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E2F6 protein levels modulate drug induced apoptosis in cardiomyocytes

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ABSTRACT

The E2F/Rb pathway regulates cell growth, differentiation, and death. In particular, E2F1 promotes apoptosis in all cells including those of the heart. E2F6, which represses E2F activity, was found to induce dilated cardiomyopathy in the absence of apoptosis in murine post-natal heart. Here we evaluate the anti-apoptotic potential of E2F6 in neonatal cardiomyocytes (NCM) from E2F6-Tg hearts which showed significantly less caspase-3 cleavage, a lower Bax/Bcl2 ratio, and improved cell viability in response to $CoCl_2$ exposure. This correlated with a decrease in the pro-apoptotic E2F3 protein levels. In contrast, no difference in apoptotic markers or cell viability was observed in response to Doxorubicin (Dox) treatment between Wt and Tg-NCM. Dox caused a rapid and dramatic loss of the E2F6 protein in Tg-NCM within 6 h and was undetectable after 12 h. The level of *e2f6* transcript was unchanged in Wt NCM, but was dramatically decreased in Tg cells in response to both Dox and $CoCl_2$. This was related to an impact of the drugs on the α-myosin heavy chain promoter used to drive the E2F6 transgene. By comparison in HeLa, Dox induced apoptosis through upregulation of endogenous E2F1 involving post-transcriptional mechanisms, while E2F6 was down regulated with induction of the Checkpoint kinase-1 and proteasome degradation. These data imply that E2F6 serves to modulate E2F activity and protect cells including cardiomyocytes from apoptosis and improve survival. Strategies to modulate E2F6 levels may be therapeutically useful to mitigate cell death associated disorders.

1. Introduction

The heart has an estimated cardiomyocyte renewal rate of only ~1%, which is a major obstacle in its repair following ischemia or exposure to toxins which induce apoptosis [3]. Instead of cardiomyocyte proliferation after injury, the dead tissue is replaced with collagen secreted by fibroblasts in a process called fibrosis [32]. This causes the heart to stiffen and lose contractile force which leads to a cycle of blood not being expelled from the heart, cardiomyocyte stretching, and cell death. Thus, finding new ways to promote cardiac survival and limit apoptosis are key to treating the diseased heart.

The E2F/pocket protein pathway is a major regulator of apoptosis in all cell types [27]. The E2F family consists of eight transcription factors which regulate the expression of genes which regulate a multitude of fate decisions in the cell [33,12]. E2Fs1-5 are evolutionarily conserved family members which are negatively regulated by binding of the Retinoblastoma protein (Rb) family [33,12]. The induction of apoptosis by E2F1 has been the most extensively studied. E2F1 can induce apoptosis via direct transcriptional activation of pro-apoptotic genes including caspases, *bnip3*, and *p73* [42,15]. It can also trigger p53 dependent apoptosis via binding through its cyclin-A binding domain, or activation of the cyclin dependent kinase inhibitor, ($p14^{ARF}$) which inhibits the E3 ubiquitin ligase (MDM2) thereby stabilizing p53 [25,17,19,18]. Forced expression of E2F1 has been demonstrated to induce apoptosis in neonatal cardiomyocytes and adult myocardium [13,1,14].

E2F6 is a novel member of the E2F family which is capable of regulating E2F dependent transcription independently of Rb [8,16,34]. It has been demonstrated to be anti-apoptotic in HEK cells against UV damage and the hypoxia mimetic, $CoCl_2$, an effect which was partially attributed to its ability to out-compete the pro-apoptotic E2F1 [40,41]. The regulation of E2F6 via microRNAs, including miR-31 and miR-185, were related to the efficacy of the chemotherapeutic induction of apoptosis in prostate and triple negative breast cancers respectively [5,43,30,31]. It was recently discovered that the Epstein Barr Virus

Abbreviations: α-mhc, α-myosin heavy chain; β_2-AR, beta-2-adrenergic receptor; Bax, Bcl2 associated protein X; Bcl2, B-cell lymphoma 2; Blm2, bloom syndrome 2 homolog; Bnip3, Bcl2 interacting protein 3; Chk1, checkpoint kinase 1; c-src, proto-oncogene tyrosine protein kinase; DCM, dilated cardiomyopathy; Dnaja3, heat shock protein hsp40; Dox, Doxorubicin; ERK, extracellular receptor kinase; HEK, human embryonic kidney cell line; HeLa, immortalized human cervical cancer cell line; NCM, Neonatal Cardiomyocytes; MDM2, Mouse double minute homolog 2 (E3 Ubiquitin ligase); $p14^{ARF}$, cyclin dependent kinase inhibitor 2A; p53, tumor suppressor p53; p73, tumor suppressor p73; Rad51, recombination protein A homolog; Rb, Retinoblastoma protein; Tg, Transgenic

