Cell Research (2014) 24:254-257.

www.nature.com/cr

LETTER TO THE EDITOR

MicroRNA-155 broadly orchestrates inflammation-induced changes of microRNA expression in breast cancer

Cell Research (2014) **24**:254-257. doi:10.1038/cr.2013.137; published online 1 October 2013

Dear Editor,

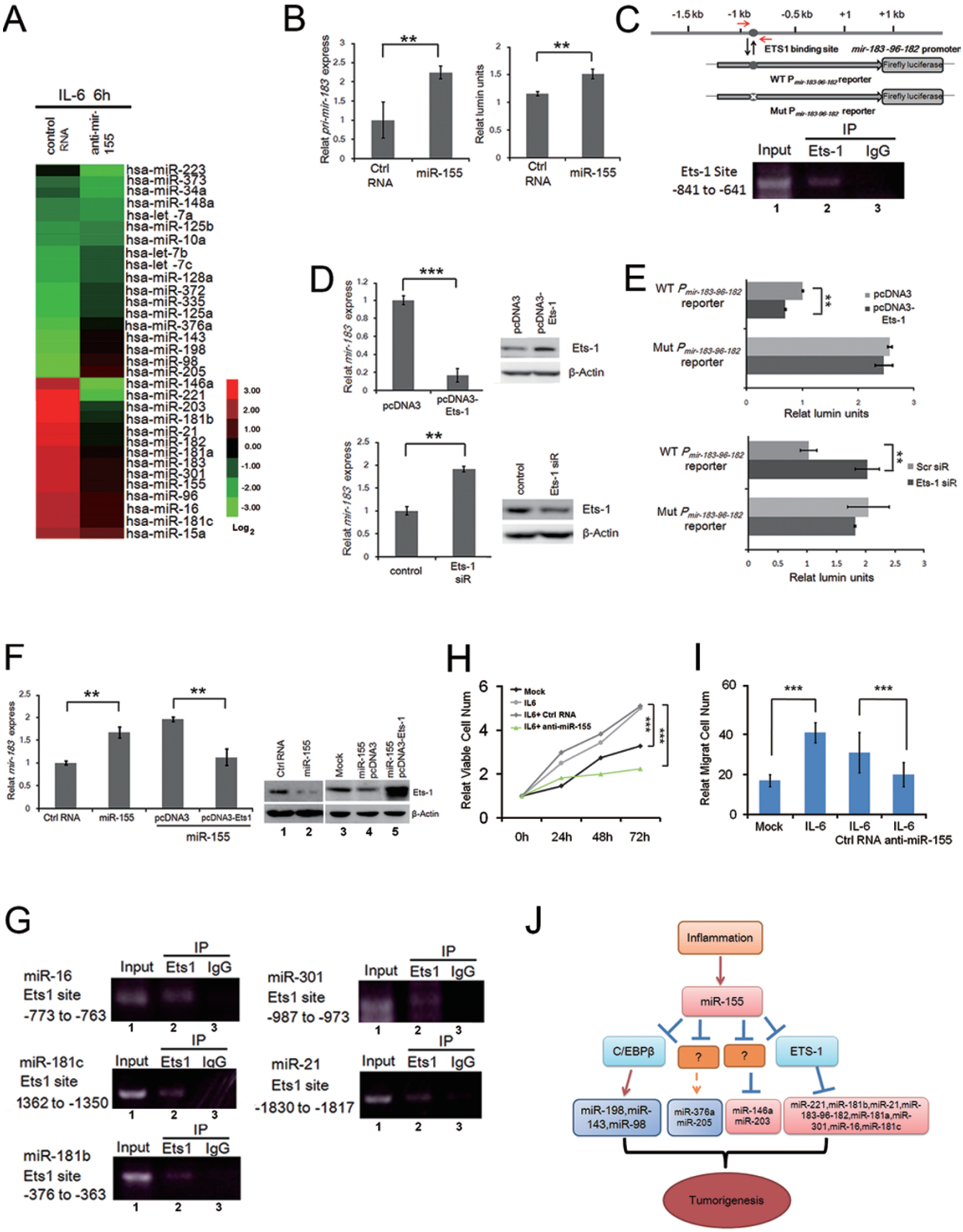
During the last decades, a number of key mediators of inflammation-induced tumorigenesis have been identified to support tumor progression in response to inflammation stimulation [1]. Recent studies show that a few cancer-related microRNAs (miRNAs) are modulated by inflammation signals [2, 3], implicating miRNAs as a new class of mediators between inflammation and cancer. However, how inflammation regulates miRNAs in cancer cells and how such miRNA mediators function in inflammation-associated tumorigenesis remain largely unexplored. miRNA-155 (miR-155) has been documented as one of the most prominent miRNAs linking inflammation to cancer [4]. On one hand, *mir-155* is ubiquitously induced by inflammation stimuli [3]; on the other hand, miR-155 leads to the constitutive activation of pro-tumorigenic inflammatory STAT3 signaling by targeting *socs1* [5] and conveys the inflammatory signals to the Warburg effect by upregulating *hexokinase-2* [6]. We previously observed that pro-inflammatory cytokine interleukin-6 (IL-6)-induced miR-155 represses a specific miRNA gene *mir-143* through targeting a transcription factor [6]; however, it is unclear how generally this mechanism might be utilized in the regulation of miRNAs by inflammation. In the present study, we show that miR-155 acts as a key regulating node linking inflammation to expression control of a number of cancer-related miRNAs, revealing a novel mechanism for the regulation of miRNAs by inflammation.

