

Original Article

C/EBP α inhibits proliferation of breast cancer cells via a novel pathway of miR-134/CREB

Jianxiang Zhang¹, Yanmei Ma², Shoujun Wang³, Fu Chen⁴, Yuanting Gu¹

Departments of ¹General Surgery, ²Gastroenterology, ³Endocrinology, ⁴Integrated TCM & Western Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China

Received August 12, 2015; Accepted October 31, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: C/EBP α plays an important role in the modulation of cell proliferation, differentiation or apoptosis in various tissues. Most recently, reduced expression of C/EBP α and growth inhibitory effect was found in primary mammary carcinomas. However, the underlying mechanism is still not fully aware. Here, we firstly identified miR-134 as a target of C/EBP α in MCF7 breast cancer cell lines. C/EBP α overexpression promoted miR-134 expression, causing suppression of apoptosis- protective genes CREB and Bcl-2, and resulted in the proliferation inhibition of MCF7 cells. Moreover, anti-miR-134 rescued the proliferation inhibition of MCF7 cells and the suppression of anti-apoptotic genes CREB and Bcl-2 caused by C/EBP α overexpression. Collectively, C/EBP α inhibited cell growth in breast cancer cells via a novel pathway miR-134/CREB.

Keywords: C/EBP α , breast cancer cell, miR-134, proliferation, CREB

Introduction

Breast cancer is a common threat to women's health. Originating in undifferentiated terminal structures of the mammary gland, breast cancer involves the clonal expansion of a transformed cell into an epithelial hyperplasia until the formation of adenocarcinomas [1]. Numerous transcription factors or other molecules were overexpressed or silenced during this process. Among the transcription factors, CCAAT/enhancer binding proteins (C/EBP) have been implicated in cellular proliferation or apoptosis in multiple tissues including mammary gland [2, 3].

C/EBP α , the founding member of the C/EBP family, plays an important role in the modulation of cell proliferation, differentiation or apoptosis in various tissues, including adipose, liver, lung and blood [2, 4-6]. Most recently, reduced expression of C/EBP α was found in primary mammary carcinomas. Overexpression of C/EBP α in breast cancer cell lines led to inhibition of proliferation [7]. However, the underlying mechanism is still not fully aware.

MicroRNAs are small non-coding RNAs that control the translation of target messenger

RNAs, thus regulating various critical biological processes. MiR-134 was discovered as a brain-specific miRNA and it played an essential role in the differentiation of the embryonic stem cell to central nervous system by suppression of Nanog [8]. It was also reported that SIRT1 regulated memory and plasticity via miR-134-mediated post-transcriptional regulation of cAMP response binding protein (CREB), an anti-apoptotic gene in a number of tissues [9]. For a long time, miR-134 was thought to only act in nervous system [10, 11]. However, much lately, a large-scale screening identified miR-134 as a candidate tumor metastasis suppressor, for its down-regulation in hepatocellular carcinoma (HCC) and significantly inhibitory effect on invasion and metastasis in HCC [12]. In addition, miR-134 was demonstrated to regulate epithelial-mesenchymal transition in lung adenocarcinoma cells [13]. These findings in non-nervous system suggested that miR-134 may play a role in more other tissues.

In this current study, we reported that miR-134 level was markedly distinct between normal and cancerous breast tissues. MiR-134 expression pattern was consistent with C/EBP α during the MCF7 breast cancer cell proliferation.

