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MicroRNA-214 suppresses growth, migration and invasion through a novel target, high mobility group AT-hook 1, in human cervical and colorectal cancer cells

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Background: *MicroRNA-214* (*miR-214*) has been shown to act as a tumour suppressor in human cervical and colorectal cancer cells. The aim of this study was to experimentally validate high mobility group AT-hook 1 as a novel target for *miR-214*-mediated suppression of growth and motility.

Methods: *HMGA1* and *miR-214* expression levels were estimated in cervical and colorectal clinical specimens using qPCR. *HMGA1* 3′ untranslated region luciferase assays were performed to validate *HMGA1* as a target of *miR-214*. Effect of altering the expression of *miR-214* or *HMGA1* on proliferation, migration and invasion of human cervical and colorectal cancer cells was investigated.

Results: *miR-214* expression was poor while that of *HMGA1* was high in cervical and colorectal cancer tissues. *miR-214*-re-expression or *HMGA1* downregulation inhibited proliferation, migration and invasion of cancer cells while *miR-214* inhibition had opposite effects. *miR-214* was demonstrated to bind to the wild-type 3′ untranslated region of *HMGA1* but not with its mutant.

Conclusions: Low expression of *miR-214* concurrent with elevated levels of *HMGA1* may contribute to cervical and colorectal cancer progression. *miR-214*-mediated regulation of *HMGA1* is a novel mechanism for its tumour-suppressive actions in human cervical and colorectal cancer cells and opens up avenues for novel therapeutic strategies for these two cancers.

Cervical cancer (CaCx) is common among women predominantly in the developing countries (Hawes and Kiviat, 2002), and in India, it is the most common cancer affecting women (Sankaranarayanan *et al*, 1996). Human papilloma virus (HPV) is the major risk factor for cervical cancer (Bouallaga *et al*, 2000), but independently, alterations in tumour-suppressor genes and/or oncogenes may also be necessary for cervical cancer progression (Zur Hausen, 1996). Although colorectal cancer (CRC) earlier had the lowest rates of incidence in Asian countries (Haggar and Boushey, 2009), in the past few years, Asia has witnessed a rapid increase in CRC incidence particularly in the developing countries, including India (Mohandas, 2011; Moghimi-Dehkordi, 2012). MicroRNAs are ~22 nucleotide long, small RNA molecules that modulate gene expression transcriptionally or posttranscriptionally by mainly binding to the 3′ untranslated region (UTR) of their target genes (Calin *et al*, 2002). miRNAs constitute unique expression signatures and modulate cellular signalling pathways in many cancers, including CaCx (Hu *et al*, 2010; Pereira *et al*, 2010) and CRC (Chen *et al*, 2015; Hur *et al*, 2015). *miR-214* functions as a tumour suppressor by downregulating oncogenes, such as *GALNT7*, *Bcl2l2* and *TFAM* in CaCx (Yang *et al*, 2009; Peng *et al*, 2012; Wang *et al*, 2013; Wen *et al*, 2014) and *FGF-1* and *ARL2* in CRC (Chen *et al*, 2014; Long *et al*, 2015), but the actual underlying mechanisms are yet to be elucidated. The high mobility group AT-hook (HMGA) proteins are non-histone chromosomal proteins involved in DNA transcription, replication,

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recombination and repair (Cleynen and Van de Ven, 2008) and, as recently shown, in the regulation of metabolism (Qiu *et al*, 2014, 2015). HMGA proteins are easily detected in embryonic, neoplastic and proliferating undifferentiated cells but not so in non-neoplastic human adult tissues (Resar, 2010). They were first discovered in CaCx cells (Lund *et al*, 1983) and subsequently shown to have an oncogenic role during CaCx initiation, progression and metastasis by cooperating with HPV18 E6/E7 oncoproteins and inactivating p53 (Bandiera *et al*, 1998; Mellone *et al*, 2008). Its enhanced expression correlates with tumorigenesis and metastasis in human CRC (Fedele *et al*, 1996; Huang *et al*, 2009). Importantly, *cis*-regulatory elements in the 3′UTR mediate posttranscriptional regulation of *HMGA1* (Borrmann *et al*, 2001). Indeed, in leukaemia, bladder and prostate cancers, *HMGA1* expression is modulated by different miRNAs targeting its 3′UTR (Kaddar *et al*, 2009; Wei *et al*, 2011; Lin *et al*, 2013). In the present study, *miR-214* is demonstrated to directly target wild-type 3′UTR of *HMGA1* and reduce its endogenous expression in CaCx and CRC cells. Downregulation of *HMGA1* expression by *miR-214* or siRNA-*HMGA1* significantly inhibits proliferation, migration and invasion of CaCx and CRC cells. Thus novel mechanistic basis for the tumour-suppressive actions of *miR-214* is revealed unraveling new therapeutic opportunities.

MATERIALS AND METHODS

Human CaCx and CRC tissue samples. Fresh CaCx tissue samples were collected from consenting patients undergoing treatment for CaCx at the Institute of Obstetrics and Gynaecology, Chennai, India. Normal fresh cervical tissues were obtained from patients undergoing hysterectomy for various non-malignant reasons. Fresh CRC tissue samples and adjacent normal tissues were collected from consenting patients undergoing treatment for CRC at the Apollo Hospitals, Chennai, India. The study was approved by the Institutional Ethics Committee of Indian Institute of Technology Madras.

Cell lines. Human cervical cancer cell lines, SiHa, CaSki and C33A, and colorectal cancer cell lines, SW480 and SW620, were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) containing 10% FBS (Life Technologies) and antibiotics (100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) in a humidified atmosphere of 5% CO_2 at 37 °C.

RNA and plasmid transfection. miRNA-control (no. CN-001000-01-20), *miR-214* mimic (no. C-301153-01), siRNA-control (no. D-001220-01-20), siRNA-*HMGA1* (no. M-004597-02), antimiR-control (no. IN-002005-01-20 or anti*miR-214* (no. IH-301153-02-0005) were obtained from GE Healthcare Dharmacon (Lafayette, CO, USA). Transient transfections of the above (with 5 nM of miRNA and antimiR mimics and 50 nM of siRNA mimics) into CaCx and CRC cells were achieved using Lipofectamine RNAiMAX (no. 13778150, Life Technologies) while pcDNA 3.1, pcDNA 3.1-*miR-214*, pIRES (vector control), pIRES-*HMGA1* (kind gift from Edward Whang, Addgene plasmid no. 13466, Addgene, Cambridge, MA, USA), 3′UTR reporter plasmid constructs were transfected using linear polyethyleneimine (no. 23966-2, MW 25 000, procured from Polysciences, Warrington, PA, USA) at a ratio of 5 : 1 to DNA. Combinations of miR-control+pIRES, *miR-214*+pIRES, miR-control+pIRES-*HMGA1*, *miR-214*+pIRES-*HMGA1* were transfected using DharmaFECT Duo (no. T-2010-01, GE Healthcare Dharmacon).