To further explore the roles of miRNAs in inflammation-associated cancer, we systematically examined the effect of IL-6 on the expression of 32 cancer-categorized miRNAs [2] using qRT-PCR assays in breast cancer cells. Consistent with our previous observation, we found that miR-155 was significantly induced by IL-6 in MCF-7 breast cancer cells, which harbor low endogenous levels of miR-155 [5], while the levels of 24 miRNAs were significantly changed in the IL-6-treated cells (by more than 2-fold) (Figure 1A), indicating that IL-6 broadly regulates cancer-related miRNAs in breast cancer cells.

Given that miR-155 plays a well-evident role in linking inflammation and cancer [4], we asked whether miR-155, induced by IL-6 via NF-κB pathway in breast cancer cells [6], is involved in the regulation of miRNAs by IL-6. To this end, we used anti-miR-155 to inhibit miR-155 function in IL-6-treated MCF-7 cells (Supplementary information, Figure S1A). Intriguingly, we found that anti-miR-155 completely attenuated the impact of IL-6 on 17 out of the 24 IL-6-affected miRNAs, including 12 IL-6-upregulated miRNAs and 5 IL-6-downregulated miRNAs (Figure 1A), indicating that miR-155 acts as an important mediator in the miRNA regulation by IL-6 in breast cancer cells.

We next asked how miR-155 regulates miRNA expression in breast cancer cells. Given that the miR-183, miR-96 and miR-182 from the *mir-183-96-182* cluster (Supplementary information, Figure S1B) were similarly modulated by the IL-6/miR-155 context (Figure 1A), we first examined how miR-155 regulates the *mirna* cluster. We found that transfection of miR-155 mimics in MCF-7 cells significantly increased the levels of *pri-mir-183*, *pri-mir-96* and *pri-mir-182* (Figure 1B, left, Supplementary information, Figure S1D and S1E) and that transfection of anti-miR-155 in MDA-MB-231 cells, which have high endogenous *mir-155* expression [5], significantly reduced the levels of all 3 transcripts (Supplementary information, Figure S1C-S1E), suggesting that miR-155 regulates the cluster at the transcriptional level. To further corroborate this, we constructed a luciferase reporter controlled by the ~1.8-kb human *mir-183-96-182* promoter $P_{mir\text{-}183\text{-}96\text{-}182}$ (Figure 1C, middle). Indeed, our reporter assays showed that the $P_{mir\text{-}183\text{-}96\text{-}182}$ activity was upregulated by cotransfection of miR-155 in MCF-7 cells (Figure 1B, right). Collectively, these results support that miR-155 activates the *mir-183-96-182* cluster at the transcriptional level.

