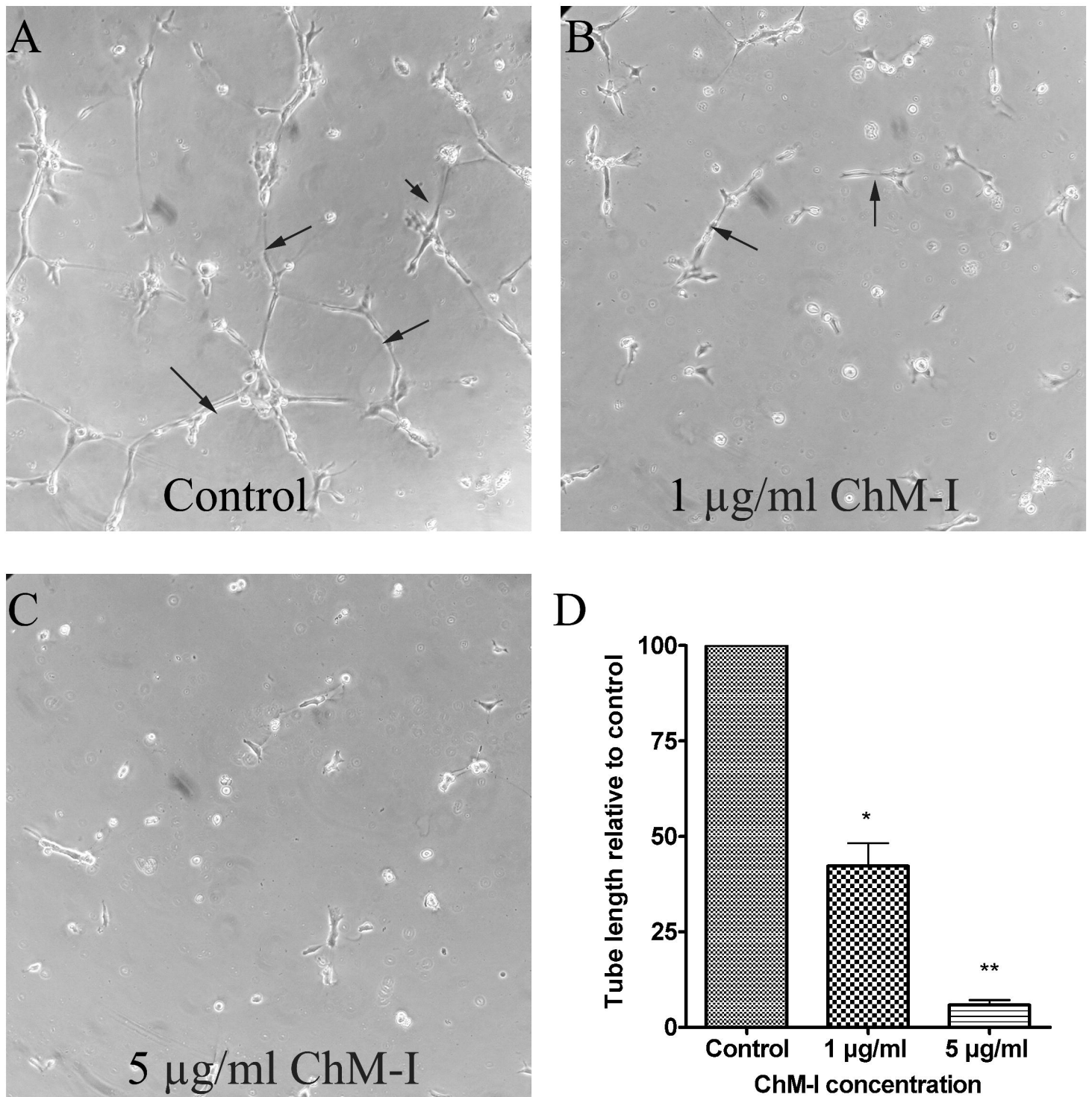


Figure 6a

	48 hour		72 hour	
Scrambled siRNA	+	-	+	-
Anti-ChM-I siRNA	-	+	-	+

ChM-I Precursor



