

VL30 retrotransposition is associated with induced EMT, CSC generation and tumorigenesis in HC11 mouse mammary stem-like epithelial cells

SOTEROULA THRASYVOULOU¹, GEORGIOS VARTHOLOMATOS², GEORGIOS MARKOPOULOS¹, DIMITRIOS NOUTSOPOULOS¹, STEFANIA MANTZIOU¹, FOTEINI GKARTZIOU¹, PANAGIOTIS PAPAGEORGIS³, ANTONIA CHARCHANTI⁴, PANOS KOUKLIS¹, ANDREAS I. CONSTANTINOU⁵ and THEODORE TZAVARAS¹

¹Laboratory of General Biology, School of Health Sciences, Faculty of Medicine, University of Ioannina;

²Laboratory of Hematology, Unit of Molecular Biology, University Hospital of Ioannina, 45110 Ioannina, Greece;

³Biological Sciences Program, Department of Life Sciences, School of Sciences, European University Cyprus,

2404 Nicosia, Cyprus; ⁴Laboratory of Anatomy-Histology-Embryology, School of Health Sciences,

Faculty of Medicine, University of Ioannina, 45110 Ioannina, Greece; ⁵Department of Biological Sciences,

Faculty of Pure and Applied Sciences, University of Cyprus, 1678 Nicosia, Cyprus

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Abstract. Retrotransposons copy their sequences via an RNA intermediate, followed by reverse transcription into cDNA and random insertion, into a new genomic locus. New retrotransposon copies may lead to cell transformation and/or tumorigenesis through insertional mutagenesis. Methylation is a major defense mechanism against retrotransposon RNA expression and retrotransposition in differentiated cells, whereas stem cells are relatively hypo-methylated. Epithelial-to-mesenchymal transition (EMT), which transforms normal epithelial cells into mesenchymal-like cells, also contributes to tumor progression and tumor metastasis. Cancer stem cells (CSCs), a fraction of undifferentiated tumor-initiating cancer cells, are reciprocally related to EMT. In the present study, the outcome of long terminal repeat (LTR)-Viral-Like 30 (VL30) retrotransposition was examined in mouse mammary stem-like/progenitor HC11 epithelial cells. The transfection of HC11 cells with a VL30 retrotransposon, engineered with an EGFP-based retrotransposition cassette, elicited a higher retrotransposition frequency in comparison to differentiated J3B1A and C127 mouse mammary cells. Fluorescence microscopy and PCR analysis confirmed the specificity of retrotransposition events. The differentiated retrotransposition-positive cells retained their epithelial

morphology, while the respective HC11 cells acquired mesenchymal features associated with the loss of E-cadherin, the induction of N-cadherin, and fibronectin and vimentin protein expression, as well as an increased transforming growth factor (TGF)- β 1, Slug, Snail-1 and Twist mRNA expression. In addition, they were characterized by cell proliferation in low serum, and the acquisition of CSC-like properties indicated by mammosphere formation under anchorage-independent conditions. Mammospheres exhibited an increased Nanog and Oct4 mRNA expression and a CD44⁺/CD24^{-low} antigenic phenotype, as well as self-renewal and differentiation capacity, forming mammary acini-like structures. DNA sequencing analysis of retrotransposition-positive HC11 cells revealed retrotransposed VL30 copies integrated at the vicinity of EMT-, cancer type- and breast cancer-related genes. The inoculation of these cells into Balb/c mice produced cytokeratin-positive tumors containing pancytokeratin-positive cells, indicative of cell invasion features. On the whole, the findings of the present study demonstrate, for the first time, to the best of our knowledge, that stem-like epithelial HC11 cells are amenable to VL30 retrotransposition associated with the induction of EMT and CSC generation, leading to tumorigenesis.

Introduction

VL30s constitute a family of murine-specific DNA sequences characterized by the typical retroviral structure 5'LTR-gag-pol-env-3'LTR. They are represented by 372 sequences, categorized as 86 full-length and 49 truncated copies, as well as 237 solo LTRs with non-random chromosomal distribution in the mouse genome (1). As regards the full-length copies, their internal sequences bear multiple stop codons and, due to the lack of protein coding capacity, are classified as non-autonomous LTR retrotransposons (2). VL30 transcription is induced by various stimuli (3-5), which justify VL30s' prominent feature as early response genes (2).

Correspondence to: Dr Theodore Tzavaras, Laboratory of General Biology, School of Health Sciences, Faculty of Medicine, University of Ioannina, 45110 Ioannina, Greece
E-mail: thtzavar@uoi.gr

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VL30 transcripts play significant roles in cellular processes regulating gene expression (6), proto-oncogene transcription and cellular proliferation (7), as well as steroidogenesis (8). Moreover, they have effects on tumorigenesis (7,8) and in induced cerebral ischemia, polyribosome-bound VL30 transcripts can lead to the inhibition of translation and cell death (9). An important feature of VL30s is that their typical 30S transcript is packaged into murine leukemia C-type helper viruses (10) and, following its reverse transcription into cDNA, can be transmitted to heterologous cells (11). Accordingly, VL30 retrotransposition occurs in a retroviral fashion mechanism. The authors have previously demonstrated the VL30s' retrotransposition competence (12), and new integrated genomic copies are characterized by 4bp-target site duplications (5). Importantly, induced VL30 retrotransposition affects genome integrity, leading to programmed cell death (13).

Retrotransposition is an intracellular phenomenon based on a retrotransposon RNA-intermediate, which following its reverse transcription into cDNA by an active reverse transcriptase, is randomly integrated into a new genomic site (14). Thus, retrotransposition is a potent mutagenic process as new retrotransposon copy-integrations can inactivate or deregulate gene expression. A low retrotransposition frequency naturally takes place mainly during oogenesis and embryogenesis (15,16); however, high frequency rates may result in the onset of genetic diseases or tumorigenesis (16).

