

# ROLE OF THE TRANSCRIPTIONAL FACTOR E2F1 IN CXCR4-MEDIATED NEUROTOXICITY AND HIV NEUROPATHOLOGY

Saori Shimizu, Muhammad Z. Khan and Olimpia Meucci

Department of Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, PA 19102, USA

## INTRODUCTION

Alterations of the Rb/E2F1 pathway have been involved in neurodegenerative disorders including NeuroAIDS (1, 2). In order to test the hypothesis that an up-regulation of E2F1 activity is involved in HIV neuropathogenesis, we have been studying E2F1 expression and transcriptional activity in primary neurons exposed to HIV-gp120 and in human brain tissue samples from HIV+ patients with (HIVHAD) or without neurological deficits (HV). Our data show that gp120 induces a time-dependent increase of the E2F1 protein levels in rat primary neurons. In these neurons, E2F1 is predominantly (if not exclusively) localized to the nucleus, and gene reporter assays indicate that gp120 promotes transcription of E2F1-dependent genes. In line with these observations, treatment of neurons with gp120 also increases E2F1 transcriptional targets, namely *cdc2* and *Puma*. Importantly, primary neurons derived from E2F1 deficient mice were resistant to gp120-induced neurotoxicity, and showed no changes in *Cdc2* and *Puma* protein levels following gp120 treatment. Finally, the studies with brain tissue samples showed increased expression of E2F1 in the nucleus of neurons of HAD patients, but not in the HIV+ patients without dementia or in control (i.e. HIV negative) patients. These data suggest that aberrant expression of E2F1 transcriptional targets in post-infectious neurons is one of the mechanisms involved in the apoptotic neuronal cell death observed in HIV neuropathology.

## METHODS

**Immunohistochemistry:** Sections from frontal cortex of human brain were de-paraffinized and re-hydrated, and endogenous peroxidase was quenched. Antigen retrieval was performed at 95°C for 40 min. Sections were incubated with anti-E2F1 antibody (Santa Cruz, #381, 1:50) and HIV-1 gp120 as secondary antibody (Jackson ImmunoResearch, 1:200), detected with Vectastain (Vector Laboratories). For E2F1 applications, sections were incubated using TSA Protein (Dako). For double staining with neuronal cytoplasmic marker MAP2, sections were incubated with MAP2 antibody (1:1000 Chemicon), followed by anti-mouse secondary antibody (1:200), detected with DAB (Vector Laboratories). E2F1 (+) neurons were counted in 10 fields of each patient by MAP2-label control neurons.

**Neuronal cultures:** Mouse primary cortical neurons were cultured in the presence of a glial feeder layer as previously described (2,3). Cells were treated with gp120 (200 μM, 24h) on the 7th day in vitro (DIV). When indicated, CXCR4 antagonist AMD (100 ng/ml) was added to the medium 15 min before gp120.

**SH-SY5Y:** Human neuroblastoma cells were obtained from ATCC and maintained in MEMEM 12 medium+10% FBS. Cell differentiation was induced by the addition of retinoic acid (10 μM) to the culture medium (3 days before experiment).

**Western Blots:** After treatment, cells were washed with cold balanced salt solution, scraped in lysis buffer and processed for SDS-PAGE (2). Primary antibodies were used at 1:1000 (E2F1 K9-20, Sigma), 1:1000 (Puma, Cell Signaling), 1:500 (Cdc2, Santa Cruz), 1:500 (CXCR4, Santa Cruz) followed by appropriate secondary antibodies, and detected using SuperSignal West Pico Plus kit (Pierce).

**Immunocytochemistry:** Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X, and incubated with primary antibodies: E2F1 (1:50, Sigma), and cleaved caspase-3 (1:50, Cell signaling) overnight, followed by appropriate secondary antibodies and nuclear counterstaining (Dapi 33342, Molecular Probes). Staining was observed using an epifluorescence microscope (Olympus IX-70) connected to a CCD camera (Mossam), and images were acquired and analyzed by the software Metamorph (Universal Imaging) (5).