To dissect the molecular mechanism for the transcriptional activation of *mir-183-96-182* cluster by miR-155, we used TransFac and Genomatix softwares [7] to search for potential transcription-factor-binding sites in the promoter and found a putative binding site for Ets-1,


A
IL-6 6h
control RNA
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hsa-miR-223
hsa-miR-373
hsa-miR-34a
hsa-miR-148a
hsa-let -7a
hsa-miR-125b
hsa-miR-10a
hsa-let-7b
hsa-let -7c
hsa-miR-128a
hsa-miR-372
hsa-miR-335
hsa-miR-125a
hsa-miR-376a
hsa-miR-143
hsa-miR-198
hsa-miR-98
hsa-miR-205
hsa-miR-146a
hsa-miR-221
hsa-miR-203
hsa-miR-181b
hsa-miR-21
hsa-miR-182
hsa-miR-181a
hsa-miR-183
hsa-miR-301
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C
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+1 kb
ETS1 binding site
mir-183-96-182 promoter
Firefly luciferase
WT P mir-183-96-182 reporter
Mut P mir-183-96-182 reporter
IP
Input
Ets-1
IgG
Ets-1 Site
-841 to -641
D
Relat mir-183 express
pcDNA3
pcDNA3-Ets-1
Ets-1
β-Actin
control
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WT P mir-183-96-182 reporter
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miR-16
Ets1 site
-773 to -763
miR-301
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-987 to -973
miR-181c
Ets1 site
1362 to -1350
miR-21
Ets1 site
-1830 to -1817
miR-181b
Ets1 site
-376 to -363
J
Inflammation
miR-155
C/EBPβ
?
?
ETS-1
miR-198,miR-143,miR-98
miR-376a miR-205
miR-146a miR-203
miR-221,miR-181b,miR-21,miR-183-96-182,miR-181a,miR-301,miR-16,miR-181c
Tumorigenesis

Figure 1 miR-155 regulates miRNA expression in breast cancer cells by targeting *ETS-1* and *C/EBPβ*. **(A)** qRT-PCR analyses of the effects of IL-6 and miR-155 on the expression of 32 cancer-related miRNAs in MCF-7 breast cancer cells. Twenty-four hours post transfection of anti-miR-155 or negative control RNA (Ctrl RNA) into IL-6-treated MCF-7 cells, the miRNA levels were measured using qRT-PCR analyses, with U6 serving as the internal reference. The levels of miRNAs in Ctrl RNA/IL-6- or anti-miR-155/IL-6-treated cells were normalized to their expression in mock cells, which was set to 1.0 for each tested miRNA. The heatmap represents differentially expressed miRNAs, with upregulated miRNAs in red and downregulated miRNAs in green. **(B)** The effect of miR-155 on the expression of the primary *mir-183* transcript (left) and the activity of the $P_{mir\text{-}183\text{-}96\text{-}182}$ reporter in MCF-7 cells (right). **(C)** Schematic representation of the predicted Ets-1-binding site in the *mir-183-96-182* promoter (top) and constructions of wild-type $P_{mir\text{-}183\text{-}96\text{-}182}$ and Mut $P_{mir\text{-}183\text{-}96\text{-}182}$ reporters (middle). ChIP analyses of the *mir-183-96-82* promoter using antibodies against Ets-1 were shown at the bottom. The primers for ChIP-PCR were indicated by arrowheads in schematic promoter (top), and their sequences were provided in Supplementary information, Table S1. **(D)** The effects of *Ets-1* overexpression (top) or knockdown (bottom) on *mir-183* expression in MCF-7 cells. **(E)** Modulation of the $P_{mir\text{-}183\text{-}96\text{-}182}$ promoter activity by *Ets-1* overexpression (top) or knockdown (bottom). **(F)** Ectopic expression of *Ets-1* reversed the upregulation of *mir-183* by miR-155. Left, qRT-PCR analyses of miR-183 levels; right, western blot analyses of the Ets-1 protein, with β-actin serving as a loading control. **(G)** ChIP analyses of the indicated miRNA promoters using antibodies against Ets-1. **(H**, **I)** Anti-miR-155 overrides the pro-tumorigenic effects of IL-6 in MCF-7 cells. IL-6-treated MCF-7 cells were transfected with Ctrl RNA or anti-miR-155, and the MTT **(H)** and transwell migration assays **(I)** were performed 24 h post transfection. **(J)** Model of miR-155 linking inflammation to miRNA expression in cancer cells. The mean values ± SD of 3 separate experiments were plotted. $^{**}P < 0.01$, $^{***}P < 0.001$. Results shown are representative of three independent experiments.

