Supporting Information

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SI Materials and Methods

Retroviral Infection. The Retro-X Tet-Advanced System (Clontech) was used according to the manufacturer's instruction. Clones stably expressing *miR-155* were prepared from MDA-MB-231 cells and SW620 cells following manufacturers' instructions. In brief, cells were infected first with the *pRetroX-tight-Pur-miR-155* response virus. Colonies resistant for puromycin then were infected with the *pRetroX-Tet-On Advanced* regulator virus and selected for resistance to both puromycin and Geneticin. Throughout the selection process, cells were grown in medium containing Tet-FBS (Clontech) that does not contain any tetracycline residue. A fraction of double-resistant clones then was treated with 500 ng/mL doxycycline for 2 d before *miR-155* expression was analyzed by qRT-PCR. HCT116 cells were transiently infected with a viral suspension containing both the *pRetroX-Tet-On Advanced* regulator vector and the *pRetroX-tight-Pur* response vector containing the construct of interest and then were left to recover for 2 d in regular medium before the addition of doxycycline.

Preparation of Expression Constructs. The *WEE1* reporter construct was prepared by inserting the 3′ UTR of human *WEE1*, prealably amplified by PCR from HEK-293 cells' genomic DNA, downstream of the Luciferase gene in the XbaI site of the *pGL3*-Control vector (Promega). The mature *miR-155* and *miR-155* precursor (*premiR-155*) were cloned in the *pRetroX-tight-Pur* vector following digestion by NotI and EcoRI of double-strand DNAs prepared by reannealing the following primers: *miR-155* mature: V155MatForward: 5′-ATAGCGGCCGCTTAATGCTAATCGTGATAGGGGTGAATTCGCG -3′ and V155MatReverse: 5′-CGCGAATTCACCCCTATCACGATTAGCATTAAGCGGCCGCTAT-3′; *premiR-155*: V155PreForward: 5′-ATAGCGGCCGCCTGTTAATGCTAATCGTGATAGGGGTTTTTGCCTCCAACTGACTCCTACATATTAGCATTAACAGGAATTCGCG-3′ and V155PreReverse: 5′-CGCGAATTCCTGTTAATGCTAATATGTAGGAGTCAGTTGGAGGCAAAAACCCCTATCACGATTAGCATTAACAGGCGGCCGCTAT-3′.

Selection of 6-TG–Resistant Colonies. To eliminate any preexisting *HPRT* mutants, cells were grown in 100 μM hypoxanthine, 400 nM aminopterin, and 16 μM thymidine (HAT medium) Sigma) for 3 d. The MDA-MB-231 cells used for the experiment reported in Fig. 2*C* were cleansed in HAT medium for 15 d. After three washes, cells were resuspended and incubated in regular medium for another 3 d. T47D, HCT116, SW620, or MDA-MB-231 cells then were treated with macrophage-conditioned medium or doxycycline as required. Two days later, HCT116 cells were plated in 96-well round-bottomed plates (1,000 cells per well), and T47D, SW620, or MDA-MB-231 cells were plated in 48-well plates (10^6 cells per plate) in selection medium containing 30 μM 6-TG. *HPRT* mutants then were selected based on their resistance to 6-TG. During the selection process, cells containing the retroviral constructs were constantly stimulated with doxycycline. After 2–3 wk of selection on 6-TG medium (with 6-TG–contaning medium changed every 3 d), plates were stained with crystal violet to allow the visualization and counting of 6-TG–resistant colonies.

Estimation of Mutation Rates. For the experiments with SW620 and MDA-MB-231 stable clones (Fig. 1 and Table S1), mutation rates were adjusted for cell growth and were estimated based on a modified version of fluctuation analysis (18). The cell growth-adjusted mutation rate was analyzed based on the formula $r = f \times \tau/t$, where f is the mutation frequency (mutations per cell), τ is 1/cell division rate (in cell divisions per day), and t is the length of *miR-155* induction (in days). For the experiments with MDA-MB-231 cells (Fig. 2*C*), the estimated mutation rate was based on the average mutant frequency and population doubling (23, 24) according to the schema shown in Fig. S5. Mutant frequency and population doubling were estimated at each of the steps shown thereafter (i.e., right after HAT cleansing, 3 d after HAT cleansing, 3 d after mock (control) or TNF/LPS treatment, and 3 d after the end of the treatment). Cells were plated in 6-TG–supplemented medium at a density of 1.5×10^6 cells per 10-cm dish. Additionally, plating efficiency (PE) at the time of selection was determined by plating 500 cells per 10-cm dish in triplicate in RPMI medium without hypoxanthine. Cells were incubated for 14–20 d, and colonies were visualized by staining with 0.5% crystal violet in 4% paraformaldehyde (Sigma). Mutant frequency (MF) then was determined as follows: $MF = a/(60 \times 10^6 \times [b/1.5 \times 10^3])$, where a is the total number of 6-TG–resistant colonies, and b is the total number of colonies on all three plates (23, 24). PE and the exact number of cells subcultured were used to calculate population doubling (PD) as follows: $PD = (\ln[\text{total number of cells}] - \ln[\text{number of cells plated} \times PE])/\ln 2$. Mutation rate was estimated by plotting the observed mutant frequencies as a function of PD and calculating the slope by linear regression. This slope yields the mutation rate (mutations per cell per generation) (23, 24).

