

## Analysis of expression levels of E2F transcription factors in lineage negative hematopoietic stem cells of young and old mice

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### Abstract

**Background and aims:** The E2F family of transcription factors is encoded by at least eight genes, E2F1-8. These proteins are important targets of the retinoblastoma protein (RB) contributed in regulation of transcription, cell cycle and apoptosis. The aim of this study was to investigate the expression levels of E2F protein family including E2F1, E2F2, E2F7, E2F8 and RB1 in the lineage negative (Lin<sup>-</sup>) hematopoietic stem cells (HSCs) of young and aged black mice using Real Time RT-PCR and western blot techniques.

**Methods:** Lin<sup>-</sup> HSCs of 4 young (7-10 weeks old) and 3 aged (76 weeks old) mice have been isolated from their bone marrow cells using MACS column and after RNA extraction of culturing cells and cDNA preparation, samples were then analyzed by Real Time RT-PCR and western blot techniques.

**Results:** The E2F7 and E2F8 expression levels of the Lin<sup>-</sup> HSCs of old mice were only the transcriptional factors significantly decreased when compared with young mice.

**In conclusion:** It seems the functional roles of important E2F7 and E2F8 transcription factors in moderating potentially destructive activity of E2F1 and regulation of cell cycle have been diminished in Lin<sup>-</sup> HSCs of aged mice. Hence, the apoptotic activity of the E2F1 would affect to the most HSCs, reinforcing bone marrow to proliferate the HSCs in old mice.

However, Real Time RT-PCR data showed that the expression level of the E2F1 in those cells was not increased significantly as expected. This is the first report in this regard and further investigation with more samples need to reconfirm these data.

**Key words:** E2Fs; lineage negative; HSCs; Expression levels

### Introduction

The E2F family proteins as the important transcription factors in regulating cell cycle progression can be divided into two groups: traditional ones including E2F1, E2F2, and E2F3a which are potent activators of transcription, while E2F3b, E2F4, E2F5, E2F6, E2F7 and E2F8 are thought to be repressors for gene expressions

( Muller et al., 2004; DeGregori et al. 2006; Dimova et al., 2005; Muller et al., 2001 ). These genes are expressed at low levels during quiescence and are induced whenever a series of activations push the cell from a G<sub>0</sub> to a G<sub>1</sub> state and enters the cell cycle (Iaquinta et al., 2007). Meanwhile, retinoblastoma gene (RB1) which is a key regulator in cell-cycle entry (transition G<sub>1</sub>/S) sequesters E2Fs for transcription of many genes involved in cell-cycle regulation, DNA replication and other functions like as activation of the apoptotic pathway ( Calzone et al., 2008). Each of the E2F proteins has also additional functions,

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including effects on the cell differentiation and cell survival or apoptosis, such as E2F1 has been known to be a potent stimulant of apoptosis (Sahin et al., 2010). However, two novel E2F family members, E2F7 and E2F8, provide a very important tool to control of the excessive E2F1 activation ( Li et al., 2008; Moon et al., 2008; Lammens et al., 2009; De Bruin et al., 2003; Di Stefano et al., 2003). The ability of the E2F proteins to proliferate or kill the cells implies that E2F-dependent transcription needs to be carefully controlled ( Moon et al., 2008). Although the role of traditional E2F such as E2F1 and E2F2 have been extensively described and well established but the mechanism of functions of novel E2Fs such as E2F7 and E2F8 in repression or activation of cell cycle progression is unclear ( Di Stefano et al., 2003; Maiti et al., 2005; Christensen et al., 2005). Transcription factors also play a major role in all stages of hematopoiesis, from commitment to the hematopoietic lineage, to emergence and maturation of hematopoietic stem cells (HSCs) as well as the lineage choice ( Dykstra et al., 2008). HSCs demonstrate a high level of cell cycle activity during fetal life, but these cells reside in the bone marrow (BM) and enter a mainly quiescent ( $G_0$ ) state to maintain blood homeostasis during adult life. It has been reported that all that activities of HSCs restricted to the fraction of actively cycling lineage-negative ( $Lin^-$ ) BM cells remain in only 3 week-old weanling mice and then HSCs rapidly switch to a quiescent state by 4 weeks of age, with only about 5% of total HSCs actively in the cell cycle during adult life (Bowie et al., 2006; Pietras et al., 2011; Morrison et al., 1996). The present investigation was focused on the active fraction of HSCs and expression levels of the classical E2F1, E2F2 and novel E2F7, E2F8 together with RB1 were assessed in the  $Lin^-$  HSCs of young and old mice as a model to address some of the aforementioned challenges.

