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Hypoxia reduces MAX expression in endothelial cells by unproductive splicing

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ABSTRACT

The MYC–MAX–MXD network is involved in the regulation of cell differentiation and proliferation. Hypoxia affects the expression levels of several members of this network, but changes specific to *MAX* expression have so far not been shown. We found that in endothelial cells, hypoxia induces alternative splicing of *MAX*, thereby increasing the expression of two *MAX* isoforms that differ from the wild type in their 3′ end. Isoform C is degraded by nonsense-mediated decay and isoform E encodes a highly unstable protein. The instability of isoform E is conferred by 36 isoform-specific amino acids, which have the capacity to destabilize heterologous proteins. Both splicing events are therefore unproductive and serve the purpose to downregulate the wild type protein.

1. Introduction

MAX (MYC associated factor X) is a ubiquitously expressed transcription factor and the central part in a network of several transcriptional regulators involved in apoptosis, cell differentiation, proliferation and transformation. MAX forms heterodimers with MYC oncoproteins (c-MYC, L-MYC, N-MYC) stimulating cell proliferation as well as several MYC antagonists (MXD1–4, MNT, MGA), which inhibit proliferation and contribute to differentiation [1]. In addition, MAX can form homodimers, but it is unclear if homodimerization impacts on its function in vivo [2]. All network members encode a basic Helix-Loop-Helix Leucine Zipper domain (bHLHLZ) for DNA-binding and dimerization. The different MAX complexes compete for the same DNA motif (E-box) in the promoter region of a vast number of target genes. Usually MAX/MYC complexes activate gene expression by histone modification and increased transcription elongation, whereas MAX/MXD complexes repress gene expression. *MYC* expression is deregulated in various types of cancer and its tumorigenic potential seems to depend on the expression of other network members [1]. *MAX* mutations are associated with pheochromocytoma and small-cell lung cancer [3,4].

MYC proteins are short lived (~15–20 min), their levels heavily regulated on the transcriptional and posttranscriptional level [5]. In contrast, MAX wild type (WT) protein is very stable (>24 h) and abundantly expressed, with only few examples of posttranscriptional gene regulation [6]. Two miRNAs (miR-22 and miR-193b) have been shown to downregulate *MAX* expression and thereby inhibit cell cycle progression in normal and cancer cells [7–9]. Further, alternative splicing of exon 2 gives rise to a long (p22) and a short (p21) protein differing by a nine amino acid (aa) insertion at the N-terminus and the tendency to homodimerize [10]. In addition, mRNA isoforms encoding C-terminal truncated MAX variants have been reported [11,12]. These are created either by retention of intron 4 (isoform E) or by inclusion of a cassette exon located within intron 4 (isoform C) (Fig. 1A). Both C-terminal isoforms have been shown to enhance the transforming activity of c-MYC in vivo, whereas WT MAX reduces it.

To increase chances of survival under prolonged hypoxic conditions, cells decrease their energy consumption, amongst other measures, by decreasing their proliferation rate. MYC protein is downregulated by decreased translation efficiency and destabilization of the protein [13]. In addition, its function is counteracted by displacement of MAX/MYC complexes from promoter binding sites and upregulation of its antagonist *MXD2* (also MXI1) [14]. Until now, no regulation of *MAX* has been reported under hypoxia. Recently, we discovered that the mRNA of isoform E is upregulated

