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Tbx20 promotes H9c2 cell survival against oxidative stress and hypoxia *in vitro*

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Sustained and cardiac injury specific overexpression of Tbx20 provide cardiac protection of adult heart by preserving cardiac function increasing its survival rate post myocardial infarction (MI). However, the molecular mechanism underlying this protective pathway is largely unknown. Thus, in the current study, we examined Tbx20 and associated protective signaling pathway against two specific injury inductions. Injury inductions were done by imparting the cultured cardiac H9c2 cells with oxidative stress and hypoxia. Both stresses resulted in increased Tbx20 expression which activated the level of *Nmyc1* and *Bmp2* showing increased cellular proliferative rate. However, it was not sufficient to overcome the stress responses owing to increased apoptosis. The sustained overexpression of Tbx20, prior to injury induction, showed further enhancement in the expression patterns of *Nmyc1* and *Bmp2* that accelerated the proliferation rate, thus promoting the formation of increased number of viable cells, reducing the cellular apoptosis post injury. Moreover, overexpressed Tbx20 inhibits cellular hypertrophy post injury by increasing the activation level of *Yap1* and together may follow a feedback loop mechanism downregulating the *p-Akt* activity. Therefore, Tbx20 overexpression has been found to be sufficient to impart cardiac protection post injury by triggering its associated signaling molecules.

Keywords: Apoptosis, Hypertrophy, Injury, Proliferation

At present heart failure (HF) is a major worldwide concern[1]. Moreover, cardiovascular diseases (CVDs) are posing a major threat globally, estimated to be affecting more than 23.6 million people by 2030[2]. Cardiomyocyte (CM), a major cell type of the myocardial region, aiding in the development of fetal heart and maintain its homeostasis[3], shows a gradual decreased rate in its proliferation activity in mammal as the development shifts from embryo to postnatal to adult life[3,4]. This is caused by the CMs withdrawal from the cell cycle[5,6] resulting in their binucleation conditions along with increased expression of cell senescence markers and adult contractile protein isoforms[5-7]. Hence, the limited proliferative capacity of the adult CMs (<1%/year)[8], cannot overcome the huge loss of functional CMs in adult heart post myocardial infarction (MI). Moreover, physiological hypertrophy, undergone by CMs post MI in order to overcome the massive loss of CMs, as an adaptive mechanism may proved to be fatal giving rise to pathological hypertrophy if sustained for long[9-11]. All these pave the way for the onset of CVD and HF in mammals, including humans[1,12].

Tbx20, a member of the Tbx1 subfamily of T-box transcription factor plays an important role in cardiogenesis and is also expressed in multiple organs, including the developing and adult heart[13]. Mutations within the Tbx20 coding region results in the onset of human congenital heart diseases, and the loss of Tbx20 in a wide variety of model systems leads to cardiac defects and eventually HF[14]. Both in mice and in humans, TBX20 gain- and loss- of function mutations resulted in the occurrence of vast range of cardiac abnormalities[13,15-17]. Moreover, in adult mice, CM specific ablation of Tbx20 leads to severe cardiomyopathy with associated arrhythmias followed by death[18]. Tbx20 promotes CM proliferation via induction of *Nmyc1* and increased Bmp10-pSmad 1/5/8 signaling *in vivo* in fetal mice and Bmp2-pSmad 1/5/8 and PI3K/Akt/GSK3β/β-catenin signaling pathways in adult and neonatal

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Abbreviations: Akt, Protein kinase B; Bmp2, Bone morphogenic protein 2; CM, Cardiomyocyte; HF, Heart failure; H_2O_2, Hydrogen peroxide; HY, Hypoxic stress; OS, Oxidative stress; $Tbx20^{OE}$, Overexpressed Tbx20; Yap, Yes associated protein.

CMs. Furthermore, Tbx20 is found to play important role in adult cardiac protection both pre and post injury[13,14,19]. Sustained overexpression of Tbx20 in adult mice heart results in increased numbers of small, proliferative, mononucleated CMs, marked by persistent expression of fetal genes with reduced cardiac hypertrophy or fibrosis[19]. Likewise, adult heart post MI shows significant improved survival upon selective over-expression of Tbx20 in adult CMs with increased level of proliferation activators and fetal contractile proteins[20]. Although several efforts have been made to induce repair or regeneration of adult heart following injury, the molecular mechanisms underlying the adult CM proliferation remains largely unexplored.

Furthermore, it has been shown that Tbx20 augments cardiac protection by preserving cardiac function, reducing infarct size and inhibiting cardiac remodelling post MI[20]. But the molecular mechanism through which Tbx20 imparts these processes post injury is largely unknown. Generation of reactive oxygen species (ROS) and oxygen depletion (hypoxia) are common phenomena observed during adult cardiac injuries like MI or ischemia-reperfusion (I/R) or aortic banding (AB)[21-23]. Therefore, in our current study we tried to demonstrate Tbx20 associated signaling pathways upon cardiac specific injury induction to come out with some mechanistic insight. In addition, sustained overexpression of Tbx20 is found to overcome the stress level imparted by injury by showing increased number of viable cell populations enhanced by increased cellular proliferation, reducing the apoptotic rate. This is triggered by *Nmyc1* and *Bmp2*, the known downstream targets of Tbx20. On the other hand, increased Tbx20 level results in inhibiting cellular hypertrophy by activating Yes associated protein (Yap) post injury. Furthermore, our study reveals the reduction in the cellular hypertrophy is also favoured by reduction in the activated level of Akt. Thus, sustained over-expression of Tbx20 in cultured cardiac cells aids to compensate the damage caused by cardiac injury by forming increased number of viable cell types by increased proliferation and reduced apoptosis, inhibiting cellular hypertrophy thus offering a novel therapeutic approach towards cardiac protection.

Thus, in our work, we have tried to understand the aspect of Tbx20 in cardiac protection by targeting its downstream signaling molecules and cross-talk with other transcription factor. And for this two specific injury inductions were imparted in cardiac H9c2 cells *in vitro*.

Materials and Methods

Cell culture and induction of oxidative stress and hypoxia

H9c2, undifferentiated rat ventricular myoblast cells[24] were cultured in Dulbecco's modified Eagle's medium (DMEM 1X, 11995-065 Invitrogen) supplemented with 10% fetal bovine serum (10270-106 Invitrogen), 10,000 units/mL penicillin and 10000 µg/mL streptomycin (15140-122 Invitrogen) maintained under a sterile humidified atmosphere of 5% CO_2 at 37°C. Every 2-3 days, the cells were fed and were subcultured once they reached 70-80% confluence. Cells were seeded at $1X10^6$cells/ml for experimentation in 60mm cell culture dishes (628-160 cell star Greiner Bio-one) in DMEM-10% FBS complete media. After adhering overnight, oxidative stress was induced by treating the cells with 200 µM concentrations of hydrogen peroxide (H_2O_2) for 30 min in serum free DMEM in 5% CO_2 incubator at 37°C[5,26]. After the required incubation time, media was aspirated and the cells were replenished with fresh serum free DMEM media for 18h in 5% CO_2 incubator at 37°C. Likewise, for hypoxia induction, H9c2 cells were incubated in an anaerobic container that contained an Anaero Gas Pack (LE002A HiMedia) according to the manufacturer's instructions. Under hypoxic condition, the cells were incubated with glucose free DMEM (11966-025 Invitrogen) without serum and kept for overnight 18 h followed by 5 h of reoxygenetion in medium with 10% FBS-containing DMEM (reoxygenetion medium) in the 5% CO_2 incubator at 37°C. For normoxic conditions the cells were incubated with glucose containing DMEM-10% FBS for the same time point in the 5% CO_2 incubator at 37°C[31]. After the required incubation period of both experimental setups, cultures were washed with phosphate-buffered saline (DPBS 1X 14040-133 Invitrogen) and harvested for RNA isolation, immunostaining and protein extraction.

Experiments were also carried out in HEK293 (Human Kidney cell line) cells which were also maintained under the same conditioning media and similar sterile conditions as mentioned previously for H9c2 cells.

Overexpression of Tbx20 in cultured cells by transfection

For overexpression of Tbx20 in H9c2 and HEK293 cells, the cells were seeded in 60mm cell culture dishes at $1X10^6$ cells/ml and after overnight of adhering they were transfected with ViaFect Transfection Reagent (E498 1/2/3 Promega) at 2:1 ratio of transfection reagent and plasmid containing mouse full length cDNA cloned in frame with pcDNA3.1 of 2 µg concentration according to the manufacturer's instructions. For control groups, the cells were transfected with the transfection reagent without the desired plasmid. Next the transfected experimental groups were incubated for 20 h in the 5% CO_2 incubator at 37°C followed by the respective stress induction as described previously. Next after the treatment, the cultures were washed with DPBS and harvested for RNA isolation, immunostaining and protein extraction.