Western blotting. Total cell lysates were prepared by incubating cells in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate and 1% SDS) on ice for 1 h, and protein concentration was quantified by Bradford's method according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). Samples (50 μg protein) were resolved on 10% SDS-PAGE and transferred to a PVDF membrane (Immunoblot, Bio-Rad) using a Bio-Rad Mini PROTEAN III apparatus. Anti-HMGA1 (no. 7777S) and anti-ACTB (no. A5441) antibodies were purchased from Cell Signaling (Danvers, MA, USA) and Sigma-Aldrich (St Louis, MO, USA), USA, respectively, while anti-mouse IgG-peroxidase-conjugate (no. 115-035-003) and anti-mouse IgG-peroxidase-conjugate (no. 111-035-003) secondary antibodies were bought from Jackson Laboratories (West Grove, PA, USA). Bands detected using the Enhanced Chemiluminescence Kit (Bio-Rad) were visualised using ChemiDoc (Bio-Rad) and analysed by densitometry (Image Lab, Bio-Rad). ACTB was used as an internal control. Experiments were repeated at least once to confirm the results obtained earlier.

RNA isolation and real-time quantitative PCR. Tissue samples were ground and RNA was extracted using the manufacturer's protocol (TRIzol, Life Technologies) and RNA was also isolated from cells using TRIzol (Life Technologies). Mature miRNA levels were estimated by performing stem-loop reverse transcription followed by quantitative PCR; reverse transcription by MMLV reverse transcriptase (Life Technologies) was performed using *miR-214*-specific and *RNU6*-specific stem-loop primers. PCR amplification of *miR-214* or *RNU6* was performed using a forward primer specific for *miR-214* or *RNU6* (internal control) and a universal reverse primer. For estimating *HMGA1* mRNA levels, reverse transcription was carried out by MMLV reverse transcriptase (Life Technologies) using oligo-dT and amplified using appropriate gene-specific PCR primers. Detection and quantitation of *HMGA1* or *ACTB* (internal control) was carried out using the DyNAmo ColorFlash SYBR Green qPCR Kit reagent (no. F416L, Thermo Scientific, Waltham, MA, USA) on Eppendorf realplex4 Mastercycler epgradient S (Eppendorf, Hamburg, Germany). Relative expression levels of genes analysed were calculated using $2^{-\Delta CT}$ (tissue samples) or $2^{-\Delta\Delta CT}$ (cancer cells) method.

3′UTR luciferase assays. *HMGA1* 3′UTR that contains putative binding sites for the *miR-214* was amplified from human genomic DNA using Phusion high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) and cloned into the 3′UTR of Renilla luciferase gene in the psiCHECK-2 reporter vector (Promega, Madison, WI, USA). The *miR-214*-binding site was mutated by substituting five out of the six bases in the miRNA-binding sequence (seed sequence) in the 3′UTR of HMGA1 using appropriate primers and the mutant construct thus synthesised was used as a negative control. CaCx or CRC cells were co-transfected with pcDNA3.1 or pcDNA3.1-*miR-214* and the wild-type or mutant 3′UTR luciferase constructs in a 24-well format, and 24 h posttransfection, cells were lysed using Passive Lysis Buffer, and Renilla luciferase activity was measured using the Dual Luciferase Assay Kit (no. A2492, Promega) and a luminescence plate reader (Molecular Devices Inc., Sunnyvale, CA, USA), wherein firefly luciferase acted as the internal control.

Migration and invasion assays. Migration assays were performed by transfecting CaCx or CRC cells with *miR-214* or siRNA-*HMGA1* or anti*miR-214* or respective controls and then seeding 5×10^4 cells in DMEM onto the upper part of each Transwell chamber (BD Biosciences, Franklin Lakes, NJ, USA) and adding 10% FBS containing DMEM to the lower part of the chamber. Cells adhering to the bottom of the Transwell membrane were stained with 0.1% crystal violet 48 h later and images were obtained using an Olympus TL4 inverted light microscope (Shinjuku, Tokyo, Japan). In addition, stain was collected from stained cells by washing with 10% acetic acid and quantified by measuring absorbance at 595 nm (Saito *et al*, 1997) using a Bio-Rad Model 680 microplate reader (Bio-Rad, Shinagawa-ku, Tokyo, Japan). Invasion assays were performed in a similar manner but by

allowing the cells to migrate through a GelTrex-coated (no. A15696-01, Life Technologies) layer in the upper part of a Transwell chamber.

BrdU incorporation assays. Proliferation assays were performed by transfecting 0.7×10^4 cells with *miR-214* or anti*miR-214* or siRNA-*HMGA1* or respective controls and, 48 h posttransfection, were examined using the BrdU Cell Proliferation Assay Kit (no. 6813S, Cell Signaling) according to the manufacturer's protocol.

TCGA data analysis. TCGA open access data directory (http://cancergenome.nih.gov/) was used to obtain miRNA and mRNA expression data sets for human CaCx and CRC tumours. Normalised TCGA level 3 miRNA-seq and RNA-seqV2 data were compiled using R studio and used for assessing the expression of miRNA and mRNA. These linear sequencing data expression values were then used to compute Pearson product–moment correlation coefficient.

Statistical analysis. qPCR was performed in duplicates for all the clinical tissue specimens and mean expression was calculated within normal or cancer tissue subsets. As paired normal specimens were available only in the case of colorectal tumour, *miR-214* or *HMGA1* expression in a tumour was normalised to its paired normal counterpart. One-way ANOVA test was performed to evaluate fold expression relative to mean. In the case of cervical clinical specimens, mean values for each data set (tumour or normal) were calculated. In the case of miR-control-, *miR-214*-, siR-control-, si-*HMGA1*-, antimiR-control- or anti*miR-214*-transfected cells, three independent experiments were conducted for RNA quantitation (*in vitro*), BrdU incorporation, migration and invasion assays, and after appropriate normalisation, s.e.m. was plotted. After performing unpaired *t*-tests, *P*-values were calculated and represented as $^*P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$ or $^{****}P \leqslant 0.0001$.

