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MiR203 mediates subversion of stem cell properties during mammary epithelial differentiation via repression of ΔNP63α and promotes mesenchymal-to-epithelial transition

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During reproductive life, the mammary epithelium undergoes consecutive cycles of proliferation, differentiation and apoptosis. Doing so relies on the retained proliferative capacity, prolonged lifespan and developmental potency of mammary stem cells (MaSCs). ΔNp63α, the predominant *TP63* isoform in mammary epithelia, is robustly expressed in MaSCs and is required for preservation of self-renewing capacity in diverse epithelial structures. However, the mechanism(s) underlying subversion of this activity during forfeiture of self-renewing capacity are poorly understood. MicroRNAs (miRNAs) govern critical cellular functions including stem cell maintenance, development, cell cycle regulation and differentiation by disrupting translation of target mRNAs. Data presented here indicate that expression of miR203, a miRNA that targets ΔNp63α and ΔNp63β is activated during luminal epithelial differentiation and that this pattern is observed in the murine mammary hierarchy. In addition, we present evidence that the transcription factor Zeb1 represses miR203 expression, thus enhancing ΔNp63α protein levels. Furthermore, ectopic miR203 suppresses ΔNp63α expression, proliferation and colony formation. The anti-clonogenic effects mediated by miR203 require suppression of ΔNp63α. In addition, ectopic miR203 promotes mesenchymal-to-epithelial transition and disrupts activities associated with epithelial stem cells. These studies support a model in which induction of miR203 mediates forfeiture of self-renewing capacity via suppression of ΔNp63α and may also have anti-tumorigenic activity through its reduction of EMT and cancer stem cell populations.

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The majority of mammary gland development occurs after birth and is governed by a precise sequence of hormonal and morphogenic signals that regulate ductal branching and elongation of the nascent mammary gland.[1] Mammary organogenesis is driven by the mitotic activity and extravasation of the terminal end bud through the stromal fat pad. Following mammary organogenesis and near the completion of puberty, the terminal end bud regresses and the mammary gland enters a stage of regenerative homeostasis characterized by periodic, successive regenerative cycles composed of distinct waves of proliferation and cell death.[2] This pattern of regenerative stasis relies on the presence and activity of mammary stem cells (MaSCs), which have been prospectively isolated from primary mouse and human mammary epithelial cells.[3–6] The epithelial portion of the mammary gland is a bilayered ductal system. The inner most layer consists of luminal epithelia that line the mammary ducts and the milk-producing lobuloalveolar units. The basal/myoepithelial layer separates the luminal epithelia from a basement membrane and contains cells that co-express ΔNp63α,[7] nestin[8] and other markers associated with stem cell activity. Several studies have demonstrated that these cells co-enrich with serial mammary regenerative capacity, indicating that the basal/myoepithelial layer may represent a specialized MaSC niche.[3,4,9]

Abundant evidence implicates ΔNp63 isoforms in preservation of self-renewal and tissue stasis in diverse epithelial structures, including the mammary gland. Targeted ablation of ΔNp63 isoforms leads to broad-spectrum epithelial hypoplasias characterized by stem cell depletion and ablated regenerative kinetics.[10–12] Although the precise mechanisms by which ΔNp63α preserves self-renewing capacity are not fully understood, substantial evidence indicates that it potently inhibits cellular senescence.[13] In addition, haploinsufficiency of TP63 confers a premature aging phenotype associated with a sharp increase in cellular senescence.[13–15] In basal breast cancers and head and neck squamous cell carcinomas, ΔNp63α acts as a pro-survival factor and a mediator of chemoresistance that actively represses expression of pro-apoptotic effectors.[16,17] These studies provide compelling evidence that ΔNp63α is critical for preservation of replicative capacity,

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Abbreviations: DIP, Dexamethasone, Insulin, Prolactin treatment; EMT, Epithelial-to-mesenchymal transition; FACS, Fluorescence activated cell sorting; IMEC, immortalized mammary epithelial cells; KRT, Cytokeratin; MaSC, mammary stem cells; MiR-CON, Synthetic microRNA control; MS, mammospheres
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prolonged life span and survival that are characteristic of adult and cancer stem cells. They further suggest that specific mechanisms exist to subvert these activities during lineage commitment and cellular differentiation.

MicroRNAs (miRNAs) are a class of endogenous small RNA molecules that are approximately 22 nucleotides in length.[18] MiRNAs govern diverse cellular activities including proliferation, apoptosis, differentiation, development and tumorigenesis by targeting the RNA-induced silencing complex to the 3′-UTR of target mRNAs.[19,20] MiR203 was identified as a stemness inhibiting miRNA that is highly expressed in the epidermis where it targets α and β isoforms of TP63 to promote epidermal differentiation.[21,22] In addition to its role in normal epithelial biology, miR203 has also been shown to be aberrantly expressed in several types of human cancers including bladder, colon, pancreatic, liver, prostate and lung.[23–28] Interestingly, miR203 is repressed by the transcriptional repressor zinc-finger E-box binding homeobox 1 (ZEB1), a repressor of multiple key mediators of epithelial differentiation[29] and a potent activator of epithelial-to-mesenchymal transition (EMT).[30] EMT is a key developmental program that can be re-activated during cancer development and has been linked to tumor invasion, metastasis and chemo-resistance.[31] In addition, cancer cells have been reported to utilize EMT to acquire cancer stem cell properties in part through the modulation of miRNAs.[32–34] These reports implicate miRNAs as mediators of EMT, stemness and the acquisition of an aggressive cancer phenotype.[33,34] These findings, coupled to reports linking ΔNp63α to MaSC renewal and breast cancer aggression suggest that miR203 may have important roles in the mammary regenerative hierarchy as well as in breast cancer.

The goal of this study was to determine the functional significance of miR203 in MaSC activity and luminal epithelial cell fate in the mammary gland. Results indicate that expression of miR203 is induced during lactogenic differentiation and increases during luminal epithelial differentiation. Data presented here indicate that in mammary epithelia, miR203-mediated suppression of ΔNp63α reduces proliferation, clonogenic potential and transcriptional suppression of HBP1, a pro-differentiation gene transcriptionally repressed by ΔNp63α.[35] In a panel of mammary epithelial and breast cancer cell lines, miR203 expression correlates with the degree of luminal differentiation, as it is lowest in basal/triple negative and highest in luminal-like breast cancer cell lines. Finally, ectopic miR203 expression is able to mediate a mesenchymal-to-epithelial transition (MET) that was associated with a reduction in the cancer stem cell population ($CD44^+CD24^-$) and an increase in the expression of the luminal cell marker CD24. Together, these studies suggest that miR203 is a potent stem cell regulator and has a significant role in the acquisition of luminal epithelial cell fate through the forfeiture of MaSC activity in the normal mammary gland and breast cancer stem cells.