Semi-quantitative Reverse Transcriptase PCR and Quantitative Real Time RT-PCR analysis

Total RNA was isolated from the experimental cultured cells by using TRIzol® Reagent (15596026 Invitrogen) according to the manufacturer's protocol. About 1.0 µg of total RNA was used to generate cDNA with iScript™ Reverse Transcription Super-mix synthesis kit (170-8841 Biorad) according to the manufacturer's instructions. One µl of synthesized cDNA was used for analysis of semi-quantitative reverse transcriptase PCR and quantitative RT-PCR (Sso Fast Evagreen Super-mix 172-5203 AP Biorad) as described previously[15]. Oligonucleotide primer sequences (rodent) used for quantification of *Catalase*[27], *Sod2*[27], *Tbx20*[28], *Nmyc1*[15], *Bmp2*[29], *Gapdh*[29] were described previously. Corresponding oligonucleotide primers were designed using Primer3 Input software which are provided in Table 1.

Amplification reactions were performed with 34 cycles as described previously[15] and normalized with Gapdh[29]or *β-actin*. Normalized threshold cycle values of samples amplified in at least triplicate were used to calculate the relative gene expression levels.

Immunostaining and fluorescence microscopy

For immunofluorescence staining the control and treated cell samples were fixed in 1% paraformaldehyde solution (PFA) for 3 min followed by 4% PFA for the next 10 min at 24°C and then washed with PBS. Prior to the addition of primary antibody the fixed cells were rinsed in PBS containing 0.05% Tween 20 solution followed by blocking in 1X NP40 blocking solution for 1h. After the incubation in blocking reagent the samples were once again rinsed in PBS with 0.05% Tween 20 solution.

The primary antibody used for immunofluorescence staining: karyokinesis marker Ki67 (1.0 µg/mL ab 15580 Abcam) and microfilaments (F-actin) marker phalloidin conjugated with Alexa Fluor® 488 (A12379 Invitrogen). Following the primary antibody incubation for overnight, the cells were washed in PBS and again rinsed with PBS + 0.05% Tween 20 solution and incubated with the corresponding Alexa Fluor-488 goat antirabbit secondary antibody (1:1000 ab 181448 Abcam). After the required incubation for 1-2h was over, the cell nuclei were counter stained with DAPI (ab104139 Abcam) by incubating for 5min at 24°C in dark and mounted for microscopic image detection. As a negative control, all slides were incubated with only secondary antibody but with no primary to detect the background fluorescence. Immunofluorescence was detected using Zeiss Lab.A1 Fluorescent microscope. Images were obtained by using ISC capture Software.

Determination of cell viability and proliferative index

The average numbers of the viable cell populations were determined by counting the total number of DAPI positive cells in the respective control, H_2O_2 treated, normoxic, hypoxic and transfected followed by respective injury induced cultured sample groups. Likewise, proliferative indices of the same cultured samples were calculated as the percentage of the total number of Ki67 positive nuclei/the total number of DAPI positive nuclei[19]. The total number of Ki67 positive and DAPI positive nuclei were counted from at least 200 numbers of H9c2 cells in each experiment where each experimental condition was carried out, at least in triplicate.

Table 1—Primers used for real time quantitative PCR to detect gene expression in H9c2cells

Gene	Annealing Temperature (°C)	Product size, (bp)	Primer sequences
Hif1α	58	218	5'-CCAGCAGACCCAGTTACAGA-3'5'-TTCCTGCTCTGTCTGGTGAG-3'
Bax	56.1	176	5'-TCATGAAGACAGGGGCCTTT-3'5'-CTGCAGCTCCATGTTGTTGT-3'
Bcl_2	56.1	174	5'-CTTCAGGGATGGGGTGAACT-3'5'-CAGCCTCCGTTATCCTGGAT-3'
Yap1	58	234	5'-CGCTGAGTTCCGAAATCCTG-3'5'-AGAGCAGAGCAACAGTGGAT-3'
Nppa	56.1	168	5'-GGAAGTCAACCCGTCTCAGA-3'5'-TGGGCTCCAATCCTGTCAAT-3'
Nppb	53.8	172	5'-AGTCCTAGCCAGTCTCCAGA-3'5'-GTCTCTCCTGGATCCGGAAG-3'
β-actin	55.0	145	5'-CCTCTATGCCAACACAGTGC-3'5'-CCTGCTTGCTGATCCACATC-3'

Determination of cell size

To determine the total surface area of individual H9c2 cells, the cells were first fixed with 1% PFA after washing with PBS. Then the cells were washed with 1X NP40 followed by blocking in same for 1h. The microfilaments (F-actin) of the cells were marked by phalloidin. After incubation for 2h at 4°C, the nuclei of the cells were counterstained with DAPI for additional 5min at room temperature in dark. Fluorescence images were next captured using 40X objective as described previously[19]. Entire surface area of individual cells were quantified using Image J (NIH) for a minimum of 300-350 numbers of H9c2 cells with centrally located nuclei of each experimental groups including respective controls.

Protein isolation and western blotting

Protein lysates were isolated from control and treated H9c2 cultured cell samples using ice-cold RIPA lysis buffer as described previously[30]. Western blots were performed as described previously[19]. Immuno-blots were developed using chemi-fluorescent detection with the Clarity™western ECL substrate (170-5060 Biorad) and scanned using ChemiDocTMMP imaging system (Biorad). Signal intensities were quantified using Image J (NIH). p-Akt (60 kDa) (1:1000; #4058 Cell Signaling), total Akt (60 kDa) (1:1000; #4685 Cell Signaling), p-Yap (65-75 kDa) (1:1000; #13008 Cell Signaling) and total Yap (65-75 kDa) (1:1000; #14074 Cell Signaling) primary antibodies were used for immunoblot analyses. The Total Akt and Total Yap were used as the loading controls and experiments were carried out at least in triplicate.

Statistical analysis

Microsoft office Excel 2007 was used to perform statistical analyses. Quantitative values are presented as mean ± standard error (mean ±SEM). Statistical significance between two groups was performed using Student's *t* test. Differences were considered to be statistically significant when P <0.05(*); P <0.01(**) and P <0.001(***).

Results and Discussion

Oxidative stress and hypoxia inductions in cultured H9c2 cells

Sustained over-expression of Tbx20 in adult mouse hearts result in persistent expression of fetal genes without any hypertrophy or fibrosis[19] and selective overexpression of Tbx20 preserves cardiac function, reduces infarct size and inhibits cardiac remodeling post MI[20]. But the specific injury mediated reactivation mechanism through which Tbx20 mediates this cardiac protective processes post injury is largely unknown.ROS generation and oxygen depletion are common phenomena observed during adult cardiac injuries, such as MI or I/R or aortic banding[21-23]; Hence, to know the detail understanding of cardiac injury specific protective mechanism of Tbx20 and its associated downstream signaling targets, we induced oxidative stress upon the H9c2 cells by treating them with 200 μM concentration of hydrogen peroxide (H_2O_2)[26]. Initially, the cells were treated with a gradient of H_2O_2 concentrations (50, 100 and 200 μM) with the most enhanced expression of Tbx20 was noted at 200 μM concentration of H_2O_2, thus 200 μM concentration of H_2O_2 was chosen for the required sets of oxidative induction experiments. The successful induction of oxidative stress on the H9c2 cells is shown by the increased expression of antioxidant marker genes, *Catalase* and *Sod2* approximately by 1.84-folds (P <0.01) and 3.5-folds (P <0.05), respectively (**Fig. 1 A-C**) compared to their respective controls. Likewise hypoxia was induced separately on the H9c2 cells as described previously[31] and it is evident by the significant upregulated expression of hypoxia indicator, *Hif1α* (1.2-folds; P <0.001) (**Fig. 1 D & E**). Thus, from the increased expression of respective stress indicators it is evident that both the oxidative stress and hypoxia are induced successfully in H9c2 cells. ROS