a known miR-155 target [8], located within $P_{mir\text{-}183\text{-}96\text{-}182}$ (Figure 1C, top). Chromatin immunoprecipitation (ChIP) assays using anti-Ets-1 antibodies in MCF-7 cells, which exhibit a higher endogenous level of the Ets-1 protein (Supplementary information, Figure S2A), showed a significant enrichment of $P_{mir\text{-}183\text{-}96\text{-}182}$ fragment (Figure 1C, bottom). Moreover, *mir-183* expression in these cells was strongly reduced by ectopic expression of *Ets-1* but was significantly enhanced by *Ets-1* knockdown (Figure 1D), indicating that Ets-1 acts as a transcription repressor of *mir-183*. To further substantiate this, we generated a Mut $P_{mir\text{-}183\text{-}96\text{-}182}$ reporter by mutating the putative Ets-1-binding site (Figure 1C, middle). This mutant promoter obtained a ~2-fold increase in the activity compared with the wild type in MCF-7 cells (Figure 1E). As expected, the wild-type $P_{mir\text{-}183\text{-}96\text{-}182}$ reporter was significantly repressed by *Ets-1* overexpression and stimulated by *Ets-1* knockdown, whereas the Mut $P_{mir\text{-}183\text{-}96\text{-}182}$ was only marginally affected (Figure 1E), further supporting that Ets-1 is a transcriptional repressor of *mir-183-96-182*. We next examined whether miR-155 indeed upregulates *mir-183* through targeting *Ets-1*. We found that miR-155 overexpression significantly increased miR-183 expression and also reduced Ets-1 protein level, whereas the upregulation of *mir-183* by miR-155 was completely reversed when a miR-155-resistant form of *Ets-1* was coexpressed in these cells (Figure 1F). Collectively, these results strongly support that miR-155 upregulates *mir-183* via targeting *Ets-1*.

We next asked whether targeting *Ets-1* is also involved in the miR-155-mediated upregulation of other miRNAs in breast cancer cells. Strikingly, we found that, except the *mir-146a* promoter, all the other 8 promoters of miR-155-upregulated miRNAs contain one or more putative Ets-1-binding site(s) (Supplementary information, Figure S2B). ChIP assays in MCF-7 cells showed that five out of the eight promoter fragments, including $P_{mir\text{-}16}$, $P_{mir\text{-}181c}$, $P_{mir\text{-}181b}$, $P_{mir\text{-}301}$ and $P_{mir\text{-}21}$, were significantly enriched by the anti-Ets-1 antibody but not by control IgG (Figure 1G). Interestingly, knockdown of *Ets-1* in MCF-7 cells significantly enhanced the expression of 10 out of the 12 miR-155-upregulated miRNAs (Supplementary information, Figure S2C), supporting that targeting *Ets-1* represents a common mechanism for the upregulation of miRNAs by miR-155 in breast cancer cells. We reasoned that the lack of $P_{mir\text{-}181a}$ and $P_{mir\text{-}221}$ enrichment by the anti-Ets-1 antibody is likely due to transient interactions between the Ets-1 protein and the two promoters; however, it cannot exclude an indirect repression effect of Ets-1 on *mir-221* or *mir-181a*. Consistently, we found that IL-6 treatment reduced the Ets-1 protein in MCF-7 cells (Supplementary information, Figure S2D) and that ectopic expression of *Ets-1* significantly attenuated the IL-6-induced upregulation of miRNAs (Supplementary information, Figure S2E). Nevertheless, we noted that the expression of miR-146a and miR-203 was marginally altered by *Ets-1* knockdown (Supplementary information, Figure S2C), suggesting that other mechanism(s) might also be involved in the regulation of miRNAs by miR-155.