Epithelial-to-mesenchymal transition (EMT) occurs mainly during normal embryonic development. In this process, epithelial cells lose their cell-to-cell contacts, exhibit cytoskeletal remodeling, gain migratory properties and switch to a mesenchyme-like gene expression program. In addition, EMT promotes cancer progression by facilitating invasion and metastasis (17-19). EMT induction is associated with the transcriptional repression of epithelial cadherin (E-cadherin) triggered by transforming growth factor- β 1 (TGF- β 1), epidermal growth factor (EGF) and wingless-related integration site/ β -catenin (Wnt/ β -catenin) signaling pathways (20,21). The majority of these pathways function through pleiotropically acting transcription factors, such as members of the zinc finger protein snail (Snail-1), snai2-snail homolog 2 (Slug), basic helix-loop-helix E47 and twist-related protein 1 (Twist) families (22). Accumulating evidence suggests a link between EMT and stem cells in cancer (23,24).

Stem cells are characterized by an indefinite cell division capacity and a potential to self-renew, enabling their maintenance, regeneration and differentiation into adult tissues. A functional ductal tree deriving from the mammary epithelial tissue indicates the presence of stem cells (25). Such cells are relatively dormant and are activated only during mammary gland development supported by ductal and alveolar progenitors (26), which have stem cell-like properties (27). The Wnt (28) and Notch (29) signaling pathways, playing important roles in self-renewal, can regulate stem cells. A trait of mammary stem cells is their ability to form mammospheres, observed *in vitro*, which retain the undifferentiated, multi-potent and proliferative state (30). Human breast tumors bear a small population of cancer stem cells (CSCs), identified by a CD44^{high}/CD24^{-low} antigenic phenotype, also termed as tumor-initiating cells (31). Consistent with this, EMT, cancer and mammosphere formation are linked with the EMT-induced phenotype in

transformed human mammary epithelial cells characterized by an increasing CD44⁺/CD24^{-low} cell subpopulation, which exhibits enhanced mammosphere formation (24).

Given the deleterious effects of retrotransposition, host organisms have evolved diverse defense mechanisms to silence retrotransposons. Among others, DNA methylation is an epigenetic defense mechanism which can block retrotransposition in differentiated cells by silencing retrotransposon RNA expression (32). In addition, epigenetic programs play a key role in the function and differentiation of stem cells where several genes are expressed as a result of a hypo-methylation state (33). In the present study, the question of whether mouse mammary epithelial HC11 cells characterized by stem-like/progenitor properties (34-36) have the potential to elicit VL30 retrotransposition events was addressed. Upon transfection with an engineered VL30 retrotransposon, it was demonstrated that HC11 cells provide an amenable cellular environment for the occurrence of VL30 retrotransposition events. Notably, the outcome of VL30 retrotransposition was associated with the induction of EMT, CSC generation and tumorigenesis. The findings of the present study are the following: i) VL30 retrotransposition as a tumorigenesis agent, and ii) the induced EMT/CSC/tumorigenesis properties of HC11 cells may be considered as a marker of such cells.

Materials and methods

Cells and cell culture. HC11 is an immortalized mouse mammary epithelial cell line (34) with stem-like or progenitor cell properties (36). C127 are mouse non-tumorigenic mammary epithelial cells. HC11 and C127 cell lines were purchased from Life Technologies. J3B1A cells are a clonal derivative from the spontaneously immortalized mouse mammary epithelial cell line Eph4 (37) (obtained from Dr Priscilla Soulie, Medical School Center, Geneva, Switzerland). C127 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 g/l), 10% FBS, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 mg/ml). J3B1A cells were grown in DMEM containing low glucose (1 g/l), 10% FBS, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 mg/ml). HC11 cells were cultured in Roswell Park Memorial Institute medium (RPMI)-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 5 mg/ml insulin and 10 ng/ml EGF (34). NIH3T3 mouse embryo fibroblasts (CRL-1658, ATCC) were grown under standard conditions in DMEM growth medium supplemented with 10% (v/v) FCS, 2 mM glutamine, and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin).

Transfection and cell cloning. Test cells (0.25×10^6) were transfected with 2.5 μ g DNA of plasmid NVL-3*/EGFP-INT/hygromycin B (12) using Polyfect[®] (Qiagen). Two independent transfections were performed and 20 single or massive HC11-VL30, J3B1A-VL30 and C127-VL30 cell clones were isolated following selection with 50-80 μ g/ml hygromycin B for 18 days. A total of 2.5 μ g DNA of the enhanced green fluorescence protein (EGFP-N1) plasmid (BD Biosciences) was used for transfection of the HC11 cells and the selection of neomycin-resistant clones was performed using G418

at 400 $\mu\text{g/ml}$ for 16 days. Single clone refers to a particular population of cells isolated from one well-defined cell focus, grown onto a cell-culture treated plate, produced following antibiotic selection. Massive clones refer to the remaining antibiotic-resistant foci isolated from the same plate and further pooled.

Measurement of retrotransposition frequency and fluorescence microscopy. The retrotransposition frequency of isolated single or massive clones was measured by fluorescence-activated cell sorting (FACS) using trypsinized subconfluent clone cells. Normal HC11 or J3B1A or C127 cells were used as respective controls to evaluate EGFP-background fluorescence, setting intensity threshold values up to 99.60% to be considered as negative and 0.4% as false-positive. VL30 clone samples with values $>0.4\%$ were scored as retrotransposition-positive. Obtained data were analyzed using BD CellQuest v.3.1 software as previously described (38). The detection of EGFP-positive cells was performed with fluorescence microscopy (12,13).

