Mutator activity induced by microRNA-155 (*miR-155*) links inflammation and cancer

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Infection-driven inflammation has been implicated in the pathogenesis of ∼15–20% of human tumors. Expression of microRNA-155 (*miR-155*) is elevated during innate immune response and autoimmune disorders as well as in various malignancies. However, the molecular mechanisms providing *miR-155* with its oncogenic properties remain unclear. We examined the effects of *miR-155* overexpression and proinflammatory environment on the frequency of spontaneous hypoxanthine phosphoribosyltransferase (*HPRT*) mutations that can be detected based on the resistance to 6-thioguanine. Both *miR-155* overexpression and inflammatory environment increased the frequency of *HPRT* mutations and down-regulated WEE1 (WEE1 homolog-S. pombe), a kinase that blocks cell-cycle progression. The increased frequency of *HPRT* mutation was only modestly attributable to defects in mismatch repair machinery. This result suggests that *miR-155* enhances the mutation rate by simultaneously targeting different genes that suppress mutations and decreasing the efficiency of DNA safeguard mechanisms by targeting of cell-cycle regulators such as WEE1. By simultaneously targeting tumor suppressor genes and inducing a mutator phenotype, *miR-155* may allow the selection of gene alterations required for tumor development and progression. Hence, we anticipate that the development of drugs reducing endogenous *miR-155* levels might be key in the treatment of inflammation-related cancers.

genome stability | LPS | TNF | proliferation | transgenic mice

It now is well established that chronic and persistent inflammation contributes to cancer development (1–3). Infection-driven inflammation is involved in the pathogenesis of ∼15–20% of human tumors, and tumor-infiltrating leukocytes, such as monocytes/macrophages, T lymphocytes, and neutrophils, are prime regulators of cancer inflammation (1–3). Furthermore, even tumors that are not epidemiologically linked to pathogens are characterized by the presence of an inflammatory component in their microenvironment.

MicroRNAs (miR) are a class of short, noncoding RNAs that are implicated in many aspects of cell biology. *MiR-155* is implicated in hematopoiesis, the innate and acquired immune response, and autoimmune disorders (4–9). A direct link between elevated levels of *miR-155* and the formation and development of tumors such as leukemias and breast, lung, or gastric cancers has been established recently (4, 5, 9–12). Thus, transgenic mice overexpressing *miR-155* in B cells or hematopoietic stem cells show enhanced proliferative disorder that results in high-grade lymphoma (13, 14). Being oncogenic and implicated in inflammation, *miR-155* is the prototype of microRNAs that stand between inflammation and carcinogenesis. However, the molecular mechanisms behind this link remain unclear. Because *miR-155* targets transcripts implicated in DNA repair (15), we evaluated the mutator activity of *miR-155* and that of the *miR-155*–linked inflammatory environment as a potential mechanism connecting inflammation and cancer. In this study we report that *miR-155* and inflammatory stimuli increase the spontaneous mutation rate.

Results

Overexpression of *MiR-155* Results in Enhanced Mutation Rate. *MiR-155* targets core components of the DNA mismatch repair (MMR) machinery, among other mutator pathways (15), suggesting that elevated levels of *miR-155* might enhance the rate of spontaneous mutations. To measure the mutation rate, we took advantage of the hypoxanthine phosphoribosyltransferase (HPRT) locus, which is a well-established method for estimating mutation rate. The HPRT enzyme catalyzes the conversion of guanine into guanine monophosphate and hypoxanthine into inosine monophosphate in the purine salvage pathway (16, 17). The loss of HPRT function confers resistance to 6-thioguanine (6-TG), because 6-TG becomes cytotoxic only after phosphoribosylation by HPRT. This resistance can be used to identify cells that have acquired mutations at the HPRT locus (16, 17). Because the acquired mutations are thought to occur randomly, the HPRT gene can be used as a reporter gene, and the frequency of mutation at the HPRT locus can be used as an estimate of global genomic instability. To measure the effects of *miR-155* on mutation rate, we first developed stable clones of SW620 colorectal adenocarcinoma cells and MDA-MB-231 breast adenocarcinoma cells expressing mature *miR-155* under the control of the *Tet-On* inducible system. Incubation of SW620 clones 8A, 22C, and 23A with doxycycline increased *miR-155* expression by 2.94 ± 0.23-, 5.58 ± 0.43-, and 8.10 ± 0.65-fold, respectively (mean ± SD) (Fig. S1 and Table S1). Similarly, doxycycline treatment increased *miR-155* expression by 12.01 ± 2.34-, 26.42 ± 1.11-, and 32.07 ± 3.27-fold in MDA-MB-231 clones 9C, 2B, and 19B, respectively.

