Developmental Biology 403 (2015) 150–161

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

MicroRNA-200c-141 and ΔNp63 are required for breast epithelial differentiation and branching morphogenesis

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ARTICLE INFO

Article history:
Received 6 October 2014
Received in revised form
18 April 2015
Accepted 5 May 2015
Available online 9 May 2015

Keywords:
MiR-200c-141
ΔNp63
Breast cancer
Breast morphogenesis
Stem cells
EMT

ABSTRACT

The epithelial compartment of the breast contains two lineages, the luminal- and the myoepithelial cells. D492 is a breast epithelial cell line with stem cell properties that forms branching epithelial structures in 3D culture with both luminal- and myoepithelial differentiation. We have recently shown that D492 undergo epithelial to mesenchymal transition (EMT) when co-cultured with endothelial cells. This 3D co-culture model allows critical analysis of breast epithelial lineage development and EMT. In this study, we compared the microRNA (miR) expression profiles for D492 and its mesenchymal-derivative D492M. Suppression of the miR-200 family in D492M was among the most profound changes observed. Exogenous expression of miR-200c-141 in D492M reversed the EMT phenotype resulting in gain of luminal but not myoepithelial differentiation. In contrast, forced expression of ΔNp63 in D492M restored the myoepithelial phenotype only. Co-expression of miR-200c-141 and ΔNp63 in D492M restored the branching morphogenesis in 3D culture underlining the requirement for both luminal and myoepithelial elements for obtaining full branching morphogenesis in breast epithelium. Introduction of a miR-200c-141 construct in both D492 and D492M resulted in resistance to endothelial induced EMT. In conclusion, our data suggests that expression of miR-200c-141 and ΔNp63 in D492M can reverse EMT resulting in luminal- and myoepithelial differentiation, respectively, demonstrating the importance of these molecules in epithelial integrity in the human breast.

Introduction

Maintaining tissue integrity while allowing remodeling capacity in organs depends on the presence of tissue stem cells. This is particularly applicable in tissue with fast cellular remodeling such as blood, skin, gastrointestinal epithelium and the breast gland that is subjected to periodic tissue remodeling and branching morphogenesis from the onset of menarche to menopause (Raouf et al., 2012; Reya et al., 2001; Villadsen et al., 2007; Woodward et al., 2005). The breast epithelium consists of an inner layer of polarized luminal epithelial cells and an outer layer of myoepithelial cells that can be discriminated by marker expression (Gudjonsson et al., 2002a; Pechoux et al., 1999). Luminal cells express simple keratins (K) such as K18 and K19, the adhesion molecules E-cadherin and EpCAM and tight junction proteins such as claudin, occludin and ZO-1. In contrast myoepithelial cells express basal keratins such as K5/6, 14 and 17, P-cadherin, vimentin, alpha-smooth muscle actin and the basal cell marker p63 (Gudjonsson et al., 2002a; Pechoux et al., 1999). Although phenotypically and functionally different, luminal- and myoepithelial cells are generally thought to arise from common stem cells (Gudjonsson et al., 2002b; Pechoux et al., 1999; Raouf et al., 2012; Villadsen et al., 2007; Woodward et al., 2005). Furthermore, these stem cells or their downstream progenitors are believed to be the preferred targets of cancer initiation (Raouf et al., 2012; Smalley and Ashworth, 2003).

Epithelial to mesenchymal transition (EMT) is a conserved developmental process where epithelial cells loose epithelial properties and adapt a mesenchymal phenotype. This is observed during gastrulation, neural crest formation and wound healing. EMT is also a driving force in tumor cell invasion and metastasis manifested by loss of cell-to-cell adhesion and increased migration potential

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http://dx.doi.org/10.1016/j.ydbio.2015.05.007

(Hanahan and Weinberg, 2011). In addition, accumulating evidence suggests that EMT may provide differentiated epithelial cells with stem cell properties and contribute to cancer stem cell formation (reviewed in Ansieau (2013)).

MicroRNAs (miRNAs) have moved into the spotlight as master regulators in stem cell biology and fate decision (Hilmarsdottir et al., 2014; Yang and Rana, 2013; Zapata et al., 2012). Recent studies have shown that the miR-200 family plays a crucial role in regulating epithelial integrity and loss of its expression may drive cells through EMT (Hanahan and Weinberg, 2011; Howe et al., 2012). Members of the miR-200 family target the EMT associated transcription factors ZEB1 and ZEB2, which in turn suppress E-cadherin (Olson et al., 2009). The miR-200 gene family is located on two distinct clusters (miR-200ba-429 and miR-200c-141) on chromosomes 1 and 12, respectively (Mongroo and Rustgi, 2010).

The p63 transcription factor is expressed in the basal cells of various tissues, such as the skin, lung, prostate, salivary glands and breast (Blanpain and Fuchs, 2007). p63 is a member of the p53 family and has two major isoforms, ΔNp63 and TA-p63. The ΔN isoform is the dominant form in most tissues and has been identified as a regulator of basal cell associated markers such as K5, K14 and P-cadherin (Romano et al., 2007, 2009; Shimomura et al., 2008). The role of ΔNp63 has mostly been studied in the skin where it is crucial for adult stem cell maintenance. ΔNp63 has also been identified as an essential factor for epithelial stratification in the skin (Senoo et al., 2007) and lung (Daniely et al., 2004; Pellegrini et al., 2001). We have recently shown that knock-down of p63 in lung epithelial basal cells result in increased senescence and lack of pseudostratification (Arason et al., 2014).

The breast epithelial progenitor cell line D492 forms branching structures in 3D culture with differentiated luminal and myoepithelial cells (Gudjonsson et al., 2002b). Furthermore, in 3D coculture with endothelial cells the cell line displays two main morphotypes: branching colonies and spindle-like EMT colonies. The D492-mesenchymal (D492M) cell line was established from a single EMT colony (Sigurdsson et al., 2011, 2013). In this study, we show that D492 and D492M differ greatly in terms of miRNA expression. In particular the miR-200 family is downregulated in D492M. Methylation analysis shows that the promoter area of miR-200c-141 is methylated in D492M only. When introduced into the D492M, miR-200c-141 only reestablishes the luminal epithelial phenotype. Overexpression of miR-200c-141 in D492 and D492M prevents the endothelial-induced EMT demonstrating the importance of miR-200c-141 in preserving epithelial integrity. The missing myoepithelial phenotype was reestablished by introduction of ΔNp63 into D492M. Co-expression of both miR200c-141 and ΔNp63 in D492M reestablished the luminal- and myoepithelial expression seen in D492 and restored branching potential in 3D culture.

Materials and methods

Cell culture and 3D cultures

D492 and D492M were maintained in H14 medium as described previously (Sigurdsson et al., 2011). Primary luminal epithelial cells ($EpCAM^+$) and myoepithelial cells ($EpCAM^-$) were isolated by magnetic cell sorting (MACS) and maintained in CDM3 and CDM4 as described previously (Pechoux et al., 1999). Primary human breast endothelial cells (BRENCs) were isolated from breast reduction mammoplasties and cultured on endothelial growth medium (EGM-2) (Lonza)+5% FBS (Invitrogen) (Sigurdsson et al., 2006). Growth factor reduced reconstituted basement membrane (rBM, purchased as Matrigel, BD Biosciences) was used for 3D culture. 3D monocultures were carried out in 24 well culture plates (BD Falcon). 1×10^4 $D^4$92 cells were suspended in 300 μl of rBM. Co-culture experiments were carried out with 1×10^3 cells mixed with 1×10^5 BRENCs. 300 μl of mixed cells/rBM were seeded in each well of a 24 well plate and cultured on H14 (monoculture) or EGM5 (coculture) for 16 days. For detailed description of 3D cell culture see Sigurdsson et al. (2011).

Pre-cluster assay was adapted from Hirai et al. (1992). Briefly, 50,000 cells were mixed in 500 μl of EGM5 media and cultured on a 24 well low adhesion plate (plate coated with 12 μg/ml polyhema, for 24 h at 37 °C). After 24 h, cell clusters were collected with brief centrifugation and resuspended in 500 μl H14 media and 50 μl of preclustered organoids embedded in 100 μl of matrigel and seeded in 8 well chamber slite. The branching phenotype of cell clusters was determined after cultivation for 8 days.

Branching, solid and spindle-like structures were isolated from 3D cocultures with gentle shaking on ice in PBS-EDTA (5 mM) solution as described previously (Lee et al., 2007). Newly formed Mesenchymal and branching colonies were sorted under a stereomicroscope for RNA isolation.