PCR, reverse transcription-quantitative PCR (RT-qPCR) and reverse transcription-PCR analysis (RT-PCR). PCR analysis for the detection of the 342bp VL30 retrotransposition diagnostic band was performed with isolated clone DNAs and EGFP primers (12). For real-time PCR analysis, 1 μg of total RNA isolated (using the RNeasy Mini kit, Qiagen) was converted into cDNA using the QuantiTect reverse transcription kit (Qiagen). After 1:10 cDNA dilution, reactions were performed in PCR multi-plate wells containing 5 μl SYBR PCR Master Mix 2 (KAPA Biosystems), 1 μl cDNA, 3 μl ddH₂O and 1 μl primer pair-mix (5 pmol/ μl , each primer). The synthesized pairs of primers, designed using Primer3 primer software were as follows: TGF- β 1 forward (F), 5'-tga gtggctgtctttgacg-3' and reverse (R), 5'-agccctgtattccgtctct-3'; Slug F, 5'-tctgcagaccactctgatg-3' and R, 5'-agcagccagactct catgt-3'; Snail-1 F, 5'-tgagaagccattctctgct-3' and R, 5'-cttcac atccgagtggtt-3'; Twist F, 5'-cggacaagctgagcaagatt-3' and R, 5'-gcaggacctggtacaggaag-3'; homeobox protein nanog (Nanog) F, 5'-aagcagaagatgctgactgt-3' and R, 5'-atctgctggaggctgagg ta-3'; octamer-binding transcription factor 4 (Oct4) F, 5'-ccaatc agcttgggctagag-3' and R, 5'-ctgggaaaggtgtcctgtga-3'; and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) F, 5'-gcagtg gcaaaagtgagatt-3' and R, 5'-gaattgcccgtgagtggagt-3'. Each reaction was performed in triplicate under the following thermal cycling conditions: Step 1: 95°C/2:00, Step 2: 95°C/0:02, Step 3: 60°C/0:20, Step 4: 60°C/0:01, repeat steps 2-4 (x39), melting curve: 72°C to 95°C, increment 0.5°C for 0:05 using the CFX96 Real Time System, C1000 Thermal Cycler (Bio-Rad Laboratories, Inc.). Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed with previously used VL30 (39) and degenerated endogenous reverse transcriptases (enRTs) primers (5). In detail, the nucleotide sequence of the degenerated enRT primers was as follows: 5'-SE, 5'-T GGA (AC)(CT)(AG) (GT)(ACT)(CT) T(GAC)C C(AC)C AGG G(AT)-3'; and 3'-SE, 5'-A (AG)(GAC)A G(AGT)A (AT) GT CAT C(CT)A (CT)(AG)T A-3' designed at the conserved domains 4 and 5 of amino acid sequences identified in the amino-terminal coding regions of most known RT polymerases including that of murine leukemia virus (MuLV) (40).

Western blot analysis, indirect immunofluorescence analysis and immunofluorescence staining for FACS. For western blot analysis, cells were harvested in 150 μl RIPA lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate and 0.1% (w/v) SDS] in the presence of 1 mM PMSF, 1 $\mu\text{g/ml}$ pepstatin and 1 $\mu\text{g/ml}$ leupeptin. The concentration of total extracted proteins was determined by the Bradford method (Bio-Rad Protein assay kit II #5000002#). Protein samples of 40 μg were resolved by electrophoresis using 10% polyacrylamide-0.1% (w/v) SDS gels. The proteins transferred to nitrocellulose membranes (Schleicher & Schuell) were finally analyzed by western blotting. The membranes were blocked in 5% fat-free milk in PBS for 3 h at room temperature and were subsequently incubated with the follow primary antibodies: Rabbit polyclonal epithelial cadherin (E-cadherin; sc-7870), rabbit polyclonal neural cadherin (N-cadherin; sc-7939), mouse monoclonal vimentin (sc-32322) and mouse monoclonal GAPDH (sc-32233) (all from Santa Cruz Biotechnology, Inc.) at a 1:250, 1:400, 1:500 and 1:400 dilution, respectively. For protein band visualization, compatible horseradish peroxidase-conjugated secondary antibodies, namely goat anti-rabbit IgG-HRP (sc-2004) and goat anti-mouse IgG-HRP (sc-2005), dilution 1:5,000 and 1:2,000, respectively, were used for enhanced chemiluminescence (using ECL SuperSignal West Pico Chemiluminescent Substrate; Pierce, Thermo Fisher Scientific, Inc.).

For indirect immunofluorescence analysis confluent normal or clone cells grown on glass coverslips were fixed with methanol and reacted for 1 h at room temperature with a mixture of two primary antibodies: Mouse monoclonal fibronectin (sc-59826) and rabbit polyclonal E-cadherin (sc-7870) (both from Santa Cruz Biotechnology, Inc.) at a dilution of 1:100 and 1:50, respectively. The samples were then incubated for 1 h at room temperature with a mixture of two secondary antibodies Fluorescein (FITC)-AffiniPure goat anti-mouse IgG (H+L) (Cat. no. 115-095-003) and CyTM5 AffiniPure Goat Anti-Rabbit IgG (H+L) (cat. no. 111-175-144) (both from Jackson ImmunoResearch, UK) at a dilution of 1:100 and 1:200, respectively, and propidium iodide (PI) following RNase treatment. Stained samples were observed and photographed using a confocal microscope (Leica SP).