Results

Expression of miR203 correlates with differentiation in the mammary epithelium. Substantial evidence indicates that ΔNp63α is required for preservation of MaSCs. However, the mechanism(s) by which this activity is subverted during forfeiture of self-renewing capacity and developmental commitment are not well understood. MiR203 directly targets sequences within exon 15 of TP63 that encode the 3'UTR of α and β isoforms.[22] This finding coupled to the fact that ΔNp63α is required for MaSC preservation suggests that increased expression of miR203 may promote differentiation in the mammary regenerative hierarchy. To test this, enriched fractions of MaSCs ($Lin^-/CD24^+/CD29^{high}/CD61^+$), luminal progenitors ($Lin^-/CD24^+/CD29^{low}/CD61^+$) and mature luminal epithelia ($Lin^-/CD24^+/CD29^{low}/CD61^-$) were isolated (Figure 1a) and analyzed for expression of miR203. Cytokeratin profiling of these fractions revealed that $Lin^-/CD24^+/CD29^{low}/CD61^-$ fractions were enriched for the luminal epithelial cytokeratins KRT18 and KRT19 (Supplementary Figure S1a), whereas $Lin^-/CD24^+/CD29^{high}$ was highly enriched for expression of basal epithelial markers, KRT14 and KRT5 (Supplementary Figure S1b). In addition, GATA3 expression was highest (Supplementary Figure S1c) in mature luminal epithelia ($Lin^-/CD24^+/CD29^{low}/CD61^-$), which is consistent with previous studies indicating that expression of CD61 segregates luminal epithelial cells,[35] and that this progression requires GATA3.[36,37] Analysis of miR203 levels indicated a sharp increase in luminal progenitors and differentiated luminal epithelial cells relative to the enriched MaSC fraction (Figure 1b). Previous studies have implicated Zeb1 as a potent repressor of miR203 and other miRNAs, and can promote tumorigenic capacity by maintaining self-renewal capacity.[38] Consistent with these findings, Zeb1 expression was highest in the enriched fractions of MaSCs and declined in committed populations (Figure 1c). To determine if the observed increase in miR203 correlated with a decline in ΔNp63α protein levels, $Lin^-/CD24^+/CD29^{high}/CD61^+$ cells and $Lin^-/CD24^+/CD29^{low}/CD61^+$ cells, which enrich for mammary epithelial stem and luminal epithelial progenitor cells, respectively, were stained for total p63. Results indicate that p63 is robustly expressed in the enriched fractions of MaSCs and undetectable in the luminal progenitors (Figure 1d). Finally HBP1, a pro-differentiation HMG-box transcriptional regulator[39,40] whose expression is repressed by ΔNp63α was observed to increase with differentiation (Figure 1e). Together, these data support a model in which Zeb1 represses miR203 expression thereby preserving ΔNp63α expression in MaSCs and that this repression is relieved during the loss of self-renewing capacity. Consistent with this model, we noted a strong correlation between miR203 levels and the degree of differentiation in breast cancer cell lines (Figure 1f and Supplementary Figure S1D).

MiR203-mediated suppression of ΔNP63α is required for lactogenic differentiation. The previous data demonstrate a correlation between miR203 expression and differentiation in the mouse mammary epithelium as well as in breast cancer. To determine if miR203 expression is induced during luminal epithelial differentiation and to determine the role of Zeb1 in this process we adopted an established luminal differentiation system in which the immortalized murine mammary epithelial cell line, HC11 is first deprived of EGF and then treated with dexamethasone, insulin and prolactin