C/EBP α inhibits cell proliferation via miR-134

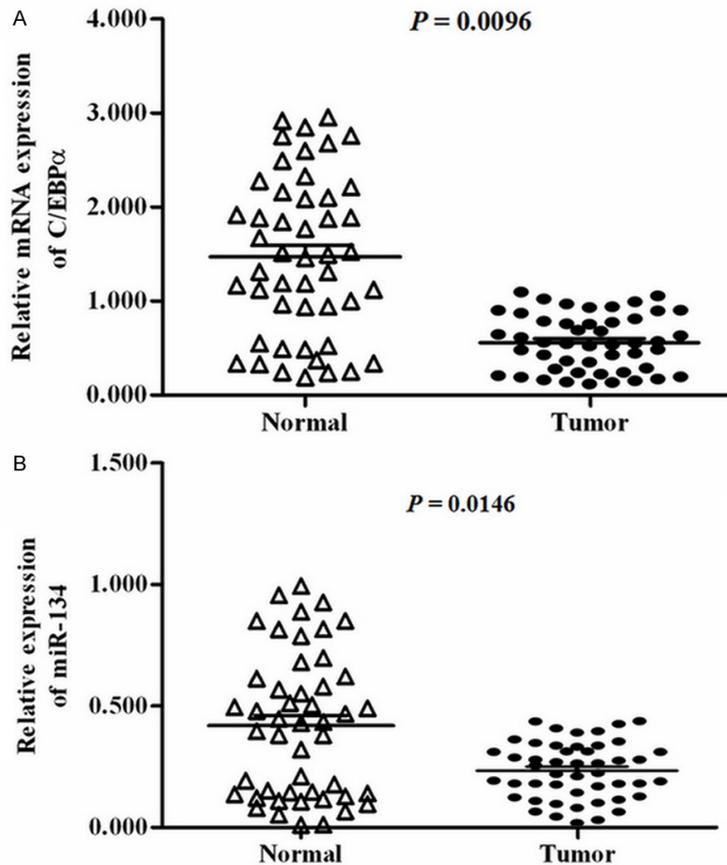


Figure 1. Expression of C/EBP α and miR-134 in normal and cancerous breast tissue. Relative expression levels of C/EBP α (A) and miR-134 (B) are shown in 48 normal breast tissues, 48 primary breast cancer samples. The relative level of C/EBP α was normalized so that the mean ratio of the 48 cancerous breast samples equals a value of 0.5. The relative level of miR-134 was normalized to the mean value of 18S RNA in the 48 normal breast tissues.

Moreover, overexpression and silence of C/EBP α caused elevation and descent of miR-134 level respectively. Simultaneously, the anti-apoptotic gene CREB and B-cell lymphoma-2 (Bcl-2) were down- and up-regulated response to the alternation of miR-134 level. Collectively, we found a novel pathway that C/EBP α induced breast cancer cell apoptosis via miR-134/CREB.

Materials and methods

Samples

RNA was excreted from the mammary cells of 48 healthy and excised primary breast tumors of 48 women treated at Affiliated Xijing Hospital, Fourth Military Medical University, Xi'an, China, from 2012 to 2014. The samples were examined histologically for the presence of tumor cells.

Cell culture

MCF-7 breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA) and were cultured in DMEM supplemented with 10% FBS (Gibco, Invitrogen, Carlsbad, CA) and 1% penicillin, streptomycin, and neomycin antibiotic mixture. Cells were grown in a humidified incubator with an atmosphere of 95% air-5% CO₂ at 37°C.

Transfection of oligonucleotides and plasmids

The pcDNA-C/EBP α expression plasmid was constructed and tested to be effective by Dingguo Changsheng Bio-technology Co. LTD. (Beijing, China). Oligo antagonist and mimic for miR-134 were designed and synthesized by Ribobio Biotechnology Co. LTD. (Guangzhou, China).

For transfection, MCF7 cells were seeded into 6-well plates. When growing to 80% of confluence, their medium was changed to MEM without FBS and antibiotic mixture. After starvation for 4 hours, the

medium was changed for DMEM supplemented with 10% FBS. Immediately, 6 μ g of pcDNA-C/EBP α , or 100 nm miR-134 mimic, or 100 nm Oligo antagonist for miR-134 were co-transfected or transfected alone into the cells with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instruction. After another 6-8 h, the medium was changed for fresh DMEM supplemented with 10% FBS.

RNA extraction and RT-qPCR

Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The resultant RNA was applied in the reverse transcription reaction to obtain the first chain cDNA. Real-time qPCR reactions were carried out in a final volume of 25 μ l, using SYBR Premix Ex Taq (TaKaRa), 0.4 mM of each primer, and 200 ng of cDNA template. Each