The cell growth-adjusted *HPRT* mutation rate, estimated based on a modified version of fluctuation analysis (18), increased with *miR-155* levels in both SW620 and MDA-MB-231 cell clones (Fig. 1 *A* and *B*). Constant elevated expression of *miR-155* the enhanced mutation rate by up to 3.39-fold in SW620 clones and up to 3.47-fold in MDA-MB-231 clones (Table S1). Moreover, HCT116 colorectal carcinoma cells transiently overexpressing *miR-155* by 19.57 ± 0.62-fold under the control of *Tet-On* inducible system (Fig. S2) showed a 2.81-fold higher mutation rate (Table S1). These results establish a direct link between mutation rate and *miR-155* levels.

As expected, the basal spontaneous cell growth-adjusted mutation rates of SW620 and MDA-MB-231 cells was comparable ($0.75 \pm 0.27 \times 10^{-7}$ and 1.28×10^{-7} mutations per cell, respectively) and were ∼440-fold lower than the spontaneous mutation

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Data deposition: Microarray data have been submitted to the MIAMExpress (Minimum Information About a Microarray Experiment, www.ebi.ac.uk/miamexpress) with accession no. E-TABM-1118.

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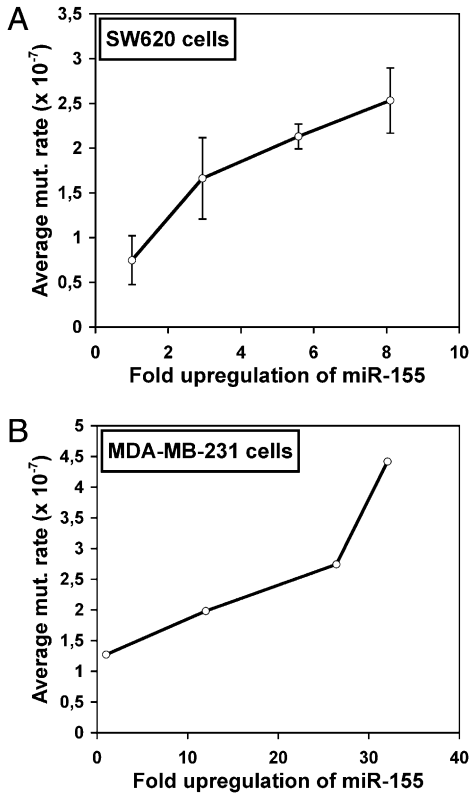

Fig. 1. The average mutation rate in SW620 and MBA-MD-231 cells increases with the rate of *miR-155* expression. (*A* and *B*) Average mutation rates of 6-TG–resistant colonies from SW620 (*A*) and MDA-MB-231 clones (*B*) stably expressing *miR-155* and either mock-treated or treated with doxycycline, plotted against the fold upregulation of *miR-155* values. Fold upregulation 1 value corresponds to mock-treated controls.

rate of HCT116 cells (560×10^{-7} mutations per cell) (Table S1), because HCT116 cells contain a deletion of the *hMLH1* (human MutL homolog 1) MMR gene (19). The deletion of the *hMLH1* MMR gene decreased the *miR-155*-induced mutator activity only partially, suggesting that *miR-155*–induced mutator activity is not very sensitive to the basal level of mutation rate (i.e., to the integrity of the DNA safeguarding machinery) and that *miR-155* targets additional transcripts implicated in DNA repair and/or genome stability.