β -Actin

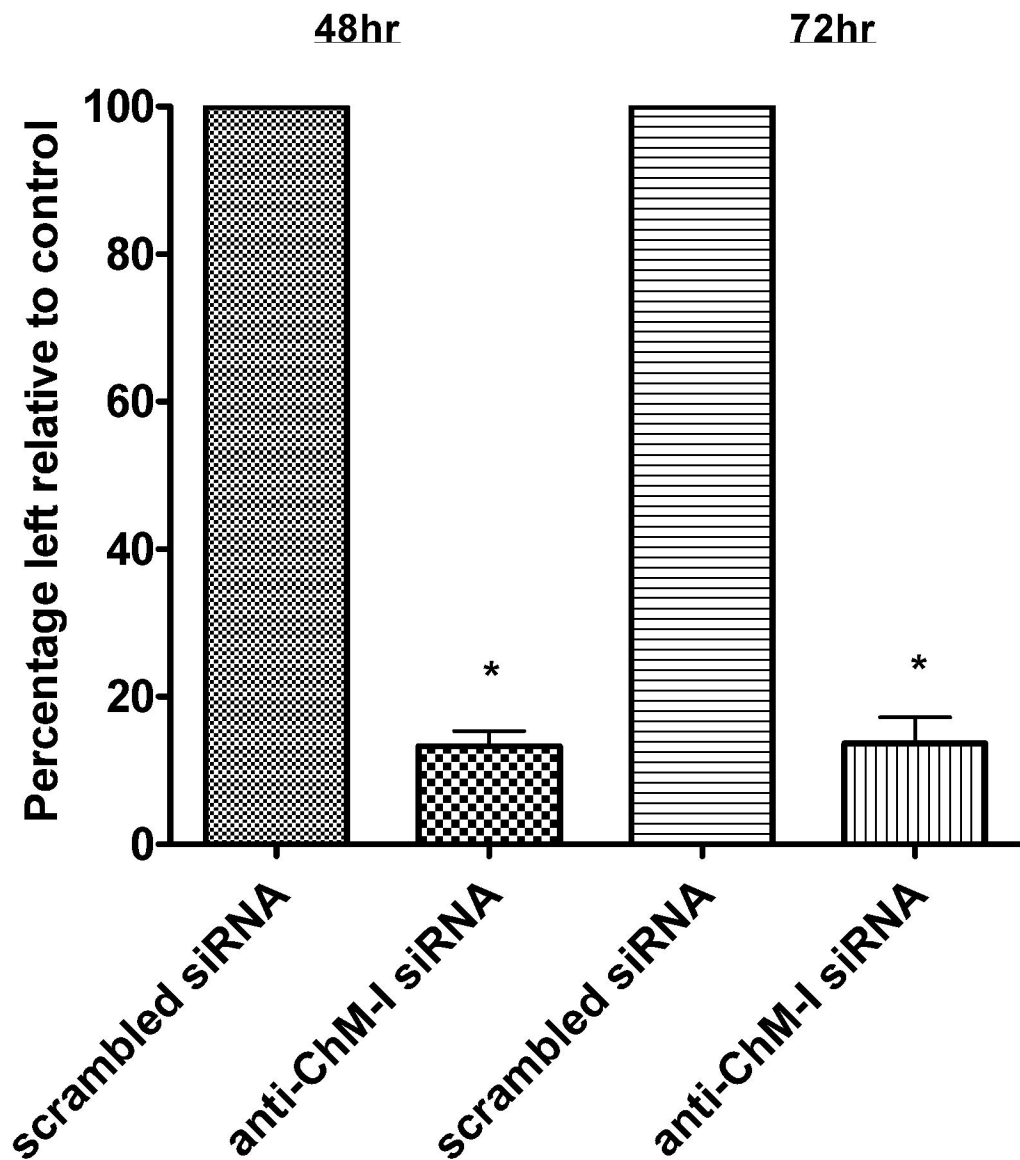
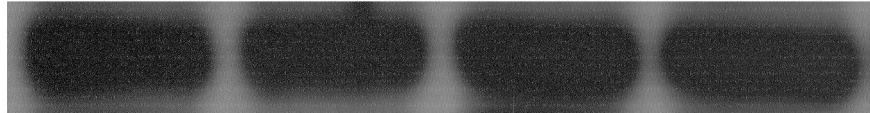
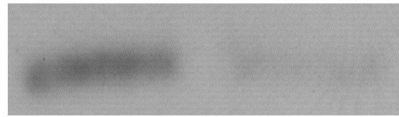