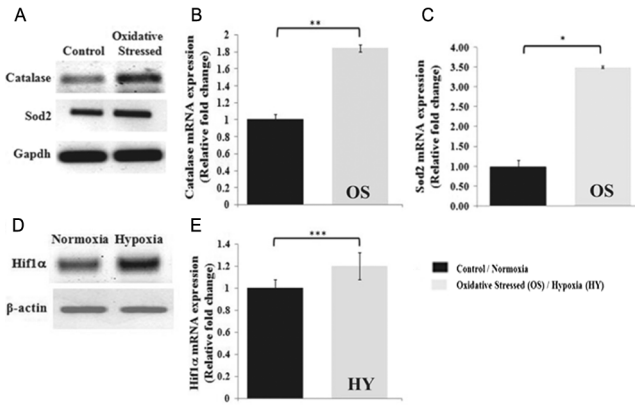


Fig. 1 — Oxidative and hypoxic stress inductions in cultured H9c2 cells. Semiquantitative and real time PCR analyses (A-C) reveal increased expression of antioxidant markers, *Catalase* and *Sod2* by1.84-folds (P=0.002) and 3.5-folds (P=0.023), respectively in the oxidative stress induced (200 μM H_2O_2) cultured H9c2 cells compared to the control ones (n=3); and (D & E) show increased expression of hypoxic indicator *Hif1α* by 1.2-folds (P=0.001) in the hypoxia induced H9c2 cells compared to the normoxic cultured cells. [*Gapdh* and β-actin are used as the loading controls for oxidative and hypoxic stress inductions, respectively. (n=3) (*P =<0.05 *vs*. the control, **P =<0.01 *vs*. the control, ***P=<0.001 *vs*. the control)]

generation leads to amplification of Akt signaling pathway[31] and Akt is a downstream signaling target of Tbx20[22]. Besides oxidative stress, hypoxia also leads to ROS generation as evident from the increased expression of *Catalase* and *Sod2* by 3.4-folds (P <0.01) and 1.5-folds (P <0.001), respectively (Suppl. Fig. 1 A and B. *All supplementary figures are available only online along with the respective paper at repository. http://nopr.res.in*) indicating both oxidative stress and hypoxia may trigger Tbx20 in the similar fashion with its associated downstream targets. In contrast, *Hif1α* also showed an increased trend under H_2O_2 treated condition (Suppl. Fig. 1C) indicating *Hif1α* may not be restricted within hypoxic condition only.

Sustained over-expression of Tbx20 promotes increased cell viability post injury induction

To determine Tbx20 mediated signaling pathways that are activated after injury induction in cultured cells, first Tbx20 mRNA expression is detected post oxidative stress and hypoxia induction. Interestingly, Tbx20 is increased under both the stress conditions by 3.05-folds (P <0.05) (**Fig. 2A**) and 4-folds (P <0.05)

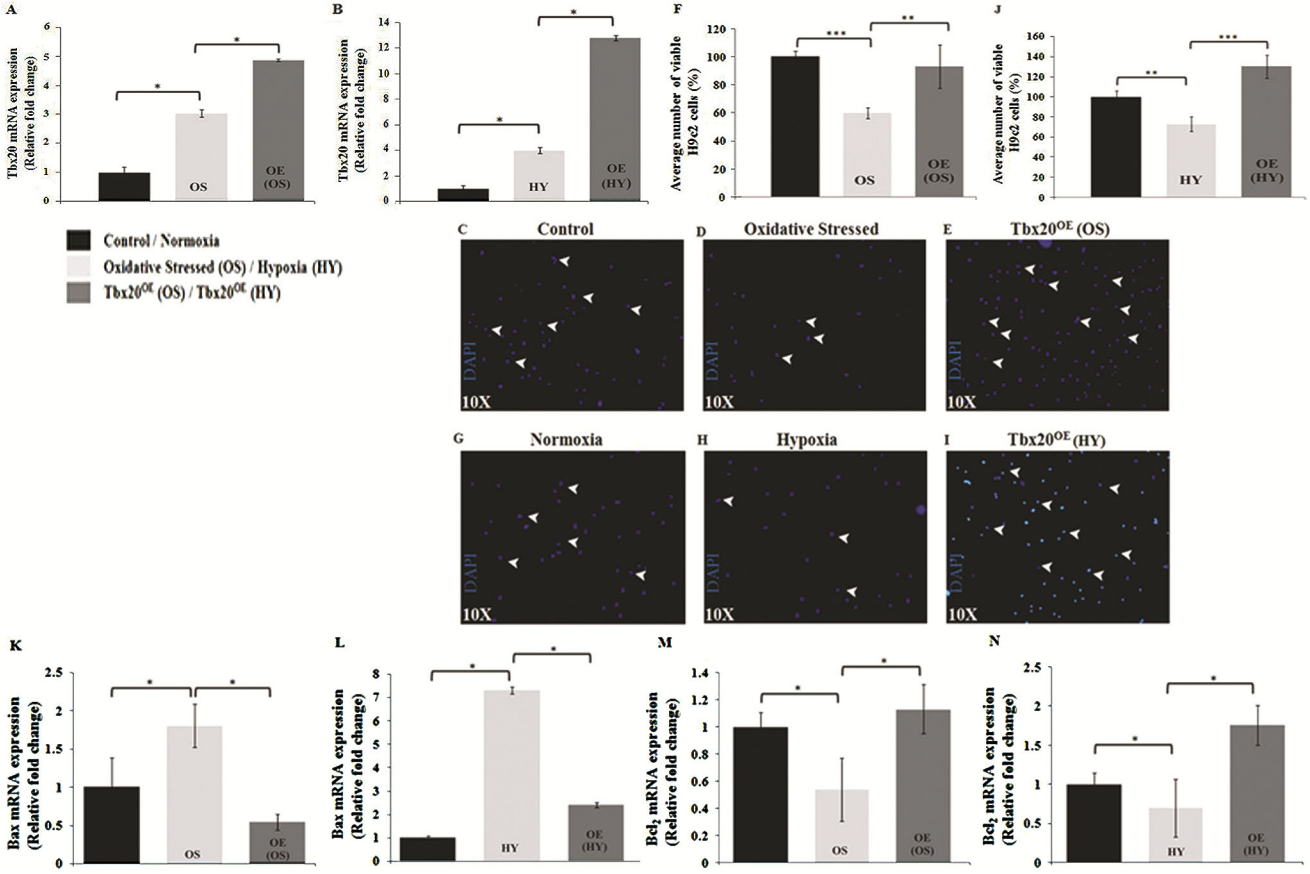


Fig. 2 — Sustained overexpression of Tbx20 can overcome post injury stresses by showing increased cell viability. (A & B): Quantitative real time PCR analyses reveal increased *Tbx20* expression in the oxidative stress induced and hypoxia induced H9c2 cells by 3.05-folds (P=0.023) and 4-folds (p=0.02) respectively in comparison to the untreated cells (n=6). Furthermore, after transfecting the cultured H9c2 cells with plasmids containing mouse full length Tbx20 cDNA cloned in pCDNA 3.1 followed by respective oxidative and hypoxic stress inductions, *Tbx20* expression is further upregulated by 1.6-folds (p=0.028) and 3.2-folds (P=0.04) in the Tbx20 transfected H9c2 cells compared to their respective non-transfected just injury induced conditions (n=6); (C-E; G-I): The viable number of cells as determined by the DAPI positive nuclei observed at 10X magnification shows decreased number of viable cells under oxidative and hypoxic stress injured conditions but strikingly with Tbx20 overexpression followed by injury induction showed increased population of viable cell types; (F & J): Statistical analyses reveal the viable cell numbers decreased by 40% (P=0.001) and 27% (P= 0.011), respectively under stress injured conditions and increased by 55% (P=0.0084) and 78% (P=0.001) in Tbx20 overexpressed condition compared to just injured scenario (n=5-6). (K-N) Quantitative real time PCR analysis reveals increased expression of pro-apoptotic indicator *Bax* by 1.8-folds (P=0.02) (K) and 7.3-folds (P=0.03) (L) upon oxidative and hypoxic injury inductions; likewise anti-apoptotic indicator Bcl_2 showed decreased expression by 46% (0.54-folds) (M) and 30% (P=0.03) (N), respectively under the same conditions. Again with the sustained over-expression of Tbx20 prior to injury followed by respective stresses, the data shows decreased mRNA level expression of *Bax* 70% (P=0.05) (P <0.02) (K & L) in both and increased Bcl_2 expression by 2.1-folds (P=0.02) (M) and 2.51-folds (P=0.02) (N) in comparison to their respective injury induced controls. [*Gapdh* and β-actin are used as the loading controls for oxidative and hypoxic stress inductions, respectively. (n=6) (*P =<0.05 *vs*. the control, **P =<0.01 *vs*. the control, ***P=<0.001 *vs*. the control)]