## Materials and methods

### Animals

C57 black male mice (C57BL/6) were used in this study. Four young (7-10 weeks old) and three aged (76 weeks old) mice were maintained on a standard laboratory diet and

housed at the controlled temperature (23°C) with daily exposure to a 12:12 hour light: dark cycle. All animal experiments were approved by the Animal Care Committee, Institute of Molecular Medicine and Max-Planck Research group on stem cell aging, University of Ulm, Germany

### Isolation of bone marrow cells

Femurs and tibiae were removed from the black mice and whole BM cells were obtained by flushing the marrow cavities using phosphate-buffered saline (PBS) with an Insulin syringe-needle. The cells were washed twice with PBS and 2% fetal bovine serum (FBS), filtered through a coarse pre-separation filter and centrifuged at 1200 g for 5 min at 4° C.

### Isolation of lineage negative progenitor HSCs

$Lin^-$  HSCs were separated from whole BM cells using MACS (Magnetic Activated Cell Sorting, Miltenyi Biotec GMBH, Germany) system. Pellets of aforementioned cells were resuspend with PBS and 2% FBS and after incubation with MACS microbeads, the cell suspension is loaded onto the LD column of MACS system. Briefly, resuspend whole BM cells were incubated for 30 min on ice with a cocktail of biotinylated antibodies including rat anti-mouse CD3, CD4, CD8, CD11b, B220, Mac-1, Gr-1, TER-119 and FACS buffer and after washings to remove excess antibodies, the cells were incubated with magnetized microbeads that bind and eliminate the antigen-bound antibodies. Unbound  $Lin^-$  HSCs were purified by magnetic separation using LD column of MACS system. The number of  $Lin^-$  HSCs was counted on the automated cell-viability analyzer (Beckman Coulter, Inc. USA).

### Total RNA isolation, Reverse transcription polymerase chain reaction (RT-PCR) and Real Time RT-PCR

Total RNA was isolated from the  $Lin^-$  HSCs of young and old mice using Ambion kit (Invitrogen) following the manufacturer's instructions. Each sample of isolated total RNA was further treated with DNase I enzyme (Invitrogen). The yield and

quality of extracted total RNA were assessed by optical density at 260nm and denaturing agarose gel electrophoresis. Reverse transcription polymerase chain reaction (RT-PCR) was performed using the Script® VILO™ (Invitrogen), RevertAaid™ First Strand cDNA Synthesis kit according to the manufacturer's instructions. Standard curves were performed using cDNA to determine the linear range and PCR efficiency of each primer pair. Reactions were done in duplicate, and relative amounts of

cDNA were normalized to GAPDH. Quantitative real-time RT-PCR (qRT-PCR) was performed on the samples with an ABI (Applied Biosystems, Weiterstadt, Germany) apparatus, 7300 Real-Time PCR system, using SYBR Green PCR Master Mix (with ROX, passive reference dye, Applied Biosystems) detection method. All reactions were done in triplicate and relative amounts of cDNA were normalized to GAPDH as an internal control gene. The qRT-PCR primers have been shown in Table 1.

**Table 1** Sequence of primers used in Real-Time RT-PCR.

Gene		Sequence of primers	Product size (bp)	Annealing temperature
Mouse E2F1 ( mE2F1) NM_007891.4	Forward	5'- CTGGAGCAAGAAGCAGTATT-3'	193	53°
	Reverse	5'- CCTCGAGACCAAAGTGATAG-3'		
mE2F2 NM_177733.6	Forward	5'- CGTCGTCTCTAAAGTTCACC-3'	175	53°
	Reverse	5'- TGGTAGAGACCAAGGACATC-3'		
mE2F7 NM_178609.4	Forward	5'- TACACACTGCTTGCTTTGAC-3'	185	53°
	Reverse	5'-TGGTCAATTCACAAACAGAA-3'		
mE2F8 NM_001013368.5	Forward	5'- CACAAGTCCTTCCTTCTG -3'	250	53°
	Reverse	5'- CCATTTTGTCAAATTCCTCAT -3'		
mRb1 NM_009029.2	Forward	5'-AGAAGAAGAACGTTGTCCA-3'	236	53°
	Reverse	5'-AGTTGGCTTCCACTTTGATA -3'		
Mouse GAPDH NM_008084.2	Forward	5'- GTCGTGGAGTCTACTGGTGT-3'	163	53°
	Reverse	5'- TGCTGACAATCTTGAGTGAG-3'		