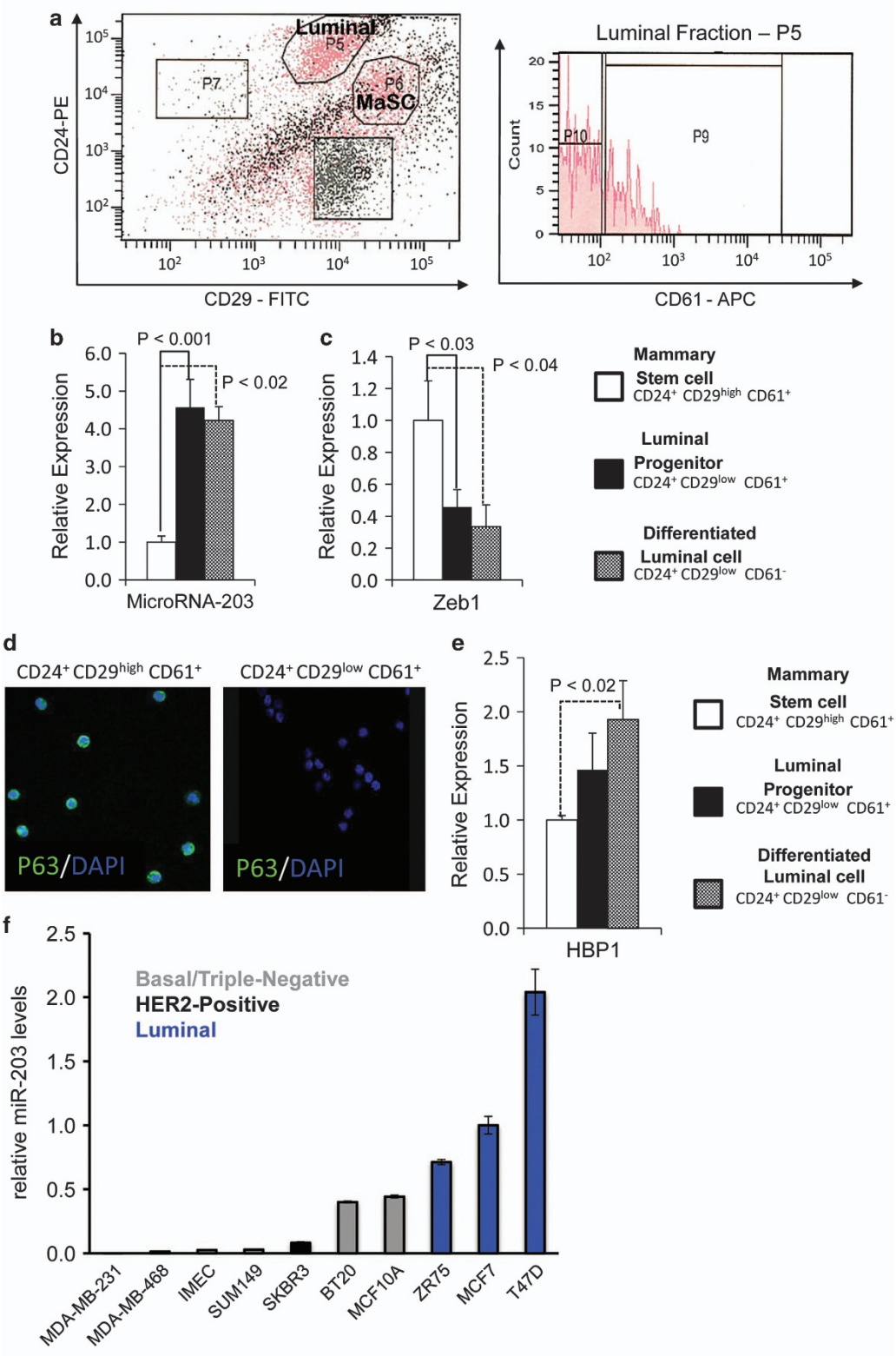

Figure 1 Expression of miR203 correlates with differentiation in the mammary epithelium. (**a**) Fluorescence-activated cell sorting (FACS) of negative lineage selected mammary epithelial cells excised from the mouse mammary gland and labeled with CD29, CD24 and CD61. Labeled mammary epithelial cells were gated for isolation of MaSCs, luminal progenitor and differentiated luminal cell fractions based on CD29, CD24 and CD61 status. (**b**) qRT-PCR analysis of isolated mammary cells fractions for detection of mature miR203 expression. (**c**) Total RNA was isolated from mammary cell fractions for qRT-PCR analysis on ZEB mRNA expression. (**d**) Immunofluorescent staining against P63 from mammary epithelial stem cells (left) and luminal mammary cells (right) applied to glass microscope slides. (**e**) qRT-PCR analysis of isolated mammary cell fractions for detection of HBP1 mRNA (transcriptionally repressed by ΔNP63α). (**f**) Mature miR-203 expression levels normalized to SnU6 from RNA lysates of 10 mammary and breast cancer cell lines using two-step TaqMan assays. All data are means ± S.E.; $n = 2$, *P*-values are indicated

(DIP).[41] These cultures undergo lactogenic differentiation and express high levels of β-casein in response to EGF withdrawal and treatment with DIP (Figure 2e). Analysis of cells treated with vehicle or DIP for 72 h revealed that DIP caused decreased expression of Zeb1 (Figure 2a), increased expression of miR203 (Figure 2b), suppression of ΔNp63α protein (Figure 2c), while ΔNp63α mRNA remained unchanged (Supplementary Figure S2), and increased expression of HBP1 (Figure 2d). These results are consistent with the patterns of gene expression observed in the murine mammary epithelial hierarchy (Figure 1). They also suggest that the induction of HBP1 and β-casein may require suppression of ΔNp63α. To test this, ΔNp63α was expressed with a heterologous 3′UTR that is insensitive to miR203 in HC11 cells and treated with vehicle or DIP. QRT-PCR-based analysis indicated that the DIP-dependent induction of HBP1 was disrupted by ectopic ΔNp63α (Figure 2f). This result is consistent with previous studies indicating that HBP1 is directly repressed by ΔNp63α.[35] Similarly, DIP-dependent luminal epithelial differentiation, as measured by induction of β-casein, was disrupted by ectopic ΔNp63α indicating that suppression of ΔNp63α is required for DIP-induced luminal differentiation (Figure 2g). Together, these results support a model in which induction of miR203 and suppression of ΔNp63α expression are required for luminal epithelial differentiation in the DIP-induced HC11 model system (Figure 2h).

MiR203 induces G0/G1 arrest and blocks colony formation capacity that is rescued by ΔNp63α. To determine if induction of miR203 associated with mammary epithelial differentiation mediates repression of ΔNp63α and forfeiture of self-renewal; miR203 was transfected into two immortalized mammary epithelial cells (IMECs), IMEC and HC11, both of which have features of MaSCs including high levels of ΔNp63α. Western analysis of transfectants revealed that miR203 significantly reduced expression of ΔNp63α in IMECs and HC11 cells (Figure 3a). In these studies, it was also noted that transfection of miR203 but not miR-CON (control) resulted in a substantial reduction in cell number in the HC11 cultures within 48 h of transfection (Figure 3b). Similar, but less dramatic results were observed in IMECs (data not shown), which is likely due to the slower proliferation rates of IMECs. This observation coupled to studies indicating that ΔNp63α is required for retention of proliferative potential,[11] suggested that the reduction in cell number within miR203 transfectants may be the result of a proliferative arrest. To test this, HC11 cells were transfected with miR-CON or miR203 and cell cycle distribution was analyzed by propidium iodine staining at 72 h after transfection. Results indicated a substantial accumulation of cells in the 2N state (Figure 3c), suggesting that cells had either undergone proliferative arrest or had become quiescent. This result suggests that miR203 has a potent anti-proliferative effect, which is consistent with the observed increase in miR203 expression during cellular differentiation. To determine if the anti-proliferative effects of miR203 required suppression of ΔNp63α, IMEC cells were infected with retroviruses programmed to express nothing (pLPC) or the open reading frame of ΔNp63α (pLPC-ΔNp63α^{ORF}), which renders it resistant to miR203-mediated repression, and then transfected with miR-CON or miR203. Results indicated that ectopic miR203 prevented colony formation (Figure 3d). Importantly, the anti-clonogenic effect of miR203 was potently rescued by expression of ΔNp63α, indicating that miR203 is able to suppress ΔNp63α in a manner that is consistent with forfeiture of proliferative capacity in mammary epithelium.

MiR203 opposes the activities of normal and cancer stem cells. The ability of miR203 to repress ΔNp63α expression (Figure 3a) and disrupt ΔNp63α-mediated clonogenicity (Figure 3d) suggests a role in the forfeiture of stem cell properties. A common feature of epithelial stem cells is the ability to form multicellular structures that can proliferate in an attachment-independent manner. These structures, referred to as mammospheres, are derived from MaSCs and this culture system has been used to enrich for tumorigenicity within breast tumors and breast cancer cell lines.[42] The ability to enrich for subpopulations with stem cell features coupled to the observed increase in miR203 levels in non-stem populations (Figures 1 and 2) suggested that miR203 levels would be reduced in mammospheres relative to their parental monolayer culture. To test this, miR203 levels were measured in MCF7 cells derived from monolayer and mammosphere cultures. Results indicated miR203 levels are reduced in the mammospheres (Figure 4a). To determine the effects of miR203 on mammosphere forming capacity, IMEC cells were either mock transfected or transfected with miR-CON or MiR203 and 25 000 cells from each transfection were cultured under low attachment conditions for 14 days. Visual inspection of the cultures indicated that ectopic miR203 completely ablated mammosphere formation (Figure 4b) and this was quantified by counting cells following gentle trypsinization of the mammospheres (Figure 4b). These data suggest that miR203 is sufficient to disrupt mammosphere forming capacity, which is consistent with a model in which miR203 subverts the stem cell phenotype. Several studies have demonstrated a substantial enrichment of stem cells within the $CD44^+/CD24^-$ fraction of breast tumors and breast cancer cell lines.[43,44] To determine the effects of miR203 on this feature of stem cells, IMEC cells were mock transfected or transfected with miR-CON or miR203 and $CD44^+/CD24^-$ fractions were quantified by flow cytometry. IMECs were chosen for this study because of their high proportion of $CD44^+/CD24^-$ cells and their low endogenous levels of miR203. Results indicated that ectopic miR203 caused a reduction in the $CD44^+/CD24^-$ population from 97.5 to 79.3% relative to the miR-CON transfectants (Figure 4c). This reduction was due in large part to an increase in $CD44^+/CD24^+$ cells suggesting that miR203 caused an increase in the number of cells that had forfeited self-renewing capacity. Together, these data indicate that miR203 is sufficient to subvert the phenotypic properties of MaSCs.