**Survival assays:** Neuronal survival was analyzed by Hoechst 33342 and cleaved caspase 3 staining (Cell signaling).

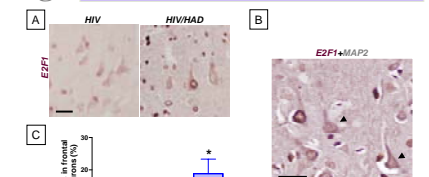
**Luciferase Assay:** Rat cortical neurons were transfected with a luciferase expression vector containing the E2F1 promoter along with the pEGFP using Lipofectamine 2000 (Invitrogen). An E2F1 expression plasmid was used as a positive control. The Single-Glo™ Promoter was used for dual luciferase expression assay after transfection. All luciferase measurements were normalized using the expression of GFP.

**EMSA:** Nuclear extracts from differentiated SH-SY5Y cells were incubated with 3<sup>32</sup>P-labeled oligonucleotides containing a consensus binding site for E2F1. After incubation, samples were bound on a 4% polyacrylamide non-denaturing gel, and transferred to the membrane. DNA was cross-linked to the membrane by UV light, and DNA-protein complexes were detected by chemiluminescence (Pierce).

**Genotyping:** DNA was extracted from the cerebellum of each animal by phenol/chloroform method. PCR was performed with the primers as previously described (6), and the products were run on 3% agarose gel.

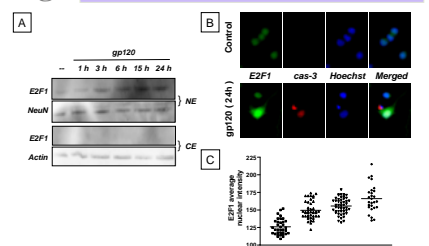
## RESULTS

### Fig.1 Up-regulation of E2F1 in the nucleus of cortical neurons in the brain of HIV/HAD patients.



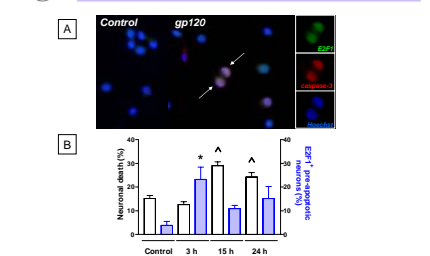
In the frontal cortex of HIV/HAD patients, E2F1 (dark red staining) is expressed in the nucleus of cortical neurons (labeled with MAP2). This staining pattern was not observed in HIV patients without dementia nor in control patients (C, ANOVA,  $p < 0.05$  vs Control and HIV). Arrowhead represents E2F1 negative neurons. A: E2F1 single staining. B: E2F1 and MAP2 double-staining. C: Graph bars represent percentage of E2F1 (+) cells. Scale bars: 5.0 μm.

### Fig.2 HIV gp120 treatment increases E2F1 nuclear level in rat cortical neurons



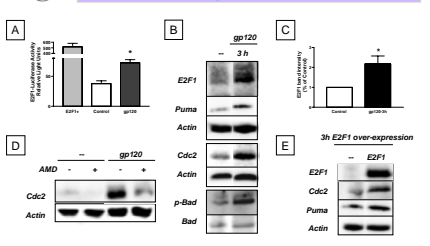
E2F1 is up-regulated in the nucleus of rat cortical neurons treated with gp120<sub>120</sub> (200 μM) for 3 h, and continues to be over-expressed at 24 h. Fluorescence intensity of E2F1 nuclear staining after gp120 treatment increased in time dependent manner (B). Each scatter plot shows intensity level in individual neurons from two or more random fields at each time point (C).