Figure 6b

Scrambled siRNA	+	-
Anti-ChM-I siRNA	-	+
Hydroquinone	+	+

ChM-I Precursor



β -Actin

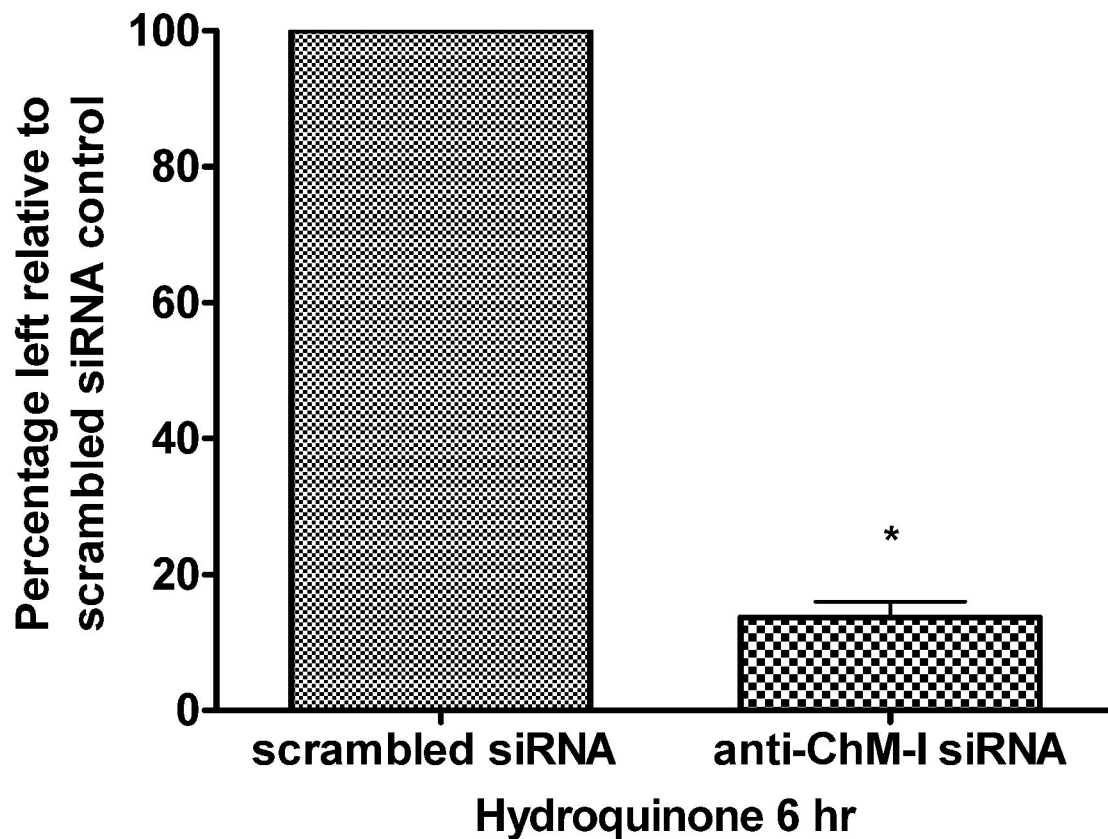
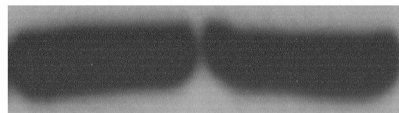