MiR203 is negatively regulated by Zeb1 and is inversely expressed in mammary and breast cancer cell lines. The previous data (Figures 1 and 2) coupled to a recent study indicating that Zeb1 promotes a stem cell phenotype via

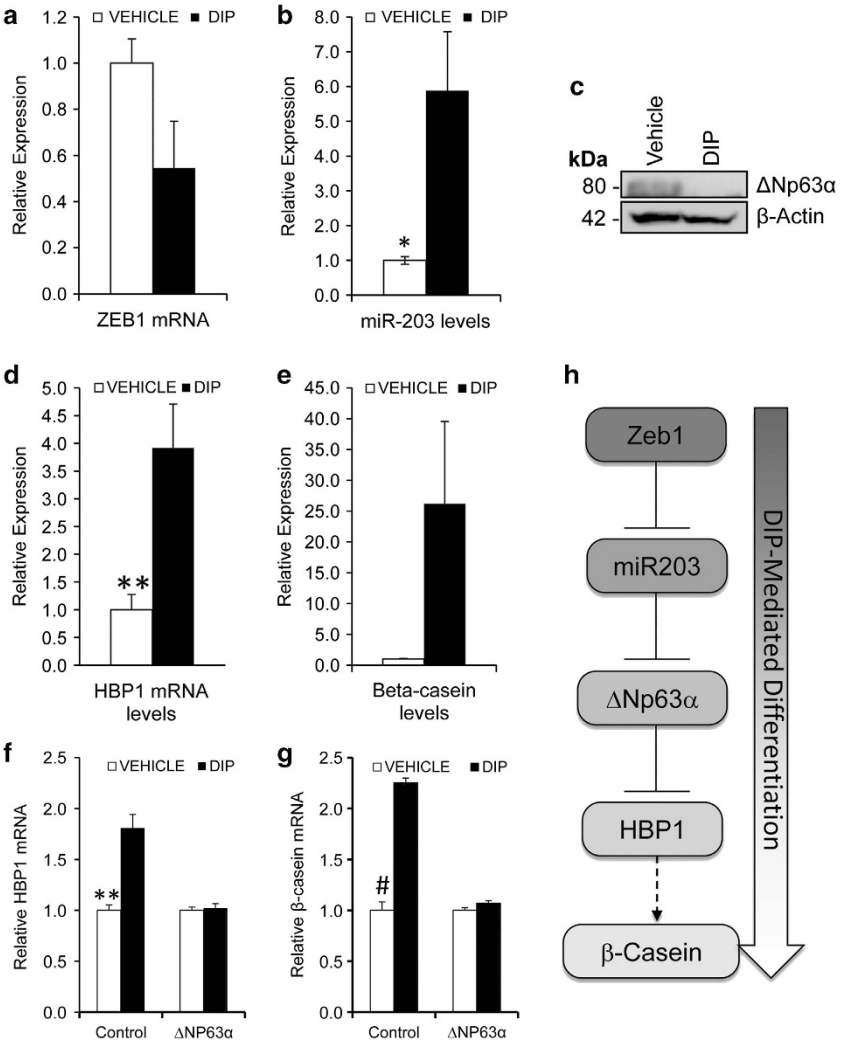

Figure 2 Modulation of the ZEB1-miR203-ΔNP63α pathway during luminal epithelial differentiation. The mouse mammary epithelial cell line HC11was plated and on confluence were then withdrawn of EGF for 48 h. Cells were then treated with differentiation inducing media (DIP) for 72 h. Cells were collected for RNA and protein analysis. Total RNA was isolated from cells for qRT-PCR analysis to detect (**a**) ZEB1 mRNA, (**b**) mature miR203, (**d**) HBP1 mRNA and (**e**) β-casein mRNA. (**c**) Western blot analysis was performed on HC11 protein lysates to detect ΔNP63α protein expression levels. (**f** and **g**) HC11 cells were treated with DIP differentiating media for 72 h in the presence or absence of an adenovirus expressing the ORF of ΔNP63α (lacking the UTR's), which cause resistance to miR-203 translational inhibition. qRT-PCR analysis were performed on total RNA lysates to detect mRNA expression levels of HBP1 (**f**), and β-casein (**g**). Data are means ± S.D.; $n = 3$, $^{*}P < 0.00004$, $^{**}P < 0.02$ and $^{\#}P < 0.005$. (**h**) Schematic of the regulatory pathway that mediates the downregulation of ΔNP63α protein during luminal mammary cell differentiation

transcriptional repression of miRNAs suggest that transcriptional repression of miR203 by Zeb1 promotes ΔNp63α expression.[38] To test this prediction and determine its relevance to breast cancer, a panel of immortal and transformed mammary epithelial cell lines representing poorly differentiated to well differentiated states was evaluated for Zeb1 mRNA levels and miR203 levels. Results indicate that Zeb1 expression is highest in poorly differentiated cells and miR203 expression is highest in well-differentiated cells (Figure 5a). This inverse correlation suggests that Zeb1 promotes expression of ΔNp63α via repression of miR203. Si-RNA-mediated suppression of Zeb1 resulted in a robust increase in miR203 levels (Figure 5b) and sharply lowered levels of ΔNp63α protein (Figure 5c). Conversely, gain-of-function studies in MCF10A cells, which were chosen for their low levels of Zeb1, indicated that ectopic Zeb1 repressed expression of miR203 (Figure 5d) and increased ΔNp63α levels (Figure 5e). Together, these studies support a model in which Zeb1-mediated suppression of miR203 enhances expression of ΔNp63α.