Inflammatory Stimuli Up-Regulate *MiR-155* in Breast Cancer Cells. Infection-driven inflammation has been associated with cancer (20). A number of studies have shown that *miR-155* is a key component of the inflammatory response (4–7). We thus screened human cell lines for the effects of proinflammatory environment on *miR-155* expression. We included colon cancer cell lines (SW480, SW620, HCT15, HCT116, and RKO), because a fraction of colorectal cancers appear linked to the inflammatory environment (20), breast (MDA-MB-231, T47D, 453, 436, and MCF7) and lung cancer (A459) cell lines, because *miR-155* is up-regulated in these types of cancers (12), and four other cell lines for comparison. Cells were treated overnight with the supernatant of LPS-stimulated human THP-1 monocytic cells, namely LPS-stimulated macrophage-conditioned medium (LSMCM), which contains many inflammatory cytokines such as TNF, IL-6, IL-8, and IL1-β (1, 3). Based on quantitative RT-PCR (qRT-PCR) analyses, *miR-155* expression in colon and lung cancer cell lines was affected only slightly by LSMCM (Fig. 2*A*). In contrast, *miR-155* levels increased by 9-, 17-, and 21-fold in MDA-MB-231, BC-453, and T47D breast cancer cell lines, respectively. We also analyzed the expression of *miR-146a*, a microRNA that is up-regulated in certain tumors (10, 11) and in LPS-challenged THP-1 cells. It has been proposed that *miR-146a* controls the termination of the immune response (21). In sharp contrast with *miR-155*, the highest *miR-146a* levels were found in HCT15 and HCT116 colon cell lines (not shown), indicating that the up-regulation of *miR-155* and *miR-146a* by inflammatory stimuli occurs independently in the above cancer cell lines and probably is tissue specific. Because both TNF (22) and LPS can induce *miR-155* (6, 7), we analyzed the effects of these two molecules in MDA-MB-231 cells. As expected, stimulation with either TNF or LPS or with both increased *miR-155* expression (Fig. 2*B*). We therefore used TNF/LPS to mimic the effects of a proinflammatory environment.

Inflammatory Stimuli Enhance the Mutation Rate. In MDA-MB-231 cells without stimulation, the mutation rate was calculated to be 0.69×10^{-7} mutations per cell per generation, a value similar to that previously found in SW480 cells (0.75×10^{-7}) (23). The estimated mutation rate was based on the average mutant frequency and population doubling (23, 24). Of note, both MDA-MB-231 and SW480 cells have intact DNA-repair machinery, unlike HCT116 cells. Mutant frequencies already had became significantly different ($P = 0.038$) 3 d after treatment, with mutation rate increasing by 2.52-fold to 1.73×10^{-7} mutations per cell per generation (Fig. 2*C*) in TNF/LPS-treated MDA-MB-231 cells vs. untreated control cells. The treatment lowered the rate of cell proliferation, probably because TNF induces growth arrest in breast cancer cells (25). Accordingly, one or two LSMCM stimulations of T47D cells increased the frequency of 6-TG–resistant colonies by 50% and 150%, respectively (Fig. 2*D*). Thus, proinflammatory signals resulting in the up-regulation of *miR-155* expression induce a significant, although moderate, mutator phenotype, which might be enhanced with chronic inflammation.

Characterization of HPRT Mutants. We then analyzed *HPRT* mutations found in cDNAs prepared from RNA extracted from T47D, HCT116, and MDA-MB-231 6-TG–resistant colonies to determine the mutation signature (Table 1 and Table S2). As expected, *HPRT* mutations from doxycycline-treated HCT116 cells displayed single base deletions or insertions of the type generally found in DNA MMR-deficient cells. The majority displayed a frameshift, transition, and transversion mutation signature consistent with an MMR defect. There also was an increase in insertions and exon deletions with *miR-155* overexpression; this increase generally has been ascribed to altered recombination repair (26). In contrast, deletion mutations consistent with recombination repair defects accounted for the majority of *HPRT* mutations in LSMCM-stimulated T47D cells and doxycycline-treated MDA-MB-231 cells, regardless of conditions (Table 1 and Table S2). These results are consistent with a role for recombination repair, exemplified by breast cancer 1 (BRCA1) and breast cancer 2 (BRCA2) mutations, in these breast tumor cell lines (27). Of note, these types of mutations have been found previously in several T-cell leukemic cell lines (16, 28). However, we noted a modest increase in transitions and transversions consistent with decreased MMR in these cells.