MicroRNA expression array

RNA was isolated from D492 and D492M at 50% and 90% confluency in monolayer culture using RNeasy minikit (Qiagen). Experiments were conducted in triplicate, on three different time points (resulting in a total of 36 samples). RNA integrity was analyzed using the Agilent 2100 Bioanalyzer (Agilent). Microarray analysis was carried out at the SCIBLU Genomics Centre at Lund University (Lund, Sweden) using Illumina Human v2 MicroRNA Expression BeadChip (Illumina). To filter out unexpressed miRNAs, the 599 miRNA probes detected in at least 3 of the 36 samples (detection p-value ≤ 0.01) were selected for further analysis. For each probe, negative intensities after background correction were replaced by missing values, and then intensities were log-transfomed (base 2) followed by mean centering across samples to generate miRNA expression levels. To identify differentially expressed miRNA genes we used the MeV software (http://www.tm4.org) and the significance of microarrays (SAM) method (Tusher et al., 2001) using 10,000 permutations. The miRNA expression data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE60524 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60524).

RT-PCR analysis

Total RNA was extracted with Tri-Reagent (Ambion). The RNA was DNAase treated and reverse transcribed with Hexanucleotide primers using RevertAid (#K1622, Fermentas). Resulting cDNA was used as template for RT-PCR performed using the primers listed in Supplementary Table 2.

qRT-PCR analysis

Quantitative RT-PCR of ΔNp63, ZEB1 and ZEB2 was performed on the same cDNA as the RT-PCR, described above. For quantification of p63, K14, K19, E-cadherin and N-cadherin SYBR green real-time PCR assay were used (Tp63 from IDT: HS.PT.58.38930512 other primers in Supplementary Table 2) and GAPDH as endogenous reference gene (primers in Supplementary Table 2). Primer pairs and probes from Applied Biosystems (TaqMan) were used for ZEB1 (Hs00232783_m1) and ZEB2 (Hs00207691_m1) quantification. GAPDH (4326317E) was used as endogenous reference gene.

Quantitative RT-PCR analysis of miRNAs was performed using miRCURY LNA™ microRNA PCR System (Exiqon). Gene expression levels were quantified using primers for hsa-miR-141 (#204504) and hsa-miR-200c (#2044852) (Exiqon). Normalization was done

with U6 RNA (#203907) or SNORD48 (#203903) (Exiqon). Relative expression differences was calculated with the $2^{\Delta Ct}$ method.

Immunochemistry

The following primary antibodies were used: Fibronectin (LabMab, (gift from D.E. Mosher (Chernousov et al., 1991))), K19 (ab7754, Abcam), K14 (NCL-LL002, NovoCastra), E-Cad (#610182, BD), N-Cad (#610920, BD), EpCAM (NCL-ESA, Novocastra), and p63 (NCL-p63, Novocastra). For double and triple labeling experiments we used fluorescence isotype specific secondary antibodies (Invitrogen) and fluorescent nuclear counterstain, TO-PRO-3 (Invitrogen). Specimens were visualized on a Zeiss LSM 5 Pa laser-scanning microscope (Carl Zeiss) and Olympus fluoview 1200.

Western blotting

Equal amounts (5 μg) of proteins in RIPA buffer were separated on 10% NuPage Bis-Tris gels (Invitrogen) and transferred to a PVDF membrane (Invitrogen). Antibodies: E-Cad (BD), Ncad (BD), K5/6 (Zymed), K8 (Abcam), K14 (Abcam), K17 (Dako), K19 (Abcam), Vimentin (Dako), Snail (Abcam), p63 (Abcam) actin, and EpCAM (Abcam) were used. Secondary antibodies were mouse or rabbit IRDey (Li-Cor) used at 1:10,000 and detected using the Odyssey Infrared Imaging System (Li-Cor). Fluorescent images were converted to gray scale.

5-Aza treatment

D492 and D492M were grown to 70% confluency. Cells were treated with 5 mM of 5-azacitidine (Sigma, A2385) in H14 medium and cultured for 4 days before IF staining or DNA, or protein isolation.

Bisulfite sequencing

Bisulfite conversion of DNA was performed with the Quiagen Epitech bisulfate kit (59,104) using 0.5–1 μg of DNA. The target DNA sequence was amplified using Nested PCR. Primer sequences are in Supplementary Table 2. DNA methylation analyses of bisulfite PCR amplicons were performed using Sequence scanner V1.0. DNA methylation level was scored as percentage methylation of individual CpG units in each sample.

miR-200c-141 and ΔNp63 overexpression

A miR-200c-141 construct was created by cloning the genomic region into a pLVTHM lentiviral vector purchased from Addgene (plasmid #12247 (Wiznerowicz and Trono, 2003)). The miR-200c-141 insert was amplified from genomic DNA using nested PCR (primers in Supplementary Table 2) and the sequence confirmed with sequencing. An empty vector was used as a control. The pLVTHM vector contains a green fluorescent protein (GFP) selection marker. Viral particles were produced in HEK-293T cells using Arrest-In transfection reagent (ATR1740; Open Biosystems) according to instructions. Virus-containing supernatants were collected 48 h after transfection and target cells were infected in the presence of 8 μg/μl polybrene. Stable, D492 and D492M with miR-200c-141 and control (empty) cells were isolated by flow-sorting, selecting GFP expressing cells using FACSaria. deltaNp63alpha-FLAG (Addgene, plasmid #26979 (Chatterjee et al., 2008)) was used to clone ΔNp63 into a pCDH lentiviral vector (System Biosciences), containing both RFP and a puromycin selection marker and viral particles were produced as described above. $D492M^{miR\text{-}200c\text{-}141}$ cells and $D492M^{empty}$ where transduced with the ΔNp63 overexpressing vector and $D492M^{empty}$ with empty pCDH vector as a control ($D492M^{2\times empty}$). Stable $D492M^{2\times empty}$, $D492M^{\Delta Np63}$ and $D492M^{miR\text{-}200c\text{-}141\Delta Np63}$ cells were selected using 2 μg/ml puromycin.

miR-200c-141 inhibition and ΔNp63 downregulation

miRCURY LNA™ microRNA Inhibitors from Exiqon were used to inhibit miR-200c-141 function, hsa-miR-200b, hsa-miR-200c (#450012-2) and hsa-miR-141, hsa-miR-200a (#450012-3). Exiqon negative control A inhibitor (#199004-04) was used as a control. Briefly, 75.000 D492 cells were seeded in each well of a 24 well plate and cultured on H14 for 24 h. Following the 24 h incubation media was removed and replaced with H14 without antibiotics, containing 25 nM inhibitor (#450012-2) and 25 nM inhibitor (#450012-3) or 50 nM negative inhibitor (#199004-04). Transfection was carried out by using 1.5 μl of Lipofectamine® RNAiMAX (Life Technologies #13778-075) per well. Cells were incubated for additional 24 h followed by RNA isolation, RT-PCR and qRT-PCR analysis. Samples were run in biological triplicates. ΔNp63 expression was downregulated using shp63alpha pLKO.1 puro lentiviral vector (Addgene plasmid 19,120 (Godar et al., 2008)) and pLKO.1 shSCR lentiviral vector (Addgene plasmid 17,920 (Saharia et al., 2008)) was used as control. Viral particles were produced in HEK-293 T cells using TurboFect transfection reagent (R0531; Life Technologies) according to manufacturer's instructions. Virus-containing supernatant was collected 48 h after transfection and target cells were infected in the presence of 8 μg/μl polybrene. Stable $D492^{Scr}$ and $D492^{p63KD}$ cells were selected using 2 μg/ml puromycin.

Statistical analysis

Data are presented as means with standard deviations of measurements. Statistical differences between samples were assessed with Student two-tailed *T*-test. *p*-Values below 0.05 were considered significant ($^{***}p \le 0.001$, $^{**}p \le 0.01$, $^{*}p \le 0.05$).

Results

Endothelial-induced EMT in D492 breast epithelial stem cell line is associated with reduced expression of the miR-200 family

We have recently shown that endothelial cells can induce EMT in D492, a breast epithelial cell line with stem cell properties (Sigurdsson et al., 2011, 2013). A mesenchymal subline of D492, referred to as D492M shows irreversible EMT as evidenced by spindle-like phenotype in 2D and 3D culture, downregulation of E-cadherin, increased expression of N-cadherin and loss of K14 and K19 expression (Fig. 1A). In order to evaluate differences in miRNA expression profiles we carried out analysis of microRNA expression in D492 and D492M cells cultured in monolayer. We identified 186 miRNAs (false discovery rate = 0.002) differentially expressed between D492 and D492M, out of 599 analyzed miRNAs demonstrating large differences between D492 and D492M (Supplementary Table 1). Of the ten most downregulated miRNAs in D492M, four belonged to the miR-200 family (miR-141, -200a,-200c,-200b) (Fig. 1B). These microRNAs are emerging as major regulators of epithelial integrity in human and murine cells (Davalos et al., 2012; Gregory et al., 2008; Mongroo and Rustgi, 2010; Wellner et al., 2009). In particular, miR-200c and miR-141 have both been strongly linked to epithelial integrity (Davalos et al., 2012; Mongroo and Rustgi, 2010). miR-200c and miR-141 are colocalized on chromosome 12 (Fig. S1) and their expression is regulated from the same promoter (Mongroo and Rustgi, 2010).