MiR203 promotes MET. The observation that Zeb1 actively represses miR203 coupled to its role in promoting EMT suggests that miR203 may be differentially expressed during this transition. To test this, CDβ-Geo cells, a derivative of HC11 cells, were treated with TGFβ to induce EMT.[45] Immunofluorescent analysis of these cells indicated that TGFβ treatment resulted in a sharp decrease in E-cadherin expression, a hallmark feature of EMT (Figure 6a). Post-EMT

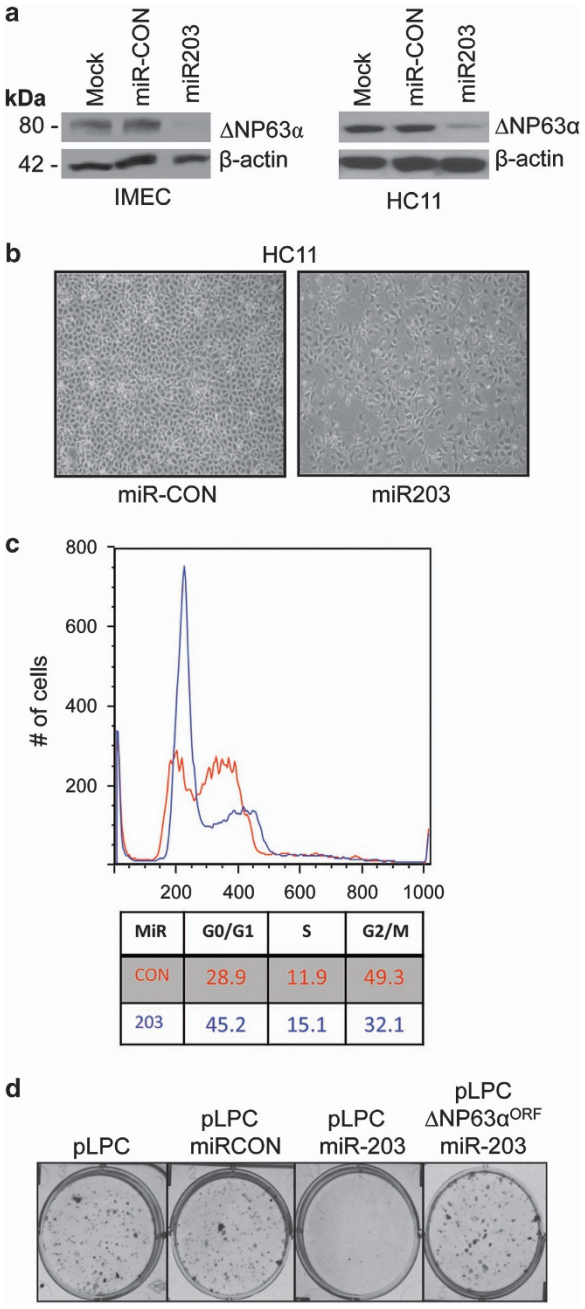

Figure 3 MiR203 induces G0/G1 arrest and blocks colony formation capacity in a manner that is rescued by ΔNP63α. (**a**) Western blot analysis of protein lysates from IMEC (left) and HC11 (right) cells transiently transfected with mature miR203 (60 nm) for 48 h and probed with an anti-P63 antibody (4A4). (**b**) 10 × Phase contrast microscopy of HC11 cells transiently transfected with mature miR203 (60 nm) for 72 h. (**c**) PI cell cycle analysis was performed via flow cytometry of HC11 cells transiently transfected with mature miR203 for 72 h (cells were treated with nocodizole to synchronize cells before PI cell cycle analysis). (**d**) IMEC cells were either infected with an empty retrovirus or a retrovirus expressing the open reading frame of ΔNP63α (pLPC-ΔNP63α^{ORF}) and then transiently transfected with either a miR-control or mature miR203 (60 nm) and then plated for colony formation assay. Colonies were stained with crystal violet and imaged. All experiments were performed in triplicate ($n = 3$)

cells expressed significantly less miR203 compared with untreated CDβ-Geo cells (Figure 6b). Conversely, Zeb1 mRNA expression was increased in post-EMT CDβ-Geo cells, which suggest that it may be a contributing factor to the reduction of miR203 expression (Figure 6b). This observation suggests that suppression of miR203 levels is required for EMT and that miR203 may oppose EMT. Consistent with this prediction, transfection of miR203 into IMECs resulted in a sharp induction of E-cadherin and a corresponding decrease in vimentin demonstrating that miR203 opposes EMT and promotes MET (Figure 6c). These results propose that miR203 may restrict cell motility, a hallmark of EMT. To test this, MDA-MB-231 cells, which display features of EMT including enhanced cell motility, were cultured to confluence and mock transfected, or transfected with miR-CON or miR203. At 24 h after transfection, uniform scratches were etched onto the monolayer and cells were visually monitored over 48 h to observe the rates at which cells re-colonize the scratch. Digital imaging (Figure 6d) and quantification (Figure 6e) indicated that transfection of miR203 potently inhibited the ability of cells to recolonize the scratch, suggesting that miR203 restricts cell motility. Together, these data indicate that expression of miR203 is reduced in cells that have undergone EMT and that miR203 is sufficient to promote MET and reduce cell motility.

Discussion

Data presented here support a model in which induced expression of miR203 is an early event in the mammary epithelial hierarchy that mediates forfeiture of self-renewing capacity by suppressing ΔNp63α. Our data indicate that miR203 correlates with differentiation in breast cancer cell lines and in the mammary epithelial hierarchy. In addition, miR203 expression increases in response to induction of differentiation and that differentiation depends on suppression of ΔNp63α. Importantly, ectopic expression of miR203 in HC11 and IMEC cells was not sufficient to induce differentiation as indicated by β-casein expression (data not shown), however, it did suppress ΔNp63α, which lead to induction of the pro-differentiation gene HBP1 (Supplementary Figures 3A and B). These data are consistent with miR203 functioning early in the differentiation pathway to mediate the forfeiture of self-renewing capacity. In addition, gain-of-function studies indicate that miR203 is anti-proliferative, disrupts self-renewal and is potently anti-clonogenic. This latter feature is rescued by ectopic ΔNp63α, indicating that this effect is mediated via suppression of ΔNp63α. Suppression of miR203 correlates with expression of Zeb1, which is a negative prognostic factor in breast cancer. These findings suggest a mechanism by which Zeb1 contributes to poor prognosis and suggests that miR203 may function as a tumor suppressor that promotes an orderly progression through the mammary epithelial hierarchy. Additional studies will be necessary to determine if miR203 has tumor-suppressor activity.

We have described a regulatory pathway that is differentially activated during cellular differentiation (Figure 2 and Supplementary Figure 3C). In the undifferentiated stem and progenitor cells, expression of Zeb1 suppressed miR203 expression thereby increasing ΔNp63α protein and suppressing expression of HBP1 (Figures 1 and 2). Conversely, in a differentiated state, Zeb1 expression is repressed resulting in increased miR203 levels, suppression of ΔNp63α and