Overexpression of *miR-155* Enhances Cell Proliferation. Remarkably, *miR-155* up-regulation increased the size of HCT116 (Fig. 3*A*) and MDA-MB-231 *HPRT* mutant colonies and allowed them to appear earlier during the selection process. Based on a forward scatter comparison, larger colonies of MDA-MB-231 clone 19B, that presented a 32-fold up-regulation of *miR-155* after doxycycline treatment, did not arise from the presence of larger cells (not shown). In contrast, carboxyfluorescein succinimidyl ester (CFSE) staining suggested that these cells underwent at least one extra round of cell division within 4–5 d as compared with untreated cells (Fig. 3*B*). These results correlate with reports showing that *miR-155* promotes proliferation in transgenic mice (13, 14). Thus, the larger size of *HPRT* mutant colonies overexpressing *miR-155* probably arises from enhanced cell proliferation.

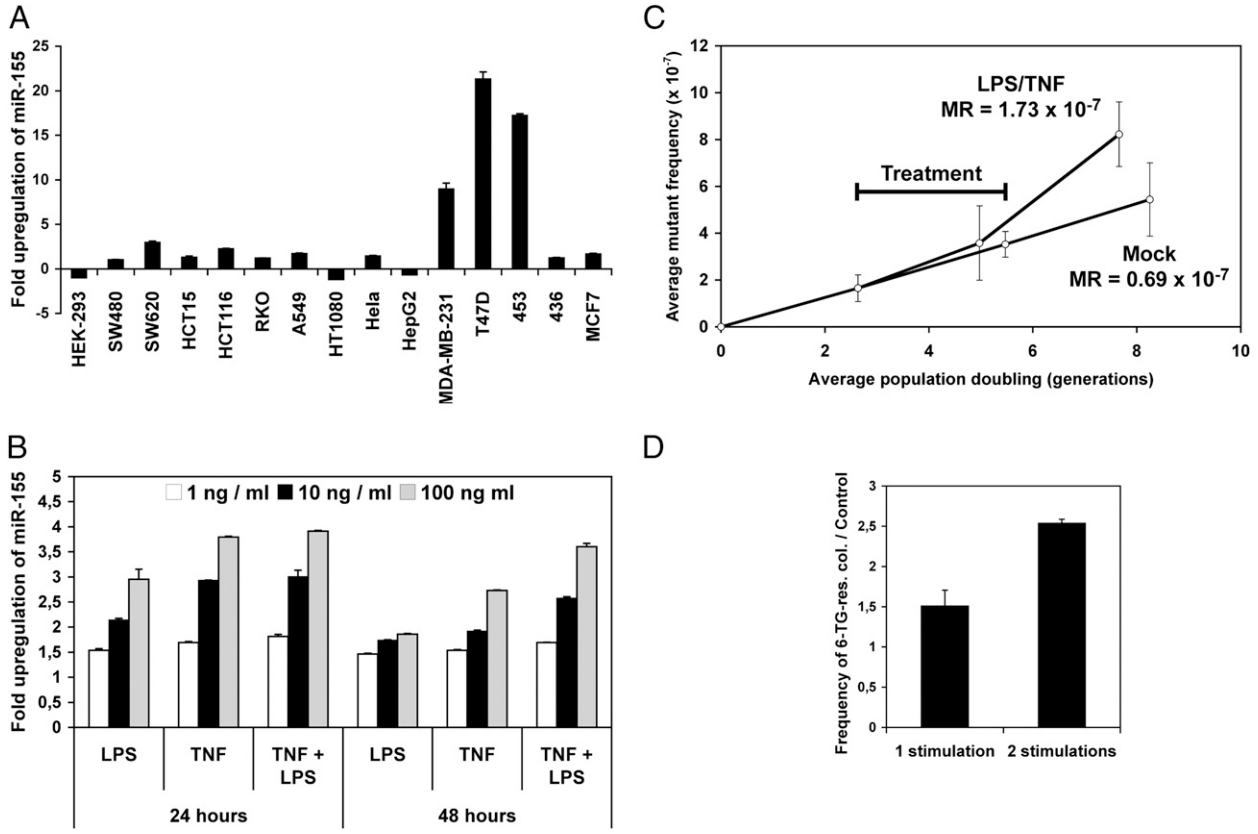

Fig. 2. Proinflammatory environment increases the mutation rates in MDA-MB-231 cells and the frequency of mutant colonies in T47D cells. (*A*) The relative up-regulation of *miR-155* in the indicated cell lines either mock treated or treated with LSMCM for 48 h were determined using qRT-PCR. The figure gives the ratios LPS-stimulated/unstimulated. Values represent mean ± SD (n = 3). (*B*) The relative up-regulation of *miR-155* in MDA-MB-231 cells treated as indicated was determined using qRT-PCR. (*C*) The mutation rates (MR) in MDA-MB-231 cells were estimated by calculating the slopes of the curves following mock and TNF/LPS treatment (n = 4 estimations of mutation frequency). (*D*) Ratios of 6-TG–resistant colonies in T47D cells treated with LSMCM for 48 h versus control mock-treated cells.