(**Fig. 2B**) as compared to control under ROS generation and oxygen depletion. Since, Tbx20 aids in CM proliferation[13,14,19], we tried to observe the number of viable cells. But surprisingly, when the viable cell population is visualized by DAPI, it is decreased under both the injury conditions by 40% (P <0.001) (**Fig. 2 C, D & F**) and 27% (P <0.01) (**Fig. 2 G, H & J**), respectively as compared to control. Therefore, injury dependent and reactivated Tbx20 is not sufficient to overcome the consequences of the injury mediated cell death. Likewise, the respective injuries resulted in increased level of cellular apoptosis that are also quantified with mRNA expression of *Bax* (Pro-apoptotic marker) and Bcl_2 (Anti-apoptotic marker) (**Fig. 2 K-N**). Under both oxidative stress and hypoxia; *Bax* is found to be upregulated by 1.8-folds (P <0.05) (**Fig. 2K**) and 7.3-folds (P <0.05) (**Fig. 2L**), respectively in comparison to controls. Likewise, Bcl_2 showed decreased expression by 46% (0.54-folds) (P <0.05) (**Fig. 2M**) and 30% (0.7-folds) (P <0.05) (**Fig. 2N**), respectively. Next, to determine the effect of over-expressed Tbx20 in the H9c2 cells (Suppl. Fig. 2 A & B), we have transfected H9c2 cells with plasmids containing mouse full length Tbx20 cDNA prior to injury followed by respective stress induction. Furthermore, the data reveals that upon induction of oxidative stress the Tbx20 expression is increased by 1.6 folds in the Tbx20-transfected H9c2 cells as compared to just oxidative stressed induced cells (P <0.05) (**Fig. 2A**). Similarly Tbx20 expression in the Tbx20-transfected cells undergone hypoxia is over-expressed by 3.2-folds (P <0.05) (**Fig. 2B**) compared to just hypoxic cells. Moreover, we found that the cells over-expressing Tbx20 either undergoing oxidative stress or hypoxia show increased number of viable cells by 55% (P <0.01) (**Fig. 2 D-F**) and 78% (P <0.001), respectively as compared to untransfected controls (**Fig. 2 H-J**). This increased viability of cells is caused by reduction in the cellular apoptosis in the Tbx20 transfected followed by oxidative stress and hypoxia induced conditions to that of injury only prevailing situations with endogenous basal level Tbx20, which is evident from the decreased level of *Bax* by 70% (0.3-folds) (P <0.05) (**Fig. 2 K & L**) in both the conditions respectively. Furthermore, Bcl_2 is also found to be upregulated under the Tbx20 over-expressed oxidative stress and Tbx20 overexpressed hypoxia conditions by 2.1-folds (P <0.05) (**Fig. 2M**) and 2.51-folds (P <0.05), respectively (**Fig. 2N**) in comparison to their respective injury induced only controls. Thus, not basal but sustained over-expression of Tbx20 results in increased number of viable cell population, reducing the cellular apoptosis thus aiding to overcome the stress level post injury.

Increased Tbx20 over basal level helps to inhibit cellular hypertrophy post injury

CMs undergo increased cell size or hypertrophy post cardiac injury which is an adaptive feature to make up the CMs cell number loss[32]. But if increase in CM cell size is sustained for long it proves to be fatal (pathological hypertrophy)[32,33]. Therefore, we have examined the size of H9c2 cells post injury. Data reveals post oxidative stress and hypoxia, H9c2 cells are increased in surface area by 56% (P <0.01) (**Fig. 3 A, B & D**) and 54% (P <0.001) (**Fig. 3 E, F & H**), respectively as compared to controls indicative of cellular hypertrophy. Interestingly, upon over-expression of Tbx20 it is found that the cells though have undergone injury still shows reduced cell size by 40% (P <0.001) (**Fig. 3 B-D**) post oxidative stress and 47% (P <0.001) (**Fig. 3 F-H**) post hypoxia compared to their injury induced only conditions. This is consistent with the *in vivo* study that states increased Tbx20 promotes increased number of small dividing mononucleated CMs post injury[20]. To ascertain if Tbx20 helps to maintain CM cell size, we analyzed the expression of another transcription factor *Yap1* (Yes associated protein 1) which aids in maintaining CM cell size and growth[32]. Furthermore, Yap1 activation attenuates hypertrophic conditions of adult heart post MI[33]. Thus, upon analyzing the expression of *Yap1* it is found that *Yap1* is increased by 2.2-folds (P <0.05) and 1.6-folds (P <0.05) in the overexpressed Tbx20 post oxidative and hypoxia groups compared to injury induced only conditions, respectively (**Fig. 3 I & J**). Furthermore, our study reveals that the hypertrophic indicators, *Nppa* and *Nppb* both showed a downward trend in their mRNA expression level by 20% (0.8-folds) (P <0.05); (P <0.07) (**Fig. 3K-L**), respectively in Tbx20-overexpressing cells upon induction of oxidative stress as compared to untransfected control cells. Similarly, *Nppa* and *Nppb* are downregulated by 60% (0.4-folds) (P <0.05); (P <0.001) (**Fig. 3 M & N**) in the Tbx20 overexpressing cells upon hypoxia induction as compared to untransfected controls. Therefore, our data suggests Tbx20 may play an important role in maintenance of cell size by reducing

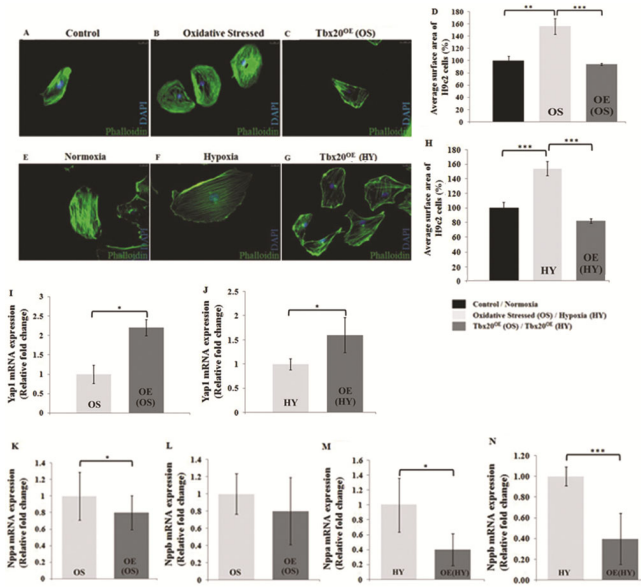


Fig. 3 — Increased Tbx20 over basal level helps to inhibit cellular hypertrophy post injury (A-C; E-G): The surface area of the cells were determined by staining the microfilaments of the cultured H9c2 cells by phalloidin and the nuclei of the cells were counterstained by DAPI. It shows that the surface area is increased under the oxidative and hypoxic stress induced conditions but with the overexpression of Tbx20 interestingly the cell sizes have got decreased (scale bar: 0-25 µm). (D & H): Statistically, it is found that both oxidative and hypoxic stress induction results in increase in cell size by 56% (P=0.011) and 54% (P=0.001), whereas; the cells though undergoing injury but having elevated Tbx20 level shows decrease in cell surface area by 40% (P=0.001) and 47% (P=0.001) compared to their respective injury induced conditions (n=3). (I & J): Quantitative real time PCR analyses reveal increased level of mRNA expression of *Yap1* by 2.2-folds (P=0.02) and 1.6-folds (P=0.02) with sustained overexpression of Tbx20 prior to injury followed by oxidative and hypoxic stress induction, respectively compared to their controls of non-transfected just injury induced cells (n=3). (K-N): Moreover, quantitative analysis of hypertrophic indicators, *Nppa* and *Nppb* show decrease in their mRNA level expression by 20% (0.8-folds) (P=0.03); (P=0.06) (K-L) and 60% (0.4-folds) (P=0.02); (P=0.001) (M-N) in the Tbx20 transfected injury induced cultured cells with respect to just injury induced non-transfected cells. [(n=6) (*P =<0.05 *vs*. the control, **P =<0.01 *vs*. the control, ***P=<0.001 *vs*. the control]

cellular hypertrophy post injury by activating *Yap1*.But whether there lies a direct crosstalk between these two transcription factors is unknown and currently under investigation.