Decreased expression of miR-200c and miR-141 in D492M compared to D492 was confirmed by qPCR (Fig. 1C). In addition,

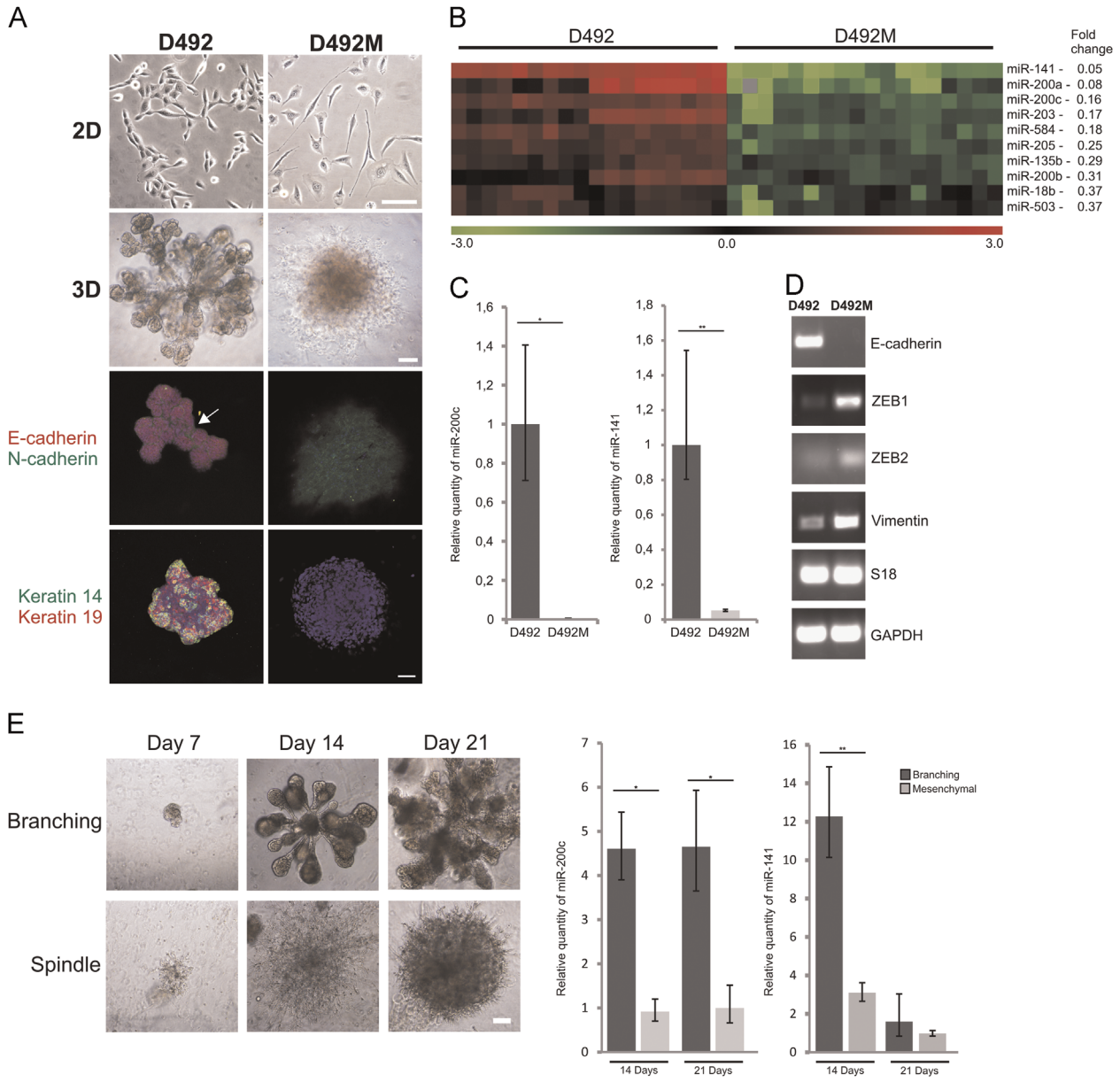

Fig. 1. The miR-200 family is downregulated in breast epithelial stem cells undergoing EMT. (A) D492 and D492M generate branching and mesenchymal-like structures in 3D culture, respectively. D492 a breast epithelial cell line with stem cell properties forms cuboidal epithelial phenotype in 2D culture and elaborate branching structures in 3D culture in reconstituted basement membrane (rBM) matrix. In contrast, D492M forms a spindle-like phenotype in 2D and 3D cultures. Immunostaining shows reduced expression of E-cadherin (red) and increased expression of N-cadherin (green) in D492M. K14 (green) and K19 (red) that are present in the branching structures of D492 in 3D culture are lost in the spindle-like colonies of cultured D492M cells. Note, the N-cadherin expression in the branching structures of D492 is restricted to the interface between lobular units (arrow) compared to the overall staining in D492M. Cells were counterstained with TO-PRO-3 nuclearstain. Bar = 100 μM. (B) miRNA expression analysis shows drastic difference between D492 and D492M. Heatmap of differentially expressed miRNAs shows a drastic difference between D492 and D492M. Of the top 10 downregulated miRNAs in D492M, four where from the miR-200 family (miR-200a, miR-200b, miR-200c and miR-141). There was not significant difference in the expression of the fifth miR-200 family member, miR-429. (C) Validation of miR-200c and miR-141 expression in D492 and D492M. miR-200c and miR-141 downregulation in D492M was verified with qPCR. The expression of miR-200c was shown to be more than 500 fold in D492 relative to D492M. miR-141 expression was almost 20 fold higher in D492 than D492M. miRNA levels were normalized to U6. (D) Expression of ZEB1 and ZEB2 is increased in D492M. PCR expression analysis of selected targets regulated by miR-200c-141 shows different expressions between D492 and D492M. E-cadherin was lost in D492M accompanied by increased expression of ZEB1, ZEB2 and vimentin. S18 and GAPDH as loading control. (E) miR-200c and -141 are downregulated in newly formed mesenchymal structures. D492 cells were embedded into matrigel in coculture with endothelial cells in order to form branching and spindle like structures. After 14 and 21 days in culture, RNA was isolated from newly formed mesenchymal colonies. qPCR for miR-200c and -141 demonstrated significantly higher expression of these miRNAs in newly formed branching colonies than in mesenchymal colonies. miRNA levels were normalized to SNORD48. Bar = 100 μM.

RT-PCR shows down-regulation of E-cadherin in D492M accompanied by up-regulation of downstream targets for miR-200c such as ZEB1, ZEB2 and vimentin (Fig. 1D). These data show that loss of miR-200c-141 is accompanied by increased expression of EMT regulators, reduced expression of epithelial markers and gain of mesenchymal phenotype.

We next validated the differences in miR-200c-141 expression in newly formed branching and mesenchymal structures from 3D cultures. D492 and BRENCS were co-cultured in 3D rBM and RNA was isolated from branching and spindle-like colonies at two different time points, after 14 and 21 days in culture (Fig. 1E). These two time points represent different phases of the branching process where extensive branching is occurring on day 14, but on day 21 colony growth and branching has stopped. qPCR confirmed that miR-200c and miR-141 are downregulated in mesenchymal structures relative to branching structures at day 14 but on day 21 miR-141

expression is downregulated in both branching and mesenchymal structures while miR-200c remains unchanged. The reason for downregulation of miR-141 during branching at this time point is unclear. It has however been shown that miRNAs biogenesis is under strict control and subject to many post-transcriptional regulation mechanisms (Ha and Kim, 2014).

The promoter region of miR-200c-141 is methylated in D492M but not in D492

Gene silencing by CpG methylation is widely used by cells to suppress gene expression. Recent papers have shown that the expression of the miR-200 family can be regulated through methylation of its CpG rich promoter region (Vrba et al., 2010; Wiklund et al., 2010). To see if this was the case in D492M we bisulfite sequenced the CpG islands in the promoter area of the miR-200c-141 and as shown in Fig. 2A methylation occurs only in D492M, suggesting that methylation might play a role in the differential expression. The promoter of E-cadherin is commonly methylated during EMT but bisulfate sequencing showed that neither D492 nor D492M were methylated at the E-cadherin promoter (Fig. 2A). CpG islands in the other miR-200 family promoters, miR-200ba-429 were methylated in both D492 and D492M (Fig. S2A). These data are consistent with previous reports, showing that the miR-200c-141 cluster is regulated by promoter methylation while histone methylation is more important for repression of the miR-200ba-429 cluster (Davalos et al., 2012).