C/EBP α inhibits cell proliferation via miR-134

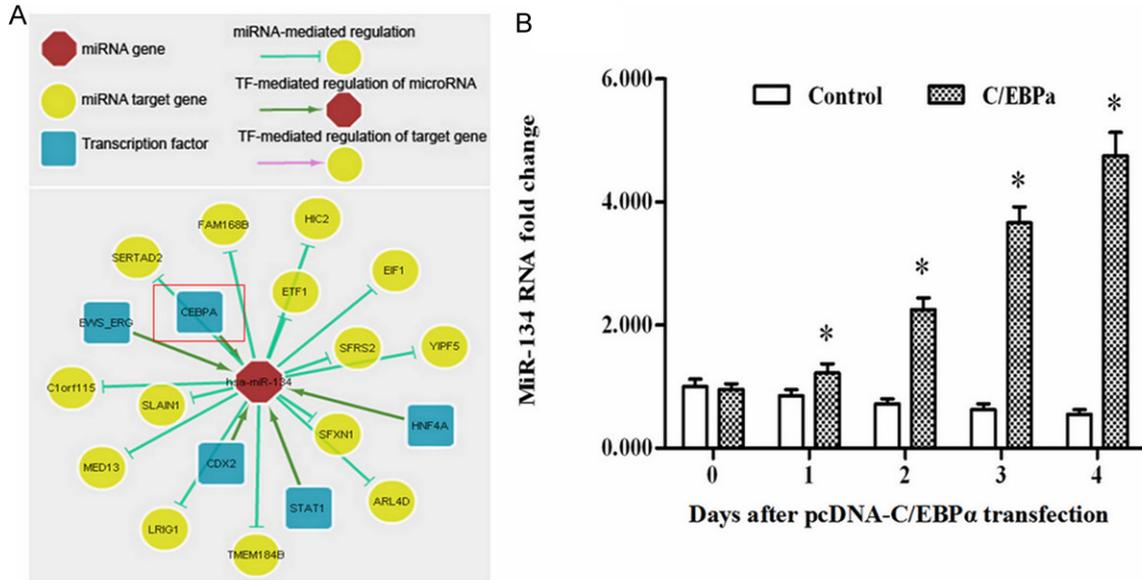


Figure 2. C/EBP α promoted expression of miR-134 in MCF-7 breast cancer cells. A. The regulation relationship MiR-134 is predicted to be promoted by C/EBP α using the online server ChIPBase. B. Overexpression of C/EBP α elevated the level of miR-134 in MCF-7 cells. The pcDNA-C/EBP α was transfected into MCF-7 cells. At time points of d 0, d 1, d 2, d 3, d 4, the level of miR-134 in the cells was determined by real-time qPCR. * $P < 0.05$.

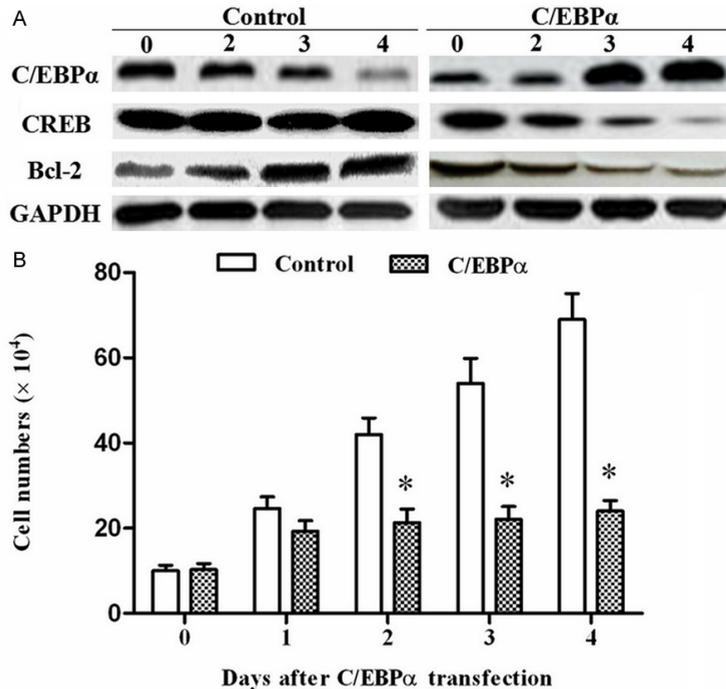


Figure 3. Overexpression of C/EBP α decreased cell proliferation of MCF-7 breast cancer cells. A. Overexpression of C/EBP α decreased expression of CREB and Bcl-2. At time points of day 0, day 2, day 3, day 4, expression of the anti-apoptotic genes CREB and Bcl-2 in the MCF-7 cells were determined by Western blotting. B. MCF-7 cell proliferation was decreased by C/EBP α overexpression. After transfection, the cell numbers were counted by Cellometer[®] Cell Counters and Cell Analysis Systems (Nexcelom Bioscience LLC, Lawrence, MA). * $P < 0.05$.