RESULTS

Expression of *miR-214* is lower while that of *HMGA1* is higher in both human cervical and colorectal tumours than in their corresponding normal tissues. *miR-214* is a known tumour-suppressor miRNA in cervical (CaCx) and colorectal (CRC) cancers and acts by downregulating a few oncogenes as is the case with many tumour-suppressor miRNAs. A putative, conserved, 6-mer, *miR-214*-binding sequence located at 308–314 bases downstream in the *HMGA1* 3′UTR was identified using TargetScan. *HMGA1* was of particular interest among the target genes identified because of its positive roles in cancer cell proliferation and invasion in CaCx and CRC cells. Prompted by the inverse correlation in the expression of this miRNA–target pair deduced from previous reports that studied *miR-214* or *HMGA1* expression separately (Fedele *et al*, 1996; Bandiera *et al*, 1998; Yang *et al*, 2009; Chen *et al*, 2014), these two were analysed together by TCGA data analysis or quantitative PCR in patient samples to strengthen the possibility of the existence of a regulatory mechanism. TCGA analysis showed a clear inverse correlation between *miR-214* and *HMGA1* both in CaCx and CRC (Figures 1A and B). Pearson correlation coefficients of -0.239 ($P = 0.0019$) and -0.274 ($P = 0.0005$) were obtained for CaCx and CRC tumour samples, respectively. When a few samples of CaCx and CRC were analysed to check if this correlation existed in a local population, the same trend was found. Expression levels of mature *miR-214* analysed in various samples fell into a lower range in CaCx tissues compared with normal tissues (Figure 1C), with mean values of 20.26 and 66.13, respectively. *HMGA1* mRNA levels were found in a relatively higher, narrow range (Figure 1D) in CaCx relative to normal tissues with mean values of 22.6 and 8.2, respectively. Similarly, *miR-214* was found to be poorly expressed (mean 0.68 ± 0.3), whereas *HMGA1* expression was higher (mean 11.52 ± 5.4) in CRC tissues than in their paired normal tissues (Figures 1E and F). Expression levels of *miR-214* were higher in 4 out of the 20 CRC samples, whereas *HMGA1* expression was lower in 1 out of the 20 tumours when compared with their paired normal tissues. These results showing an inverse relationship between the expression levels of *miR-214* and *HMGA1* in human CaCx and CRC together with the identification of a *miR-214*-binding site in the *HMGA1* 3′UTR suggested that *miR-214* may directly target and regulate *HMGA1*.

***HMGA1* is directly targeted by *miR-214*.** As the physiological effects of endogenous *miR-214* are difficult to ascertain owing to their low levels in CaCx and CRC cells, the miRNA was first re-expressed ectopically. CaCx and CRC cells transfected with *miR-214* mimic showed 3–6 and 2–5 fold more expression, respectively, than their corresponding miR-control-transfected cells (Figure 2A). Maintaining this range of expression, the effect of reintroducing *miR-214* on endogenous *HMGA1* mRNA and protein levels was determined, and the results show that *miR-214* decreased *HMGA1* both at mRNA (Figure 2B) and protein (Figure 2D) levels in human CaCx and CRC cells. To confirm whether *HMGA1* is targeted by *miR-214* by binding to its 3′UTR (Figure 2C), *HMGA1* 3′UTR luciferase assays were performed and it was observed that re-expression of *miR-214* decreased wild-type *HMGA1* 3′UTR-regulated luciferase activity by ~30% in C33A, ~46% in CaSki, ~30% in SiHa, ~25% in SW480 and ~33% in SW620 cells but not in cells transfected with *HMGA1* 3′UTR-containing mutant *miR-214*-binding sites (Figure 2E), confirming that *miR-214* binds specifically to the 3′UTR of *HMGA1* to repress gene expression. Together, these results suggest that *miR-214* negatively regulates endogenous *HMGA1* expression by binding to its 3′UTR in CaCx and CRC cells.

Re-expression of *miR-214* inhibits proliferation, migration and invasion in CaCx and CRC cells. To evaluate whether *miR-214*-mediated targeting and the consequent downregulation/repression of *HMGA1* has a role in tumorigenesis, first the effect of ectopic expression of *miR-214* on cell proliferation was studied using BrdU incorporation assay in CaCx (C33A and SiHa) and CRC (SW480 and SW620) cells. Re-expression of *miR-214* inhibited cell proliferation significantly in C33A by ~28%, SiHa by ~13%, SW480 by ~23% and SW620 by ~18% (Figure 3A). As the CaCx and CRC cells used in the current study are known to exhibit migratory and invasive properties, these cells were next tested by re-expressing *miR-214* and it inhibited migration in C33A by ~31%, SiHa by ~23% and SW480 by ~25% (Figure 3B) as well as invasion in C33A by ~25%, SiHa by ~25% and SW480 by ~33% (Figure 3C). These results confirm that reintroduction of *miR-214* suppresses the aggressive behaviour of CaCx and CRC cells by inhibiting their proliferation, migration and invasion.

***HMGA1* knockdown inhibits proliferation, migration and invasion in CaCx and CRC cells.** Given that *miR-214* may have many potential targets in CaCx and CRC cells, the antiproliferative effect of *miR-214* may not be limited to repression of *HMGA1*. To check whether suppression of *HMGA1* would simulate *miR-214*-mediated effects, siRNA-mediated knockdown of *HMGA1* was performed in CaCx and CRC cells (Figure 4A). Under these conditions, cell proliferation was inhibited by 10–40% (Figure 4B). Similarly, diminished levels of *HMGA1* reduced migration (Figure 4C) by 30% as well as invasion (Figure 4D) by 20–50%. From these data, it is inferred that merely downregulating *HMGA1* expression can inhibit proliferation, migration and invasion in CaCx and CRC cells, producing phenotypes comparable to *miR-214* reintroduction.

Inhibition of *miR-214* enhances proliferation, migration and invasion in CaCx and CRC cells. Although the ectopic expression of *miR-214* effectively inhibited proliferation, migration and invasion in

CaCx and CRC cells, whether the endogenous *miR-214* contributes to these phenomena remained to be elucidated. When the endogenous *miR-214* was inhibited using an antimiR, there was an increase in HMGA1 levels (Figure 5A) and cell proliferation (Figure 5B), migration (Figure 5C) and invasion (Figure 5D) were also enhanced by 40–60%, 30–45% and 20–60%, respectively. From these data, it is ascertained that downregulating *miR-214* enhances endogenous *HMGA1* levels concurrently stimulating proliferation, migration and invasion of CaCx