For immunofluorescence staining, trypsinized cells were reacted with conjugated allophycocyanin (APC) anti-mouse/human cluster of differentiation 44 (CD44) (cat. no. 103011, BioLegend) and phycoerythrin (PE) anti-mouse cluster of differentiation 24 (CD24) antibodies (cat. no. 101807, BioLegend) at 0.1 μg of antibody per 0.5×10^6 cells per 100 μl final volume. Samples were incubated on ice for 15-20 min in the dark, and following washing and re-suspension in cell staining buffer, were analyzed by FACS using the flow cytometer BD FacsCalibur (BD Biosciences). The calculation of retrotransposition frequency values was performed using the BD CellQuest V.3.1 software.

Real-time cell analysis (RTCA). RTCA was performed with 5,000 cells, in a volume of 100 μl RPMI medium containing 1.25-5% fetal bovine serum (FBS), transferred into wells of an E-16 plate. The rate of proliferation (cell index) was measured by a microelectronic biosensor system [xCELLigence[®] real-time cell analysis (RTCA) DP] up to 3 days.

Next-generation DNA sequencing of retrotransposition-positive clones. High-molecular weight genomic DNA isolated from retrotransposition-positive clone cells was fragmented by digestion with restriction enzymes and double-stranded linkers were ligated onto the DNA ends. Nested PCR was conducted using primers designed at the cytomegalovirus-immediate early (CMV IE) promoter of the EGFP-based retrotransposition cassette (38), and the 3' LTR of pNVL-3*/EGFP-INT (12), respectively. For the first PCR reaction, the forward primer anneals to the CMV IE promoter sequence, being downstream of the 3' LTR, while the reverse one onto the linker. For the second PCR reaction, the forward primer anneals to the 3' VL30 LTR and the same reverse primer was used (that anneals to poly-linker) at a ratio of 5:1 forward/reverse primer. PCR products of the second reaction were used for library preparation and sequencing, according to the Ion Ampliseq library preparation protocol. Obtained reads were aligned against the LTR-reference sequence using the bowtie2 algorithm (41) and LTR sequences were filtered out, using the Cutadapt program (42) [<http://journal.embnet.org/index.php/embnetjournal/article/view/200/479>].

Xenografts and tumor analysis. Balb/c mice were purchased from Harlan (UK) and were housed and kept in specific pathogen-free sterile conditions, at the Transgenic Mouse Facility (TMF) of the Cyprus Institute of Neurology and Genetics in Nicosia, Cyprus, which is licensed by the Cyprus Veterinary Services (C.EXP.101). All the bedding and water for the mice were sterilized by autoclaving. The experiments were performed under the animal project license (CY/EXP/PR.L6/2011) provided to A.I.C., issued and approved by the Cyprus Veterinary services, which is the Cyprus national authority for monitoring animal research for all academic institutions according to the regulations contained in the Cyprus Law N.55 (I)/2013 and the EU Directive 2010/63/EU. Two groups of fourteen (14) female (6-8-week-old) Balb/c mice (15-20 g weight) were inoculated by injecting $1-5 \times 10^6$ either normal HC11 or massive clone retrotransposition-positive cells per group into the fat pads near the posterior mammary gland. Developed tumors along with surrounding tissues were removed, immediately fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin using standard procedures. Paraffin-embedded tumors were cut in 5- μ m-thick sections, stained either with hematoxylin-eosin alone or analyzed by immunohistochemistry using a 1:300 diluted anti-pan cytokeratin antibody [AE1/AE3] (ab27988, Abcam) and hematoxylin staining.

Statistical analysis. GraphPad Prism 5.0 version was used for statistical analysis. Comparisons between multiple groups of retrotransposition frequency values were determined by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison post hoc-test. A two-tailed paired sampler Student's t-test was used to examine the differences between groups of real-time PCR data. In both cases, differences were considered statistically significant at $P < 0.05$.

Results

Transfection of a VL30 retrotransposon in mouse mammary epithelial cells elicits retrotransposition events. In order

to investigate whether VL30 retrotransposition occurs in progenitor mouse mammary epithelial cells, the present study used HC11 cells that have stem-like or progenitor cell properties (36), as well as differentiated J3B1A and C127 cells considered as controls. The engineered recombinant pNVL-3*/EGFP-INT vector (12) was also used, which carries both a VL30 retrotransposon tagged with an EGFP-based retrotransposition indicator cassette (38), and a hygromycin B gene. Notably, this VL30 retrotransposon truncated in the internally presumed gag, pol and env sequences retains its functional retroviral replication signals primer binding (-PBS), psi (Ψ) packaging signal and poly-purine tract (PPT), and it is able to retrotranspose (12). The retrotransposition indicator cassette, cloned in opposite transcriptional orientation to VL30 sequences, consists of a CMV promoter driving the expression of an EGFP gene, γ -globin intron in opposite orientation to the EGFP gene interrupting its expression, and a tkpoly(A) signal. The rationale of detection of a retrotransposition event is based on the structure of the NVL-3*/EGFP-INT recombinant. Before retrotransposition, EGFP transcripts originating from the CMV promoter contain the γ -globin intron and cannot produce the green fluorescent protein. The detection of EGFP expression and concomitant fluorescence can only occur, after a retrotransposition event, following intron removal by splicing and reverse transcription of the resultant transcript further integrated in the genome. Thus, the frequency of retrotransposition of a cell population can be measured using FACS analysis. In addition, the retrotransposition-positive cells can be observed by fluorescence microscopy through EGFP expression and further documented with PCR analysis based on a 342-bp band, diagnostic of new retrotransposon copies integrated in the genome (12,13).