individual sample was run in triplicate wells. The reactions were initially denatured at 95°C for 3 min followed by 35 cycles of 95°C for 15 s, 60°C for 1 min. Quantification of amplicons was done using ABI 7300 software (Applied Biosystem). The primers applied in the reactions are as follows: C/EBP α (F: 5'-TGG ACA AGA ACA GCA ACG AG-3', R: 5'-TTG TCA CTG GTC AGC TCC AG-3'), CREB (5'-TCA GCC GGG TAC TAC CAT TC-3', 5'-TTC AGC AGG CTG TGT AGG AA-3'), Bcl-2 (5'-ATT GTG GCC TTC TTT GAG TTC G-3', 5'-CAT CCC AGC CTC CGT TAT CC-3'), 18S (F: 5'-AAA CGG CTA CCA CAT CCA AG-3', R: 5'-CCT CCA ATG GAT CCT CGT TA-3'). MiR-134 Stem-loop primer and quantitative primers were designed and produced by Ribobio Biotech.

Western blotting

Cells were washed with PBS, and lysed in NP40 lysis buffer

C/EBP α inhibits cell proliferation via miR-134

(50 mM Tris-HCl, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 10% glycerol) with protease inhibitors cocktail (Sigma). Proteins were separated in SDS-PAGE, transferred, and immunoblotted with various antibodies. The antibodies used were anti-C/EBP α (dilution 1:500; Abcam), anti-CREB (dilution 1:500; Cell Signaling), anti-Bcl-2 (dilution 1:500; Cell Signaling), and anti-GAPDH (dilution 1:5000; Sigma).

Web servers

The regulation relationship was predicted by the online server ChIPBase_ (<http://deepbase.sysu.edu.cn/chipbase/>), which is an integrated resource and platform for transcriptional regulation of long non-coding RNAs (lncRNAs), microRNAs, other ncRNAs and protein-coding genes based on ChIP-Seq data.

Statistical analysis

All data were expressed as mean \pm S.E.M. of three or more biological replicates. Two-tailed Student's t-test was employed to determine *P*-values. The significance was set at *P* < 0.05, and extremely significance at *P* < 0.01.

Results

C/EBP α and miR-134 were both down-regulated in cancerous breast tissues

Relative levels of C/EBP α and miR-134 in 48 normal breast tissues and 48 primary breast cancer samples were compared. The real-time qPCR analysis showed that the level of C/EBP α mRNA in primary breast cancer samples was just about 1/3 of that of normal breast samples (**Figure 1A**). Interestingly, we detected a similar expression pattern of miR-134. The level of miR-134 in the cancerous samples was about 1/2 of that of the normal ones (**Figure 1B**). C/EBP α and miR-134 were both down-regulated in cancerous breast tissues, hinting that there may be some association between C/EBP α and miR-134.

Mir-134 was promoted by in MCF-7 breast cancer cells

Then we tried to clarify the regulatory relationship between C/EBP α and miR-134. The predict output of the online server ChIPBase, an integrated resource and platform for transcriptional regulation of non-coding RNAs and pro-

tein-coding genes based on ChIP-Seq, showed that miR-134 was likely to be a target of C/EBP α (**Figure 2A**).

The pcDNA-C/EBP α was transfected into MCF-7 cells. At time points of d 0, d 1, d 2, d 3, d 4, the level of miR-134 in the cells was determined by real-time qPCR. The data indicated overexpression of C/EBP α promoted miR-134 expression in MCF-7 cells (**Figure 2B**). CREB, target gene of miR-134, also an important anti-apoptotic gene in many processes, was decreased response to the increase of miR-134 (**Figure 3A**). Another apoptosis-inhibitory factor Bcl-2 was also minimized after transfection (**Figure 3A**). Simultaneously, cell count analysis showed the cell proliferation was dramatically inhibited by C/EBP α overexpression (**Figure 3B**).

Anti-miR-134 rescued the proliferation inhibition by C/EBP α overexpression

To testify the inhibition was associated with the elevation of miR-134. The pcDNA-C/EBP α , or miR-134 mimic, or miR-134 antagonist (anti-miR-134) were transfected alone or co-transfected into MCF7 breast cancer cell lines. At day 4 of transfection, the cell numbers were counted by the Cell Counters and Cell Analysis Systems. The result pointed that mimic miR-134 has the same effect with C/EBP α overexpression on inhibition of MCF7 cells and anti-miR-134 rescued the inhibition (**Figure 4A**). In consistent with the result of the cell count analysis, the suppression of anti-apoptotic genes CREB and Bcl-2 was also alleviated (**Figure 4B**). Therefore, C/EBP α inhibited cell proliferation via regulating miR-134/CREB.