***MiR-155* and Inflammatory Environment Down-Regulate WEE1, a Cell-Cycle Inhibitor.** *MiR-155* might enhance cell proliferation by targeting cell-cycle regulators. Indeed, in T47D cells, both LSMCM treatment and *miR-155* overexpression reduced the levels of WEE1, a kinase that catalyzes the inhibitory tyrosine phosphorylation of Cdc2/cyclin B, blocking cell-cycle progression at

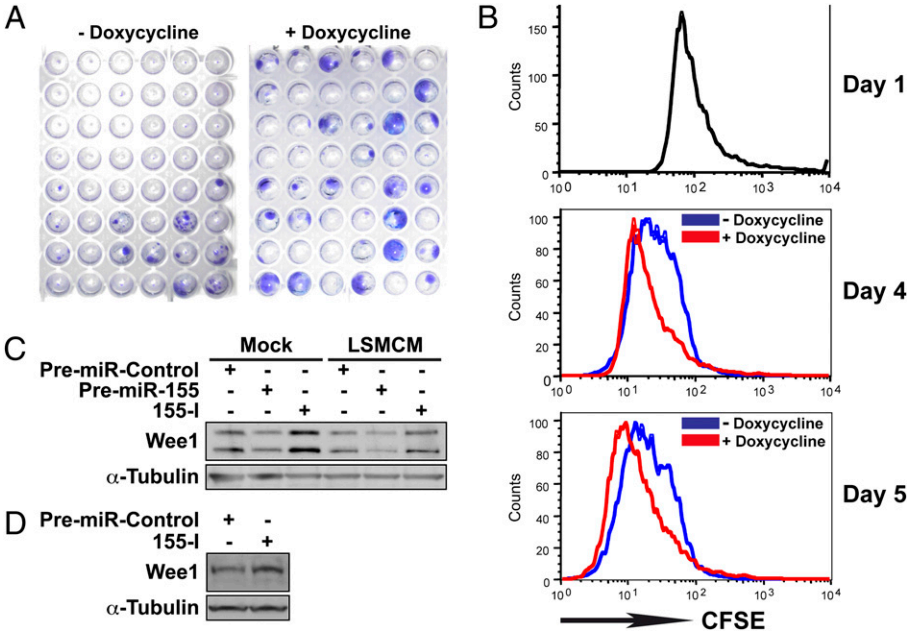

Fig. 3. Elevated *miR-155* levels increase the rate of proliferation by targeting *WEE1* transcripts. (*A*) Phenotypes of 6-TG–resistant HCT116 colonies following 48-h mock treatment (– doxycycline) or 48-h doxycycline treatment (+ doxycycline). HCT116 cells were transiently infected with *pRetroX-Tight-Pur-miR-155* and *Tet-On* constructs before doxycycline treatment. (*B*) Cells from MDA-MB-231 clone 19B were stained with CFSE before induction of *miR-155* expression by doxycycline treatment. The proliferation rate was analyzed by flow cytometry 4 and 5 d later. The experiment was repeated two times with similar results. (*C*) The levels of WEE1 in T47D cells transfected with premiR-Control, *premiR-155*, or antisense *miR-155* inhibitory RNA (*155-I*) and subsequently either mock treated or treated with LSMCM for 48 h were determined by Western blotting. (*D*) The levels of Wee1 in primary B cells isolated from the spleen of *Eμ-miR-155* transgenic mice after transfection with premiR-Control or antisense *miR-155* inhibitory RNA (*155-I*).