Figure 6c

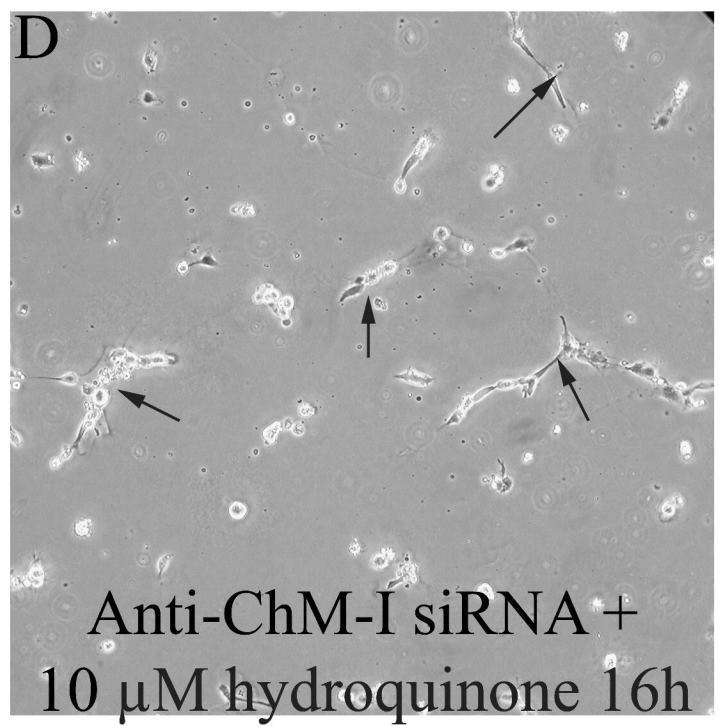
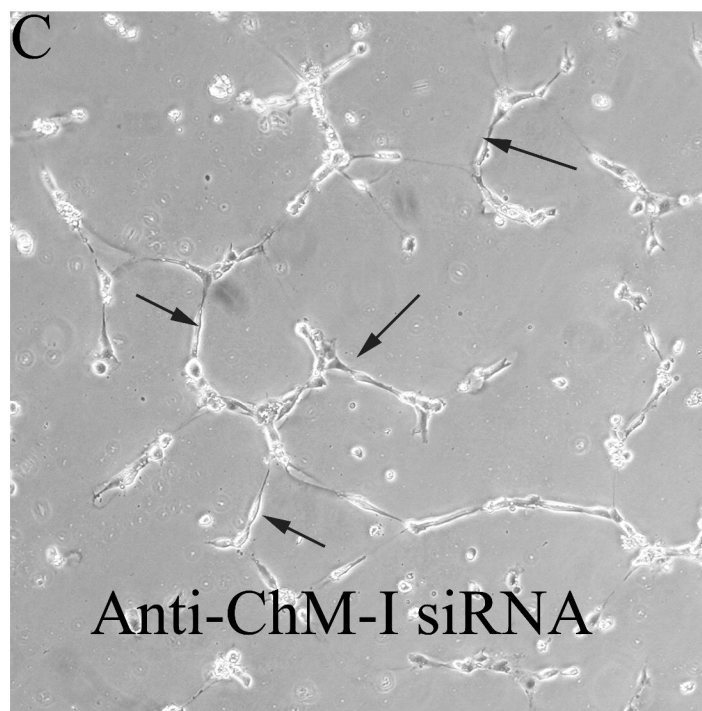