### Protein isolation and Western blot

Cell culture media were suctioned off and the cell surfaces washed three times, 5 min each with cold PBS. Protein extraction of the Lin<sup>-</sup> HSCs was performed on ice with lysis buffer and cocktail protease inhibitors (Fermentas, Maryland, USA) were added immediately to the samples and protein concentration was assessed by Bradford assay. Equivalent amounts of each cell lysate and the proportional amount of serum-free conditioned medium protein were diluted in the 4X sample buffer, boiled at 95°C for 10 min and then subjected to SDS-PAGE using two separate gradient gels running from 4 to 12% gel. After electrophoresis, one of the gels was visualized by silver stain method. Another gel was transferred onto the nitrocellulose membrane (Millipore, Billerica, MA, USA) by semi-dry method (BioRad, GmbH, Munich) and then, the

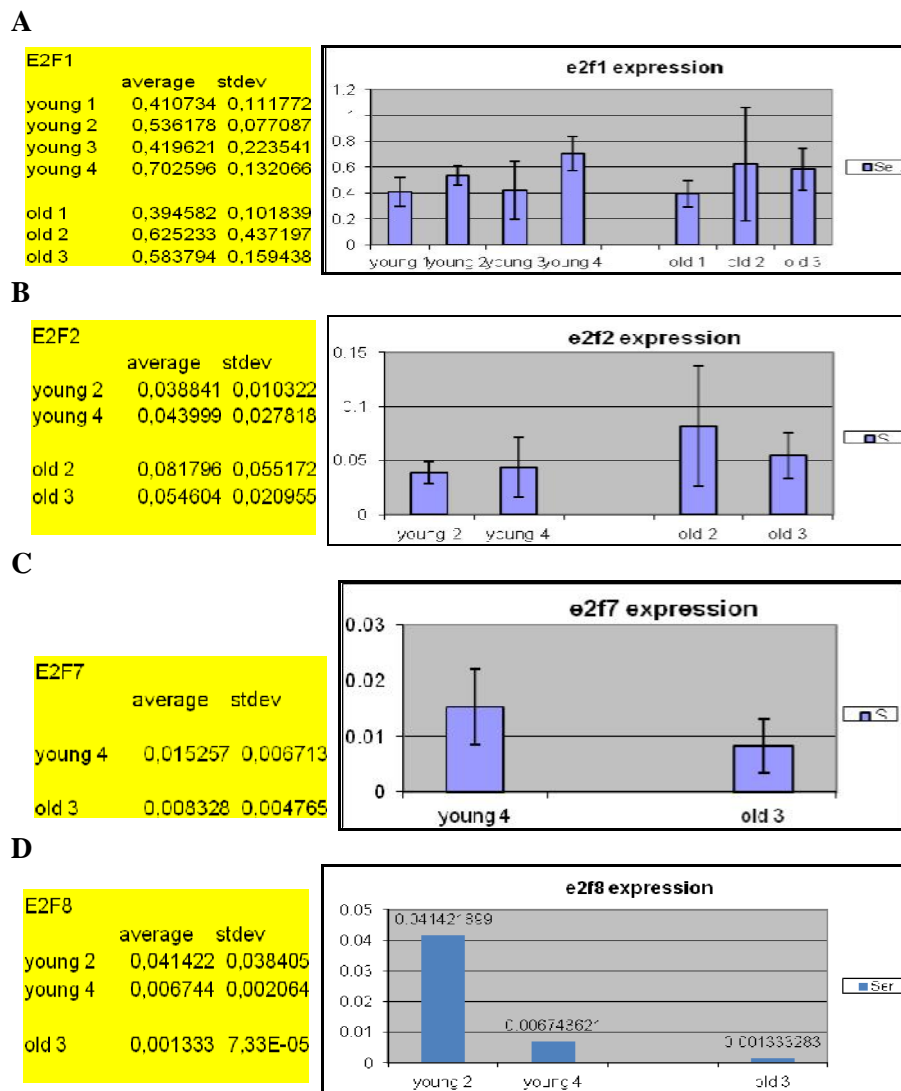
membrane was blocked for one hour at 37°C in Tris-Buffered Saline-Tween 20 (TBST) containing 5% blocking solution (Amersham, Buckinghamshire, UK). The membrane was then incubated for one hour at room temperature in the TBST containing 5% blocking solution and 1:500 dilution of mouse monoclonal anti-E2Fs as a primary antibody (Santa Cruz Biotechnology, Santa Cruz, Texas, USA) and then washed three times, 15 min each with TBST. It was then incubated for one hour in TBST containing 5% blocking solution and 1:10,000 dilution of horse radish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz) and after washing for three times, 15 min each with TBST; the membrane was then immersed in a mixture of equal volume of ECL (Enhanced Chemiluminescence) detection solutions A and B (Amersham) and exposed to X-ray film in a dark

room. The exposed films were developed by specific solutions and then digitally photographed.