Table 1. Mutations found in HPRT cDNAs prepared from 6-TG–resistant colonies of T47D, HCT116, and MDA-MB-231 cells

Cell lines	Treatment	Number of clones analyzed	Clones without mutation in the coding sequence	Clones with frameshift mutation(s)	Clones with deletion(s)	Clones with only a single deletion	Clones with one or more exons lacking	Clones with other types of deletions	Clones with insertion(s)	Clones with transition(s)	Clones with transversion(s)
T47D	Mock	24	0	12	14	17	24 Del ex. 3: 11 Del ex. 2+3: 9 Del ex. 4+5: 4	4 Del 1: 7	1 ins 1: 1	4 Transitions: 5	0
T47D	LSMCM	40	0	20	40	25	38 Del l ex. 3: 14 Del ex. 2+3: 16 Del ex. 4 + 5: 5 Del ex. 2+3+4: 1 Del ex. 6+7+8: 1 Del ex. 5: 1	11 Del 17: 3 Del 1: 21	3 Ins 1: 3	7 Transitions: 7	1 Transitions: 3
HCT116	– Doxycycline	14	1	13	13	0	0	13 Del 2: 1 Del 1: 12	4 Ins 1: 5	0 Transitions: 7	2 Transversions: 3
HCT116	– Doxycycline	14	1	13	13	0	0	13 Del 2: 1 Del 1: 12	4 Ins 1: 5	0	2 Transversions: 2
HCT116	+ Doxycycline	25	4	18	11	0	2 Del ex. 3: 1 Del ex. 2+3: 1	9 Del 1: 16	13 Ins 1: 17	6 Transitions: 6	6 Transversions: 8
MDA-MB-231	– Doxycycline	11	3	8	10	5	8 Del ex. 2+3: 1 Del ex. 7: 8	3 Del 1: 5	0	1 Transitions: 1	0
MDA-MB-231	+ Doxycycline	24	2	17	21	11	17 Del ex. 2: 1 Del ex. 2 + 3: 1 Del ex. 7: 16	9 Del 1: 17	0	4 Transitions: 6	2 Transversions: 2

Ex., exon; LSMCM, LPS-stimulated macrophage-conditioned medium; Mock, unstimulated macrophage-conditioned medium.

the G_2/M phase (Fig. 3*C*) (29). In contrast, an antisense *miR-155* inhibitory RNA (*155-I*) increased WEE1 accumulation. Both LSMCM and *miR-155* overexpression also reduced the expression of a luciferase reporter construct containing the *WEE1* 3′ UTR (Fig. S3). Accordingly, *155-I* increased Wee1 levels in primary B cells isolated from *Eμ-miR-155* transgenic mice that overexpress *miR-155* in B-cell lineage (13), thus confirming that Wee1 is a bona fide *miR-155* target (Fig. 3*D*). Taken together, these results show that inflammatory stimuli down-regulate WEE1 through up-regulation of *miR-155*. Because WEE1 depletion rapidly induces DNA damage in newly replicated DNA (30), these results suggest that *miR-155* overexpression may shorten the period required for selection of cancer-associated mutations. Furthermore, Affymetrix microarrays revealed that transcripts coding for several factors controlling cell cycle, DNA repair, and genome stability were affected by LSMCM in both T47D and MDA-MB-231 cell lines (Table 2). This result suggests that the ability of inflammatory stimuli to induce defective checkpoints and genomic instability, similar to *miR-155*, might contribute to tumorigenesis.

Discussion

In this study we analyzed the mutator activity of *miR-155* and of the *miR-155*–related proinflammatory environment. Cells in which inflammatory stimuli resulted in the up-regulation of *miR-155* showed a two- to threefold increase in the mutation rate as deduced by HPRT assay. Furthermore, inducible expression of *miR-155* resulted in a similar increase in mutation rate, suggesting that the up-regulation of the mutation rate by the inflammatory stimuli is *miR-155* dependent. The mutation rate was not increased in cells in which inflammatory stimuli up-regulated only *miR-146*, another microRNA implicated in the innate immune response (data not shown).