To assess the methylation effects on the EMT phenotype in D492M, we treated D492M cells with the demethylation agent 5-azacytidine (5-aza). Phenotypic characterization after treatment showed that the epithelial phenotype could be partially restored in D492M as demonstrated by expression of EpCAM, E-cadherin, K14 and K19 and reduced expression of N-cadherin (Fig. 2B). Similarly miR-200c expression was significantly upregulated in D492M after 5-aza treatment (Fig. 2C) but there was no change in the expression of miR-141 which remained very low before and after treatment. The expression of p63 was unchanged after 5-aza treatment. Bisulfate sequencing showed reduction of methylation at the miR-200c-141 promoter after 5-aza treatment (Fig. S2B).

Having shown that both miR-200c and miR-141 are markedly down-regulated in D492M and that the miR-200c-141 promoter locus is methylated, suggesting a regulated event, we decided to ectopically express the miR-200c-141 locus in both D492 and D492M and monitor the effect on morphogenesis, epithelial integrity and stemness in our breast stem cell model.

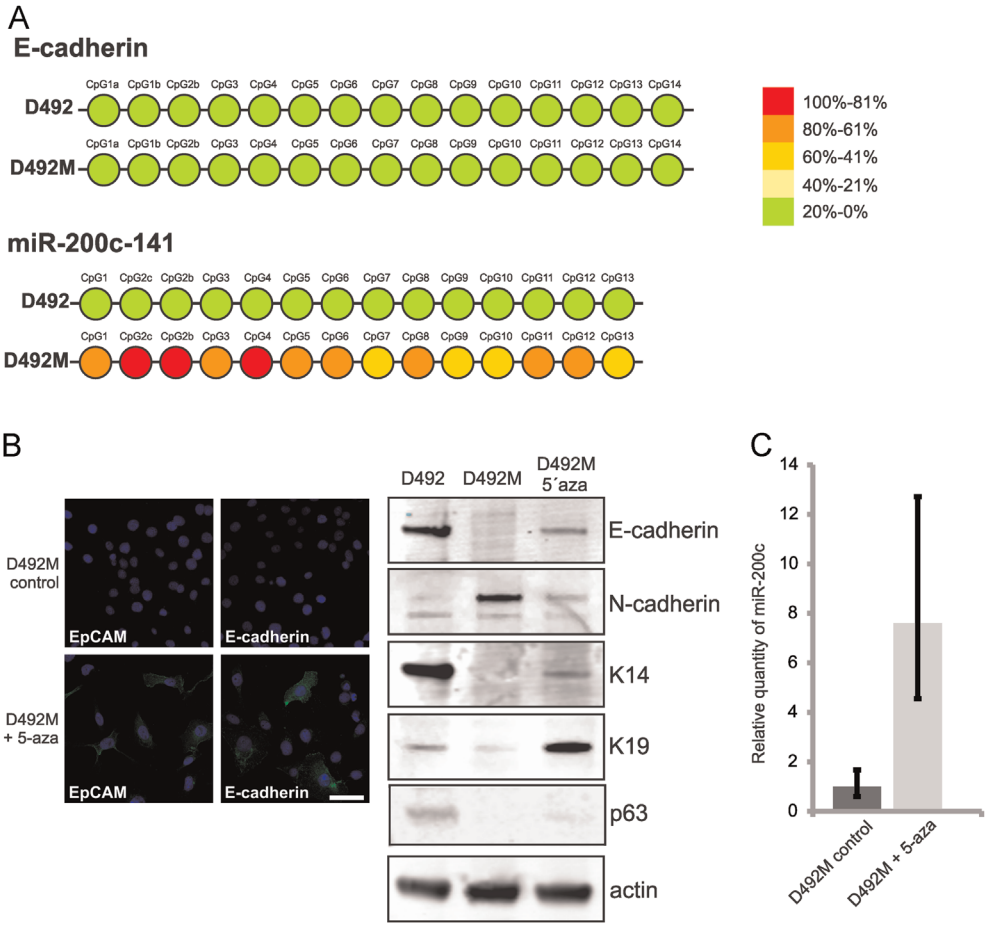

Fig. 2. The promoter region of miR-200c-141 is methylated in D492M. (A) The promoter area of miR-200c-141 is methylated in D492M. Bisulfite sequencing shows no methylation at the promoter region of E-cadherin in D492 and D492M. In contrast the promoter region of miR-200c-141 is methylated D492M only, where miR-200c and -141 expression is reduced. The color column on the right indicates percentage levels of methylation at CpG island promoter areas in E-cadherin and miR-200c-141. (B) 5-Azacytidine (5-Aza) treatment of D492M partially reverses the EMT phenotype. Immunostaining for E-cadherin and EpCAM (green) (left) demonstrates that expression is partially reestablished in D492M after treatment with a demethylation agent (5-aza). Cells were counterstained with TO-PRO-3 nuclearstain. Western blot demonstrated gain of epithelial phenotype in 5-aza treated cells as seen by increased expression of E-cadherin, K14 and K19 and decreased expression of N-cadherin but no change in p63 expression (right). Actin was used as a loading control. (C) 5-Aza treatment of D492M induces expression of miR-200c. U6 as loading control.

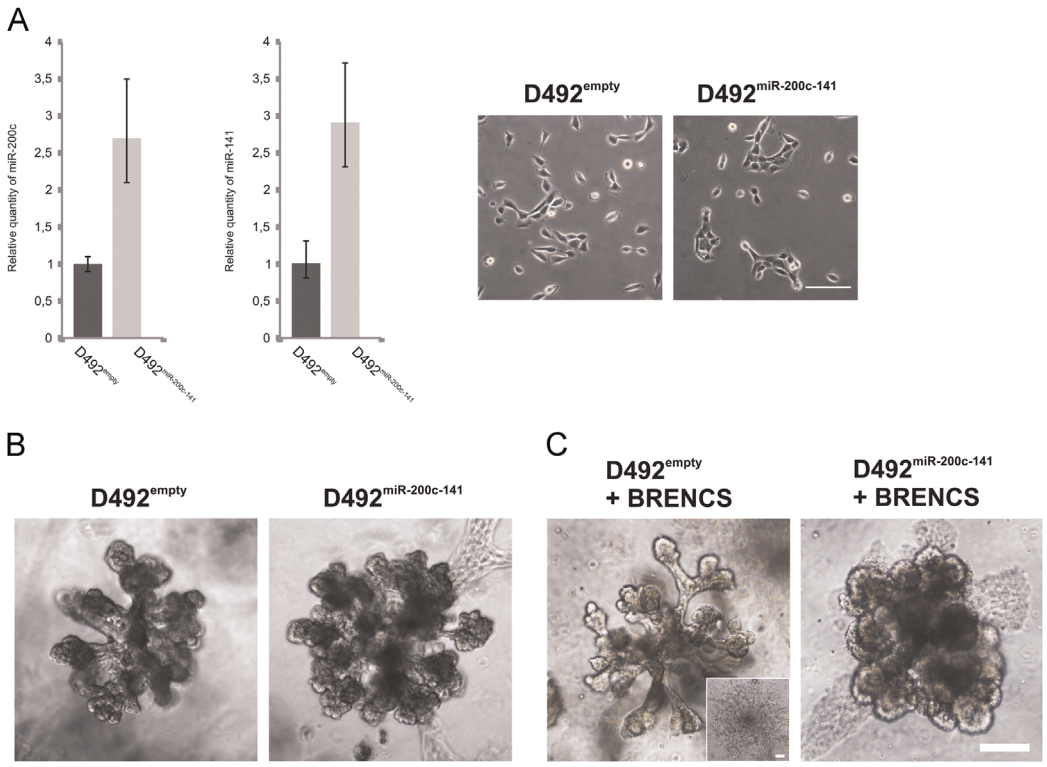

Fig. 3. Overexpression of miR-200c-141 in D492 inhibits EMT, but does not affect branching. (A) qPCR analysis verifies the overexpression of miR-200c and -141 in D492. D492M cells were transduced with a lentiviral construct containing miR-200c-141 ($D492^{miR\text{-}200c\text{-}141}$). Expression levels of miR-200c and -141 were 2–3 fold higher in $D492^{miR\text{-}200c\text{-}141}$ than in $D492^{empty}$ cells. miRNA levels were normalized to U6. (B) $D492^{miR\text{-}200c\text{-}141}$ cells cultured in rBM monoculture form branching colonies similar to $D492^{empty}$. (C) $D492^{miR\text{-}200c\text{-}141}$ are resistent to EMT in coculture with endothelial cells. $D492^{miR\text{-}200c\text{-}141}$ only form branching structures in coculture with BRENCS while $D492^{empty}$ form branching and mesenchymal colonies in coculture.