Noticeably, five miRNAs were significantly repressed by miR-155 (Figure 1A), suggesting a double face of miR-155 in the regulation of miRNA expression in breast cancer cells. Given our previous finding that IL-6 and miR-155 repress *mir-143* via targeting its transcriptional activator *C/EBPβ* in MDA-MB-231 cells [6], which also occurred in MCF-7 cells (Figure 1A and Supplementary information, Figure S3A), we hypothesized that targeting *C/EBPβ* might be responsible for the miR-155-mediated repression of the other 4 miRNAs. To test this hypothesis, we searched the human $P_{mir\text{-}376a}$, $P_{mir\text{-}198}$, $P_{mir\text{-}98}$ and $P_{mir\text{-}205}$ and found that, similar to $P_{mir\text{-}143}$, these 4 promot-

ers all contain a putative C/EBPβ-binding site (Supplementary information, Figure S3B). However, only $P_{mir\text{-}198}$ fragment was enriched by the anti-C/EBPβ antibody in ChIP assays (Supplementary information, Figure S3C), whereas the expression of miR-98 and miR-198 was significantly reduced by *C/EBPβ* knockdown (Supplementary information, Figure S3D). Neither $P_{mir\text{-}376a}$ and $P_{mir\text{-}205}$ fragments were enriched by anti-C/EBPβ antibody, nor their expression was significantly affected by *C/EBPβ* knockdown (Supplementary information, Figure S3D), implicating that additional mechanism(s) may be involved in the miR-155-repressed miRNA expression besides via targeting *C/EBPβ*.

We noted that all the five miR-155-downregulated miRNAs appear to be tumor-suppressive [9] and that several miR-155-upregulated miRNAs (i.e., miR-21, miR-301 and miR-221) are well-documented oncomiRs [2], implicating that the IL-6-driven miR-155-mediated effects would be of functional importance in tumorigenesis. Intriguingly, we found that IL-6 treatment led to a significant increase in cell proliferation and transwell migration of MCF-7 cells, whereas transfection of anti-miR-155 completely overrode the impact of IL-6 on both cell proliferation and migration (Figure 1H and 1I), suggesting that upregulating *mir-155* expression represents an important mechanism of the pro-tumorigenic activity of IL-6. We further tested the clinical relevance of the above findings in human primary breast tumors and found that 8 out of 10 miR-155/Ets-1-regulated miRNAs were significantly upregulated in miR-155 highly expressed breast tumors (n = 16) compared with miR-155 lowly expressed tumors (n = 15) (Supplementary information, Figure S4), supporting that the regulatory axis of miR-155/Ets-1/miRNAs uncovered in our study is of clinical relevance in breast cancer.

Taken together, our data suggest that miR-155 conveys inflammation to tumorigenesis through regulating many cancer-related miRNAs (Figure 1J); mechanistically, miR-155 does so by targeting the key transcription factors, i.e., upregulating a set of miRNAs by targeting their transcriptional repressor *Ets-1* and downregulating others by targeting their transcriptional activator *C/EBPβ*. In summary, our study here shows that miR-155 functions as a master miRNA to link inflammation to the expression of cancer-related miRNAs, providing new connections among inflammation, miRNAs and cancer, and direct supports for miR-155 as a potential target for cancer prevention and therapeutics [2].

Acknowledgments

This work was supported by grants from the Ministry of Science and Technology of China (2011CB811303, 2012CB910802, 2014CB964802) and the National Natural Science Foundation of China (91219306, 31270840, 31170754, 30970621).

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)