Although *miR-155* levels in MDA-MB-231 cells were increased consistently by 12- and 32-fold during doxycycline treatment, they increased transiently by only approximately fourfold after LPS/TNF treatment (Figs. 1*B* and 2*B*). Nevertheless, the mutation rate increased by 1.56- to 3.47-fold after doxycycline treatment and by 2.52-fold after TNF/LPS treatment (Figs. 1*B* and 2*C*). This result suggests that increased *miR-155* levels resulting from chronic inflammation (4–7), autoimmune diseases (8, 9), or the deregulation of endogenous genetic circuitries with the onset of cancer (4) might produce a significant mutator phenotype. These results also suggest that other inflammatory signaling pathways may work in synergy or in parallel with *miR-155*. Of note, *miR-155* has been shown to target tumor suppressor genes such as Fas-associated via death domain (*FADD*), Jumonji AT-rich interactive domain 2 (*JARID2*), and Src homology 2-containing inositol phosphatase-1 (*SHIP1*) (Table S3). In addition, other microRNAs with mutator activity might potentially be up-regulated by LPS signaling.

The increased mutation rate in HCT116 cells that lack the hMLHl DNA repair enzyme suggested that this increase occurs through *miR-155* targeting of other transcripts involved in DNA repair, recombination, or cell-cycle checkpoints. Because mutations accumulate during the S phase, when the replication of the DNA takes place right before the G2/M check point, we looked for transcripts that are predicted targets of *miR-155* and act as inhibitors of G2/M transition because their reduced expression might be associated with an increased mutation rate. In addition these transcripts should be targets of LPS/TNF signaling. We concentrated on WEE1 kinase, because it fulfilled all these criteria. In T47D cells, overexpression of *miR-155* or treatment with LSMCM resulted in the down-regulation of WEE1 expression. By targeting WEE1 and consequently facilitating G2/M transition, *miR-155* potentially allows cells that have not yet repaired the DNA to proceed to mitosis, resulting in accumulated mutations. Akt kinase also is known to function as a G2/M initiator and to inactivate WEE1 by phosphorylation, thus promoting the cell-cycle transition. It was reported recently that Akt (v-akt murine thymoma viral oncogene homolog 1) is implicated in LPS signaling by modulating the levels of *miR-155*, among other microRNAs (31). It is possible that oncogenic Akt and onco-inflammatory *miR-155* cross talk at the level of WEE1 during inflammation. We consider that the increased mutation rate associated with inflammatory signals is a combinatorial effect of *miR-155* targeting of WEE1 and other DNA repair enzymes that are down-regulated by LPS and are either direct or indirect targets of *miR-155*. It is believed that cancer results from the accumulation of mutations in somatic cells, and this study suggests that, by increasing the mutation rate, the inflammatory *miR-155* might be the key player in inflammatory-induced cancers in general.

The control of cell-cycle progression and DNA repair in eukaryotes are highly conserved. However, in the event of an infection the cells must respond quickly by producing cytokines, chemokines, and other inflammatory components of the immune defense. During this robust response, it is possible that the DNA repair machinery and cell-cycle checkpoints are put on hold. At this stage the up-regulation of *miR-155* by inflammatory stimuli to clear the antigen quickly also results in an increased mutation rate. Furthermore, regardless of the primary cause of a mutation, there is a high probability that, in the event of an infection, the mutation will be fixed. In conclusion, we believe that simultaneous *miR-155*–driven suppression of a number of tumor suppressor genes combined with a mutator phenotype allows the shortening of the series of steps required for tumorigenesis and represents a model for cancer pathogenesis (Fig. S4). Thus, the up-regulation of *miR-155* by chronic inflammation appears to

Table 2. Transcripts encoding factors related to DNA replication and maintenance whose levels decrease significantly following treatment of T47D and MDA-MB-231 cells