Overexpression of miR-200c-141 in D492 prevents endothelial induced EMT

Partial EMT (p-EMT) is a process previously described in wound healing and mammary tubulogenesis during branching morphogenesis (Leroy and Mostov, 2007). Our data shows that miR-200c and miR-141 are highly expressed in D492 to a level of approximately two fold that of primary breast epithelial cells (Fig. S3A). However miR-200c-141 could be locally downregulated at branching tips during branching morphogenesis as consequence of p-EMT. Local downregulation is not expected to influence levels of total miRNA and hence would not be detected in qPCR (Fig. 1E). To test if local downregulation of miR-200c and miR-141 is necessary for branching we constitutively overexpressed miR-200c-141 in D492 (Fig. 3A) thereby inhibiting possible partial downregulation by subpopulation of cells. $D492^{miR\text{-}200c\text{-}141}$ cells showed no differences in morphology in 2D culture (Fig. 3A) or in expression of critical epithelial markers (Fig. S3B). When embedded into rBM $D492^{miR\text{-}200c\text{-}141}$ showed a similar phenotype as $D492^{empty}$ (Fig. 3B). However, in contrast to $D492^{empty}$, $D492^{miR\text{-}200c\text{-}141}$ was unable to undergo EMT when cocultured with BRENCS and only formed branching structures, showing the efficiency of miR-200c-141 to maintain epithelial integrity and prevent EMT (Fig. 3C). We conclude that down-regulation of miR-200c-141 is necessary to undergo EMT, but not for branching morphogenesis in the breast.

Ectopic expression of miR-200c-141 in D492M induces luminal epithelial differentiation

To understand the importance of miR-200c-141 silencing in maintaining the mesenchymal phenotype seen in D492M, we constitutively overexpressed miR-200c-141 in D492M (Fig. 4A). Re-expression of miR-200c-141 in D492M reversed the mesenchymal phenotype allowing reemergence of cuboidal epithelial phenotype in monolayer culture and increased expression of luminal epithelial markers (EpCAM, E-cadherin, K19) and decreased expression of N-cadherin (Fig. 4A and B). Interestingly, $D492M^{miR\text{-}200c\text{-}141}$ does not express the myoepithelial keratins K5/6 and K14. Faint expression of K17 can be seen on western blot (Fig. 4B). Furthermore, $D492M^{miR\text{-}200c\text{-}141}$ fails to reestablish expression of the myoepithelial transcription factor p63 that is known to regulate expression of basal markers such as K14 and P-cadherin (Romano et al., 2007; Shimomura et al., 2008). Further characterization showed that the level of the EMT transcription factors, and targets of miR-200c and -141, ZEB1 and ZEB2 were decreased (Fig. S4A). These data indicate that miR-200c-141 is particularly important for maintaining the luminal epithelial phenotype. In order to support this further we analyzed the expression of miR-200c and -141 in purified primary luminal- and myoepithelial cells. The expression was 15–260 fold higher in the EpCAM positive luminal epithelial cells compared to the EpCAM negative fraction containing the myoepithelial cells (Fig. 4C). Having shown that forced expression of mir-200c-141 in D492M reestablished the luminal epithelial phenotype we evaluated its effect on the branching potential of D492M and the ability of the cell line to undergo endothelial-induced EMT.

D492MmiR-200c-141 cells form epithelial-like colonies in 3D culture and are resistant to endothelial-induced EMT

To evaluate the morphology of $D492M^{miR\text{-}200c\text{-}141}$ in 3D culture we seeded cells into rBM. In contrast to $D492M^{empty}$ that only generates mesenchymal/spindle-like structures, $D492M^{miR\text{-}200c\text{-}141}$ forms epithelial structures that, however, are not as complex as those generated from the original D492 cell line (compare Fig. 4D upper panel and Fig. 1A). Immunocytochemistry demonstrated a typical mesenchymal phenotype in the $D492M^{empty}$ cells as shown

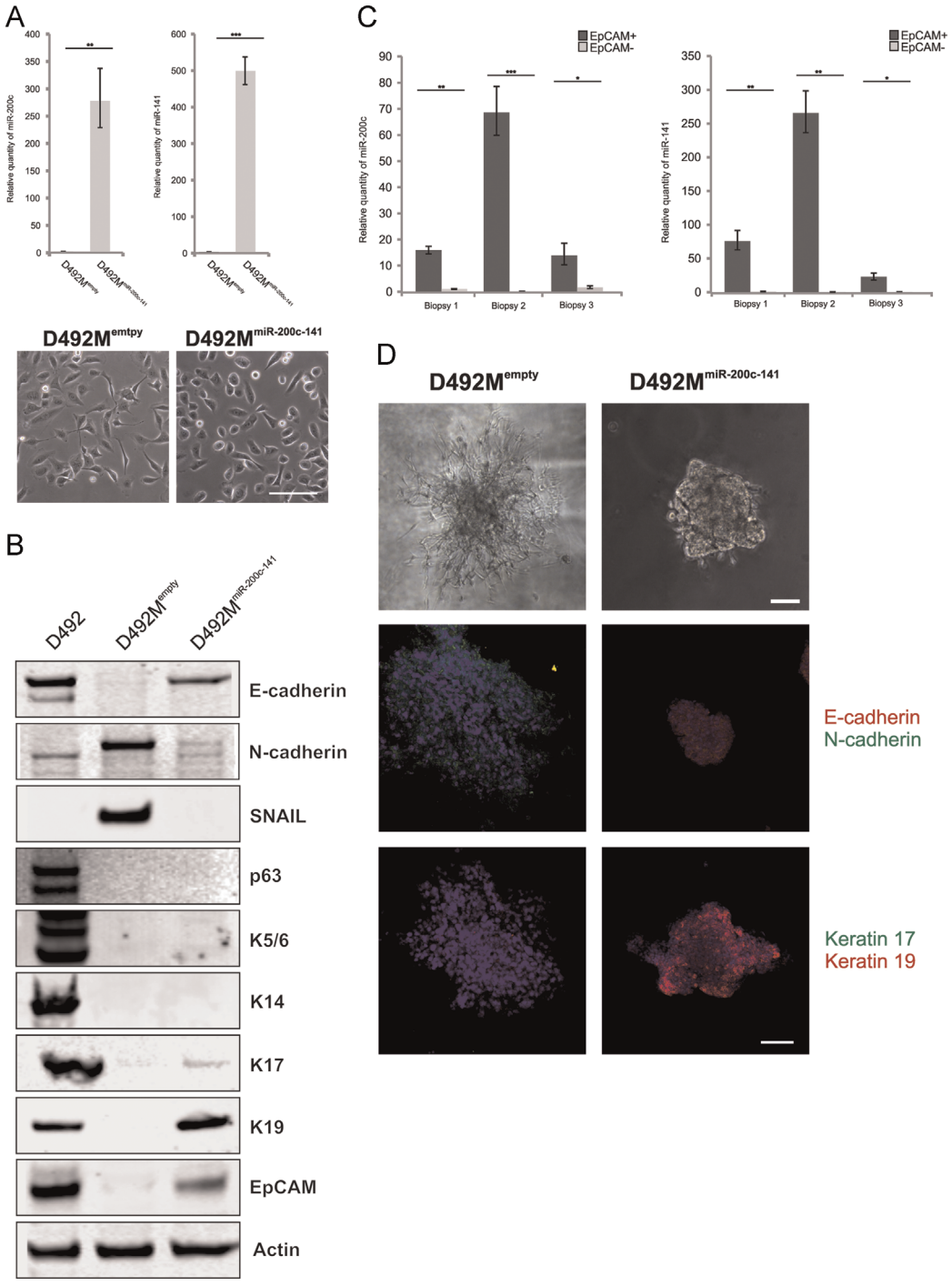

Fig. 4. Forced expression of miR-200c-141 induces luminal epithelial differentiation. (A) Overexpression of miR-200c-141 in D492M induces epithelial morphology in 2D culture. D492M cells were transduced with a lentiviral construct containing miR-200c-141 (D492M$^{miR\text{-}200c\text{-}141}$). The expression was 300–500 fold higher in D492M$^{miR\text{-}200c\text{-}141}$ than in D492M^{empty} cells. Phase contrast image of D492M$^{miR\text{-}200c\text{-}141}$ shows cuboidal epithelial phenotype in culture. miRNA levels were normalized to U6. Bar = 100 μM. (B) D492M$^{miR\text{-}200c\text{-}141}$ acquires luminal epithelial phenotype. Western blot demonstrates expression of E-cad, EpCAM and K19 in D492M$^{miR\text{-}200c\text{-}141}$ cells. In contrast little or no expression of p63, K14, K5/6, or K17 is seen. Also, the expression of the EMT markers N-cad and SNAIL is reduced compared to D492M^{empty}. Actin was used as a loading control. (C) miR-200c and miR-141 are luminal epithelial markers in the breast. qPCR analysis shows that EpCAM positive cells (luminal), express 5–7 fold more miR-200c and 40–120 fold more miR-141 than EpCAM negative cells (myoephithelial). Measurement was done in paired luminal and myoepithelial cells from three different biopsies. miRNA levels were normalized to SNORD48. (D) D492M^{empty} and D492M$^{miR\text{-}200c\text{-}141}$ form spindle and irregular branching like structures in 3D rBM culture, respectively. Immunostaining shows vague expression of N-cad (green) and strong E-cad (red) expression in D492M$^{miR\text{-}200c\text{-}141}$. Note the lack of polarity in N-cad expression (compared with Fig. 1A). D492M$^{miR\text{-}200c\text{-}141}$ retain K19 (red) expression in 3D culture but lack expression of K17 (green). Cells were counterstained with TO-PRO-3 nuclearstain. Bar = 100 μM.