### Fig.3 E2F1 up-regulation is an early event in gp120-induced cell death.



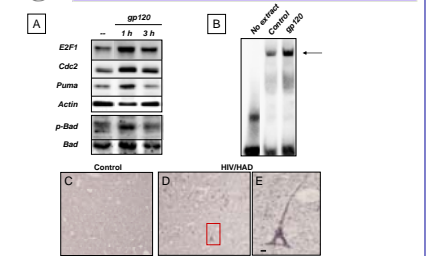
E2F1 (+) (caspase-3 (-) neurons showing healthy morphology (A, arrows) were observed at 3h (blue bars in graph B, ANOVA,  $p < 0.001$  vs control), while apoptotic cells (defined by condensed nuclei and cleaved caspase-3 positive) were found at later time points (white bars in graph B, ANOVA,  $p < 0.001$  vs Control).

### Fig.4 HIV gp120 increases transcriptional activity and levels of E2F1 targets in rat cortical neurons.



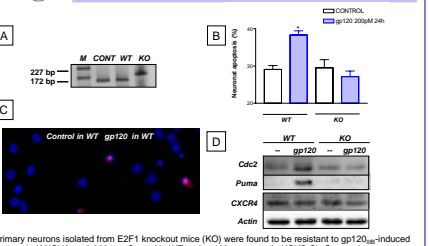
A. gp120 increased transcriptional activity in rat primary neurons (1-hst,  $p < 0.01$  vs Control). The bar of E2F1+ represents cells transfected E2F1 plasmid with expression vector. B-E: Levels of E2F1 downstream targets were increased after gp120<sub>120</sub> treatment (B/D) and E2F1 over-expression (E) in rat cortical neurons, and blocked by pre-treatment with the CXCR4 antagonist AMD3100 (D). C: Quantification of E2F1 level at 3 h after gp120<sub>120</sub> treatment (n=3, Hist,  $p < 0.05$  vs Control).

### Fig.5 Effect of HIV gp120 on E2F1-dependent proteins in human neurons.



A: Levels of E2F1 downstream targets were increased after gp120 treatment in human neuroblastoma cells (SH-SY5Y). B: Binding of nuclear proteins from differentiated SH-SY5Y to a specific E2F1 consensus sequence was increased by gp120<sub>120</sub> treatment (200 μM, 24 h) as assessed by EMSA. C-E: Over-expression of Cdc2 was observed in the cytoplasm of the frontal cortical neurons in HIV/HAD patient. Scale bars: 5.0 μm.

### Fig.6 E2F1 is necessary for gp120-induced apoptotic cell death.



Primary neurons isolated from E2F1 knockout mice (KO) were found to be resistant to gp120<sub>120</sub>-induced apoptosis (ANOVA,  $p < 0.0001$  vs Control in WT and gp120 treatment in KO)(B,C). Genotyping was performed to confirm the absence of E2F1 in the KO (A). E2F1 downstream targets, Cdc2 and Puma, were up-regulated after gp120 treatment in wild type (WT) mice, whereas no changes of these proteins were observed in the KO mice under the same experimental conditions (D). No difference of CXCR4 expression levels were observed between WT and KO mice, suggesting that gp120-induced alteration of E2F1 targets is not due to receptor difference (D).

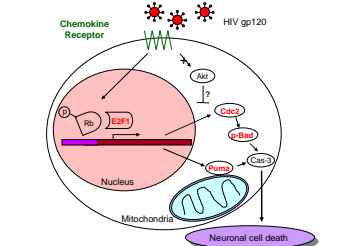
## SUMMARY AND CONCLUSIONS

The findings presented here indicate that:

- E2F1 is over-expressed in the nucleus of cortical neurons under pathological conditions in vivo and in vitro.
- The HIV gp120 increases levels of direct E2F1 pro-apoptotic targets in primary neurons as well as neuronal cell lines. E2F1 targets, Cdc2 and Puma (7), are also over-expressed in HIV/HAD patients.
- E2F1 is necessary for gp120 induced neurotoxicity.

Our observations support the hypothesis that inappropriate activation of the Rb/E2F1 pathways may be involved in the neuronal injury and dysfunction leading to the clinical manifestations of NeuroAIDS.

## Model illustrating involvement of E2F1 in neuropathogenesis of HIV



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