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Nuclear antigen 3 (EBNA3), which is necessary for EBV immortalization and the induction of EBV cancers, binds to and stabilizes E2F6 which inhibited the pro-apoptotic E2F1 [26]. These studies suggest that E2F6 is an important player in regulating apoptosis in cancer cells. If E2F6 is indeed anti-apoptotic it may be beneficial in a variety of disease including heart failure, but there is a lack of information evaluating this potential in the heart.

We previously explored the function of the E2F pathway in the postnatal heart via expression of the repressor E2F6 in a transgenic (Tg) mouse [37]. Surprisingly, expression of E2F6 led to E2F dependent gene activation including genes involved in the cell cycle and DNA damage repair. The unexpected gene activation was attributed to E2F6 competing for DNA binding at E2F/Rb sites and the specific down-regulation of E2F3 which critically regulates cardiac development through gene regulation [37,20]. E2F6 induced a dose dependent dilated cardiomyopathy (DCM), without the normally expected pathological hypertrophic or apoptotic responses [37]. We detected the specific stimulation of a β_2-adrenergic survival signaling pathway in E2F6-Tg myocardium which induced the production of Bcl2 protein via the extracellular receptor kinase (ERK) potentially accounting for the lack of apoptosis observed [22]. In the present study we further evaluate the capacity for E2F6 to protect the heart from apoptosis by exposing neonatal cardiomyocytes (NCM) from E2F6-Tg mice to drug induced apoptotic insult.

2. Materials and methods

2.1. Mice and genotyping

All animal work was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011). The protocols were approved by the University of Ottawa's institutional animal care committee: cmm-1725, cmm-1723. All possible steps were taken to ameliorate animal suffering. Mouse pups were euthanized by decapitation. Previously described transgenic (Tg) mice with cardiac specific expression of E2F6 (B6C3F1) under control of the *α-mhc* promoter were bred with WT B6C3F1 [37].

Genotyping was performed via DNA extraction from mouse ear (adult) or tail (pup) clip and PCR using the Phire Tissue Direct (Thermo Scientific) kit as per the manufacturer's instructions. Primers spanning the 6th intron of E2F6 were used (ATCACAGTACATATTAGGAGCAC-sense, and GGTGCGGCTACCAGTCTACA–anti-sense) which result in the amplification of a long fragment (988 bp) in Wt mice and a long and short fragment (342 bp) in E2F6-Tg mice.

2.2. Neonatal cardiomyocyte isolation

Neonatal cardiomyocytes were isolated from Wt and Tg mouse hearts and postnatal day 1 following decapitation. Hearts were rinsed in HBSS and incubated in 0.5% trypsin dissolved in HBSS overnight at 4 °C while genotyping was performed. Hearts were digested in 0.5% Collagenase type II (Gibco) dissolved in HBSS and cells were collected after each digestion via centrifugation at 3000*g* for 3 min and re-suspended in feeding media (DMEM, 16% media-199, 10% horse serum, 5% FBS, 1% penicillin/streptomycin, and 1% non-essential amino acids). Total suspensions were plated on uncoated 10 cm dishes to remove fibroblasts. Cardiomyocytes were seeded (1×10^6/well on 6 well plates for protein analysis or 1×10^5/well on 96 well plate for viability assay) onto 0.1% gelatin coated plates and allowed to attach for 48 h in a 37 °C incubator with 5% CO_2.

2.3. HeLa cell culture and treatments

HeLa were obtained from ATCC and grown in DMEM supplemented with 10% FBS and 1% Pen/Strep. NCM and HeLa were serum starved for 24 h prior to the addition of cobalt chloride (CoCl2) (250 μM–1000 μM) or Doxorubicin (Dox) (0.5 μM–1.0 μM) for 24 h unless otherwise indicated. For proteasome inhibition HeLa were incubated with MG-132 (10 μM) (Sigma) dissolved in DMSO or DMSO alone for 1 h. For cell viability analysis cell titer blue reagent (resazurin-10 μl) was added to each well and cells were incubated at 37 °C with 5% CO_2 for 3 h as per the manufacturer's protocol (Promega). Absorbance at 570 nm and 590 nm were recorded by the Synergy H1 plate-reader (Biotek). Viability experiments were performed in triplicate ($n = 8$).