by N-cadherin expression and lack of K14, K19 and E-cadherin expression. The D492M$^{miR\text{-}200c\text{-}141}$ cells showed a weak overall N-cadherin in 3D culture staining unlike D492 cells that expresses N-Cadherin particularly at the interface between lobular units. D492M$^{miR\text{-}200c\text{-}141}$ cells are E-cadherin positive and K19 positive, but negative for K17, confirming lack of myoepithelial differentiation in the cells. These results were confirmed by western blot analysis showing expression of luminal epithelial markers but not mesenchymal or myoepithelial markers in 3D (Fig. S4B). When cocultured with BRENCs previously shown to induce EMT in D492

(Sigurdsson et al., 2011), D492M$^{miR\text{-}200c\text{-}141}$ forms branching- and solid round colonies but not mesenchymal colonies (Fig. S5A–C). To further examine the role of miR-200c-141 in epithelial differentiation we treated D492 with miRNA inhibitors for miR-200c and miR-141 for 24 h and monitored the effect on known target genes (ZEB1 and ZEB2) and epithelial markers (Fig. S6). Significant changes were seen in ZEB2 expression, but there was no change in E-cadherin, N-cadherin, K14 and K19. ZEB1 expression was absent in both miR-200c-141 inhibitor treated D492 and the control D492 cell line. This data suggests that miR-200c-141 was only partly inhibited, potentially because of their high expression in D492, resulting in moderate effect in downstream targets, but not enough to induce phenotypic changes in D492.

p63 expression is necessary and sufficient for establishment of myoepithelial differentiation in D492M

The fact that miR-200c-141 was only able to rescue the luminal epithelial differentiation in D492M left us with the open question what regulates the myoepithelial differentiation in D492. p63 is a

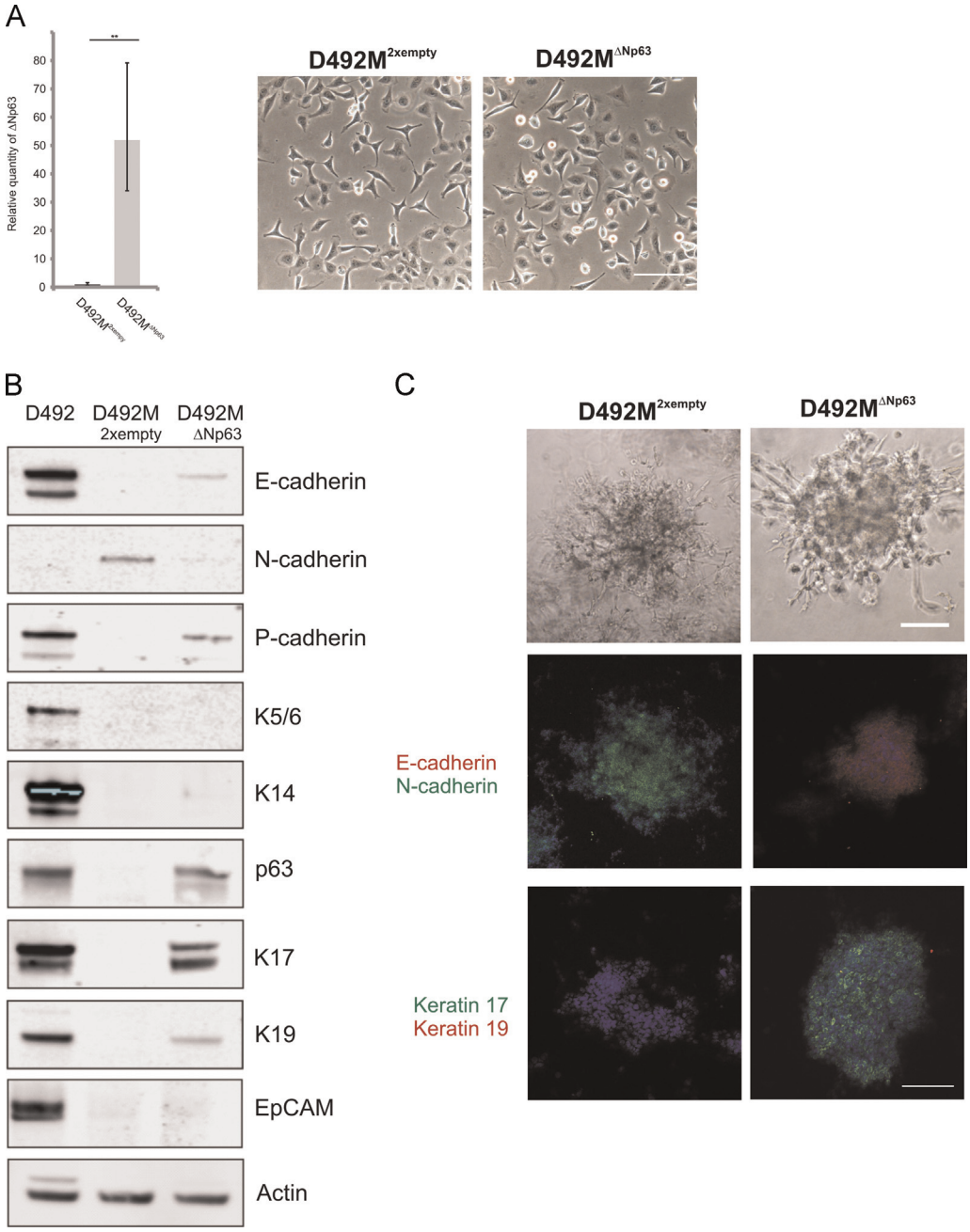

Fig. 5. Forced expression of ΔNp63 induces myoepithelial differentiation. (A) ΔNp63 overexpression has little effect on morphology of D492M in 2D culture. D492M cells were transduced with a lentiviral construct containing ΔNp63 (D492M$^{\Delta Np63}$). The expression levels of ΔNp63 were 50 fold higher in D492M$^{\Delta Np63}$ than in D492$^{2\times empty}$ cells. Phase contrast image of D492M$^{\Delta Np63}$ shows minor effect of ΔNp63 overexpression on cell morphology. miRNA levels were normalized to U6. Bar = 100 μM. (B) D492M$^{\Delta Np63}$ acquires myoepithelial phenotype. Western blot shows strong expression of the myoepithelial markers p63, K17 and P-cadherin and a vague K14 expression. Also, the expression of the EMT marker N-cad is reduced compared to D492M^{empty}. Actin was used as a loading control. (C) D492M$^{\Delta Np63}$ cell in rBM monoculture form irregular colonies. D492M$^{\Delta Np63}$ form structures with slightly more dense morphology than D492M, but retain the mesenchymal morphology of the parental cell line. IF staining shows strong expression of E-cad (red) and K17 (green) but lack of N-cad (green) and K19 (red) expression. Cells were counterstained with TO-PRO-3 nuclearstain. Bar = 100 μM.

known regulator of basal cells in number of epithelial organs such as the skin, lung, salivary glands and breast (Candi et al., 2008). Although the expression of p63 is associated with myoepithelial cells in the breast the functional role of this transcription factor in myoepithelial differentiation has not been thoroughly explored. ΔNp63 is downregulated in D492M compared to D492 (Fig. S7). We therefore overexpressed ΔNp63 in D492M (Fig. 5A). Expression of ΔNp63 in D492M resulted in a switch from N-cadherin to E-cadherin (Fig. 5B). Moreover D492M$^{\Delta Np63}$ cells expressed the myoepithelial markers K17 and P-cadherin but lack the luminal epithelial marker EpCAM. Upregulation of ΔNp63 in D492M induced expression of the luminal epithelial marker K19, but to a much lesser extent than miR-200c-141 (Fig. 4B). Surprisingly, ectopic expression of ΔNp63 did not result in K5/6 or K14 myoepithelial marker expression as reported in other organs (Romano et al., 2009). In 3D rBM culture the D492M$^{\Delta Np63}$ cells form structures with slightly more dense morphology than D492M, but retain the mesenchymal morphology of the parental cell line.