Tbx20 over-expression shows increased level of cellular proliferation post injury

Tbx20 plays important role in CM's proliferative activity both in developing and adult hearts[13,14,19]. Hence we tried to elucidate the proliferative indices of the cultured H9c2 cells post injury and found that with the

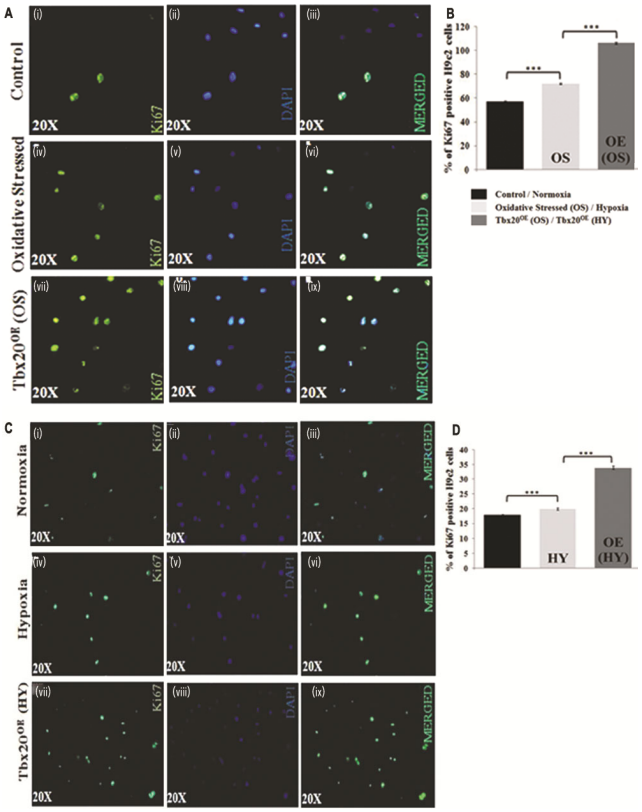


Fig. 4 — Tbx20 overexpression shows increased level of cellular proliferation post injury [A(i-ix); C(i-ix)] The proliferative cells marked as the Ki67 positive [A(i,iv,vii); C(i,iv,vii)] which are counterstained by DAPI (nuclei) [A(ii,v,viii); C(ii,v,viii)] and the merged images [A(iii,vi,ix); C(iii,vi,ix)] all are taken at 20X magnification. The proliferative rate is found to be increased in injury induced conditions, oxidative and hypoxic as seen by the increased number of Ki67 positive nuclei [A(iv-vi); C(iv-vi)] and moreover with elevated Tbx20 expression followed by stress induction showed further increase in the proliferative rate [A(vii-ix; C(vii-ix)]. (B & D) Statistical analyses reveal proliferative index as measured by the percentage of Ki67 positive cell nuclei showed increased rate by 1.3-folds (P=0.0008) and 1.11-folds (P=0.0004) under oxidative and hypoxic injury induced condition compared to control ones. Likewise, with the overexpression of the Tbx20 level the proliferative rate got accelerated by 1.95-folds (P=0.0001) and 1.9-folds (P=0.0001) followed by injury induction with respect to only oxidative and hypoxic stress induced condition respectively. [(n=3) (*P =<0.05 *vs*. the control, **P =<0.01 *vs*. the control, ***P=<0.001 *vs*. the control)]

enhanced expression of Tbx20 under both the stress conditions (**Fig. 2 A & B**), the proliferative rate measured as the percentage of Ki67 positive cells also showed slight increase in the rate approximately by 1.3-folds (P <0.001) [**Fig. 4 A(i-iii, iv-vi) & B**] and 1.11-folds (P <0.001) [**Fig. 4 C(i-iii, iv-vi & D**] in comparison to the controlled groups. This proliferative rate is found to be not sufficient to overcome the injury as the numbers of total viable cells are much less in the stressed conditions than the control groups owing to

increased cellular apoptosis (**Fig. 2 K-N**). Sustained as well as selective overexpression of Tbx20 results in increased rate of CM proliferation along with increased number of mononucleated CM cells[19,20], Hence, we overexpressed Tbx20 level in the H9c2 cells prior to injury and then subjected them to stress induction. Overwhelmingly, we found that the proliferative rate is increased by 1.95-folds (P <0.001) [**Fig. 4 A(vii-ix) & B**] and 1.9-folds (P <0.001) [**Fig. 4 A(vii-ix) & D**] in Tbx20 overexpressed cells followed by injury induction to those which were just subjected to injury. Moreover, this upregulation in the cell proliferative capacity also resulted in increase in number of viable cells (**Fig. 2 D-F & H-J**) reducing the cellular apoptosis (**Fig. 2 K-N**), overcoming the injury conditions. Again, the number of dividing cells is also found to be enhanced under Tbx20 transfected conditions post injury than in comparison to injured conditions only and also from non-injured controlled conditions (Suppl. Fig. 3 A-B). Interestingly, this observation of increased number of dividing cells with Tbx20 overexpression is found to coincide with the observations that involve Gata4 positive small nucleated neonatal CM cells undergoing proliferation with induced Tbx20 overexpression[19]. Overall, over-expressed Tbx20 activates cellular proliferation sufficient for increasing the cell viability as a repair response post injury.

Tbx20 promotes *Nmyc1* and *Bmp2* mediated proliferative signaling pathways to overcome stress induction

Nmyc1 and *Bmp2* are the known downstream targets of Tbx20[15,16,19]. Furthermore, overexpressed Tbx20 promotes the activation of Bmp2-pSmad 1/5/8 signaling pathway in adult hearts *in vivo*[19]. Therefore, to investigate whether these two signaling molecules are stimulated by Tbx20 upon injury induction, we analyzed their expression pattern and the data reveals that both *Nmyc1* and *Bmp2* show increased mRNA expression by 2.51-folds (P <0.001) (**Fig. 5A**) and 1.99-folds (P <0.01) (**Fig. 5B**), respectively upon ROS induction and 2.04-folds (P <0.05) (**Fig. 5C**) and 1.7-folds (P <0.01) (**Fig. 5D**), respectively under hypoxic condition. But enhanced expression of *Nmyc1* and *Bmp2* triggered by increased expression of Tbx20 though stimulating the proliferative rate is not sufficient to overcome the stress conditions characterized by decrease in the total number of viable cell population (**Fig. 2 C-D, F, G-H & J-N**). Therefore, sustained over-expression of Tbx20 achieved by transfection followed by oxidative stress induction resulted in activated expression of Nmyc1 and Bmp2 approximately by 2.6-folds (P <0.01) (**Fig. 5A**) and 2.2-folds (P <0.01) (**Fig. 5B**) compared to non-transfected oxidative stress induced only cells. Similarly, Tbx20 transfected cells undergoing hypoxia showed increased expression of Nmyc1 and Bmp2 by 2.7-folds (P <0.05) (**Fig. 5C**) and 1.6-folds (P <0.05) (**Fig. 5D**), respectively compared to injury induced only controls. Therefore, these data reveals sustained overexpression of Tbx20 activates both of its downstream targets, *Nymc1* and *Bmp2* activating proliferative signaling pathways to a level that is sufficient and necessary for increased cell numbers and viability thus overcoming the stresses imparted by the injuries.

Tbx20 reduces cellular hypertrophy by activating Yap and together regulating the pAkt level

Hypertrophy is a common adaptive phenomenon undergone by the CMs upon MI to make up the loss in CM numbers[9]. Akt, a serine/threonine protein kinase regulating cardiac growth[33] shows its short term activation in cardiac protection during MI by facilitating physiological hypertrophy whereas long term activation results in pathological hypertrophy and heart failure[34-36].

