

## Supplementary data

## Supplementary Materials and Methods

### *Myc-MS1 expression plasmid*

The full-length coding sequence of mouse *msl* containing a c-Myc tag situated in the N-terminus before the coding sequence of *msl* was amplified by PCR using the Roche Expand High Fidelity PCR System and cloned into the expression vector pcDNA3.1(+) (Invitrogen). The MS1 expression plasmid was sequenced to confirm authenticity.

### *Cell culture and transfection*

**H9c2 cells, derived from the ventricular compartment of the rat embryonic heart(European Collection of cell cultures), was grown in an atmosphere of 5% CO<sub>2</sub>/95% humidified air at 37°C in Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin, and 100 µg/ml Streptomycin. The H9c2 cells are strictly mononucleated myoblasts that resemble both skeletal and cardiac muscle myoblasts [1]. H9c2 cells have been extensively used in diverse stress signalling and disease states (e.g. apoptosis, hypertrophy, hypoxia-reoxygenation and cardio-protection), with similar findings compared to primary**

**cardiomyocytes [e.g. 2-6]. Of note, bioluminescent H9c2 cells (expressing BCL-2) have been used as a graft into ischemic rat heart, which increased cell survival and improved heart function *in vivo* as assessed by optical bio-imaging [7]. Collectively, these data indicates similar signalling pathways are utilised in primary cardiomyocytes and H9c2 cells. Therefore H9c2 cells were chosen as an *in vitro* model system in the current study to investigate cellular hypertrophy [8-12] and cell death [13-20].**

Transfections were performed at ~50% confluence with the reagent JetPEI (Autogen Bioclear) following the manufacturer's instructions. For immunofluorescence, cells were plated onto ethanol treated glass coverslips for 48 hours prior to transfection.

#### *Immunofluorescence Microscopy*

Twenty-four hours after transfection, cells were rinsed with phosphate-buffered saline (PBS), fixed and permeabilised using a Leucoperm kit (Serotec). During permeabilisation mouse anti c-Myc fluorescein isothiocyanate (FITC) (Serotec) was added at a 1:10 dilution in PBS and left for 30 minutes at room temperature in the dark. Thereafter, 3x 5 minute washes in PBS at room temperature were carried out and Alexa fluor 350 phalloidin (Invitrogen) was then added at 1:40 dilution in 1% bovine serum albumin (BSA) in PBS and left for 20 minutes at room temperature in the dark. This was followed by 3x 5 minute washes in PBS at room temperature and then the coverslips were mounted onto slides using Prolong Gold Antifade (Invitrogen). Images were captured using a Nikon Eclipse TE2000-E microscope and processed using Volocity 4 software (Improvision).

### *Real-time and Semi-quantitative RT-PCR*

Gene expression changes were examined 24 hours after transfection. Those genes (brain natriuretic peptide (*BNP*) and cardiac  $\alpha$ -actin) that did not alter in expression following 24 hours were also examined 72 hours after transfection. RNA was isolated from cells after transfection using TRIzol reagent (Invitrogen) and then treated with DNase I (Sigma). DNase-treated RNA was reverse transcribed using SuperScript II kit (Invitrogen). Controls were also included that contained 1  $\mu$ l dH<sub>2</sub>O instead of the 1  $\mu$ l Superscript II enzyme (200 U). Real-time quantitative PCR (RTQPCR) was performed on an Applied Biosystems Prism 7900 HT sequence detection system to quantify mRNA levels of apoptosis repressor with caspase recruitment domain (*ARC*), leukemia inhibitory factor (*LIF*), interleukin-6 (*IL-6*), adrenomedullin, jun-B and fos-related antigen-1 (*fra-1*). For *ARC*, a pre-optimised TaqMan Gene Expression assay (Applied Biosystems) was used with Tata-binding protein (*TBP*) mRNA serving as control. For the other genes RTQPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Ribosomal Protein L32 (*RPL32*) served as an internal control. Relative mRNA quantification was analysed using the Pfaffl method [21]. For *msl*, *BNP*, and cardiac  $\alpha$ -actin, semi-quantitative PCR was used to assess relative mRNA levels. The amplicons were visualised by agarose gel electrophoresis and *RPL32* served as a loading control. Relative mRNA expression was analysed by band quantification using the GeneTools software (Syngene) and standardised to *RPL32*. The primer pairs used for all the assays are shown in Table 1 (Supplementary data).