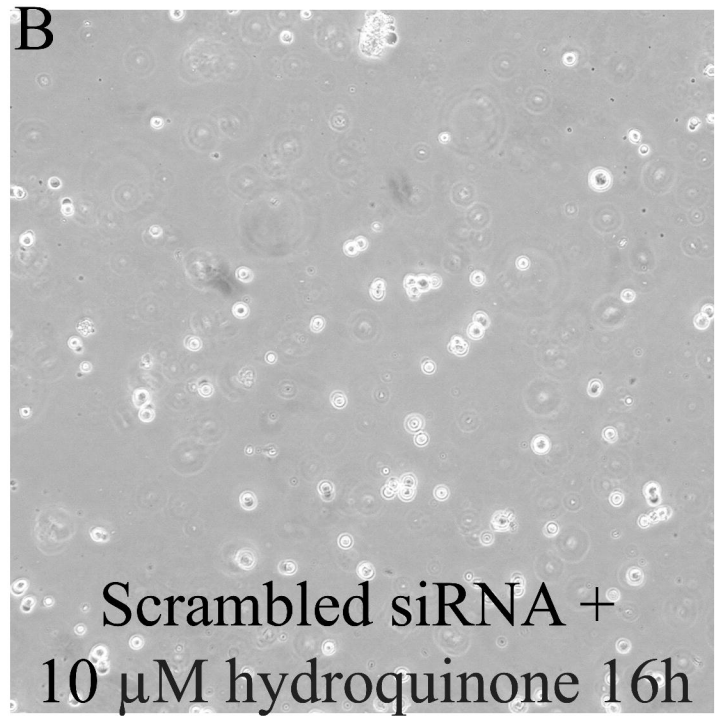
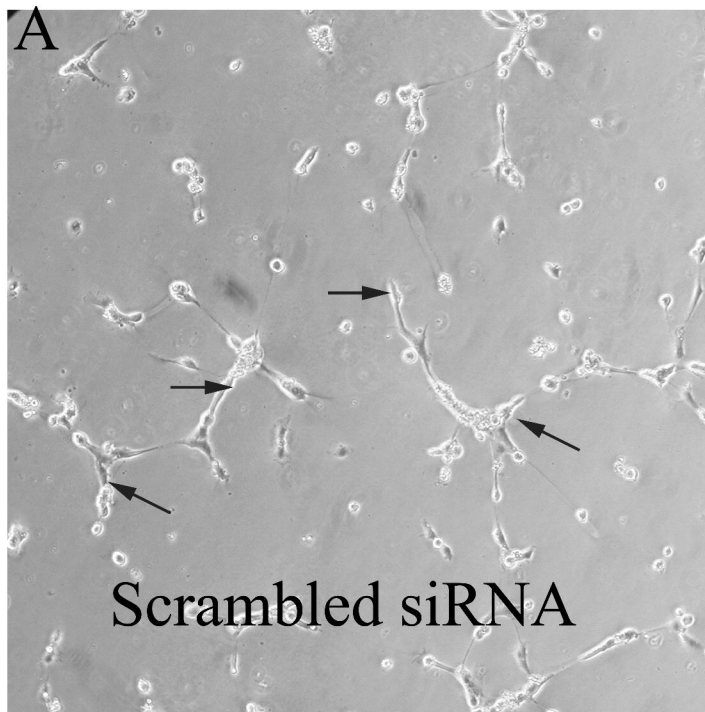