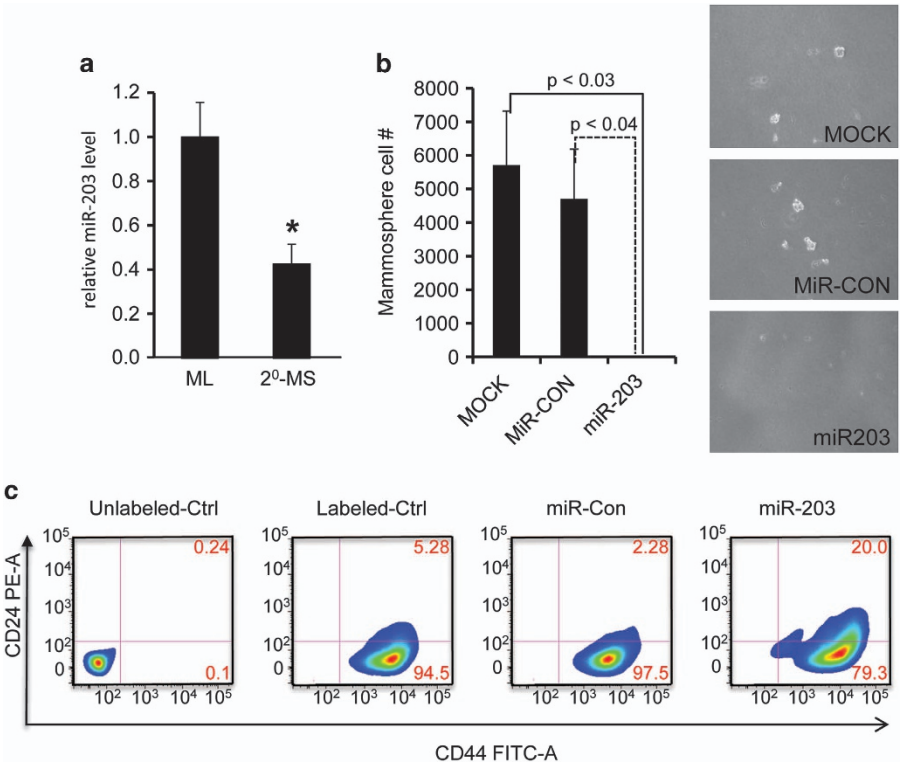

Figure 4 MiR203 opposes the activities of normal and cancer stem cells. (**a**) RT-qPCR analysis of total RNA for mature miR-203 expression levels isolated from parental (ML) and secondary mammospheres (2°-MS) of MCF-7 cells (mammospheres enrich for stem cells). Data are means ± S.D.; $n=3$, asterisk indicates $P<0.05$. (**b**) Mammosphere formation assay of IMEC cells transiently transfected with miR-control of mature miR203 (60 nM); 25 000 cells were then transferred to low binding plates and grown for 14 days. Mammospheres were trypsinized and total cells were counted per experimental group (left), Representative 10× phase contrast images of mammospheres from each experimental group (right). Data are means ± S.E.; $n=3$, *P*-values are indicated. (**c**) Flow cytometric analysis of IMECs labeled with the stem cell enrichment markers CD24 and CD44. IMECs were transiently transfected with miR-control or mature miR203 (60 nM). Seventy-two hours after transfection, cells were labeled with CD24-PE and CD44-FITC and analyzed via flow cytometry. Representative flow cytometry density plots of CD24 *versus* CD44 from experiment conduced in triplicate

increased expression of HBP1 (Figure 2). HBP1 has been previously identified as a tumor suppressor in breast cancer,[46] a target of transcriptional repression of ΔNp63α[39] and a mediator of differentiation.[47] It is also a potent inhibitor of Wnt signaling, which maintains self-renewal, suggesting that induction of HBP1 downstream of miR203 may contribute to the forfeiture of self-renewal.[46,48] In addition, in the HC11 model of luminal differentiation, HC11 cells are grown in the undifferentiated state via EGF treatment. Following EGF withdrawal, HC11 cells are induced to a committed pre-differentiated progenitor state, which are then committed to a complete differentiated state by addition of DIP. We observed initial induction of miR203 as early as the pre-differentiated state on EGF withdrawal (data not shown); which supports miR203 as an early mediator in the differentiation pathway, most likely to facilitate the forfeiture of stem cell properties. Furthermore, in a recent study, miRNA targeted gene profiling identified overlapping signatures between the undifferentiated stem cell-like HC11 state and poor prognosis where patients exhibiting a SC-like miRNA profile had lower survival rates.[49] It will therefore be of interest to determine if expression of miR203 correlates with a favorable prognosis in breast cancer patients. In addition, as miR203 targets ΔNp63α and BMI1, another protein implicated in the preservation and activity of stem cells, it will be of interest to determine if miR203 expression can identify patients at low risk for breast cancer recurrence.

We present evidence that ablation of Zeb1 leads to increased expression of miR203 and suppression of ΔNp63α. Coupled to several studies implicating Zeb1 in activation of pro-EMT transcriptional programs,[38,50] and tumor cell dedifferentiation,[29] our study implicates miR203 in the specification of epithelial cell fate. Further, it suggests that miR203 mediates forfeiture of cellular plasticity, which is a hallmark of EMT and cancer stem cell behavior.[51] In addition, ZEB1 is overexpressed in various cancer cell lines, including breast, and has been shown to promote tumor cell migration and metastasis.[52] Similarly, a miRNA expression profiling study of human metastatic cancers and paired primary tumors found miR203 to be consistently underexpressed in the metastatic tissue,[53] further implicating a relationship between ZEB1 regulation of miR203 and tumor invasiveness and metastasis. Coupled with data presented in this study, miR203 appears to have an anti-tumorigenic role, and its expression may coincide with metastatic potential.

Finally, we show that miR203 was sufficient to induce a MET and a decrease in the breast cancer stem cell population (Figures 5 and 6). Of interest, ectopic miR203 significantly increased the expression of the luminal cell surface marker CD24, further implicating miR203 as a mediator of luminal cell