### *Western blotting*

M-PER mammalian protein extraction reagent (Pierce) in the presence of 1 × complete mini protease inhibitor cocktail (Roche) was used to extract proteins from H9c2 cells following transfection. Equal amounts of protein (15 µg) were electrophoresed on denaturing 10% polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated with a c-Myc (9E10) mouse monoclonal antibody at 1:200 (Santa Cruz Biotechnology Inc.) or the loading control  $\alpha$ -tubulin (Tu-02) mouse monoclonal at 1:1000 (Santa Cruz Biotechnology Inc.), followed by an anti-mouse horseradish peroxidase conjugate at 1:5000 (Amersham). For detection, blots were processed using enhanced chemiluminescence (ECL) kit (Amersham) and the luminescent signal detected on Hyperfilm ECL (Amersham).

### *Cell Proliferation measurements*

For direct cell counting, cells were washed in PBS, trypsinized, re-suspended in fresh medium and counted with a hemocytometer. The percentage of cells in S phase and G<sub>2</sub>/M phase were determined by Vybrant DyeCycle Violet Stain (Invitrogen) using flow cytometry according to the manufacturer's instructions. The c-Myc FITC fluorescence of individual transfected cells and the percentage of cells in S phase and G<sub>2</sub>/M phase were

measured using a DakoCytomation CyAn ADP flow cytometer and analysed using the Summit v4.3 software.

## **Supplementary Results**

### *MS1 expression plasmid*

Semi-quantitative RT-PCR confirmed that H9c2 cells transfected with the Myc-MS1 expression plasmid over-expressed *ms1* mRNA (Supplementary Fig. 1A). Western blotting and immunofluorescence microscopy using a c-Myc antibody confirmed that H9c2 cells over-expressed MS1 protein (Supplementary Figs. 1B and 1C). To determine if MS1 colocalised with actin in H9c2 cells, transfected H9c2 cells were co-stained with c-Myc FITC antibody and Alexa fluor 350 phalloidin to detect MS1 and actin, respectively. Actin filaments were observed in all cells transfected with or without the Myc-MS1 expression plasmid and in cells over-expressing MS1 the MS1 signal colocalised with actin (Supplementary Fig. 1C).

### *MS1 over-expression in vitro protects against staurosporine-induced apoptotic cell death*

Cells were treated with various concentrations of staurosporine (5 nM - 25 nM) for 24 hours and the amount of apoptotic cells increased with increasing concentrations of staurosporine (5 nM, ~29%; 15 nM, ~39%; 25 nM, ~45%), see Supplementary Fig. 2.

### Supplementary Figure Legends

**Fig. 1.** Transient transfection leads to MS1 over-expression that colocalises with actin in H9c2 cells. H9c2 cells were transiently transfected with a Myc-MS1 expression plasmid (M) or empty vector control (C), n = 3. (A) Representative semi-quantitative RT-PCR analysis of *msl* mRNA. RPL32 was used as an internal control to account for inaccuracies in initial RNA levels. (B) Western blot analysis of MS1 protein in H9c2 cells. (C) Immunofluorescence microscopy of MS1 protein in H9c2 cells. Cells were co-stained with c-Myc FITC antibody and Alexa fluor 350 phalloidin to detect MS1 and actin, respectively. Merge image shows MS1 colocalisation with actin in MS1 transfected cells. Bar = 20  $\mu$ m. Magnification 40 $\times$ .

**Fig. 2.** Quantification of staurosporine-induced apoptosis in H9c2 cells by flow cytometry. H9c2 cells were left untreated or treated with staurosporine (5 nM - 25 nM) for 24 hours, stained with Vybrant DyeCycle Violet Stain to detect apoptotic cells (sub-G<sub>1</sub> phase) by flow cytometry. The percentage of apoptotic cells based on the sub-G<sub>1</sub> phase

DNA content was quantified. The results are the mean  $\pm$  SD from 3 independent experiments.

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