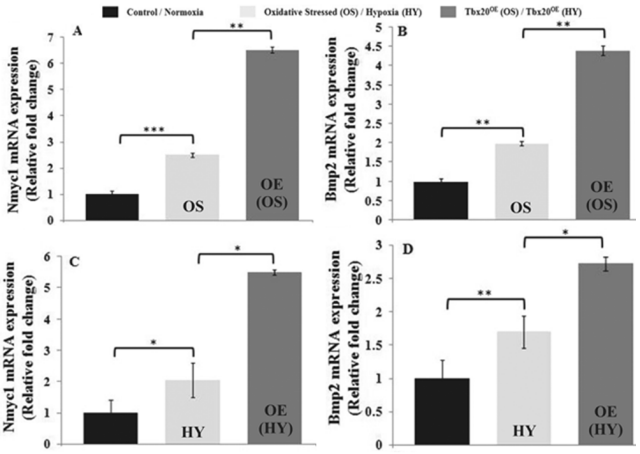


Fig. 5 — Tbx20 promotes Nmyc1 and Bmp2 mediated proliferative signaling pathways to overcome stress induction (A & B) Quantitative real time PCR analyses reveal the two downstream targets of Tbx20, *Nmyc1* and *Bmp2* showed increased mRNA expression by 2.51-folds (P=0.001) and 1.99-folds (P=0.01), respectively upon ROS induction and moreover, with sustained overexpression of Tbx20 the expression level of them both was elevated by 2.6-folds (P=0.005) and 2.2-folds (P=0.01) post injury induction compared to just stress exhibited controls (n=6); and (C & D) similarly, quantitative analyses reveal *Nmyc1* and *Bmp2* showing enhanced expression by 2.04-folds (P=0.04) and 1.7-folds (P=0.01) under hypoxia compared to normoxia. [Likewise, elevated Tbx20 level prior to injury resulted in increased *Nmyc1* and *Bmp2* transcript level by 2.7-folds (P=0.04) and 1.6-folds (P=0.03) post hypoxic injury induction compared to just hypoxia. [(n=6) (*P =<0.05 *vs*. the control, **P =<0.01 *vs*. the control, ***P=<0.001 *vs*. the control)]

Hence, the activated/phosphorylated form of Akt (p-Akt) triggers cardiac hypertrophy[37]. Moreover, this Akt signaling pathway is also triggered by another cardio-protectant protein SUR2A upon hypoxic stimulation[38-40]. Furthermore, Akt is a part of signaling cascade of both Tbx20 and Yap1[19,41]. Therefore to elucidate a mechanistic insight for reduced cellular hypertrophy by Tbx20 and Yap1 we assayed the protein expression of p-Yap to total Yap (t-Yap) and p-Akt to total Akt (t-Akt)under injured only and Tbx20 overexpressed followed by injury inductions. It is found that upon Tbx20 overexpression followed by oxidative stress and hypoxia the ratio of p-Yap/t-Yap is decreased by 24% (P <0.05) (**Fig. 6 A & B**) and 40% (P <0.05) (**Fig. 6 C & D**), respectively. Thus, clearly depicting, the activation of the Yap1 by elevated Tbx20 level, since upon phosphorylation, Yap becomes inactive[20]. On the other hand, reduction in cellular hypertrophy is found to be facilitated by the decreased p-Akt/t-Akt activation level by 45% (P <0.05) (**Fig. 6 E & F**) and 40% (P <0.05) (Fig 6 G, H) in the Tbx20 transfected cells followed by respective stress induction compared to nontransfected injury induced only control cultured

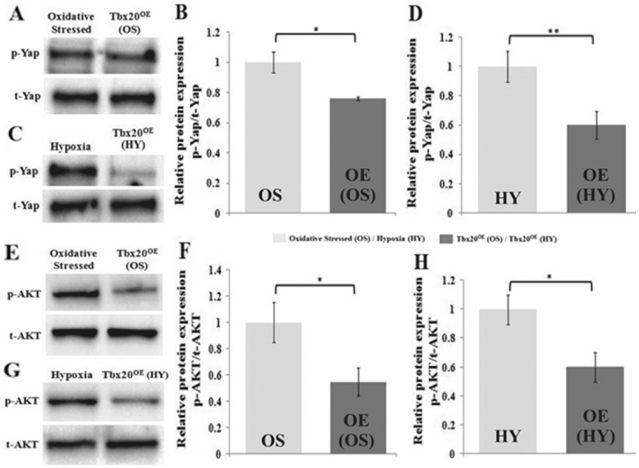


Fig. 6 — Tbx20 reduces cellular hypertrophy by activating Yap and together regulating the p-Akt level (A-H) Protein extracted from oxidative and hypoxic injury induced and Tbx20 overexpressed followed by similar injury induced cultured H9c2 cells. Western blot analysis was carried out which reveals decreased p-Yap expression in both transfected followed by injury induced condition by 24% (P=0.05) (A & B) and 40% (P=0.01) (C & D) compared to their respective non-transfected just injury stress induced controls. P-Yap was normalised to total Yap (t-Yap) (n=4). Likewise, the p-Akt showed decreased protein expression by 45% (P=0.05) (E & F) and 40% (P=0.02) (G & H), respectively in the Tbx20 transfected stress induced cells with respect to just injury induced non-transfected cultured H9c2 cells. [The p-Akt was normalised to the total Akt (t-Akt). (n=4) (*P =<0.05 *vs*. the control, **P =<0.01 *vs*. the control, ***P=<0.001 *vs*. the control)]

cells. Since it has been documented that elevated Tbx20 level promotes Yap1 activation and reduces CM hypertrophy[20]. Thus, from our current study we can suggest that the over-expressed Tbx20 level promotes the activation level of Yap1 post injury conditions and together they may aid in decreasing the activation level of Akt and in turn facilitate the inhibition of cellular hypertrophy. But the detailed mechanism needs further investigation.

Limited proliferative capacity and physiological hypertrophy best characterizes the CM in adult hearts, although the detail molecular cues controlling this transition from embryonic/fetal/neonatal to adult hearts are relatively unknown. The mononucleated, highly proliferative nature of embryonic CMs, along with the expression of fetal contractile proteins of the fetal CMs are lost when they change their fate to adult forms[3,4]. The adult CMs become mostly binucleated, relatively quiescent and express adult contractile protein isoforms and cell senescence markers[5-7]. Even the high proliferative capacity of fetal CMs get reduced to <1%/year proliferative activities in adult[8]. Furthermore, the neonatal CMs having the ability to regenerate are lost within 1 week after birth[6]. Thus, any kind of cardiac injuries harbouring over the adult heart results in massive loss of CMs and propelling the scenario to the onset of HF[14]. Therefore, search for pro-proliferative factors in adult heart post injury is worth investigating.

Sustained overexpression of Tbx20 in adult CMs results in persistence of fetal characteristics with increased mononucleation, higher proliferative rate and decrease cell senescence[19]. In addition, Tbx20 plays an important role in increasing the survival of the adult heart post MI, if it is selectively over-expressed in the adult CMs prior to injury[20]. Being a transcription factor, Tbx20 acts as an activator as well as a repressor of other signaling molecules[16,20,42-44]. In fetal CMs, Tbx20 triggers the proliferative indices by inducing *Nmyc1* and increased Bmp10-pSmad 1/5/8 signaling whereas in neonatal and adult it activates the Bmp2/pSmad 1/5/8 and PI3K/Akt/GSK3β/β-catenin signaling pathways[13,14,19]. On the other hand, Tbx20 binds directly to cell cycle repressor *p21* and *Meis1* and a novel anti-proliferative *Btg2* to repress them[20]. Moreover, overexpressed Tbx20 helps to preserve cardiac function, reduces infarct size and inhibits cardiac remodeling post MI[20]. All these suggest involvement of Tbx20 in imparting cardiac repair mechanisms, maintain cardiac homeostasis and

protection post injury. But the molecular mechanism mediated by Tbx20 underlying these processes post injury is not well understood.

In our study, we have tried to understand the Tbx20 mediated protective signaling pathways upon two specific injury inductions in cardiac H9c2 cells *in vitro*. Likewise, we have used the oxidative stress and the hypoxia, two most inevitable features prevalent during cardiac injuries[21-23]. As expected, the Tbx20 expression is found to be upregulated in both injuries compared to uninjured control cells. Furthermore, the increased Tbx20 level also activates its two known downstream targets of proliferative signaling pathway, *Nmyc1* and *Bmp2* and in turn promotes the proliferative activity, thus trying to impart protection against the injury. However, these were not sufficient enough to overcome the stress level of the injuries as was seen by reduced number of viable cell population for increased expression of apoptotic markers. Therefore, we sustainably overexpressed Tbx20 in the H9c2 cells and then subjected them to the same oxidative stress and hypoxia and notably found increased cell number of viable cell types with accelerated proliferative rate which was augmented with enhanced level of *Nmyc1* and *Bmp2*. This is consistent with elevated Tbx20 level promoting increased number of proliferative, mononucleated CMs post MI *in vivo*[20]. But furthermore our study reveals that increased level of Tbx20 also results in decrease in the apoptotic rate as evident from the Bax and Bcl_2 expression, thus rendering a protective pathway post injury (**Fig. 7**).