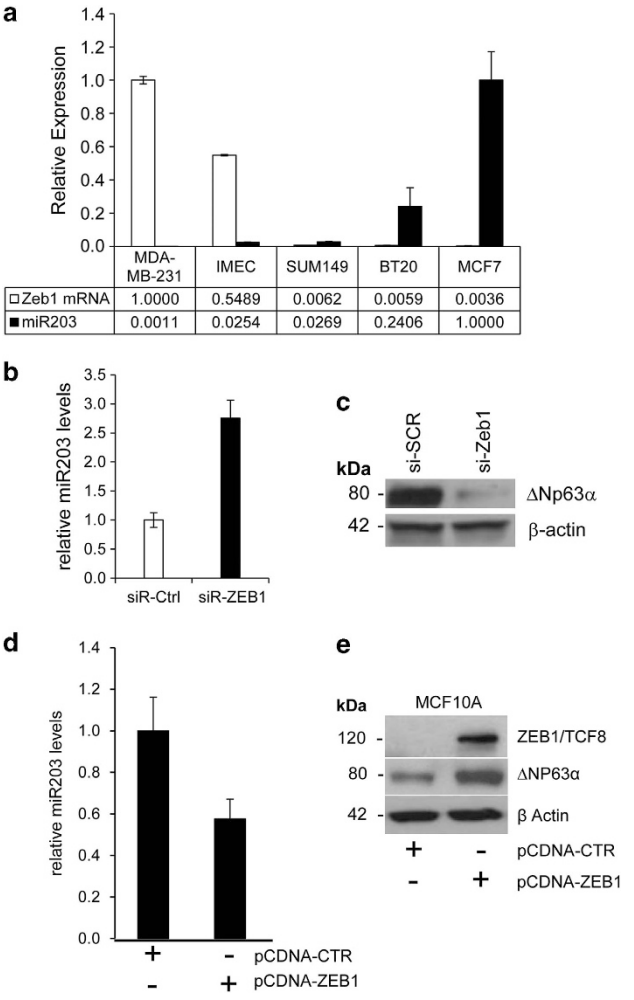

Figure 5 MiR203 is negatively regulated by ZEB1 and is inversely expressed in mammary and breast cancer cell lines. (**a**) Total RNA was isolated from five mammary and breast cancer cell lines and qRT-PCR analysis to detect ZEB1 mRNA and mature miR203 was performed. (**b** and **c**) IMEC cells were transiently transfected with siRNA against ZEB1 or scramble control (60 nm) and harvested 48 h later for qRT-PCR analysis on mature miR203 (**b**) and western blot analysis using an anti-P63 antibody (**c**). (**d** and **e**) MCF10A cells were transiently transfected with an empty mammalian expression plasmid (pCDNA-CTR) or one encoding murine Zeb1 (pCDNA-Zeb1). Cells were collected 72 h after transfection and analyzed using qRT-PCR for mature miR203 detection and western blot using anti-Zeb1 and anti-P63 antibodies. Data are means ± S.D.; $n = 3$

fate and commitment. It is possible that miR203 suppression is a mechanism by which cancer cells can acquire an EMT, stem cell-like phenotype in order to garner an aggressive, pro-metastatic phenotype. Taken together, we have described miR203 as a potent inhibitor of stem cell function through the targeted inhibition of ΔNp63α, which may have a role in breast cancer development.

Materials and Methods

Cell culture. A total of 10 mammary epithelial or breast cancer cell lines (MDA-MB-231, MDA-MB-468, SUM149, SKBR3, BT20, MCF10A, HCC1937, ZR75, T47D and MCF7) were examined in this study. Each was maintained according to ATCC guidelines. Establishment and maintenance of the IMEC line has been previously described.[7,54]

Western blot analysis. Total cell protein extracts were obtained using NETN lysis buffer (100 mM Tris-Cl (pH 7.8), 1 mM EDTA, 100 mM NaCl, and 0.1% Triton X-100) supplemented with protease and phosphatase inhibitors (Roche, Branchburg, NJ, USA). Protein concentrations were measured by Lowry protein assay and 10 μg of protein per sample was resolved on an 8% SDS-polyacrylamide gel and transferred onto a PVDF membrane. Membranes were blocked with TBST 5% non-fat dry milk for 1 h at room temperature, incubated with primary antibodies overnight at 4 °C, washed and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody.

Antibodies. For western blotting, mouse anti-p63 (4A4) (LabVision, Kalamazoo, MI, USA; 1/500 dilution), mouse anti-β-actin (Cell Signaling, Boston, MA, USA; 1/1000 dilution) antibodies were used. For sorting primary mammary epithelial cells from mice, antibodies against mouse antigens include CD24-PE (BD Biosciences, San Jose, CA, USA; 1/100 dilution), CD29-FITC (BD Biosciences; 1/100 dilution), and CD61-APC (Invitrogen, Grand Island, NY, USA; 1/100 dilution). Secondary antibodies used were anti-mouse IgG (Sigma, St. Louis, MO, USA; 1/10 000 dilution). For flow cytometry experiments, FITC-conjugated mouse anti-human CD44 and PE-conjugated mouse anti-human CD24 (BD Biosciences) were used.

RT-qPCR. RNA was isolated using RNeasy mini kit or miRNeasy mini kit for mRNA and miRNA, respectively (Qiagen, Valencia, CA, USA) as per the manufacturer's protocol. For mRNA analysis, RNA (1 μg) was reverse transcribed using random hexamers and Q-PCR was conducted using the SsoFast Eva Green master mix (Bio-Rad, Hercules, CA, USA). The $2^{-\Delta\Delta CT}$ method was used for quantification of gene expression changes. Relative changes were normalized to glyceraldehyde-3-phosphate dyhydrogenase or β-actin for differentiation experiments. The relative expression levels of miR203 were measured using a two-step Taqman assay according to the manufacturer's protocol. RNA (10 ng) for miRNA analysis was reverse transcribed using Taqman Reverse Transcriptase Kit (Applied Biosystems, Carlsbad, CA, USA) for miR203 or the internal control SnU6. Taqman real-time PCR was prepared using Universal PCR Master Mix No AmpErase UNG (Applied Biosystems) and specific primers for miR203 and SnU6 from Taqman MicroRNA Assays (Applied Biosystems). Expression levels of miR203 were based on the amount of the target message relative to that of the SnU6 transcript as a control to normalize the initial input of total RNA.

MiRNA analysis and transfections. For miRNA overexpression, 2.5×10^5 cells per well were seeded in six-well-plates. Pre-miR (Applied Biosystems) mimicking mature miR203 or control nonspecific miRNA (Pre-miR-negative control NC#1; Applied Biosystems) was transfected at 60 nM final concentration into cells using Oligofectamine transfection reagent (Invitrogen). At 24 h after transfection, cells were assessed for ΔNp63α protein content via western blot. For cell cycle analysis of HC11 cells transfected with Pre-miR203 or control, cells were split 1 : 4, 48 h after transfection and subsequently collected for propidium iodide (PI) staining 72 h after transfection. For experiments involving ZEB1 knockdown, IMEC cells were transfected with either scramble si-control or a siRNA targeted against ZEB1 at 60 nM final concentration using Oligofectamine Transfection Reagent (Invitrogen). For ZEB1 overexpression experiments, MCF10A cells were transfected with an empty control mammalian expression plasmid (pCDNA-CTR) or one encoding murine ZEB1 (pCDNA-ZEB1) using Lipofectamine 2000 Transfection reagent (Invitrogen). Cells were harvested 48 and 72 h after transfection for analysis.

Lactogenic differentiation of HC11 cells. The prolactin-responsive cell line, HC11, was split 1 : 5 and maintained in RPMI 1640 medium containing 10% fetal bovine serum, L-glutamine, 5 μg/ml insulin and 10 ng/ml epidermal growth factor. For induction of differentiation the cells were grown to confluence and then kept in the above media without EGF for 48 h to induce competence. Differentiation-competent cells were incubated in RPMI 1640 medium containing 10% fetal bovine serum, L-glutamine, 5 μg/ml insulin and supplemented with 100 nM dexamethasone, 5 μg/ml insulin, in the presence or absence of 5 μg/ml prolactin for 72 h.