Discussions

Although several reports have indicated that C/EBP α had an proliferation inhibitory effect in breast cancer [7, 14]. However, not much is known about the underlying mechanism and network mediating the anti-proliferation impacts of C/EBP α . The present study showed that C/EBP α and miR-134 were consistently down-regulated in breast cancer and had an apoptosis-inducing effect in MCF7 breast cancer cell lines. This apoptosis-inducing effect was mediated by miR-134 and its target anti-apoptotic gene CREB.

C/EBP α was most well-known as an import regulator in adipocyte differentiation [5]. MiR-134

C/EBP α inhibits cell proliferation via miR-134

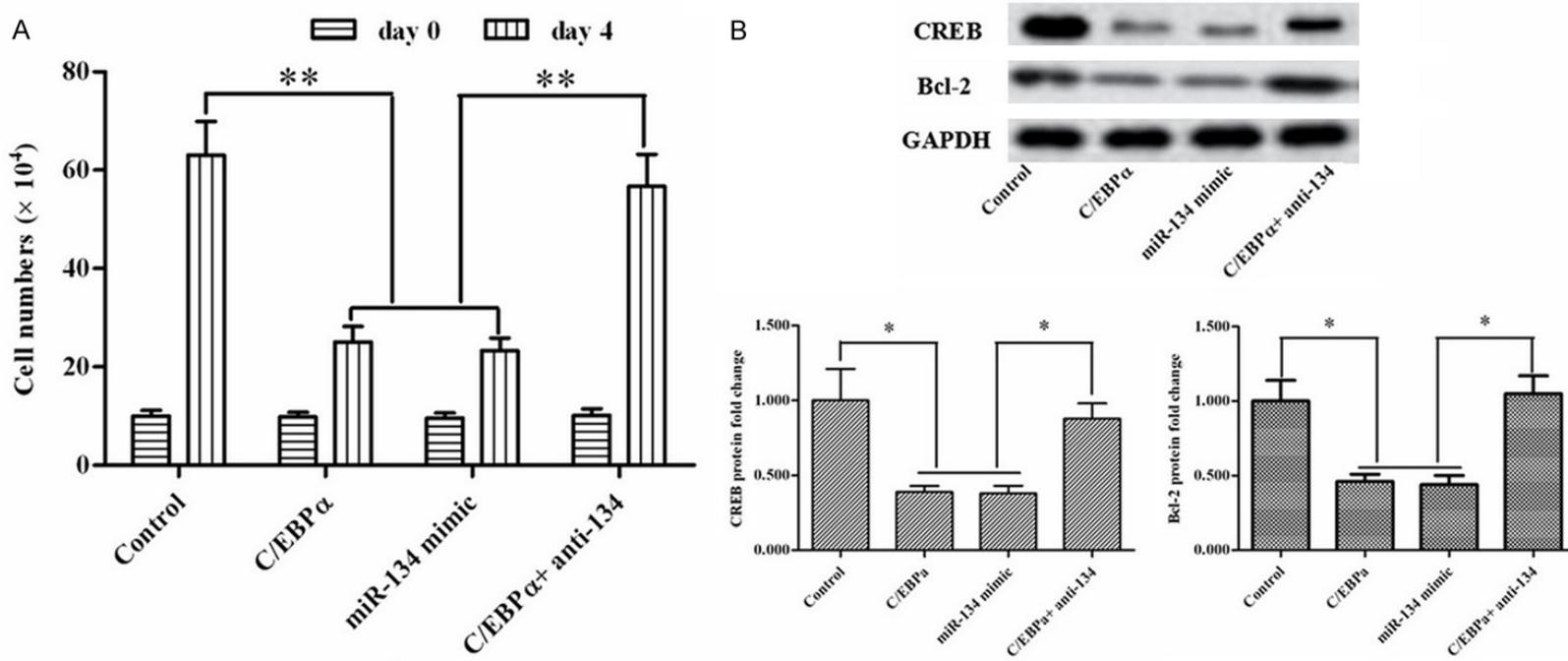


Figure 4. MiR-134 inhibitor rescued the proliferation inhibitory effect by C/EBP α overexpression. The pcDNA-C/EBP α , or miR-134 mimic, or miR-134 inhibitor were transfected alone or co-transfected into MCF7 breast cancer cell lines for 4 day. A. The cell numbers were counted by Cellometer[®] Cell Counters and Cell Analysis Systems. B. Expression of CREB and Bcl-2 proteins were detected by Western blotting. * $P < 0.05$.