2.4. RNA analysis

RNA was extracted from HeLa and NCM using the RNEasy kit (Qiagen) and from cardiac lysate using the RNEasy Fibrous Tissue Mini Kit as per the manufacturer's protocol (Qiagen). First-strand cDNA was synthesized from 1 μg RNA and oligoDT with SuperScriptII reverse transcriptase (Invitrogen) as per the manufacturer's protocol. qPCR was performed in the q-Rotor (Qiagen) using Fast Start SYBR Green (Roche). Gene expression was normalized against *18S* rRNA or *gapdh* as indicated, and fold inductions were calculated using the $\Delta\Delta C_t$ method. Primer pairs used for qPCR are:

18S: 5′-CAGTTTCAGAGAGGTCTATTGCAC-3′ (sense) and 5′-GCACTCACATGCCCATACTACATA-3′ (anti-sense),
Chk1: 5′-CGCCACATCAGGTGGTATGT-3′ (sense) and 5′-GGACACGTAGGCTGGGAAAA (anti-sense),
Rad51: 5′- TGTACATTGACACCGAGGGC-3′ (sense) and 5′-CCGCCCTGAGTAGTCTGTTC-3′ (anti-sense),
Blm2: 5′-TAAGCCTGAGTGAGGATCATGGC-3′ (sense) and 5′-TTTGTGGAGTGGAGACTCAGTG-3′ (anti-sense),
E2F6: 5′-AAGGGGCGGAGATGATGACC-3′ (sense) and 5′-GCCCCAAAGTTGTTCAGGTCAGAT -3′ (anti-sense),
E2F1: 5′-AGTCCCTTTGTACCAGTACTCCAG-3′ (sense) and 5′-GAGGGAACAGAACTG TTAGGAAAC-3′ (anti-sense),
E2F3a: 5′-CCGTATCCCTTCATTCATTGTC-3′ (sense) and 5′-CGA ACCCTCTCTCTCTCTTTTCTT-3′ (anti-sense),
E2F3b: ATGCCC ACAGCAGCAGGCAAAGC (sense) and 5′-GAGCTGAATGAACTTCTTGGTGAG-3′ (anti-sense),
human E2F6: 5′-GTATGCAGCCTTGCTGTTGA-3′ (sense) and 5′-AGTCCCTCAAGGAGCTCACA-3′ (anti-sense),
human E2F1: 5′-GGGCTCTAACTGCACTTTCG-3′ (sense) and 5′-AGGGAGTTGGGGTATCAACC-3′ (anti-sense)

2.5. Protein isolation and western blot analysis

HeLa or NCM protein lysates were collected in RIPA (50 mM Tris (pH 7.4), 1 mM EDTA, 150 μM NaCl, 0.25% deoxycholic acid, 1% NP-40) containing protease and phosphatase inhibitors (Roche) and 5 mM sodium butyrate to inhibit histone deacetylases. Lysates were centrifuged at 12800*g* for 10 min at 4 °C and protein concentrations were determined using the BCA assay (Thermo Scientific). Lysates (20–40 μg) were run on gradient (5–15%) SDS-PAGE gels in 3 × loading dye (Cell Signaling). Gels were transferred to PVDF membrane (Millipore) in transfer buffer (25 mM Tris, 190 mM Glycine, 20% methanol) overnight at 4 °C. Membranes were blocked and antibodies were diluted in TBST (1 M Tris, 290 mM NaCl, 0.1% Tween, pH 7.2) containing 5% milk. Following ECL (Roche), band signals were assessed by densitometry using Image Lab Software4.0.1 (Bio-Rad).

The following primary antibodies were used: p53(2524, 1:1000, mouse), acetyl p53 (lys379) (2570, 1:1000, rabbit), caspase 3 (9662,1:1000, rabbit), Chk1(2G1D5) (2360, mouse, 1:1000), p-Chk1(Ser345) (2348,1:1000, rabbit), and cyclin E1 (HE12) (4129, 1:1000, mouse) were purchased from Cell Signaling, Bcl2 (sc-3782, 1:500, mouse) and E2F1 (sc-251, 1:1000, mouse) were purchased from Santa Cruz Biotechnology, α-Tubulin (ab176560, 1:30,000, rabbit) and