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Original article

# Anti-apoptotic function of the E2F transcription factor 4 (E2F4)/p130, a member of retinoblastoma gene family in cardiac myocytes☆

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ABSTRACT

The E2F4–p130 transcriptional repressor complex is a cell-cycle inhibitor in mitotic cells. However, the role of E2F4/p130 in differentiated cells is largely unknown. We investigated the role of E2F4/p130 in the regulation of apoptosis in postmitotic cardiomyocytes. Here we demonstrate that E2F4 can inhibit hypoxia-induced cell death in isolated ventricular cardiomyocytes. As analyzed by chromatin immunoprecipitation, the E2F4–p130-repressor directly blocks transcription of essential apoptosis-related genes, E2F1, Apaf-1, and p73α through recruitment of histone deacetylase 1 (HDAC1). In contrast, diminution of the E2F4–p130–HDAC1-repressor and recruitment of E2F1 and histone acetylase activity to these E2F-regulated promoters is required for the execution of cell death. Expression of kinase-dead HDAC1.H141A or HDAC-binding deficient p130ΔHDAC1 abolishes the antiapoptotic effect of E2F4. Moreover, histological examination of $E2F4^{-/-}$ hearts revealed a markedly enhanced degree of cardiomyocyte apoptosis. Taken together, our genetic and biochemical data delineate an essential negative function of E2F4 in cardiac myocyte apoptosis.



## 1. Introduction

The E2f family of transcription factors plays an important role in the regulation of several intracellular processes such as proliferation, apoptosis, and differentiation. E2f family is composed of five family members (E2fs 1–5). Based on their physiological activity, E2fs 1–3 are transcription activators while E2fs 4–5 act primarily as gene repressors [1]. E2F4 is a major E2F-family member in cardiomyocytes and other differentiated and quiescent cells (collectively referred as G0 cells). E2F4 is regulated by the retinoblastoma (pRb)-related pocket protein p130 [2]. In contrast, the "activating" E2Fs (E2F1, E2F2, E2F3a) are regulated by pRb in proliferating cells [1]. The activating E2Fs induce S phase in serum starved primary fibroblasts whereas E2F4 does not [3]. Moreover, inhibitory E2F4/p130 complexes repress E2F-regulated promoters [4]. Since the activator E2Fs are not expressed in G0 they cannot account for the negative control of E2F-regulated promoters. Therefore, the E2F4/p130 transcriptional repressor complex clearly distinguishes G0 cells from G1 cells. This view is important, since the heart consists of G0 cardiac myocytes, which are differentiated, post-mitotic, and non-dividing [5–8].

In cells which has the proliferative capacity during transition from G0 to G1, E2F4 protein levels remain constant whereas p130 protein becomes phosphorylated and subsequently degraded in a proteosomal dependent manner in early G1 [2]. Furthermore, phosphorylated p130 releases E2F4 and this inactivation of the E2F4-repressor is associated with the loss of E2F4–DNA binding activity and relief from E2F4-mediated gene repression [2]. In this simplified model, promoter repression in G0 depends on E2F4/p130 but by late G1 these proteins are replaced by activating E2Fs. Notably, in G0 cells pRb does not bind to E2F-sensitive promoters (*e.g.* p107, E2F1, cdc25A, cdc6, B-myb, cyclin A, cdc2). Thus, pRb is not involved in the repression of E2F-dependent promoters in cardiomyocytes. E2F4 is held in the nucleus of G0 cells through physical association with p130 and is translocated to the cytoplasm by the nuclear export receptor CRM1 due to its nuclear export signal (NES). E2F4 lacks nuclear localization signal (NLS). Thus, the nuclear import of E2F4 is mediated by association with p130 and is not an intrinsic function of E2F4 [9]. In contrast, the activating E2Fs are constitutively localized in the nucleus independently of their association with pRb [10]. Taken together, the divergent physiological effects of E2F4 and the activating E2Fs are simply explained by their different intracellular localization and association with pocket proteins [11]. Moreover, p130 also recruits histone deacetylase 1 (HDAC1) to promoters and HDAC1 can repress gene expression by altering chromatin structure, since decreased acetylation of histone H3 and H4 residues is associated with transcriptionally inactive chromatin [12,13]. p130, but not pRb is strictly required for HDAC1-binding to E2F-responsive promoters [14]. HDAC1-activity is responsible for the underacetylated state of histones at E2F-regulated promoters and thus transcriptional repression during G0 [13]. An attractive model suggests that activating E2Fs displace E2F4/p130/HDAC1 complex from promoters. In turn, these activator