Following cell transfection with pNVL-3*/EGFP-INT and selection with hygromycin B, 20 random antibiotic-resistant single clones were isolated from each case of HC11 or C127 or J3B1A cells, as well as a 72-85 mixed clone population, thereafter referred to as massive clones. Subsequently, the retrotransposition frequency of isolated clones were measured by FACS, comparing their EGFP fluorescence profiles with those of self-fluorescence of respective non-transfected cells, as exemplified for HC11 clone 19 (HC11/cl.19) (Fig. 1A). Among the single HC11 clones, it was observed that clones 1 and 19 had a 20.8 and 21.1% retrotransposition frequency, respectively, is significantly higher to that of <4.8% measured in all other clones (Fig. 1B). In reference with the C127 cells, single clones 12 and 7 had the highest retrotransposition frequency of 5.7 and 6.9%, respectively, while in the case of J3B1A cells, the highest retrotransposition frequency was found in single clones 8 and 9 with a similar percentage of 4.6% (Fig. S1). In all other clones derived from either the HC11 or C127 or J3B1A cells, a lower retrotransposition frequency was found, as shown for a set of 10 clones (Figs. 1B and S1). Furthermore, in isolated massive clones, a higher retrotransposition frequency was observed in the case of HC11 compared to C127 and J3B1A massive clone cells with respective values of 5.48% (Fig. 1B), 3.5 and 3.2% (Fig. S1).

Subsequently, whether the retrotransposition events of retrotransposition-positive clone cells could be microscopically monitored through EGFP fluorescence was examined. Indeed, fluorescence microscopy revealed EGFP expression

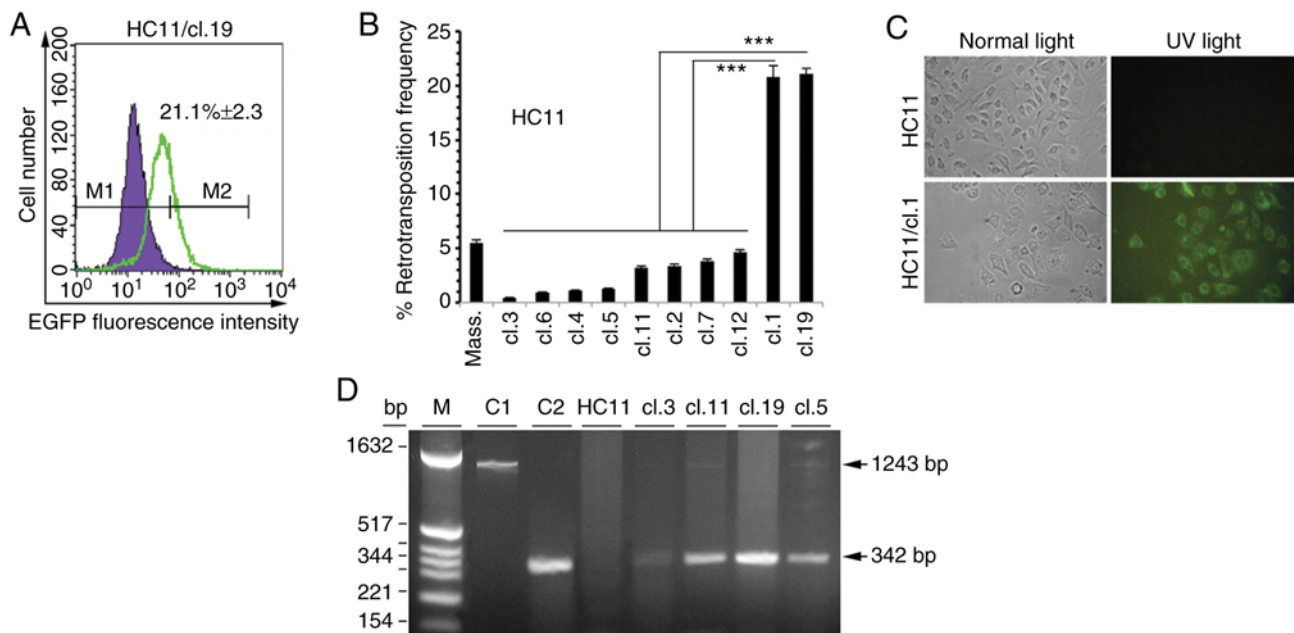


Figure 1. VL30 retrotransposition in HC11 cells. (A and B) Samples of 15,000 cells from control HC11, and isolated single or massive clones (Mass.) were measured for EGFP positivity with FACS. Overlaid violet filled-histogram and green non-filled histogram profiles in panel A represent fluorescence of control HC11 and HC11/cl.19 cells, respectively. M1 and M2 are threshold settings for control auto-fluorescence and sample fluorescence. Percentage value shown inside of histogram A, subtracted by 0.4% (false positive at M2), is the net frequency of EGFP-positive cells as the mean value \pm SD of samples in triplicate. (B) Columns represent the mean value of net retrotransposition frequencies of duplicate samples from three independent experiments with \pm SD indicated with bars. Statistical significance of compared group data shown: *** $P < 0.0001$ (Tukey's post hoc test after ANOVA). (C) HC11 and HC11/cl.1 cells grown on glass coverslips were fixed with 3.7% paraformaldehyde and photographed (magnification, $\times 40$) under normal (left panels) and UV light (right panels), respectively. (D) PCR products from DNA lysates of retrotransposition-positive clones separated on a 1.2% (w/v) agarose gel. Lanes C1 and C2 correspond to PCR reactions with pNVL-3'/EGFP-INT and pEGFP-N1 plasmids as positive controls for the 1243bp and 342bp bands, respectively. Lane HC11 represents a reaction with HC11 DNA lysate, as negative control. M denotes pBR322/HinfI molecular mass-size markers.

in HC11 or J3B1A or C127 single clones as exemplified for HC11/cl.1 (Fig. 1C). Furthermore, to gain more evidence on whether the retrotransposition events, as analyzed by FACS and observed with fluorescence microscopy, corresponded to new integrated VL30 copies into the genome, PCR analysis was performed in retrotransposition-positive clone cells. Using DNA extracted from non-transfected HC11 cells and 4 retrotransposition-positive single clones with a low (clone 3), intermediate (clones 5 and 11) and high retrotransposition frequency (clone 19) (Fig. 1B), in all cases, the expected 342 bp PCR product (Fig. 1D) was detected, diagnostic of a retrotransposition event (12).