To confirm our results implicating ΔNp63 in linage decision of D492M we used lentiviral vector to knock down p63 in D492

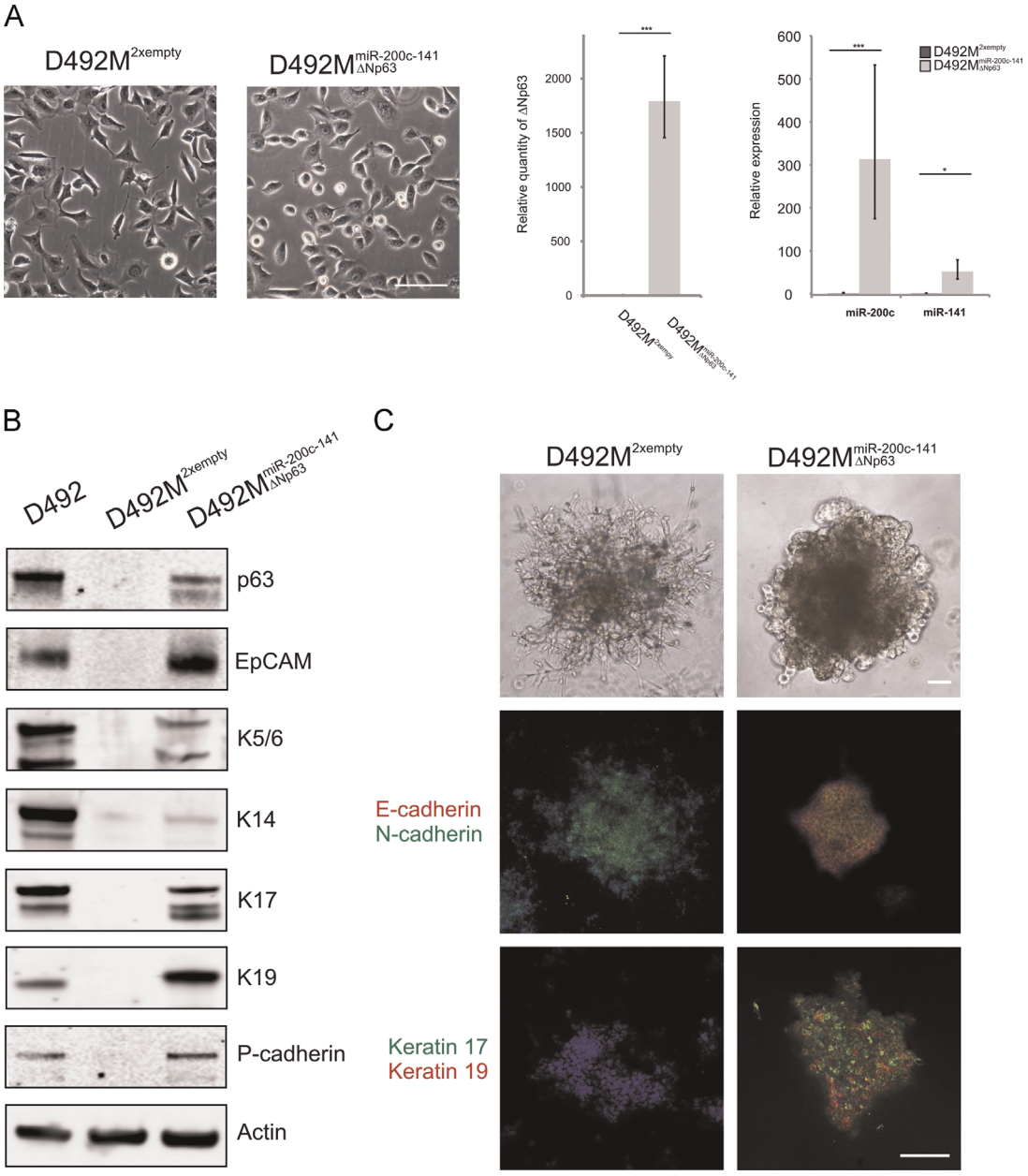

Fig. 6. Co-expression of mir-200c-141 and ΔNp63 induces LEP and MEP differentiation. (A) Co-expression of miR-200c-141 and ΔNp63 in D492M. D492M$^{\text{miR-200c-141}}$ were transduced with a lentiviral construct containing ΔNp63 (D492M$^{\text{miR-200c-141-}\Delta Np63}$). Phase contrast image of D492M$^{\text{miR-200c-141-}\Delta Np63}$ shows cuboidal epithelial phenotype in culture. miR-200c, miR-141 and ΔNp63 expression levels were 50–1700 fold higher in D492M$^{\text{miR-200c-141-}\Delta Np63}$ than in D492$^{2\times\text{empty}}$ cells, respectively. miRNA levels were normalized to U6. Bar = 100 μM. (B) miR-200c-141 and ΔNp63 coexpression induces expression of luminal and myoepithelial markers. Western blot shows strong expression of the myoepithelial markers p63, K5/6, K17 and P-cadherin and a vague K14 expression in D492M$^{\text{miR-200c-141-}\Delta Np63}$. The luminal markers EpCAM and K19 are also expressed. Actin as loading control. (C) D492M$^{\text{miR-200c-141-}\Delta Np63}$ cells in rBM monoculture form irregular branching colonies. Immunostaining shows co-expression of N-cad (green) and E-cad (red) D492M$^{\text{miR-200c-141-}\Delta p63}$. Note the lack of polarity in N-cad expression (compared with Fig. 1A). D492M$^{\text{miR-200c-141-}\Delta Np63}$ expresses both luminal marker K19 (red) and myoepithelial marker K17 (green). Cells were counterstained with TO-PRO-3 nuclearstain. Bar = 100 μM.

(Fig. S8A). Downregulation of p63 in D492 reduced expression of E-cadherin, P-cadherin and K14 but did not influence the levels of of N-cadherin and K19 (Fig. S8B and C).

Co-expression of mir-200c-141 and ΔNp63 in rescues the luminal–myoepithelial phenotype and induces branching

In an attempt to restore both luminal- and myoepthelial differentiation in D492M we transduced D492M with lentiviral vectors for the constitutive expression of miR-200c-141 and ΔNp63 (Fig. 6A). miR-200c-141 and ΔNp63 expression in D492M induced expression of various luminal and myoepithelial markers (Fig. 6B). Expression of key epithelial markers was similar to that found in D492. Interestingly, co-expression of these genes induces expression of K5/6 and K14, that were not expressed in D492M$^{miR\text{-}200c\text{-}141}$ or D492M$^{\Delta Np63}$. In 3D rBM culture the D492M$^{miR\text{-}200c\text{-}141\text{-}\Delta Np63}$ cells form epithelial structures with more mature branching than previously seen, but not as complex as D492 (Fig. 6C). Immunofluorescent staining of D492M expressing both miR-200c-141 and ΔNp63 shows induced expression of both K17 and K19 (Fig. 6C).

In regular 3D rBM culture, colonies result from the growth of a single cell. To further investigate the branching potential of D492M$^{miR\text{-}200c\text{-}141\text{-}\Delta Np63}$ we applied a cluster assay in which case the cells were preclustered in ultra-low adhesion plates for 24 h prior to seeding into matrigel. This assay allows the cells pre-arrange in multi-cell clusters before 3D culture. After 8 days culture period under this condition D492, D492M^{empty}, D492M$^{miR\text{-}200c\text{-}141}$ and D492M$^{\Delta Np63}$ form similar structures as described before, however, D492M$^{miR\text{-}200c\text{-}141\text{-}\Delta Np63}$ forms elaborate branching structures similar to D492 (Fig. 7A) which show overall expression of E-cad, K17 and K19 (Fig. 7B). Thus ectopic expression of both miR-200c-141 and ΔNp63 is necessary and sufficient to induce branching phenotype in D492M similar to D492.