Analysis of *HPRT* cDNA Mutations. The 6-TG–resistant colonies from *miR-155*-Off and *miR-155*-On infected cells were selected randomly as representative mutant clones. Clones were expanded for 1 wk before RNA extraction. Total RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor from Applied Biosystems. *HPRT* cDNAs (nucleotides 123–1,110) were amplified subsequently by PCR using the Advantage 2 Polymerase Mix from Clontech, with the forward primer 5′-GCGCGCCGGCCGGCTCCGTT-3′ and the reverse primer 5′-GGCGATGTCAATAGGACTCCAGATG-3′. In most cases, the PCR products were cloned in the *TOPO* vector (Invitrogen) and subsequently sequenced following plasmid purification. In other cases, the PCR products were purified using the PCR purification kit from Qiagen and were directly sequenced at the sequencing facility at Ohio State University using the primers 5′-GCCGGCCGGCTCCGTTATGG-3′ and 5′-ATGTCAATAGGACTCCAGATG-3′.

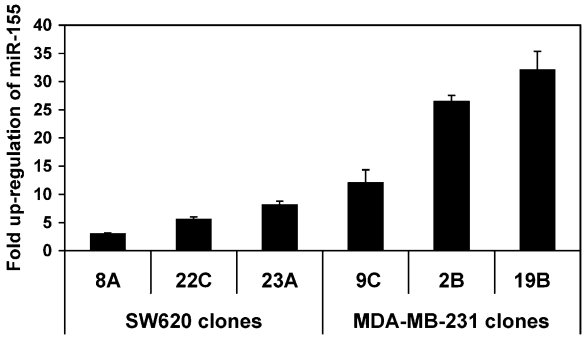

Fig. S1. The levels of microRNA-155 (*miR-155*) expression vary with the clones and the cell lines. SW620 and MDA-MB-231 clones stably transfected with a *pRetroX-tight-Pur* construct expressing mature *miR-155* were mock treated or treated with doxycycline for 48 h. The relative levels of *miR-155* were determined subsequently using quantitative RT-PCR. The figure gives the ratios of the values for mock-treated/doxycycline-treated cells. Values represent mean ± SD (n = 3).

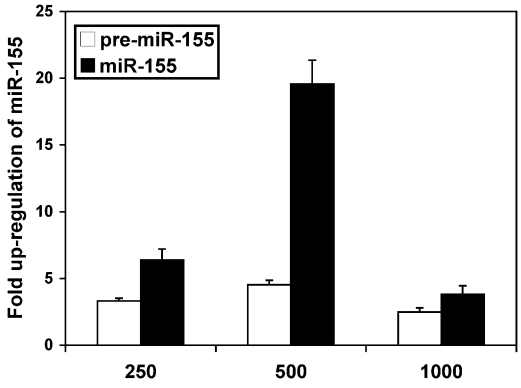

Fig. S2. Effects of increasing doses of doxycycline on the expression of *miR-155*. HCT116 cells were transiently transfected with a *pRetroX-Tight-Pur* construct expressing *miR-155* precursor (premiR-155) or *miR-155* mature form (miR-155) before 48-h treatment with the indicated doses of doxycycline (ng/mL).

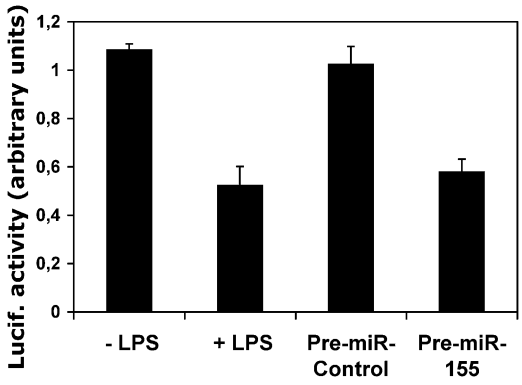

Fig. S3. *miR-155* targets the WEE1 3′ UTR. T47D cells transfected with a reporter construct containing the 3′ UTR of WEE1 downstream of the luciferase coding region were treated with an unstimulated macrophage-conditioned medium (– LPS) or with LSMCM (+ LPS) or were transfected with premiR-Control or *premiR-155*. Results were normalized to Renilla luciferase. Values represent mean ± SD (n = 5).