VL30 retrotransposition-positive HC11 cells acquire a mesenchyme phenotype. Given the potentially mutagenic effect of new VL30 copy-integrations in the genome, whether the retrotransposition-positive cells had acquired phenotypic changes was investigated. To examine this, the cell morphology of 20 retrotransposition-positive clones derived from either HC11 or C127 and J3B1A cells 45 days after clonal isolation was microscopically examined. It was found that 18 out of 20 (18/20) HC11 single clones exhibited a clear phenotypic change, as shown for clone 19 (Fig. 2A), documented by the typical elongated mesenchymal morphology. By contrast, no such change was evident in any of the J3B1A or C127 clones, even in clones with the highest retrotransposition frequency, such as J3B1A/cl.8 (Fig. 2A) and C127/clone (cl.)7 (Fig. S2). Notably, the unaltered phenotype of J3B1A and C127 clones was maintained even after a prolonged cultivation for 6 months.

Finally, to exclude the potential involvement of the plasmid sequences integrated in the genome and EGFP expression in the observed phenotypic change of retrotransposition-positive HC11 cells, we transfected HC11 cells with plasmid EGFP-N1, which expresses EGFP. Following cultivation of isolated massive EGFP-N1/G418-resistant clones for 3 months, no phenotypic change was observed (Fig. S3).

Mesenchymal phenotype of VL30 retrotransposition-positive HC11 cells is associated with the induced expression of EMT markers. To further analyze the observed mesenchymal phenotype of the retrotransposition-positive HC11 clones, the protein expression of both the epithelial marker E-cadherin and the mesenchymal markers N-cadherin and vimentin (43) was initially examined by western blot analysis in normal HC11 cells and NIH3T3 fibroblasts as a positive and negative control, respectively. In contrast to the HC11 cells, it was found that a set of 5 retrotransposition-positive clone cells exhibited a complete loss of E-cadherin expression. In addition, these clones were strongly positive for N-cadherin and vimentin expression with a protein expression profile as that of NIH3T3 fibroblasts (Fig. 2B). To gain additional evidence at the cellular level on E-cadherin loss of expression, its expression was examined along with the expression of the mesenchymal marker, fibronectin (43) by immunofluorescence analysis in HC11/cl.19 cells. Compared to the control HC11 cells, HC11/cl. 19 cells were marked by a lack of E-cadherin, as well as a strong increase in fibronectin expression at the extracellular matrix (Fig. 2C). Finally, whether the expression of the EMT-associated transcriptional factors,