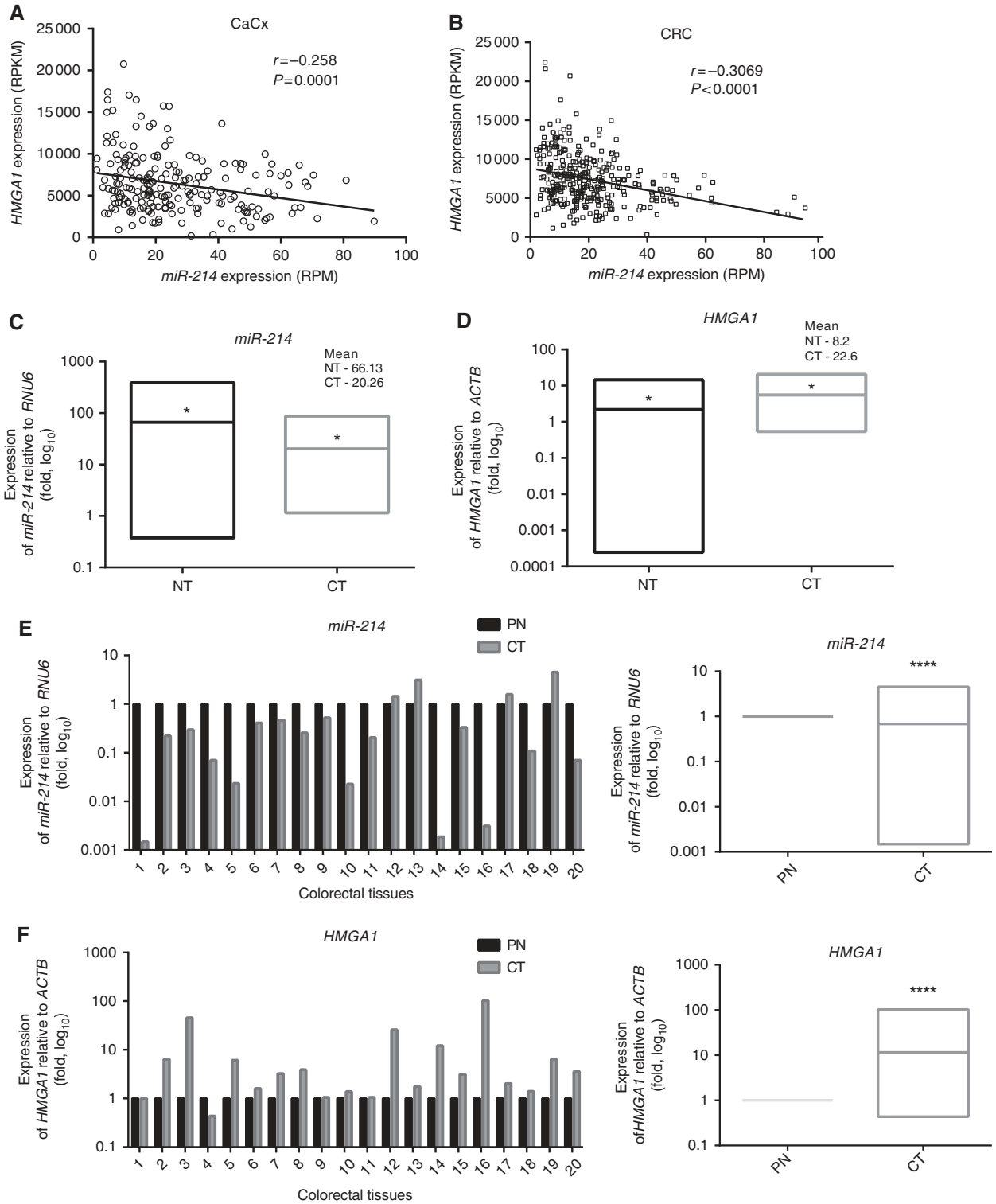

Figure 1. Relative expression levels of *miR-214* and *HMGA1* in normal and tumour cervical and colorectal tissues. TCGA database was used to plot Pearson product–moment correlation utilising normalised expression data for *miR-214* (reads per million (RPM)) and *HMGA1* (reads per kilobase per million mapped reads (RPKM)) from (**A**) 215 cervical and (**B**) t 322 colorectal umour samples. Quantitative PCR was performed to estimate the expression levels of (**C**) *miR-214* from 20 normal cervical tissue specimens (NT) and 20 cervical tumour tissue specimens (CT) or (**D**) *HMGA1* from 15 normal (NT) and tumour (CT) cervical tissue specimens. Expression levels of *miR-214* and *HMGA1*, respectively, were normalised to that of *RNU6* and *ACTB*. Similarly, 20 tumour (CT) and their paired normal (PN) colorectal tissue specimens were analysed for the expression levels of (**E**) *miR-214* and (**F**) *HMGA1*. Solid lines in box plots represent mean values. *P*-values are represented as $*P \leqslant 0.05$, $****P \leqslant 0.0001$.