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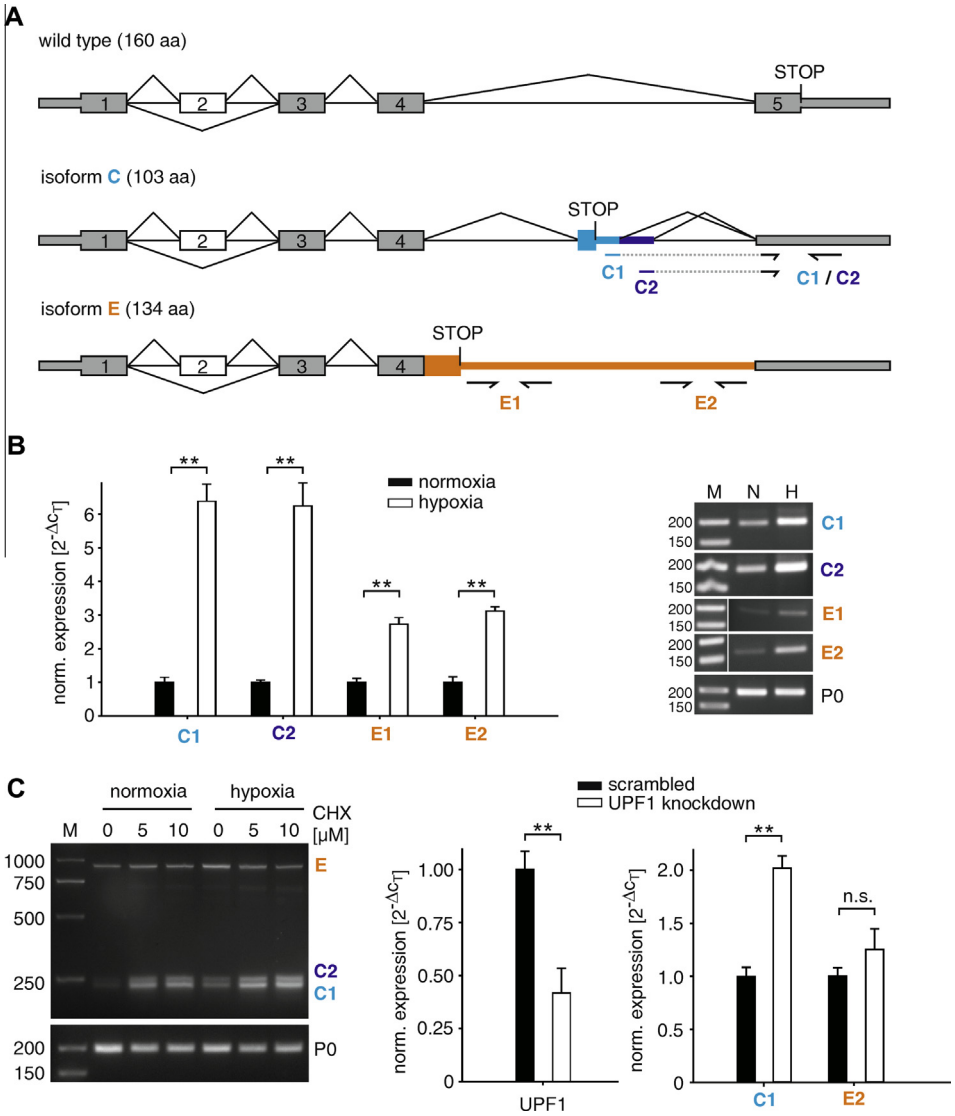

Fig. 1. C-terminal *MAX* mRNA isoforms are upregulated during hypoxia and partially targeted to NMD. (A) *MAX* mRNA isoforms coding for proteins with different C-termini. In isoform C a cassette exon (blue) located within intron 4 is retained. Due to alternatively used 5′ splice sites, the cassette exon exists in two different lengths (indicated in light and dark blue). Retention of the complete intron 4 (orange) leads to isoform E expression. Arrows show the location of primer pairs used for the qRT-PCRs in Fig. 1B and C. (B) *MAX* mRNA isoforms C and E are induced during hypoxia. (left) qRT-PCR quantification of isoforms C and E isolated from normoxic and hypoxic HUVECs. Two primer pairs binding within intron 4 up- and downstream of the cassette exon were used for the quantification of isoform E, to ensure complete intron retention. Isoforms are normalized to the total *MAX* mRNA amount. $n = 3$, $^{**}P$-value <0.01. (right) RT-PCRs of isoforms C and E. For detection of isoform C (C1 and C2), eight additional amplification rounds compared to isoform E (E1 and E2) were used, to generate a visible signal. The housekeeping gene *RPLP0* (P0) is shown as loading control. N = normoxia, H = hypoxia. (C) Isoform C is a NMD target in HUVECs. (left) RT-PCR from isoform C and E after CHX treatment in normoxic and hypoxic HUVECs. *RPLP0* (P0) is shown as loading control. (center) qRT-PCR quantification of *UPF1* mRNA after siRNA-mediated knockdown in HUVECs. (right) qRT-PCR quantification of isoforms C and E after UPF1 knockdown in HUVECs. Expression values are normalized to *RPLP0*. $n = 3$, $^{**}P$-value <0.01, n.s. = not significant.

in endothelial cells during prolonged hypoxia [15]. Here we show that during hypoxia both mRNAs encoding C-terminal truncated MAX variants are upregulated, whereas the WT is reduced on mRNA and protein level. Upregulation of these mRNAs, however, does not lead to a detectable production of the corresponding protein isoforms. Isoform C is targeted to the nonsense-mediated decay (NMD) pathway. Isoform E encodes a highly unstable protein, with the last 36 aa inducing an extremely efficient downregulation also when attached to heterologous proteins. Therefore, the sole purpose of this intricate splicing regulation seems to be the reduction of WT protein levels. As MAX is part of all MYC and MXD heterodimers, its reduction impacts on the activity of the whole network.

2. Materials and methods

2.1. Cell culture and transfection

2.1.1. Endothelial cells

Pooled HUVECs (Lonza) were grown in endothelial cell basal medium (EBM), supplemented with EGM SingleQuots (except ascorbic acid) (both Lonza) and 10% FCS (Gibco) in T75 flasks (Greiner) and used from the second or third passage. For plasmid transfection HUVECs were grown in T75 flasks to 60–70% confluence, detached with trypsin–EDTA (Gibco) and neutralized with trypsin neutralization solution (Lonza). 1×10^6 cells were transfected using the 4D-Nucleofector™ and P5 primary cell