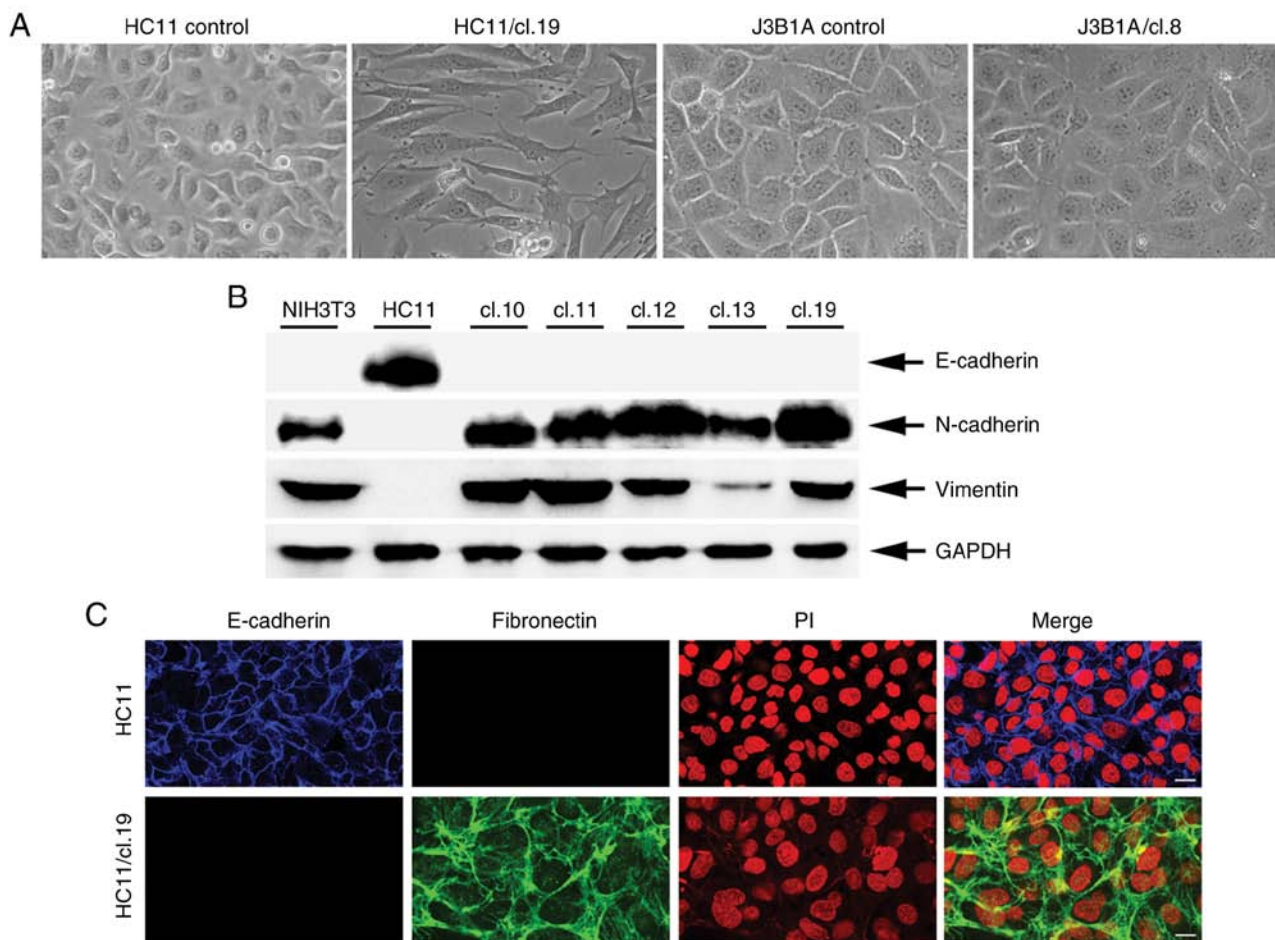


Figure 2. VL30 retrotransposition induces a mesenchymal phenotype associated with the protein expression of EMT markers in HC11 cells. (A) Fields of control HC11 or J3B1A cells and respective retrotransposition-positive clone cells (magnification, x20) grown in normal culture dishes. HC11/cl.19 and J3B1A/cl.8 panels represent cells with an induced and non-induced mesenchymal phenotype, respectively. (B) Western blot analysis of whole protein lysates from NIH3T3, HC11 and HC11 retrotransposition-positive clone cells. Arrows indicate E-cadherin-, N-cadherin- and Vimentin-antibody reactions. GAPDH refers to sample protein load. (C) Immunofluorescence of control HC11 and HC11/cl.19 cells after staining with E-cadherin and fibronectin antibodies as well as propidium iodide (PI). Scale bar, 20 μ m. Data in (B) and (C) are representative of 3 experiments.

Slug, Snail-1, Twist and cytokine TGF- β 1 (23) was affected was investigated, by examining their expression at the mRNA level. RT-qPCR analysis, using cDNA generated from normal HC11 cells and respective retrotransposition-positive massive clones, indicated that the mRNA expression of TGF- β 1, Slug and Snail-1 was strongly increased by \sim 19-, 36- and 51-fold, respectively. Of note, the RNA expression of Twist was markedly increased by \sim 341-fold (Fig. 3).

VL30 retrotransposition-positive HC11 cells exhibit properties of CSCs. A previously demonstrated, the association of EMT and gain of stem cell properties (24) prompted us to examine whether HC11 retrotransposition-positive cells, characterized by induced EMT, had acquired CSCs properties. To this end, using normal HC11 cells as a control, the proliferation rate of HC11 retrotransposition-positive single-clone cells was initially measured by RTCA in low FBS-growth medium for a period up to 72 h. It was found that clone cells had higher growth rates than the control cells when cultured in $<$ 10% FBS concentrations, as exemplified for the HC11/cl.19 cells (Fig. 4). Specifically, the cell index of the control cells supplemented with 2.5- and 5% FBS at 48 h was 0.75 and 1.0, respectively,

while the cell index of the HC11/cl.19 cells at both FBS concentrations was much higher at \sim 3.46. Furthermore, at the point of 48 h and the 1.25% FBS concentration, while the control cells could not grow, the HC11/cl.19 cells had a 2.8-cell proliferation index.

Subsequently, the observed ability of HC11 retrotransposition-positive cells to grow in low serum medium motivated us to examine whether their growth is anchorage-independent or apoptosis resistant. Thus, trypsinized cells from 5 retrotransposition/EMT-positive clones 3, 6, 11, 12 and 19 were subjected to the anoikis test for 10 days in non-adherent plates, using normal HC11 cells as a control. At 5 days post-trypsinization, the control cells failed to proliferate, exhibiting early cell death effects (Fig. 5A-a). By contrast, all clone cells were able to attach on the plate surface, proliferate and form cell clusters characterized by fibroblast-like morphology with spike-like extensions or filopodia, as well as growing small-sized cell spheroids or mammospheres on the top, as shown for the HC11/cl.12 cells (Fig. 5A-b and -c). Furthermore, after 10 days of cultivation, the size of the mammospheres was further increased and anchorage-independent/floating mammospheres were evident in the culture

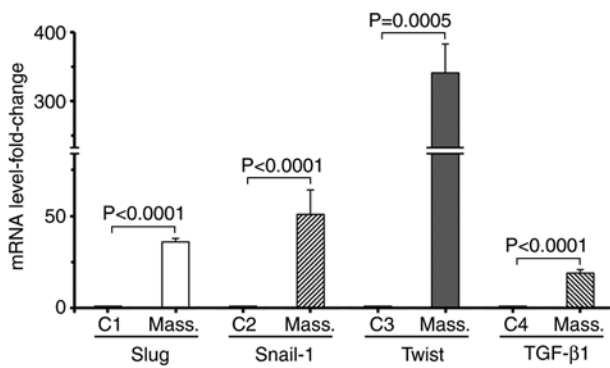


Figure 3. RNA expression levels of EMT-associated genes in HC11 retrotransposition-positive cells. Total RNA isolated from either control HC11 or retrotransposition-positive massive clone cells subjected to Real-Time PCR analysis for examining the expression of EMT-transcriptional factors using designed primers (please see Materials and methods). Columns, corresponding to each transcription factor, represent the mean value of fold-mRNA expression from three independent experiments with \pm SD indicated with bars in comparison to basal-control HC11 expression considered as 1-fold. C1-C4 and Mass. denote control HC11 and massive clone cells per respective analysis. GAPDH expression was used for cDNA template normalization. Presented P values were calculated using the paired sampler Student's t-test.