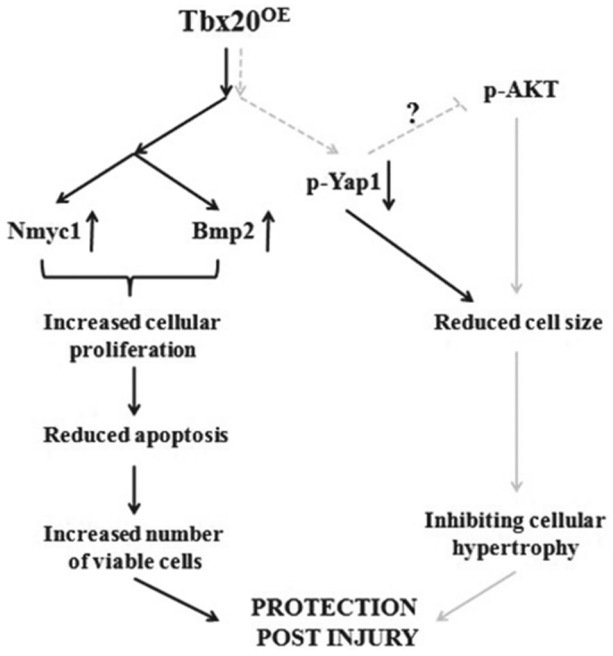


Fig. 7 — Model for Tbx20 mediated cardiac protective pathway post injury. Sustained overexpresssion of Tbx20 followed by injury induction like oxidative and hypoxic stresses results in increased number of viable cell population augmented by increased rate of cellular proliferation mediated by *Nmyc1* and *Bmp2* and reduced cellular apoptosis. Again this enhanced Tbx20 level promotes the activation of *Yap1* and together may be exhibiting a feedback loop mechanism over Akt decreasing its activation level thus reducing the cell size and inhibiting cellular hypertrophy.

Besides proliferation, CM hypertrophy also plays an important role in adult heart function and homeostasis. It is known that cardiac hypertrophy (physiological hypertrophy) which is an adaptive mechanism for CMs during MI to make up for the massive loss of CMs, may proved to be fatal if sustained for long giving rise to pathological hypertrophy[9-11]. Cardioprotection over MI induction has an important impact in CVDs[32-34]. Yap1 and Akt, the two known cardiogenic factors help to regulate and maintain CM cell size and growth[35-37]. It is known that Yap gain-of-function in adult murine hearts at a time period of 4 weeks post MI resulted in attenuation of cardiac hypertrophy with decreased heart-to-body weight ratio[35]. Whereas, the activation level of Akt is one of the prime regulators of the switch between physiological and pathological hypertrophy, depicting that increased Akt activation causes cellular hypertrophy[41]. Interestingly, it has been found that exercise induced cardiac hypertrophy was attenuated in Akt knockout mice[41]. Moreover, Tbx20 over-expression also found to reduce cardiac hypertrophy as well as Yap activation *in vivo*[17]. Therefore, in connection to all these published reports and from our experimental outcomes, our study claims a novel finding, that enhanced Tbx20 has a role in maintaining cell size by reducing cellular hypertrophy post injury. Our study shows that, this cellular hypertrophy is reduced in the H9c2 cells, documented by decreased cell surface area, which are also undergoing injury following sustainable over-expression of Tbx20. The cellular hypertrophy is reduced by the increased Tbx20 level activating the Yap1 level post injury similar to in vivo condition[20]. Surprisingly, when we observed the activated Akt level under the same scenario it is found that p-Akt is down-regulated in the cells overexpressing Tbx20 followed by injury induced conditions which are also concomitant with the known fact that decreased activation of Akt reduces cardiac hypertrophy[45-49]. Moreover, protein like SUR2A does cardiac protection by triggering the Akt pathway[38]. Interestingly, hypoxia induces phosphorylation of

AMP activated protein kinase (AMPK) with increased SUR2A levels in H9c2 cells independent of ERK1/2 or Akt phophorylation[39-40]. Likewise, we may assume from our study that increased Tbx20 level activates Yap1 in such a manner post injury that together these two transcription factors may be creating a feedback loop over Akt as it is the common downstream target for both of them[16,47] and therefore differentially regulating Akt activation level causing it to decrease and thus promoting protection post injury by inhibiting further persistence of cellular hypertrophy (**Fig. 7**). But whether there lies a direct crosstalk between these two transcription factors or the mechanism of the feedback loop by which Akt activation is altered needs further investigation.

Henceforth, we may conclude by saying that Tbx20 may pave a way for novel therapeutic approach in near future by cardiac repair and protective mechanisms which is not only limited to CM proliferation.

Conclusion

The outcome of our study states that overexpression of Tbx20 imparts protection to cultured H9c2 cells post injury by activating its two downstream targets *Nmyc1* and *Bmp2*, thus accelerating the level of cellular proliferation increasing the population of viable cells and reducing the apoptotic marker expression. Furthermore, the elevated expression of Tbx20 aids to maintain the cell size post injury by activating Yap. Together they may impart a feedback loop mechanism over the activation level of Akt, decreasing it and henceforth inhibiting the persistence of cellular hypertrophy. Thus our study may provide an extra leap towards the therapeutic novelty of Tbx20 as a cardio-protectant post injury in the adult heart.

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Conflict of Interest

There are no conflicts to disclose.

References

1 Bui AL, Horwich TB & Fonatow GC, Epidemiology and risk profile of heart failure. *Nat Rev Cardiol*, 8 (2011) 30.

2 Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, de Ferranti SD, Floyd J, Fornage M, Gillespie c, Isasi CR, Jimenez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longeneker CT, Mackey RH, Matsushita K, Mozaffarian D, Mussolino ME, Nasir K, Neumar RW, Palaniappam L, Pandey DK, Thiagarajan RR, Reeves MJ, Ritchey M,Rodriguez CJ, Roth GA, Rosamond WD, Sasson C, Towfighi A, Tsao CW, Turner MB, Virani SS, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS & Muntner P, Heart disease and stroke statistics-2017 update: A report from American Heart Association. *Circ*, 135 (2017) e146.

3 Toyoda M, Shirato H, Nakajima K, Kojima M, Takahashi M, Suzuki-Migishima R, Motegi Y, Yokoyama M & Takeuchu T, Jumonji downregualtes cardiac cell proliferation by repressing cyclin D1 expression. *Dev Cell*, 5 (2003) 85.

4 Ahuja P, Sdek P & McLellan WR, Cardiac myocyte cell cycle control in development, disease and regeneration. *Physiol Rev*, 87 (2007) 521.

5 Siedner S, Kruger M, Schroeter M, Metzler D, Roell W, Fleischmann BK, Hescheler J, Pfitzer G & Stehle R, Developmental changes in contractility and sarcomeric proteins from the early embryonic to adult stage in the mouse heart. *J Physiol*, 548 (2003) 493.

6 Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN & Sadek HA, Transient regenerative potential of neonatal mouse heart. *Science*, 331 (2011) 1078.

7 Mollova M, Bersell K, Walsh S, Salva J, Das LT, Park SY, Silberstein LE, Dos Remedios CG, Graham D, Clan S & Kuhn B, Cardiomyocyte proliferation contributes to heart growth in young humans. *Proc Natl Acad Sci. USA*, 110 (2013) 1446.

8 Bergmann O, Bhardwaj RD, Bernard s, Zdunek S, Barnabe-Heider F, Walsh CS, Zupicicih J, Alkass K, Buchholz BA, Druid H, Jovinge S & Frisen J, Evidence for Cardiomyocyte renewal in humans. *Science*, 324 (2009) 98.

9 Esposito G, Rapacciuolo A, Prasad SV Naga, Takaoka H, Thomas SA, Koch WJ & Rockman HA, Genetic alterations that inhibit in vivo pressure-overload hypertrophy prevent cardiac dysfunction despite increased wall stress. *Circ*, 105 (2002) 85.

10 Berenji K, Drazner MH, Rothermel BA & Hill JA, Does load – induced ventricular hypertrophy progress to systolic heart failure? *Am J Physiol*, 289 (2205) H8.

11 Lorell BH & Carabello BA, Left ventricular hypertrophy: pathogenesis, detection and prognosis. *Circ*, 102 (2000) 470.

12 Nrula J, Haider N, Virmani R, DiSavo TG, Kolodgie FD, Hajjar RJ, Schmidt U, Semigran MJ, Dec GW & Khaw BA, Apoptosis in myocytes in end-stage heart failure. *N Engl J Med*, 335 (1996) 1182.

13 Kirk EP, Sunde M, Costa MW, Rankin SA, Wolstein O, Castro ML, Butler TL, Hyun C, Otway R, Mackay JP, Waddell LB, Cole AD, Hayward C, Keogh CA, Macdonald P, Grifiths L, Fatkin D, Sholler GF, Zorn AM, Feneley MP, Winlaw DS & Harvey RP, Mutations in cardiac T-Box factor gene TBX20 are associated with diverse cardiac pathologies, including defects of septation and valvulogenesis and cardiomyopathy. *Am J Hum Genet*, 81 (2007) 280.