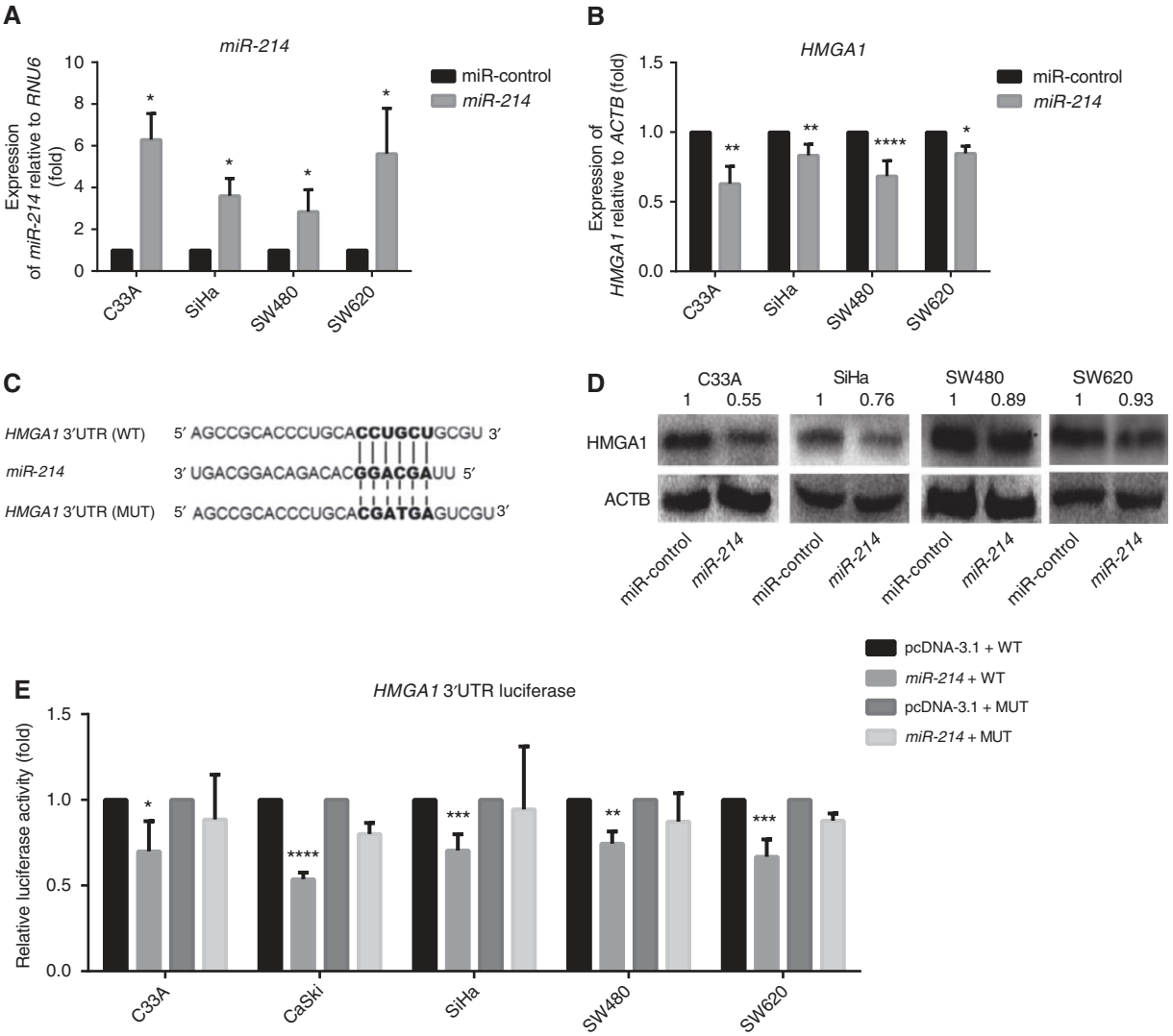

Figure 2. Changes in *HMGA1* expression upon ectopic expression of *miR-214* in CaCx and CRC cells. C33A, SiHa, SW480 or SW620 cells were transfected with control mimic (miR-control) or *miR-214* mimic and the expression levels of (**A**) *miR-214* or (**B**) *HMGA1* were estimated by qPCR using *RNU6* or *ACTB*, respectively, for normalisation, and (**D**) HMGA1 protein levels were estimated by western blotting and ACTB was used as a loading control. (**E**) psiCHECK-2 vector containing *HMGA1* 3′UTR either with wild-type *miR-214*-binding site (WT) or mutated site (MUT) was co-transfected with pcDNA 3.1 or pcDNA 3.1-*miR-214* (*miR-214*) in CaCx and CRC cells and luciferase assays were performed. Renilla luciferase activity in *miR-214*-transfected cells was normalised to that of vector-transfected cells and Firefly luciferase served as internal control. (**C**) Wild-type (WT) and mutant (MUT) *miR-214*-binding sites in *HMGA1* 3′UTR and *miR-214* binding sequence (miR-214) are shown where solid lines and broken lines connect paired and unpaired bases from WT and MUT with those of *miR-214*, respectively. *P*-values are represented as $*P \leqslant 0.05$, $**P \leqslant 0.01$, $***P \leqslant 0.001$ or $****P \leqslant 0.0001$.

and CRC cells, producing cellular phenotypes that are considerably opposite to the ones produced by reintroduction of *miR-214* or independent downregulation of *HMGA1*.

***miR-214* counters the effects of ectopically expressed *HMGA1* on proliferation, migration and invasion in CaCx and CRC cells.** As *miR-214* effectively inhibited proliferation, migration and invasion in CaCx and CRC cells, it was pertinent to test the effects of expressing its target *HMGA1* on these processes in the presence of *miR-214*. Hence, *miR-214* and a 3′UTR-less *HMGA1* (unresponsive to miRNAs) were transfected individually or in combination in CaCx and CRC cells. As expected, *miR-214* decreased HMGA1 expression and ectopic expression of *HMGA1* increased it over endogenous levels, and notably, their combined ectopic expression still led to a decrease in HMGA1 in SiHa and SW480 cells (Figure 6A). Similar effects on proliferation (Figure 6B), migration (Figure 6C) and invasion (Figure 6D) were observed when this combination was used. Although *miR-214* expression alone reduced proliferation by 14–29%, migration by 22–30% and invasion by 15–27%, ectopic expression of *HMGA1* enhanced these. Combined expression of *HMGA1* and *miR-214* reduced cell proliferation, migration and invasion by 11–29%, 15–37% and 20–25%, respectively, compared with cells transfected with miR-control and vector-control. Taken together, these results ascertain that the tumour-suppressive action of *miR-214* prevails over the protumorigenic effects of HMGA1 even if it is ectopically expressed over and above the already abundant endogenous levels in CaCx and CRC cells.

DISCUSSION

In recent years, miRNAs have been increasingly demonstrated to have crucial roles in gene regulation, cellular signalling, carcinogenesis and in related processes, including metastasis and epithelial-to-mesenchymal transition (Suzuki *et al*, 2014; Kuninty