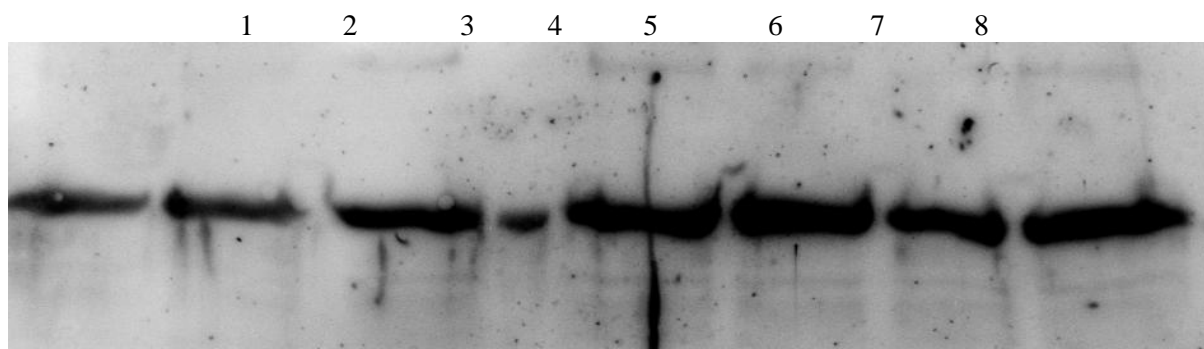
**Results**

Performing automated cell counting of the Lin<sup>-</sup> HSCs showed that we could get the enough viable cells for further study ( $4.2 \times 10^6$  cells /ml). After culturing the aforementioned cells, RNA extraction, cDNA preparation, Real Time RT-PCR and western blot techniques

have done on these samples. Data from the Real-time RT-PCR showed that only E2F7 and E2F8 expression levels of the Lin<sup>-</sup> HSCs of old mice have been significantly reduced compared with the young mice (Figure 1). These data have reconfirmed using western blot technique. Figure 2 shows the results for recognition of the E2F7 transcription factor of the young and old mice Lin<sup>-</sup> HSCs samples using western blot technique.



**Figure 1** Results of Real-Time RT-PCR on the expression levels of E2F1, E2F2, E2f7 and E2F8 in Lin<sup>-</sup> HSCs of young and old mice.



**Figure 2** Western blot analysis for detection of E2F7 protein in young and old Lin<sup>-</sup> HSCs of mice samples. Lanes 1-3 are related to the Lin<sup>-</sup> HSCs of old mice, lane 4 is also the same sample of the lane 3 which was leaked to this lane and lanes 5-8 are belonged to the Lin<sup>-</sup> HSCs of young mice.

### Discussion

There are several studies on the HSCs aging indicate that the hematopoietic system undergoes considerable changes with increasing age suggesting that self-renewal is not complete. The well-documented data of the aged hematopoietic system in mice indicate that the relatively increasing of HSCs accompanied by a loss of functional activity. Significant proportion of HSCs is indeed taken into cell cycle in old mice and the importance of this event is not yet known ( Dykstra et al., 2008; Bowie et al., 2006; Pietras et al., 2011; - Morrison et al., 1996; Sudo et al., 2000). In this regard, the roles of the E2F transcription factors should be more considered as a coordinated switch from the repressive to the activating E2Fs in every cell cycle that enables the simultaneous activation of genes involved in DNA replication and cell cycle progression. Information provided in this study on the active fragment of Lin<sup>-</sup> HSCs indicate that the expression levels of E2F7 and E2F8, which are important to moderate the excessive activity of the E2F1, are significantly reduced in old mice. Decreasing E2f7 and E2F8 expression levels at protein levels in the old mice implies that probably important regulatory factors of the cycle cell could not perform their roles in old mice and apoptotic activity of the E2F1 would cause to kill most HSCs, reinforcing bone marrow to proliferate the HSCs in old mice. In this regard, Li et al. (2008) showed

that E2F7 and E2F8 cooperate to repress E2F1 expression by using mouse-knockout technologies. This repression by E2F7/E2F8 is important for the regulation of E2F1 during S and G2 and for the suppression of E2F1-induced apoptosis. However, in our study, Real-time RT-PCR data indicated that there are no differences in the expression levels of E2F1 between young and old mice as expected. Altogether, these data indicate that the two novel and important E2Fs may have critical roles in self renewal of the HSCs in old mice and for more accurate elucidation of this effectiveness need further investigation with more samples.

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