Cell cycle distribution analysis. Cells were collected at approximately 60% confluence and fixed in 70% ethanol for 48 h at −20 °C. Cells were washed in PBS supplemented with 3% FBS and resuspended in buffer (PBS, 0.1% Triton X-100, DNase-free RNAse-A 100 μg ml^{-1}) with 50 μg ml^{-1} of PI. Following

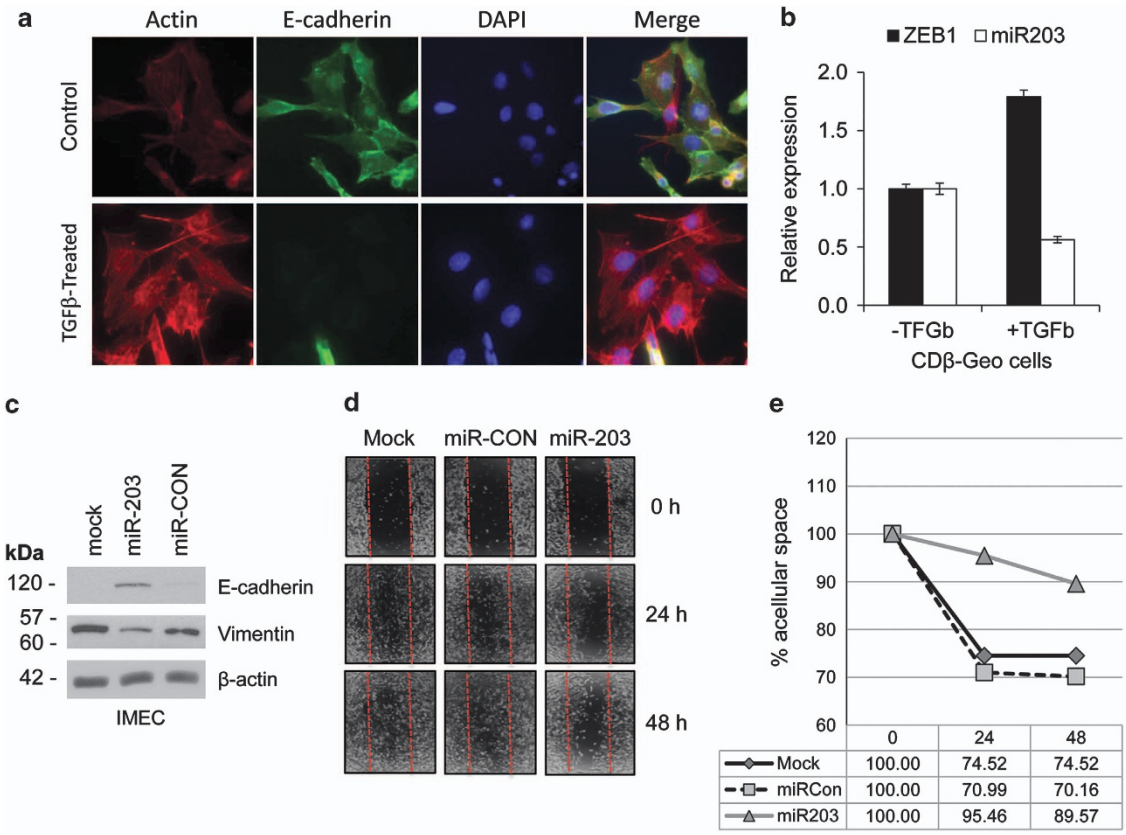

Figure 6 MiR203 promotes MET. (**a**) Immunofluorescent images stained for E-cadherin (green), β-actin (red) and DAPI for nuclear localization of CDβ-Geo cells that have been treated with vehicle (control) or TGFb. (**b**) Total RNA was isolated from CDβ-Geo cells that have not ($-$) or have undergone EMT ($+$) and qRT-PCR analysis was performed to measure Zeb1 mRNA and mature miR203 expression levels. (**c**) Western blots of protein lysates from IMEC cells transiently transfected with miR-control or mature miR203 (60 nm) for 48 h were probed with anti-E-cadherin and anti-vimentin antibodies. (**d**) MDA-MB-231 cells were transiently transfected with miR-control or mature miR203 (60 nm) and plated for wound healing assay. At 90% confluence the cells were scratched and then observed for 48 h to measure closure of the wound. 10 $\times$ phase contrast microscopy of wound healing assay at 0, 24 and 48 h after scratch (**c**) and quantification of re-migration of the cleared space between experimental controls (**e**). All experiments were performed in triplicate ($n=3$) and data are means $\pm$ S.D.

incubation at 37 °C for 1 h, PI fluorescence distribution of 20 000 single cells was measured with a FACScan Flow Cytometer (BD Biosciences). Subsequent data were analyzed with FlowJo cell cycle software (Ashland, OR, USA).

Mammosphere formation. Human IMECs were transfected with 60 nM final concentration of miR203, miR-control or mock control. At 24 h after transfection, cells were trypsinized and seeded at 25 000 cells per 10 cm plate coated with poly-2-hydroxyethyl methacrylate (20 mg/ml). Cells were grown in low binding conditions for 14 days and collected for analysis.

Retroviral infection and colony formation assay. Platinum-A retroviral packaging cell line (Cell BioLabs Inc., San Diego, CA, USA) was transfected with the mammalian retroviral expression vector pLPC (Addgene, Cambridge, MA, USA) containing the open reading frame of ΔNP63α^{ORF}, or empty vector control (pLPC). At 24 h after transfection, viral titers were collected and used to infect IMECs. Seventy-two hours after infection; IMECs were transfected with miR203 or nonspecific miR-control. At 24 h after transfection, cells were plated at colony formation density (1000 cells per well in a six-well tissue culture plate). Colonies were stained using crystal violet 12 days after plating cells.

Mammary cell preparation and FACS cell sorting. Dissection and single cell suspension of the thoracic and inguinal mammary glands from virgin female mice were prepared as previously described.[9] Negative lineage selection was performed on the RoboSep fully automated cell separator (StemCell Technologies, Vancouver, BC, Canada) using the mouse Easy-Sep MaSC enrichment kit (StemCell Technologies). Cells were subsequently labeled with antibodies against mouse antigens and include CD24-PE, CD29-FITC and CD61-APC. FACS analysis and cell sorting were performed using a FACS Aria (BD Biosciences).

Flow cytometry analysis. Cells were trypsinized and washed once with PBS containing 2% FBS (wash buffer), and resuspended in 50 μl of wash buffer. Fluorochrome-conjugated monoclonal antibodies against human CD44-FITC and CD24-PE or their respective controls were added to the cell suspension and incubated in the dark at 4 °C for 35 min. The labeled cells were then washed in wash buffer and resuspended in 500 μl PBS and subsequently analyzed on a MACSQuant Analyzer (Miltenyi Biotec, Auburn, CA, USA).

Statistical analysis. Statistical analyses were performed using Students' *t*-test. A *P*-value <0.05 was considered significant.

Conflict of Interest

The authors declare no conflict of interest.

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