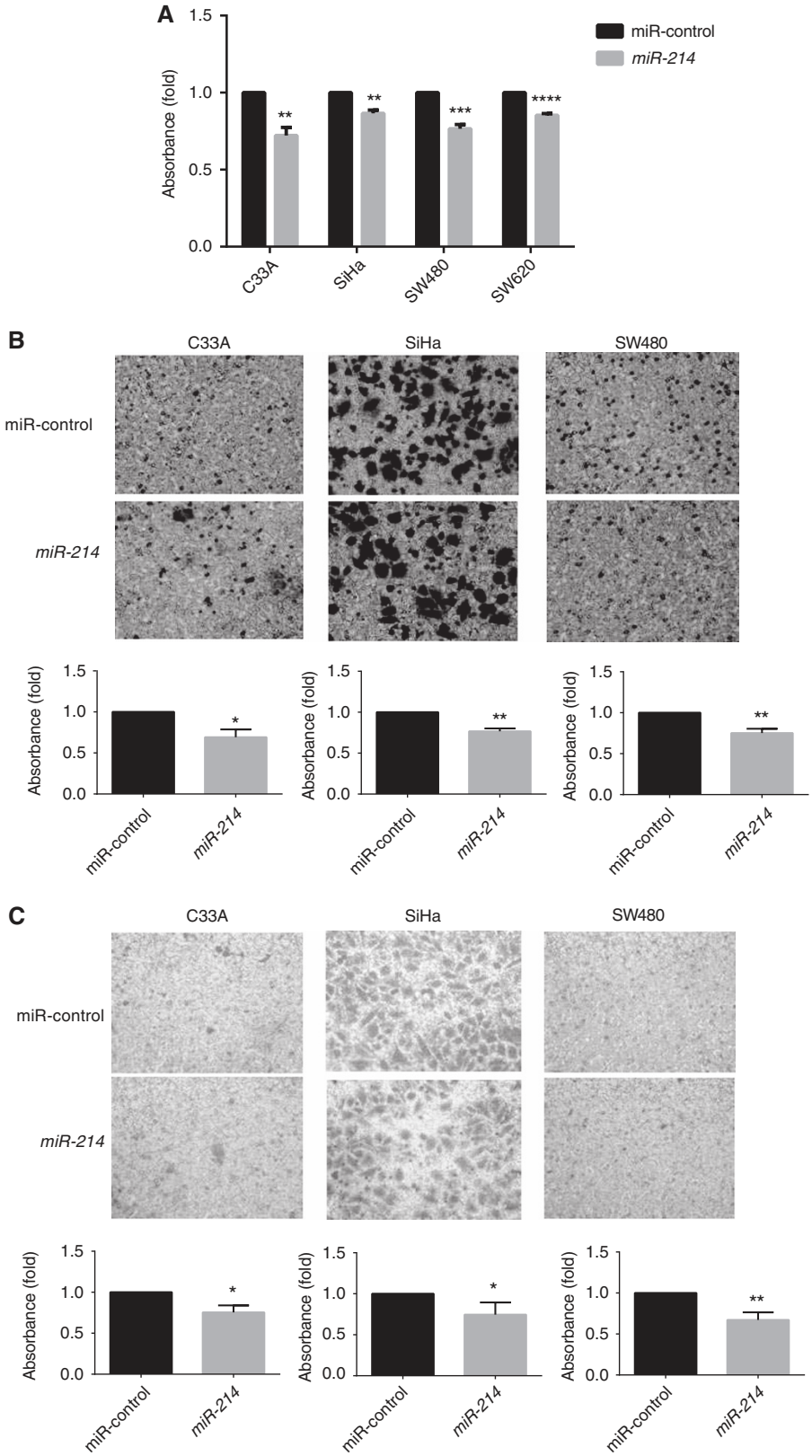

Figure 3. Effect of ectopic expression of *miR-214* on proliferation, migration and invasion. C33A, SiHa, SW480 or SW620 cells were transfected with control mimic (miR-control) or *miR-214* mimic and subsequently, (**A**) BrdU incorporation, (**B**) migration and (**C**) invasion were quantified by measuring absorbance. Absorbance values obtained with BrdU or 0.1% crystal violet for *miR-214*-transfected cells were normalised with those obtained for miR-control-transfected cells. Representative images for migration and invasion are displayed above quantitation. *P*-values are represented as $*P \leqslant 0.05$, $**P \leqslant 0.01$, $***P \leqslant 0.001$ or $****P \leqslant 0.0001$. A full colour version of this figure is available at the *British Journal of Cancer* journal online.

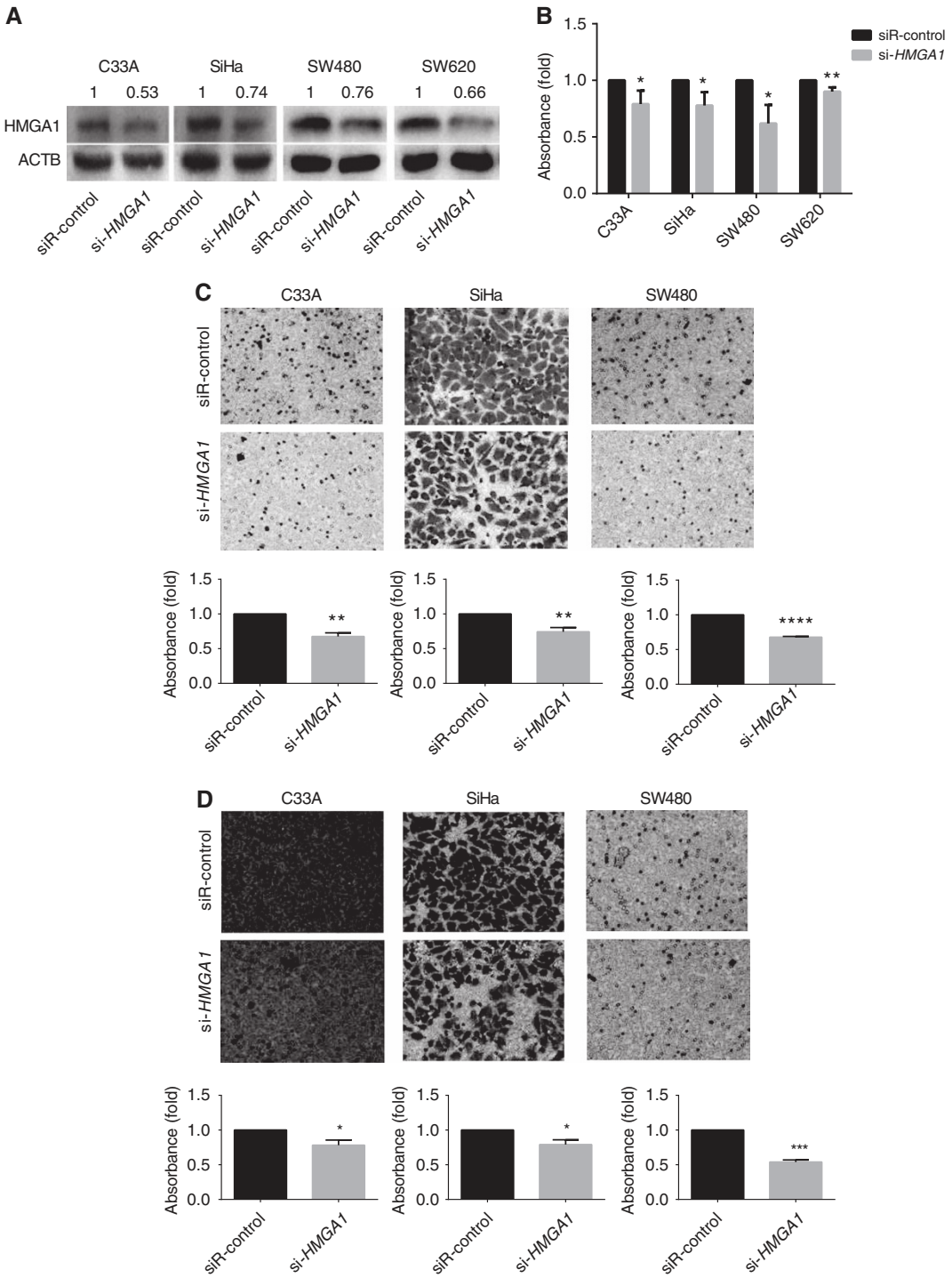

Figure 4. Changes in proliferation, migration and invasion assays upon knockdown of *HMGA1*. (**A**) CaCx or CRC cells were transfected with control siRNA (siR-control) or siRNA-*HMGA1* (si-*HMGA1*) and the expression of *HMGA1* was checked by western blotting. (**B**) BrdU incorporation, (**C**) migration and (**D**) invasion assays were quantified by measuring absorbance. Absorbance values obtained for si-*HMGA1*-transfected cells were normalised with those obtained for siR-control-transfected cells. Representative images for migration and invasion are displayed above quantitation. *P*-values are represented as $*P \leqslant 0.05$, $**P \leqslant 0.01$, $***P \leqslant 0.001$ or $****P \leqslant 0.0001$.

et al, 2016). miRNAs function by modulating the expression of several target genes simultaneously and can act as oncomirs by silencing the expression of tumour-suppressor targets and conversely can target oncogenes and behave as tumour-suppressor miRNAs (Esquela-Kerscher and Slack, 2006). Previously, it has been demonstrated that miR-29b downregulates canonical Wnt signalling and consequently inhibits colorectal cancer cell proliferation and anchorage-independent growth (Subramanian *et al*, 2014), whereas miR-106b enhances migration and invasion in non-small cell lung carcinoma cells by suppressing β-TRCP2 (Savita and Karunagaran, 2013). Present data obtained from human cervical and colorectal clinical specimens and those extracted from TCGA suggest poor expression of *miR-214* in these tumours, confirming earlier reports in these cancers. Inhibition of growth, migration and invasion in CaCx and CRC upon *miR-214* re-expression are consistent with earlier data (Peng *et al*, 2012; Chen