Figure 6d

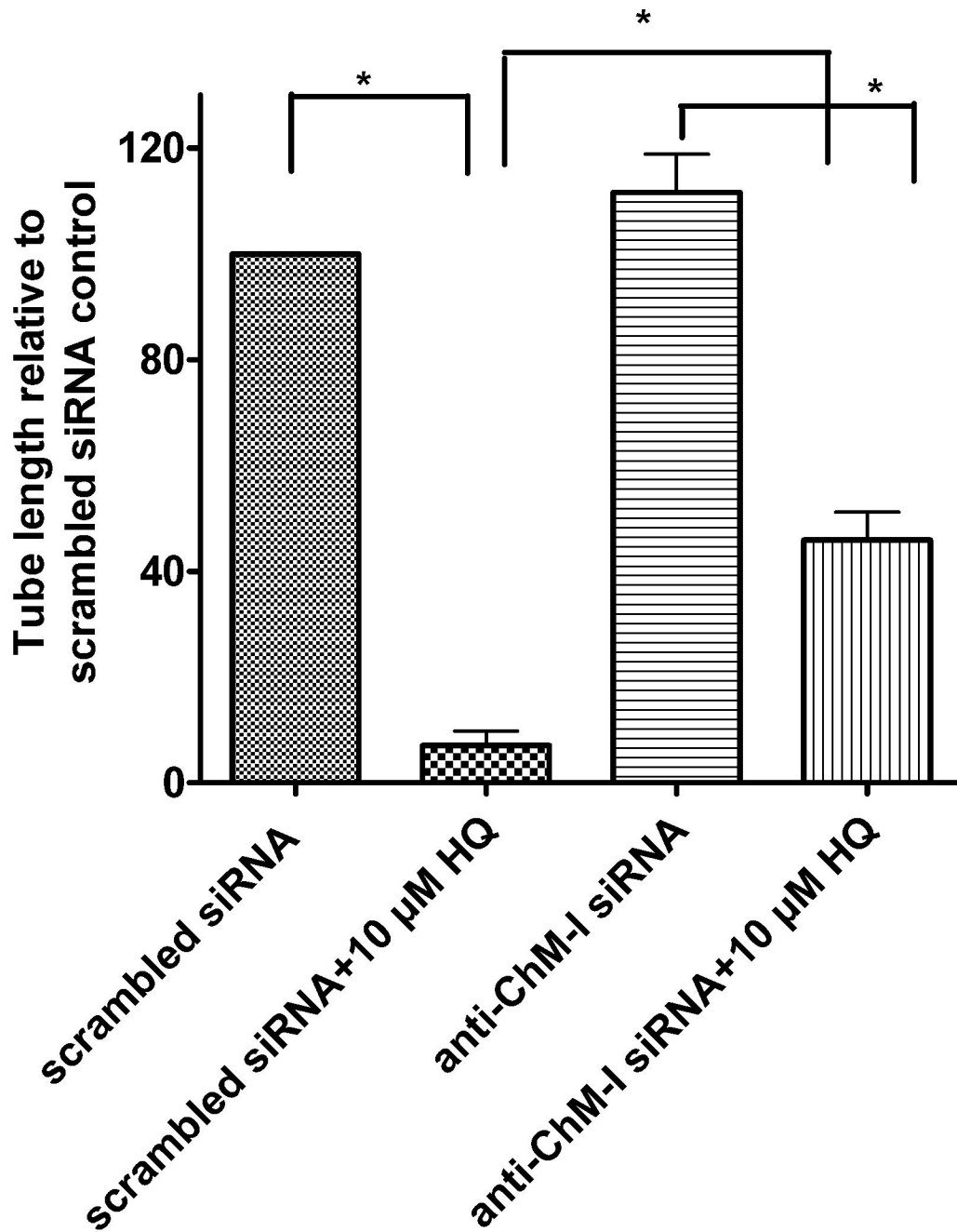


Figure 7a

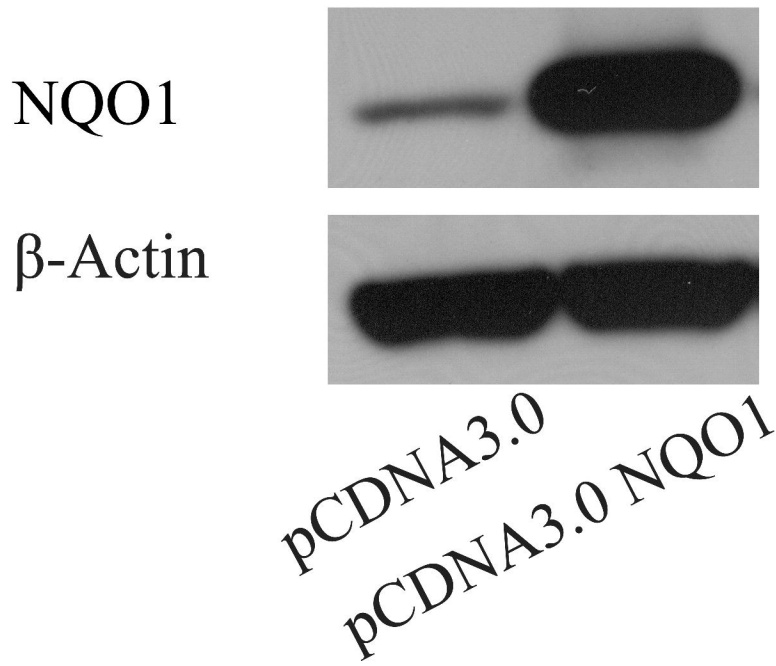