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E2Fs might recruit histone acetyltransferase (HAT) activity to promoters, enabling gene expression through relaxation of nucleosomal chromatin structure.

E2F4 was shown to be abundantly expressed in embryonic mouse cardiac ventricle and its expression decline during development and shown to be involved in mitosis [15]. Previous report shows control of pro-apoptotic gene expression in cardiomyocytes by E2F4 [16]. In neurons and other cells E2F4 plays an important role in the regulation of apoptosis [17,18]. In the heart E2F4's role is not well characterized. Therefore, to further our understanding about E2F4, we have investigated its role in the regulation of apoptosis in isolated cardiomyocytes and E2F4$^{-/-}$ heart. Here we show that E2F4/p130 suppresses pro-apoptotic gene expression in cardiomyocyte and removal of this inhibition is required for cardiomyocyte apoptosis.

## 2. Material and methods

### 2.1. Cardiomyocyte culture and E2F4 knockouts

Fisher (F344) inbred rats weighing 180–200 g were purchased from Charles River Laboratories (St. Laurent, Que.) and E2F4 knockout mice were obtained from J. Nevins (Duke University Medical Center, Durham, NC, USA) and bred as described [19]. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The local Institutional Animal Care Committee at UHN, Toronto, Ontario, ensured compliance under protocols (2011 and 1415) with the abovementioned guidelines. Rat neonatal pups and E2F4 knockout mice were anesthetized by inhalation of Isoflurane (5%) in oxygen by draw over circulation system and then their spine was cervically dislocated. Adequacy of anesthesia was ensured by inspection of respiratory rate and pattern, color of mucous membranes, corneal reflex and reactivity on toe pinch. For isolation of ventricular cardiomyocytes from 3-day postnatal Wistar rats, the hearts were dissected, minced, enzymatically digested (collagenase II, 0.5 mg/ml, Invitrogen; pancreatin 1 mg/ml, Sigma) to selectively enrich for cardiomyocytes. The resultant cell suspension ($4\times10^6$ cells) was plated onto 10 cm-collagen I (Gibco) coated dishes in culture medium DMEM/F12 containing 3 mM Na-pyruvate, 2 mM glutamine, standard antibiotics (GIBCO), 0.2% (v/v) BSA, 0.1 mM ascorbic acid, and 0.5% (v/v) insulin–transferrin–selenium (Sigma). After preplating (to eliminate adherent non-cardiomyocyte cells), myocytes were held for 36 h in the presence of 25 mM araC (inhibits proliferating non-cardiomyocyte cells) and 5% horse serum (Sigma). Cardiomyocytes were subjected to hypoxia in serum-free culture medium without BSA (hypoxia chamber; 5% $CO_2$, 95% $N_2$ in a humidified atmosphere; Labotect, Model 3015). Recombinant adenoviruses were generated with the pAdeasy vector system as per the manufacturer's instructions (Stratagene). Cardiomyocytes were transduced (100 plaque-forming units [pfu]/cell or 50 pfu/cell for double-transfections) after preplating and 36 h later exposed to hypoxic culture conditions for 24 h or other time points as mentioned.