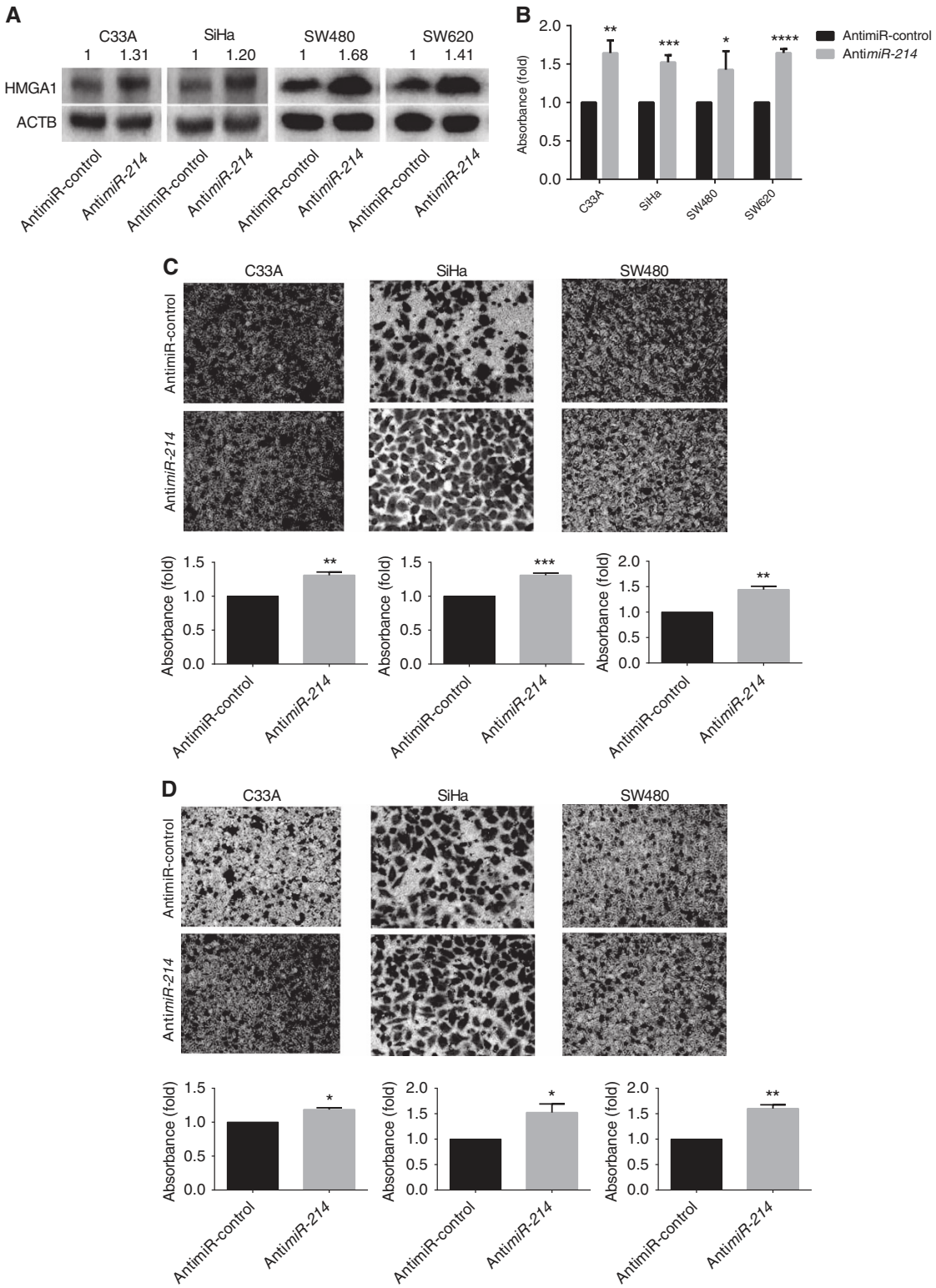

Figure 5. Effect of anti*miR-214* on proliferation, migration and invasion. (**A**) CaCx or CRC cells were transfected with control antimiR (antimiR-control) or anti*miR-214* and the expression of *HMGA1* was checked by western blotting. (**B**) BrdU incorporation, (**C**) migration and (**D**) invasion assays were quantified by measuring absorbance. Absorbance values obtained for anti*miR-214*-transfected cells were normalised with those obtained for antimiR-control-transfected cells. Representative images for migration and invasion are shown above quantitation. *P*-values are represented as $*P \leqslant 0.05$, $**P \leqslant 0.01$, $***P \leqslant 0.001$ or $****P \leqslant 0.0001$.

et al, 2014). However, *miR-214* is highly expressed in melanoma (Penna *et al*, 2013), and gastric (Yang *et al*, 2013) cancers, and in ovarian cancer, it favours cell survival and cisplatin resistance by targeting the phosphate and tensin homolog (Yang *et al*, 2008), suggesting that it may function as an oncogene as well. These contradictory results are, however, not uncommon with miRNAs as the same miRNA may act as an oncomir in one cancer type and as a tumour suppressor in another cancer. For instance, miR-155 significantly contributes to growth in B-cell lymphoma (Pedersen *et al*, 2009) but sensitises triple-negative human breast cancer cells