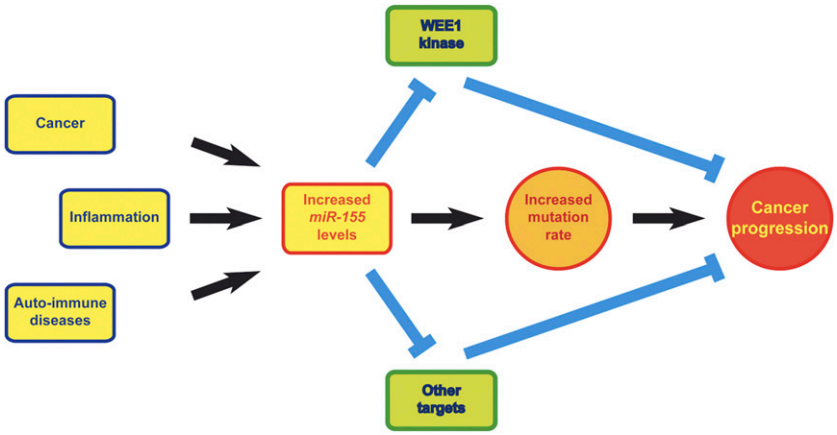

Fig. S4. Schematic representation summarizing the main results of this paper. The up-regulation of *miR-155* over a prolonged period as a consequence of chronic inflammation or the deregulation of endogenous genetic circuitries in cancer or other diseases may lead to higher mutation rates in vivo. We found that the targeting of WEE1 by *miR-155* would further extend DNA damage, as previously established (1). The up-regulation of *miR-155* also down-regulates tumor-suppressor factors and other factors controlling cell homeostasis (Table 2 and Fig. S4). Taken together, these effects can shorten the process of malignant transformation and favor cancer progression.

1. Beck H, et al. (2010) Regulators of cyclin-dependent kinases are crucial for maintaining genome integrity in S phase. *J Cell Biol* 188:629–638.

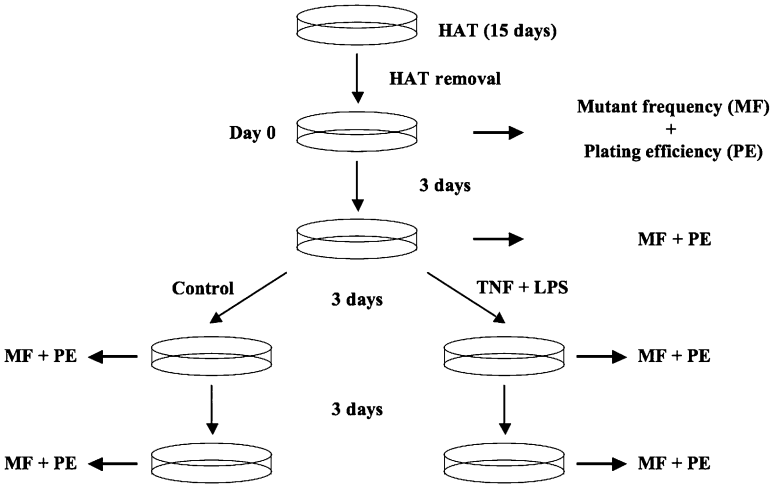

Fig. S5. Schematic representation summarizing the experimental design of Fig. 2*C*. HAT, 100 μM hypoxanthine, 400 nM aminopterin, 16 μM thymidine.

Table S1. Effects of *miR-155* overexpression on the frequency of 6-thioguanine (6-TG)–resistant colonies and the average mutation rate

Table S1

Table S2. Mutations found in *HPRT* cDNAs prepared from 6-TG–resistant colonies of human HCT116 colon cancer cells and from human T47D and MDA-MB-231 breast cancer cells after exposure to LPS-stimulated macrophage-condionned medium or doxycycline-induced overexpression of miR-155 microRN.

Table S2

Mutations found in hypoxanthine phosphoribosyltransferase (*HPRT*) cDNA prepared from 6-TG–resistant colonies of human HCT116 colon cancer cells and from human T47D and MDA-MB-231 breast cancer cells after exposure to LPS-stimulated macrophage-conditioned medium (LSMCM) or doxycycline-induced overexpression of miR-155 microRNA. The length of *HPRT* transcribed region was 1,415 nt. The *HPRT* region analyzed was nucleotides 123–1,110.
[a]Mock, unstimulated macrophage-conditioned medium.
[b]LSMCM, LPS-stimulated macrophage-conditioned medium.
[c]*HPRT* coding region, nucleotides 168–824.

Table S3. Validated targets *of miR-155* microRNA that play a role as tumor-suppressors or regulators of cell homeostasis

Table S3

The validated targets of *miR-155* have been reviewed recently (1). *APC*, adenomatous polyposis coli; *BACH1*, BTB and CNC homology 1, basic leucine zipper transcription factor 1; *CUTL1*, cut-like homeobox 1; *FADD*, Fas (TNFRSF6)-associated via death domain; *JARID2*, jumonji, AT-rich interactive domain 2; *FOXO3*, forkhead box O3; *KGF*, keratinocyte growth factor; *HIVEP2*, HIV type I enhancer-binding protein 2; *MYO10*, myosin X; *RHOA*, Ras homolog gene family, member A; *RIP1*, receptor-interacting protein kinase 1; *SHIP1*, inositol polyphosphate-5-phosphatase; *SMAD1/5*, SMAD family member 1/5; *SOCS1*, suppressor of cytokine signaling 1; *TP53INP*, Tumor protein 53-induced nuclear protein 1.

1. Tili E, Croce CM, Michaille JJ (2009) miR-155: On the crosstalk between inflammation and cancer. *Int Rev Immunol* 28:264–284.