was thought to be nervous-specific for a long time. Though most recently a few evidence showed that it also functioned in HCC (Zha et al, 2014) and lung adenocarcinoma cells (Kitamura et al, 2014), there still is no report that it has an effect on development of breast cancer. In this current study, we showed that miR-134 was diversely expressed between normal and cancerous breast tissues. Besides, elevation of miR-134 could induce cell apoptosis by decreasing CREB and Bcl-2 in MCF7 breast cancer cells. To our knowledge, this is the first report that miR-134 had an effect on breast cancer.

Since miR-134 exerts a great role in embryonic stem cell differentiation and nervous system development [15-17], regulation of its expression was also an important part of its function exploration. We predicted and testified C/EBP α was an upstream promoting gene for miR-134. Then, as a target gene of miR-134, CREB was indirectly suppressed by C/EBP α . Thus the anti-apoptotic effect of CREB was inhibited and the cells exhibited proliferation inhibition. CREB acts as an anti-apoptotic gene in multiple tissues and the molecular mechanisms varies in different tissues [18-20]. However, we still do not know how CREB functions in the breast cancer cells. Further studies will be focused on the CREB mechanism of apoptosis protection in the breast cancer cells.

In conclusion, we found a novel pathway that C/EBP α induced breast cancer cell apoptosis via miR-134/CREB.

Acknowledgements

Heartfelt acknowledgements were uttered to the Xijing Hospital for their permission of using the specimens in this study.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yuanting Gu, Department of General Surgery, The First Affiliated Hospital of Zhengzhou University, 1 Jianshe East Road, Zhengzhou 450052, China. Tel: 86-371-67967172; Fax: 86-371-67967172; E-mail: yuantingguzz@163.com

References

[1] Stephens PJ, Tarpey PS, Davies H, Van Loo P, Greenman C, Wedge DC, Nik-Zainal S, Martin

S, Varela I, Bignell GR, Yates LR, Papaemmanuil E, Beare D, Butler A, Cheverton A, Gamble J, Hinton J, Jia M, Jayakumar A, Jones D, Latimer C, Lau KW, McLaren S, McBride DJ, Menzies A, Mudie L, Raine K, Rad R, Chapman MS, Teague J, Easton D, Langerød A; Oslo Breast Cancer Consortium (OSBREAC), Lee MT, Shen CY, Tee BT, Huimin BW, Broeks A, Vargas AC, Turashvili G, Martens J, Fatima A, Miron P, Chin SF, Thomas G, Boyault S, Mariani O, Lakhani SR, van de Vijver M, van't Veer L, Foekens J, Desmedt C, Sotiriou C, Tutt A, Caldas C, Reis-Filho JS, Aparicio SA, Salomon AV, Børresen-Dale AL, Richardson AL, Campbell PJ, Futreal PA, Stratton MR. The landscape of cancer genes and mutational processes in breast cancer. *Nature* 2012; 486: 400-4.