14 Mandel EM, Kaltenbrun E, Callis TE, Zeng XX, Marques SR, Yelson D, Wand DZ & Conlon FL, The BMP pathway acts to directly regulate Tbx20 in the developing heart. *Development*, 137 (2010) 1919.

15 Chakraborty S & Yutzey KE, Tbx20 regulation of cardiac cell proliferation and lineage specialization during embryonic and fetal development in vivo. *Dev Biol*, 363 (2012) 234.

16 Cai CL, Zhou W, Yang L, Bu L, Qyang Y, Zhang X, Rosenfeld MG, Chen J & Evans S, T-box genes coordinate regional rates of proliferation and regional specification during cardiogenesis. *Development*, 132 (2005) 2475.

17 Stennard FA, Costa MW, Lai D, Biben C, Furtado MB & Solloway MJ, Murine Tbox transcription factor Tbx20 acts as repressor during heart development, and is essential for adult heart integrity, function and adaptation. *Dev*, 132 (2005) 2451.

18 Shen T, Aneas I, Sakabe N, Dirschinger RJ, Wang J, Smemo S, Westkund JM, Cheng H, Dalton N, Gu y, Boogerd CJ, Cai CL, Peterson K, Chen J , Nobrega MA & Evans SM, Tbx20 regulates a genetic program essential to adult mouse Cardiomyocyte function. *Clin Invest*, 121 (2011) 4640.

19 Chakraborty S, Sengupta A & Yutzey KE, Tbx20 promotes Cardiomyocyte proliferation and persistence of fetal characteristics in adult mouse hearts. *J Mol Cell Cardiol*, 62 (2013) 203.

20 Xiang FL , Guo M & Yutzey KE, Overexpression of Tbx20 in adult cardiomyocytes promotes proliferation and improves cardiac function after myocardial infarction. *Circ*, 133 (2016) 1081.

21 Wei C, Li H, Wang Y, Peng X, Shao H,Li H, Bi S & Xu C, Exogenous spermine inhibits hypoxia/ischemia-induced myocardial apoptosis via regulation of mitochondrial permeability transition pore and associated pathways. *Exp Biol Med*, 241 (2016) 1505.

22 Umar S & van der Laarse A, Nitric oxide and nitric oxide synthase isoforms in the normal, hypertrophic and failing heart. *Mol Cell Biochem*, 333 (2010) 191.

23 Perrino C, Feliciello A, Schiattarella GG, Esposito G, Guerriero R, Zaccaro L, Del Gatto A, Saviano M, Garbi C, Carangi R, Di Lorenzo E, Donato G, Indolfi C, Avvedimento VE & Chiariello M, AKAP121 downregulation impairs protective cAMP signals, promotes mitochondrial dysfunction, and increases oxidatives stress. *Circ Res*, 88 (2010) 101.

24 Kimes BW & Brandt BL, Properties of a clonal muscle cell line from rat heart. *Exp Cell Res*, 98 (1976) 367.

25 Chen WC, HsiehSR, Chiu CH, Hsu BD & Liou YM, Molecular identification for epigallocatechin-3-gallate mediated antioxidant intervention on the H_2O_2- induced oxidative stress in H9c2 rat cardiomyoblasts. *J Biomed Sci*, 21 (2014) 56.

26 Shen T, Yang C, ing L, Zhu Y, Ruan Y, Cheng H, Qin W, Huang X, Zhang H, Man Y, Wang S, Liu D, Bian Y, Xiao C, Zhao Y & Li J, Tbx20 functions as an important regulator of estrogen mediated Cardiomyocyte protection during oxidative stress. *Int J Cardiol*, 168 (2013) 3704.

27 Engupta A, Molkentin JD, Paik JH , DePinho RA & Yutzey KE, FoxO transcription factors promote Cardiomyocyte survival upon induction of oxidative stress. *J Biol Chem*, 286 (2011) 7468.

28 Furtado MB, Costa MW, Pranoto EA, Salimova E, Pinto AR, Lam NT, Park A, Snider P, Chandran A, Harvey RP, Boyd R, Conway SJ, Pearson J, Kaye DM & Rosenthal NA, Cardiogenic genes expressed in cardiac fibroblasts contribute to heart development and repair. *Circ Res*, 114 (2014) 1422.

29 Wu L, Feng J, Wang L, Mu Y, Baker A, Donly KJ, Harris SE, MacDougall M & Chen S, Development and characterisation of a mouse floxed Bmp2 osteoblast cell line that retains osteoblast genotype and phenotype. *Cell Tissue Res*, 343 (3) (2011) 545.

30 Gómez-Sánchez R, Pizarro-Estrella E, Yakhine-Diop SM, Rodríguez-Arribas M, Bravo-San Pedro JM, Fuentes JM, González-Polo RA & Routine Western blot to check autophagic flux: cautions and recommendations. *Anal Biochem*, 477 (2015) 13.

31 Yalcin S, Marinkovic DC, Mungamuri SK, Zhang X, Tong W, Sellers R & Ghaffari S, ROS mediated amplification of Akt/mTOR signaling pathway leas to myeloproliferative syndrome in Foxo3 (-/-) mice. *EMBO J*, 29 (2010) 4118.

32 Kavitha, John F & Indira M, Amelioration of inflammation by phenolic rich methanolic extract of *Ocimum sanctum* Linn. Leaves in isoproterenol induced myocardial infarction. *Indian J Exp Biol*, 53 (2015) 632.

33 Yousefi K, Soray H & Fathiazad F., Cardioprotective effect of methanolic extract of Marrubium valgare L. on isopoterenol induced acute myocardial infarction in rats. *Indian J Exp Biol*, 51 (2013) 653.

34 Ishwarya M, Narendhirakannan RT, Swaraj Sharma & Emilin Renitta, Effect of cardioprotective polyherbal formulation on isoproterenol induced myocardial infarction in experimental animals. *Indian J Exp Biol*, 55 (2017) 357.

35 Lin Z, von Gise A, Zhou P, Gu F, Ma Q, Jing J, Yau AL, Buck JN, Gouin KA, van Gorp PR, Zhou B, Chen J, Seidman JG, Wang DZ & Pu WT, Cardiac specific YAP activation improves cardiac function and survival in an experimental murine MI model. *Circ Res*, 115 (2014) 354.

36 Chaanine AH & Hajjar RJ, Akt signaling in the failing heart. *EUR J Heart Fail*, 13 (8) (2011) 825.

37 Shiojima I, Yefremashvili M, Luo Z, Kureishi Y, Takahashi A, Tao J, Rosenzweig A , Kahn CR, Abel ED & Walsh K, Akt signaling mediates post natal heart growth in response to insulin and nutritional status. *J Biol Chem*, 277 (2002) 37670.

38 Mohammed Abdul KS, Jovanović S, Du Q, Sukhodub A & Jovanović A, Mild hypoxia in vivo regulates cardio-protective SUR2A: A role for Akt and LDH. *Biochim Biophys Acta Mol Bas Dis*, 1852 (2015) 709.

39 Mohammed Abdul KS, Jovanović S, Du Q,Sukhodub A & Jovanović A, Upregulation of cardioprotective SUR2A by sub-hypoxic drop in oxygen. *Biochim Biophys Acta Mol Cell Res*, 1843 (2014) 2424.

40 Mohammed Adbul KS,Jovanović S, Du Q & Jovanović A, Exposure to 15% oxygen *in vivo* up-regulates cardioprotective SUR2A without affecting ERK1/2 and Akt: a crucial role for AMPK. *J Cell Mol Med,* 21 (2017) 1342.

41 Kemi OJ, Ceci M, Wisloff U, Grimaldi S, Gallo P, Smith GL, Condorelli G & Ellingsen O, Activation or inactivation of cardiac Akt/mTOR signaling diverges physiological from pathological hypertrophy. *J Cell Physiol*, 214 (2008) 316.

42 Lu F, Langenbacher A & Chen JN, Tbx20 drives cardiac progenitor formation and cardiomyocyte proliferation in zebrafish. *Dev Bio,* 421 (2017) 139.

43 Kennedy L, Kaltenbrun E, Greco TM, Temple B, Herring LE, Cristea IM & Conlon FL, Formation of a TBX20-CASZ1 protein complex is protective against dilated cardiomyopathy and critical for cardiac homeostasis. *PLoS Genet*, (2017) 13 (2017) e1007011. https://doi.org/10.1371/journal.pgen.1007011.