### 2.2. Cellular extracts and cell fractionation

NP40-buffer was used for whole cellular extracts: 50 mM Tris–HCl pH 7.5, 250 mM NaCl, 0.5% NP-40, 5 mM EDTA pH 8.0, 1 mM DTT, protease inhibitor cocktail (Roche), and phosphatase inhibitors (1 mM $Na_3VO_4$, 20 mM NaF, 10 mM β-glycerophosphate, and 1 mM $NaP_2O_7$). For subcellular cell fractions, $2.5\times10^6$ cardiomyocytes in 10 cm-dish were trypsinized and lysed in 500 μl harvest buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 500 mM sucrose, 0.1 mM EDTA, 1 mM DTT, and protease inhibitors). After centrifugation, the supernatant was designated as cytoplasmic extract. The remaining pellet was washed in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, protease inhibitors) and nuclei were lysed using 500 μl buffer C (10 mM HEPES pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, 1 mM DTT, protease inhibitors). After centrifugation ($14{,}000\times g$; 1 h; 4 °C) the supernatant was designated as nuclear fraction.

### 2.3. Reporter gene assays and chromatin immunoprecipitations

E2F1-Luc (wt) and E2F1-Luc (mt) were from J. Nevins (Duke University Medical Center, Durham, NC, USA). Luciferase activity was determined as described [20]. At 12 h after induction of hypoxia, chromatin immunoprecipitations ($4\times10^7$ cells/reaction) were done as described with minor modifications [21]. To amplify E2F-responsive promoter regions, the following primer sets were used: Rat E2F1 (GenBank XM_230765), 5′-GCCTTCGCCAGACCCCGCCACCCA-3′; 5′-CGCCGCGGCCTGCCGTCATGG-3′; and β-MHC, 5′-CAAGGAGCTACCTACCAGACAG-3′, 5′-GGCTCCAGGTCTCAGGGC-3′. PCR products were separated on 8% polyacrylamide gels. Radioactive label was analyzed in a PhosphorImager using TINA software.

### 2.4. Blotting applications, immunofluorescence, RT-PCR, and apoptosis assays

Immunoprecipitation, western- and northern blotting, immunofluorescence microscopy, RT-PCR, and apoptosis-detection were done as described in our previous publications [20,22]. p130 (sc-317), actin (sc-7210), lamin (sc-20681), p16INK4 (sc-156), HDAC1 (sc-7872), HDAC5 (sc-11419), and HDAC6 (H-300 also recognizing HDAC9) antibodies were from Santa Cruz. Acetylated histone H3 (06–599) and acetylated histone H4 (06–598) antibodies were from Upstate. E2F3 (Ab-4), E2F4 (Ab-4), cdk4 (Ab-5), and cdk6 (Ab-3) antibodies were from Thermo Scientific. E2F1 (KH129) antibody was from Neomarkers, cdk2 (610145) antibody was from Pharmingen.

### 2.5. Functional two-dimensional M-mode echocardiography

Experimental animals were randomly allotted into study groups, anesthetized and assessed by echocardiography as follows: Left Ventricle End Diastolic Distance (LVEDD), Left Ventricle End Systolic Diastolic Distance (LVESD), and Fractional Shortening (FS). All animals were pre-anesthetized in the induction chamber with mixture of oxygen and 3% of Isoflurane, and then transferred on the heating pad, secured to the pad in a left recumbent position and rectal temperature was maintained at 37.0 +/− 0.5 °C. Ultrasound system Vivid 7 system (GE Mississauga, ON) equipped with an il3L linear probe operated at 14 MHz was used, and animals imaged with 2% Isoflurane anesthesia at a room temperature. The heart was imaged in the 2-D mode in the parasternal long- and short-axis views with a depth setting of 1.0 cm and at a frame rate of 275 frames/s. M-mode echocardiography was performed by using a parasternal short-axis view at the level of the papillary muscles; images were obtained at a sweep speed of 200 mm/s. Measurements provided were done from leading edge to leading edge according to the American Society of Echocardiography guidelines (1). Wall thickness and LV dimensions were obtained from a short-axis view at the level of the papillary muscles at a frame rate of 260 Hz. FS was calculated according to the formulas $FS = [(LVEDD - LVEDS)/LVEDD]\times100$.

### 2.6. Statistical analyses

Factorial design analysis of variance (ANOVA) or τ-tests were used to analyze data as appropriate. Significant ANOVA values were followed by simple main effect analyses or *post hoc* comparisons of individual mean using the Tukey's method were appropriate. The level of significance was less than 0.05.