- [2] Lu H, Yu Z, Liu S, Cui L, Chen X, Yao R. CUGBP1 promotes cell proliferation and suppresses apoptosis via down-regulating C/EBP α in human non-small cell lung cancers. *Med Oncol* 2015; 32: 1-10.
- [3] Chen BL, Sheu ML, Tsai KS, Lan KC, Guan SS, Wu CT, Chen LP, Hung KY, Huang JW, Chiang CK, Liu SH. CCAAT-Enhancer-Binding Protein Homologous Protein Deficiency Attenuates Oxidative Stress and Renal Ischemia-Reperfusion Injury. *Antioxid Redox Signal* 2014; [Epub ahead of print].
- [4] Yoshida H, Imamura T, Fujiki A, Hirashima Y, Miyachi M, Inukai T, Hosoi H. Post-transcriptional modulation of C/EBP α prompts monocytic differentiation and apoptosis in acute myelomonocytic leukaemia cells. *Leuk Res* 2012; 36: 735-41.
- [5] Zhang ZC, Liu Y, Li SF, Guo L, Zhao Y, Qian SW, Wen B, Tang QQ, Li X. Suv39h1 mediates AP-2 α -dependent inhibition of C/EBP α expression during adipogenesis. *Mol Cell Biol* 2014; 34: 2330-8.
- [6] Bloomer SA, Kregel KC, Brown KE. Heat stress stimulates hepcidin mRNA expression and C/EBP α protein expression in aged rodent liver. *Arch Gerontol Geriatr* 2014; 58: 145-52.
- [7] Gery S, Tanosaki S, Bose S, Bose N, Vadgama J, Koeffler HP. Down-regulation and growth inhibitory role of C/EBP α in breast cancer. *Clin Cancer Res* 2005; 11: 3184-90.
- [8] Bicker S, Khudayberdiev S, Weiß K, Zocher K, Baumeister S, Schrott G. The DEAH-box helicase DHX36 mediates dendritic localization of the neuronal precursor-microRNA-134. *Genes Dev* 2013; 27: 991-6.
- [9] Gao J, Wang WY, Mao YW, Gräff J, Guan JS, Pan L, Mak G, Kim D, Su SC, Tsai LH. A novel pathway regulates memory and plasticity via SIRT1 and miR-134. *Nature* 2010; 466: 1105-9.
- [10] Siegel G, Saba R, Schrott G. microRNAs in neurons: manifold regulatory roles at the synapse. *Curr Opin Genet Dev* 2011; 21: 491-7.

C/EBP α inhibits cell proliferation via miR-134

- [11] Huang W, Cao J, Liu X, Meng F, Li M, Chen B, Zhang J. AMPK Plays a Dual Role in Regulation of CREB/BDNF Pathway in Mouse Primary Hippocampal Cells. *J Mol Neurosci* 2015; 56: 782-8.
- [12] Faraji F, Hu Y, Wu G, Goldberger NE, Walker RC, Zhang J, Hunter KW. An integrated systems genetics screen reveals the transcriptional structure of inherited predisposition to metastatic disease. *Genome Res* 2014; 24: 227-40.
- [13] Kitamura K, Seike M, Okano T, Matsuda K, Miyanaga A, Mizutani H, Noro R, Minegishi Y, Kubota K, Gemma A. MiR-134/487b/655 Cluster Regulates TGF- β -Induced Epithelial-Mesenchymal Transition and Drug Resistance to Gefitinib by Targeting MAGI2 in Lung Adenocarcinoma Cells. *Mol Cancer Ther* 2014; 13: 444-53.
- [14] Cascione L, Gasparini P, Lovat F, Carasi S, Pulvirenti A, Ferro A, Alder H, He G, Vecchione A, Croce CM, Shapiro CL, Huebner K. Integrated microRNA and mRNA signatures associated with survival in triple negative breast cancer. *PLoS One* 2013; 8: e55910.
- [15] Garofalo M, Croce CM. Role of microRNAs in maintaining cancer stem cells. *Adv Drug Deliv Rev* 2015; 81: 53-61.
- [16] Arámburo C, Alba-Betancourt C, Luna M, Harvey S. Expression and function of growth hormone in the nervous system: A brief review. *Gen Comp Endocrinol* 2014; 203: 35-42.
- [17] Meza-Sosa KF, Pedraza-Alva G, Pérez-Martínez L. microRNAs: key triggers of neuronal cell fate. *Front Cell Neurosci* 2014; 8: 175.
- [18] Mylroie H, Dumont O, Bauer A, Thornton CC, Mackey J, Calay D, Hamdulay SS1, Choo JR, Boyle JJ, Samarel AM, Randi AM, Evans PC, Mason JC. PKC ϵ -CREB-Nrf2 signaling induces HO-1 in the vascular endothelium and enhances resistance to inflammation and apoptosis. *Cardiovasc Res* 2015; 106: 509-19.
- [19] Chou CH, Lai SL, Chen CN, Lee PH, Peng FC, Kuo ML, Lai HS. IL-6 regulates Mcl-1L expression through the JAK/PI3K/Akt/CREB signaling pathway in hepatocytes: implication of an anti-apoptotic role during liver regeneration. *PLoS One* 2013; 8: e66268.
- [20] Liu B, Barbosa-Sampaio H, Jones PM, Persaud SJ, Muller DS. The CaMK4/CREB/IRS-2 cascade stimulates proliferation and inhibits apoptosis of β -cells. *PLoS One* 2012; 7: e45711.