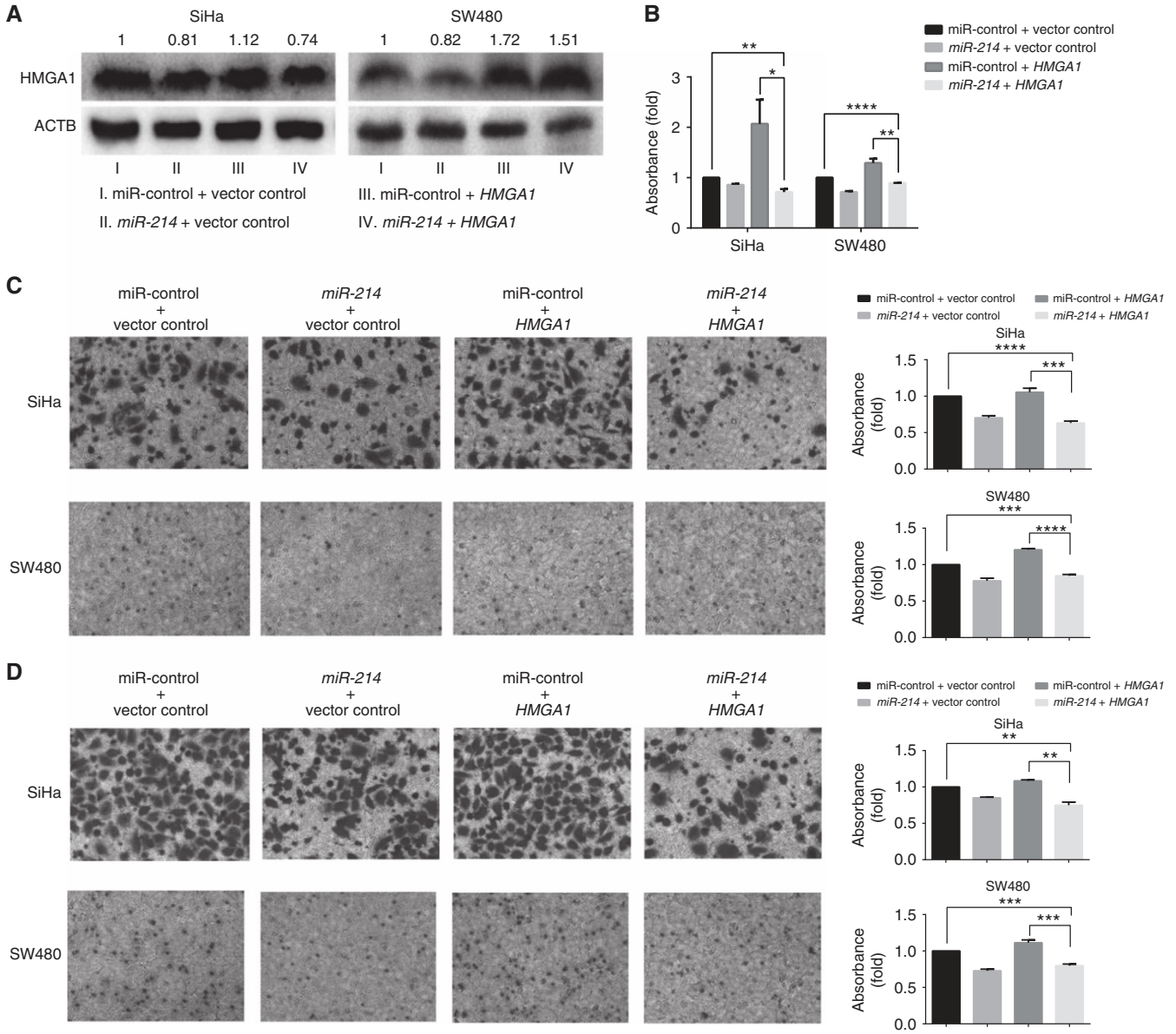

Figure 6. Changes in HMGA1-induced proliferation, migration and invasion upon reintroduction of *miR-214*. (**A**) CaCx or CRC cells were co-transfected with control miR (miR-control) or *miR-214* either with vector control or *HMGA1* and the expression of *HMGA1* was checked by western blotting. (**B**) BrdU incorporation, (**C**) migration and (**D**) invasion were quantified by measuring absorbance. Absorbance values obtained for *miR-214* + *HMGA1*-transfected or miR-214-transfected or *HMGA1*-transfected cells were normalised with those obtained for miR-control + vector control-transfected cells. *miR-214* + *HMGA1*-transfected or *HMGA1*-transfected cells were compared with miR-control + vector control-transfected cells to calculate statistical significance. Representative images for migration and invasion are shown alongside quantitation. *P*-values are represented as $*P \leqslant 0.05$, $**P \leqslant 0.01$, $***P \leqslant 0.001$ or $****P \leqslant 0.0001$.

to infrared by targeting RAD51 (Gasparini *et al*, 2014). Ectopic expression of *miR-214* inhibited cell proliferation more effectively in C33A and SW480 than in SiHa and SW620 cells, and these differential and paradoxical effects are presumably due to differences in genetic background, microenvironment and the pattern of target gene expression in each cell type. Furthermore, multiple miRNAs may exhibit cooperative binding to binding sites on a single target gene (Lewis *et al*, 2003; Krek *et al*, 2005), facilitating the formation of complex regulatory networks.

Antithetical to the impairing effect of *miR-214* re-introduction on the growth, migration and invasion of CaCx and CRC cells, the presence of HMGA1 is documented to aid tumorigenesis and its maintenance, crucial to metastasis (Reeves *et al*, 2001; Mellone *et al*, 2008; Belton *et al*, 2012; Xing *et al*, 2014). Downregulating *HMGA1* using siRNA suppressed cell proliferation, invasion and migration in this study in a manner comparable to re-expression of *miR-214*, suggesting that *miR-214* mainly acts through *HMGA1* or alternatively it emphasises a major role for HMGA1 on the regulation of tumour progression. Further, the finding in the current study that *miR-214* could target *HMGA1* 3′UTR and inhibit its expression lends credence to its tumour-suppressive role. Although downregulation of *HMGA1* by miR-16 in leukaemia (Kaddar *et al*, 2009), miR-142-3p in osteosarcoma (Xu *et al*, 2014), miR-26a in bladder cancer (Lin *et al*, 2013) and miR-296 in prostate cancer (Wei *et al*, 2011) led to inhibition of cancer cell growth and motility, present findings are the first to relate similar phenomena in CaCx and CRC cells. HMGA1 upregulates *STAT3* expression in leukaemia (Hillion *et al*, 2008), is induced by TGF-β

signalling in breast carcinoma (Zu *et al*, 2015), cooperates with NF-κB (p65 Rel-A) and bolsters HPV E6/E7 expression via a positive autoregulatory loop in CaCx (Mellone *et al*, 2008) and sustains Wnt/β-catenin pathway in CRC (Xing *et al*, 2014). Although a hitherto unknown mechanism by which *HMGA1* may be regulated in CaCx and CRC is shown, it remains to be studied whether other miRNAs predicted to target *HMGA1* may be able to regulate it simultaneously with *miR-214* or independent of it. That a potent oncogene such as *HMGA1* is most effectively regulated by *miR-214* is demonstrated by the finding that the ectopic expression of a 3′UTR-less *HMGA1* did not act as a deterrant to *miR-214* in its inhibitory effects on proliferation, migration and invasion in CaCx and CRC cells and conclusively delineates the functional relevance of the antitumorigenic role of *miR-214* in these cancers, through regulation of *HMGA1*.

Current data indicate that poor expression of *miR-214* concomitant with elevated levels of HMGA1 may contribute to malignant phenotype in CaCx and CRC. Thus the identification of *HMGA1* as a major target gene for *miR-214* in CaCx and CRC unleashes potential molecular mechanisms of tumorigenesis.

Novel and salient findings from this study in cervical and colorectal cancers are listed below.

(1) a hitherto unknown mechanism for the tumour-suppressive actions of *miR-214*.
(2) miRNA-mediated regulation of *HMGA1*, and
(3) siRNA-mediated downregulation of *HMGA1* and its effect on proliferation, migration and invasion.

CONCLUSIONS

This is the first report elaborating that *miR-214*, whose attenuated expression in CaCx and CRC has been associated with poor prognosis in previous studies, functions as a tumour suppressor by negatively regulating expression of oncogene *HMGA1*. Down-regulation of cellular *HMGA1* alone affected cancer cells in a manner comparable to that of restoration of *miR-214* while inhibition of endogenous *miR-214* resulted in elevated *HMGA1* levels and a consequent amelioration of growth, migration and invasion. Further, ectopic expression of *miR-214* was able to thwart the protumorigenic effect of ectopically expressed *HMGA1* and suppress cancer cell's growth and motility.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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