Symbol	Gene name	Fold change
RFC5	Replication factor C (activator 1) 5, 36.5kDa	0.72
NEIL3	Nei endonuclease VIII-like 3 (*E. coli*)	0.71
AURKB	Aurora kinase B	0.71
GTSE1	G-2 and S-phase expressed 1	0.64
RAD54L	RAD54-like (*S. cerevisiae*)	0.75
ARL3	ADP ribosylation factor-like 3	0.81
BCCIP	BRCA2 and CDKN1A interacting protein	0.77
SKP2	S-phase kinase-associated protein 2 (p45)	0.65
RDM1	RAD52 motif 1	0.75
PARP2	Poly (ADP ribose) polymerase 2	0.86
RAD54B	RAD54 homolog B (*S. cerevisiae*)	0.67
ERCC6L	Excision repair cross-complementing rodent repair deficiency, complementation group 6-like	0.61
DCTPP1	dCTP pyrophosphatase 1	0.78
DDB2	Damage-specific DNA binding protein 2, 48kDa	0.84
CDKN2C	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0.64
AK3L1	Adenylate kinase 3-like 1	0.84
APRT	Adenine phosphoribosyltransferase	0.84
DDB2	Damage-specific DNA binding protein 2, 48kD	0.84
BRCC3	BRCA1/BRCA2-containing complex, subunit 3	0.83
TOP2A	Topoisomerase (DNA) II alpha 170kD	0.77
CDT1	Chromatin licensing and DNA replication factor 1	0.74
RECQL4	RecQ protein-like	0.72
PARP1	Poly (ADP ribose) polymerase 1	0.86

After Affymetrix microarray analyses, comparisons were done between two pools of cells treated with either unstimulated macrophage-conditioned medium or with LPS-stimulated macrophage-conditioned medium. The two pools contained four independent replicates from both T47D and MDA-MB-231 cell lines. Transcripts are arranged according to the decreasing values of P (all $<1 \times 10^{-7}$).

indicate at least one of the missing links between cancer and inflammation.

Materials and Methods

Cell Culture, Transfection, and Treatment. Cells were grown following standard procedures. T47D cells were transfected using lipofectamine (Invitrogen). Unstimulated LSMCM were prepared from the supernatant of human THP-1 monocytic cells mock stimulated or stimulated with *Salmonella enteritidis*-derived LPS (100 ng/mL, Sigma) for 6 h. THP-1 cells subsequently were centrifuged, and the supernatant was filtrated to eliminate any remaining cells. T47D cells then were cultivated in the presence of unstimulated medium or LSMCM for 48 h. When needed, a second stimulation was conducted in the same way, after the cells had been allowed to recover 4 d in regular medium. TNF was obtained from Invitrogen. The B-cell line was established by purifying B cells from the spleen of an *Eμ-miR-155* transgenic mouse using the isolation kit from R & D Systems. B cells subsequently were cultured for 2 wk in 100 ng/mL RPMI/15% FBS/LPS and for 3 more weeks without LPS. They were electroporated using the Amaxa kit (Lonza).

Preparation of Retroviral Expression Constructs and Retroviral Infection. See *SI Materials and Methods* for information.

Selection of 6-TG–Resistant Colonies, Estimation of Mutation Rates, and Analysis of HPRT cDNA Mutations. See *SI Materials and Methods* for information.

Isolation of RNAs and qRT-PCR. RNA was extracted with TRIzol (Invitrogen) and subsequently subjected to DNase digestion (Turbo-DNase; Ambion). *MiR-155* qRT-PCR was performed using TaqMan MicroRNA Assays (Applied Biosystems). Values were normalized using *RNU-44*. Real-time PCR was run in triplicate from three different cDNAs.

Flow Cytometry Analysis. CFSE was purchased from Molecular Probes/Invitrogen. CFSE staining was carried out using manufacturer's protocol. Cells were fixed in 1% paraformaldehyde before analyses. Flow cytometry analyses were performed at the Flow Cytometry facility of Ohio State University. Data were analyzed using the software program FlowJo (Tree Star, Inc.).

Western Blots. Cells were lysed 48 h after transfection or electroporation. Anti-WEE1 and anti–α-tubulin antibodies were from Cell Signaling Technology.

Affymetrix Microarray Analyses. RNAs extracted with TRIzol (Invitrogen) were subsequently subjected to DNase digestion (Turbo-DNase; Ambion). Affymetrix microarray analyses were done at the Ohio State University microarray facility.

Luciferase Assays. Cells plated in 12-well plates (1×10^6 cells per plate) were transfected with 0.4 μg of DNA (*pGL3*-control vector or *WEE1* reporter constructs; Promega), 20 ng of Renilla luciferase control vector (*pRL-TK*; Promega), and 50 nM of either a premiR control (premiR Precursor Molecule-Negative Control #1; Ambion), *premiR-155* (*miR-155* precursor; Ambion), or *155-I* (an antisense *miR-155* inhibitory RNA; Ambion). Assays were performed 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

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