Figure 7b

HQ 10 μ M 16h

NQO1

ChM-I

Precursor

β -Actin

pCDNA3.0
pCDNA3.0 NQO1

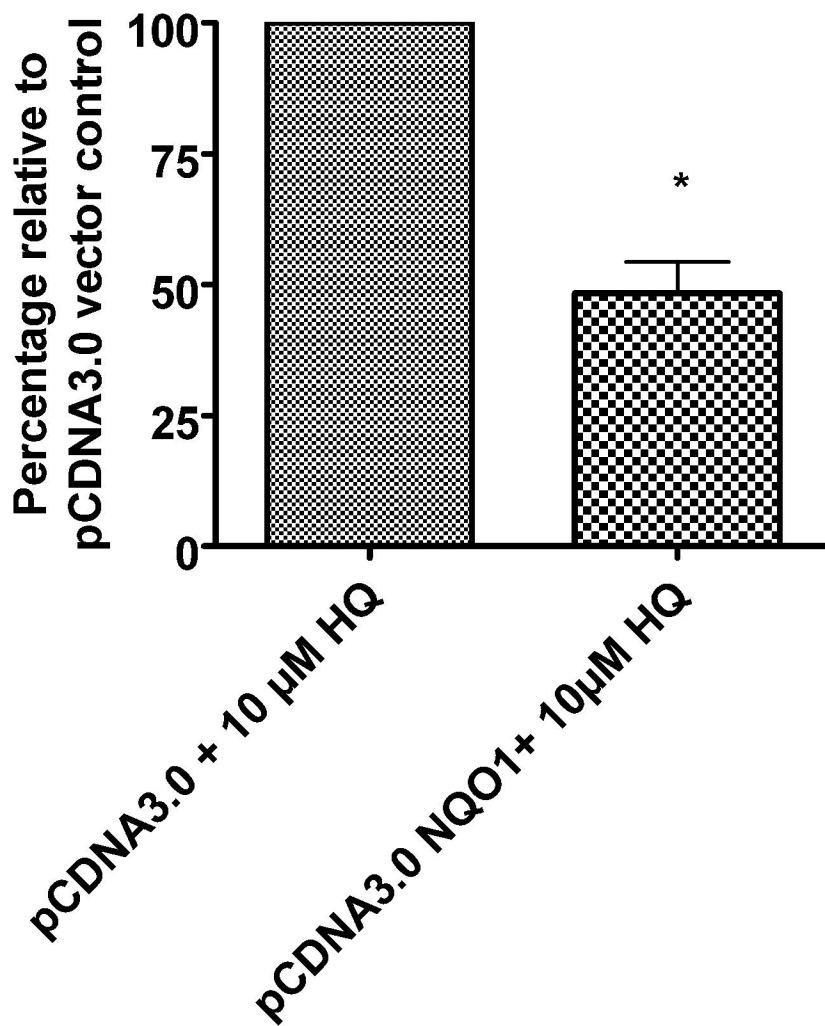


Figure 7c

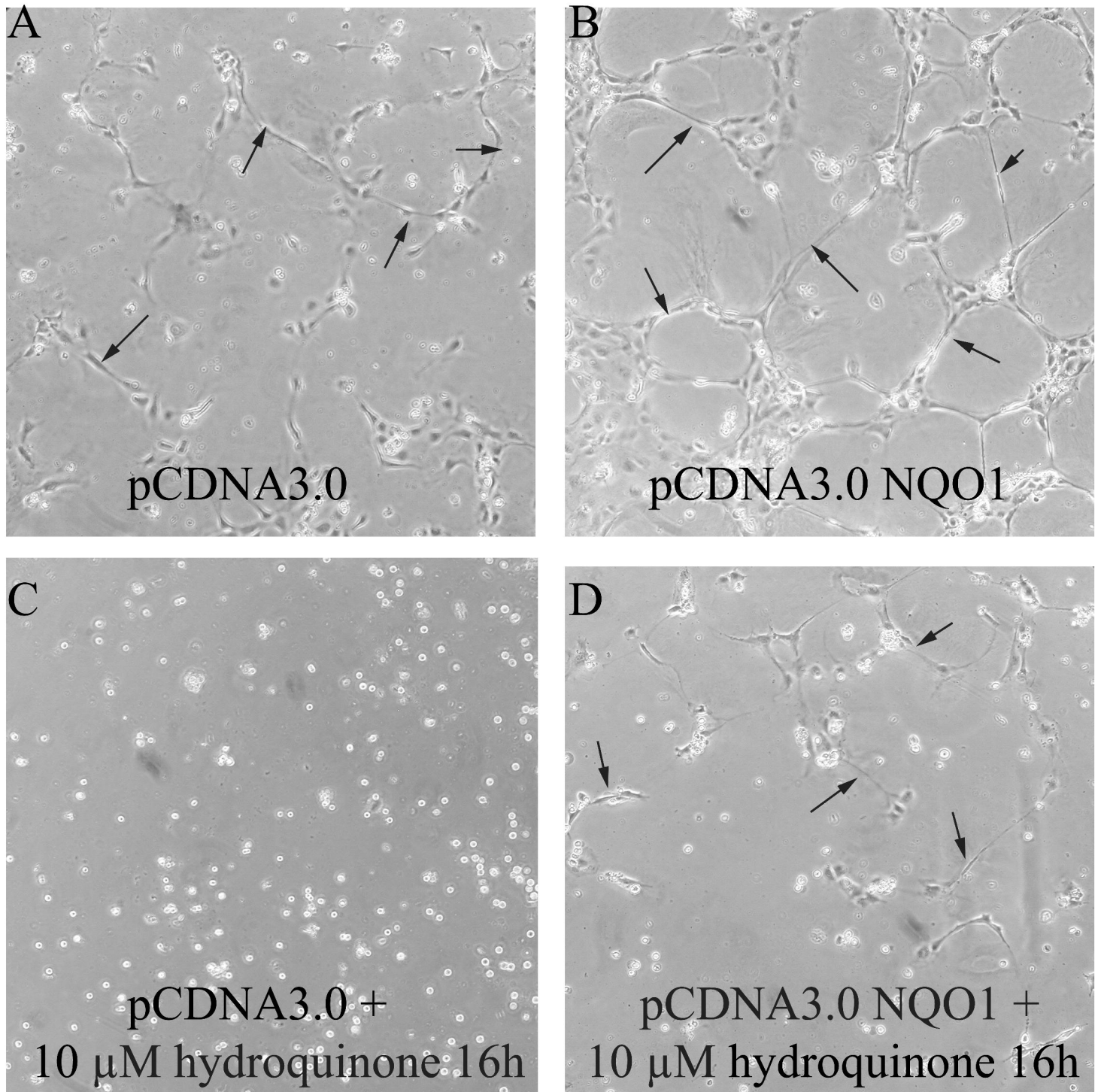


Figure 7d