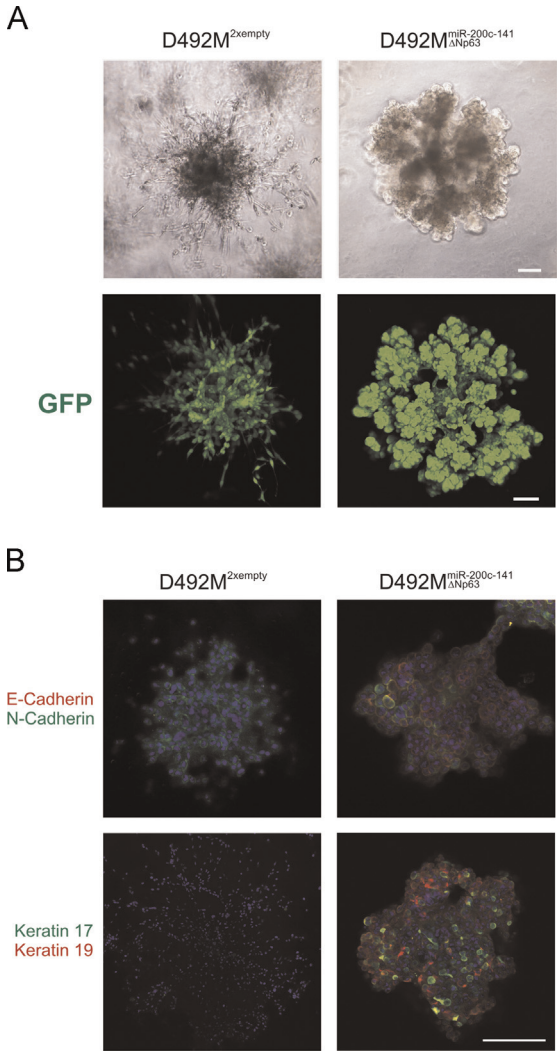

Fig. 7. Co-expression of mir-200c-141 and ΔNp63 in rescues the luminal–myoepithelial phenotype and induces branching. (A) D492M$^{miR\text{-}200c\text{-}141\text{-}\Delta Np63}$ form epithelial branching structures in cluster assay. Cells were preclustered in an ultra low adhesion plate for 24 h, embedded into matrigel and cultured for 8 days. D492M$^{miR\text{-}200c\text{-}141\text{-}\Delta Np63}$ form elaborate branching structures similar to D492 (see Fig. 1A) while D492M form mesenchymal structures in cluster assay (upper bright field, lower GFP). Bar = 100 μM. (B) Expression of luminal and myoeithelial markers in D492M$^{miR\text{-}200c\text{-}141\text{-}\Delta Np63}$. Immunostaining shows low N-cad (green) and strong E-cad (red) expression in D492M$^{miR\text{-}200c\text{-}141\text{-}\Delta p63}$ as well as K17 (green) and K19 (red) expression. Cells were counterstained with TO-PRO-3 nuclearstain. Bar = 100 μM.

Discussion

In this study we have identified critical elements controlling entry to the EMT program in breast epithelium using a model based on the human epithelial stem cell line D492. We found that forced expression of miR-200c-141 suppressed EMT in D492 and converted its mesenchymal derivative D492M towards epithelial phenotype of the luminal type. While expression of the miR-200c-141 construct did not rescue the branching potential of the original cell line, additional up-regulation of the myoepithelial marker ΔNp63 had a complimentary effect by restoring branching capacity. The results demonstrate the importance of miR-200c-141 for breast epithelial integrity in general and luminal epithelial differentiation in particular. They also underline the requirement for both luminal and myoepithelial elements for obtaining full branching morphogenesis in breast epithelium.

Our study was inspired by our initial observation showing that the permanent conversion of D492 to D492M was accompanied by dramatic down-regulation of the miR-200c-141 locus previously linked to epithelial integrity. After confirming our initial findings we demonstrated that the down-regulation is caused by methylation-based repression of the miR-200c-141 promoter. Previous studies are also in line with our data showing that GpC-rich promoter areas for miR-200c-141 are frequently methylated during EMT (Castilla et al., 2012; Vrba et al., 2010). Castilla et al. have shown that the miR-200c-141 locus is frequently hypermethylated in triple negative breast cancer and metablastic breast cancer both cancer subtypes showing prominent EMT phenotype. They also demonstrated hypermethylation of the miR-200-141 locus in cellular model of spontaneous EMT (Castilla et al., 2012). Vrba demonstrated that CpG island is unmethylated in promoter area of human miR-200/miR-141 expressing epithelial cells and in miR-200c/miR-141 positive tumor cells. They also also demonstrated that the CpG island is heavily methylated in human miR-200c/miR-141 negative fibroblasts and miR-200c/miR-141 negative tumor cells (Vrba et al., 2010).

In primary tissue, we found that expression levels of miR-200c and miR-141, was strong in luminal breast epithelium but levels were much lower in the myoepithelial compartment. This is in line with the results of Bockmeyer et al. who showed that miR-200c-141 expression in tissue from reduction mammoplasty was predominantly in the luminal epithelial compartment and to a much lesser extent in myoepithelial cells (Bockmeyer et al., 2011).

Our attempt to demonstrate the regulatory role of miR-200c-141 in EMT by forcing its expression in D492 resulted in complete inhibition of endothelial induced EMT, while allowing the cells to undergo branching morphogenesis. When the miR-200c-141 construct was expressed in D492M, it reestablished luminal epithelial

properties, accompanied with resistance towards endothelial induced EMT. However the inability of D492M$^{miR\text{-}200c\text{-}141}$ to restore full branching potential of the original cell line encouraged further search for complementary regulatory elements.

The strict commitment of D492M$^{miR\text{-}200c\text{-}141}$ to luminal differentiation suggested that either stem cell or myoepithelial/basal elements were missing for restoring the original branching potential. The p63 protein is being increasingly recognized as a master regulator of basal epithelial cells in stratified epithelium including skin, lung, prostate and the mammary gland (Blanpain and Fuchs, 2007; Senoo et al., 2007). Indeed, basal cells are considered stem cell or progenitor population in these organs (Blanpain and Fuchs, 2014). In the mammary gland, p63 is highly expressed in the basal layer that comprises both differentiated myoepithelial cells and candidate stem or progenitor cells (Yallowitz et al., 2014). Studies have revealed an important role for p63 during mammary gland development in both infant and adult mice. p63 knockout mice lack the mammary gland completely as consequence of absence of squamous epithelia and they are consequently not viable (McKeon, 2004; Yang et al., 1999). In the adult gland, Forster et al. showed that basal/myoepithelial cell specific p63 knock-out in virgin mice caused defects in luminal cell proliferation and differentiation resulting in failed lactation (Forster et al., 2014). Although expression of p63 is associated with myoepithelial and progenitor cells in the breast, its expression in the basal compartment is essential for luminal cell formation highlighting the importance of both cell types for TDLU formation in the breast. Since p63 expression is also down-regulated in D492M relative to D492 we hypothesized that restoration of the p63 protein levels would contribute to the branching potential. Forced expression of ΔNp63 in D492M resulted in increased expression of myoepithelial markers without influencing branching capacity. Simultaneous overexpression of miR-200c-141 and ΔNp63 in D492M resulted in complete reversion of the mesenchymal phenotype and D492M$^{miR\text{-}200c\text{-}141\Delta Np63}$ had the same profile of critical epithelial markers as D492. Furthermore, D492M$^{miR\text{-}200c\text{-}141\Delta Np63}$ was able to carry out complex branching morphogenesis in pre-clustered 3D assay.

In our study we demonstrate miR-200c-141 and p63 are strong regulators of luminal epithelial- and myoepithelial differentiation, respectively and coexpression of these molecules in D492M capture the branching morphogenesis seen in wild type D492. When D492 cells undergo EMT they lose stem cell properties measured by the ability to generate both luminal- and myoepithelial cells.

We have shown that in D492, EMT is associated with marked repression of the miR-200c-141 locus. Although the mesenchymal phenotype could be reversed with re-expression of miR-200c-141, the cells regain only the luminal epithelial phenotype suggesting that the bi-potential stem cell phenotype is dependent on additional factors. The reintroduction of transcription factor p63 into D492M induced mesenchymal to epithelial transition (MET) with a myoepithelial phenotype.

Recently, Shimono et al. showed that microRNAs including miR-200 family were downregulated in breast cancer stem cells and normal breast epithelial stem cells. In line with our results, they showed that miR-200c inhibited the ability of stem cells to generate mammary ducts and form tumors (Shimono et al., 2009). Although D492M$^{miR\text{-}200c\text{-}141}$ re-adapts the epithelial phenotype it is unable to generate branching structures at the same level as the parental cell line D492, possibly because of the requirement for basal/myoeptihelial factors in full branching. Interestingly, along with the fact that miR-200 family preserves epithelial integrity in normal tissue such as the breast gland and may as such act as tumor suppressors there are accumulating data showing that these miRNAs facilitate colonization and metastases of distant organs including the lung (Dykxhoorn et al., 2009; Korpal et al., 2011). This indicates that regulation of the miR-200 family may be context dependent both spatially and temporally.

In summary, our data suggest a key role for the miR-200c-141 locus in maintaining epithelial integrity, luminal differentiation and suppression of epithelial to mesenchymal transition in the human breast gland. ΔNp63, a myoepithelial marker in the breast gland, induced MET in D492M and restored myoepithelial differentiation. Co-expression of these genes resulted in complete MET and mature branching morphogenesis, underlining the importance of both luminal and myoepithelial compartments in breast morphogenesis.

Acknowledgment

This work was supported by Grants from Landspitali University Hospital Science Fund, University of Iceland Research Fund, Science and Technology Policy Council-Research Fund (Grant No. 120416022), "Göngum saman", a supporting group for breast cancer research in Iceland (www.gongumsaman.is). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2015.05.007.

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