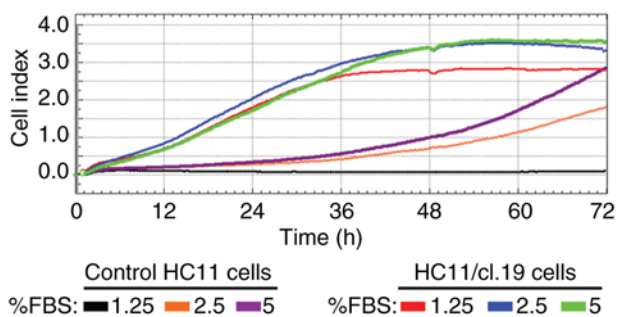


Figure 4. RTCA proliferation rates of HC11 retrotransposition-positive cells in low serum-media. 5,000 cells deriving from either control HC11 or HC11/cl.19 cells, in a volume of 100 μ l RPMI medium supplemented with low FBS concentration, transferred and cultured in wells of an E-16 plate. Samples were analyzed in duplicate (n=2). Representative colored proliferation curves correspond to each FBS concentration shown by respective color bars, at the bottom. Cell index refers to cell proliferation rate.

medium (Fig. 5A-d). Subsequently, the protein expression of the CD44 and CD24 cell surface antigen markers in EMT-positive clones was examined by FACS analysis. It was found that the expression of CD44 was significantly increased, while that of CD24 was decreased, as shown for the 3 clones, 11, 12 and 19, compared to the control HC11 cells (Fig. 5B). Among these clones, clone 19, characterized by the highest retrotransposition frequency (Fig. 1B), exhibited a relatively lower CD24 expression, while clones 11 and 12 the highest increase in CD44 expression.

Finally, mammospheres isolated from retrotransposition/EMT-positive clone 12 cells were examined at the mRNA level for the expression of Oct4 and Nanog stem cell markers, as well as their potential for self-renewal and differentiation. As regards the former, RT-qPCR analysis revealed that the mRNA expression of the Nanog and Oct4 genes was \sim 0.35- and \sim 2.3-fold higher than the levels of the control HC11 cells, respectively (Fig. 5C). With respect to the latter, floating

mammospheres were isolated, trypsinized and cultured in non-adherent plates as single cells. Following 3 days of culture, the generation of secondary small-sized mammospheres was observed, which had almost half of the initial mammosphere size in 5-7 days (Fig. 6A). Furthermore, isolated floating mammospheres, grown in cell-culture treated plates under confluent conditions for 15-20 days, formed early tissue structures distinguished by two distinct cell layers: An inner layer consisted of luminal epithelial-like cells, and a surrounding ring of myofibroblast-like cells resembling the outer layer of a mammary acinus (Fig. 6B-a-e).

New retrotransposed VL30 copies are integrated at the vicinity of cancer-related genes. Taking into account the induced CSC-like properties observed in retrotransposition-positive HC11 cells, whether the new genomically integrated VL30 copies were linked with known genes involved in EMT and cancer was examined.

To this direction, high-molecular weight DNA isolated from cells of clones 19 and 7, characterized by a respective high and medium retrotransposition frequency of 21.1% and \sim 4% (Fig. 1B), was used for targeted sequencing analysis. The strategy of sequencing analysis was based on: i) The human CMV-IE promoter as its particular sequence is part of the EGFP-etrotransposition indicator cassette (38), but not present in the mouse genome; and ii) the end of 3'VL30 LTR in locating the start of a new integration site in the genome (Fig. S4). Following sequencing analysis, 74,668 and 61,385 total reads were initially received for clones 19 and 7, respectively. Following alignment against the reference mouse genome sequence (mm10), a respective number of 273 and 230 mapped reads was received. Filtering-out the LTR sequence included in reads and from the mapped coordinates of trimmed reads, a number of 24 and 17 high-confidence new VL30 integrations were found in clone 19 and clone 7 DNA, respectively. Subsequently, whether the sum of these integrations was linked to a particular chromosome was examined. It was found that chromosomes 3, 8, 9, 10, 16 and 18 had no integrations, while the higher number of integrations was identified in chromosomes 4, 5, 11, 12 and X with a number of 6, 5, 4, 4 and 4 integrations, as presented in Table SI.

Given that LTR sequences harbor promoter and enhancer sequences that can influence gene expression, the total number of 41 integrations of both clones was further examined to determine whether there was any association with a gene involved either in breast or other types of cancer. It was found that 10 integrations were associated with genes of various types of cancer and 11 with breast cancer. Furthermore, to link the observed EMT properties of retrotransposition-positive clones (Fig. 2), these integrations were examined for any association with genes associated with EMT. Two integrations were found concerning the zinc finger protein Zfp808 and Gm13151 (or Zfp988) genes, which may be involved in EMT (44), and two additional integrations close to Akap2 or Fbxo33 genes that are associated with both cancer and EMT. Finally, two integrations were found at the vicinity of the Fam49a and Rragc genes. These genes are involved in the epidermal growth factor receptor/phosphatidylinositol-3-kinase/phosphatase and tensin homolog deleted on chromosome ten (EGFR/PI3K/PTEN), and serine-threonine protein kinase/mammalian target of

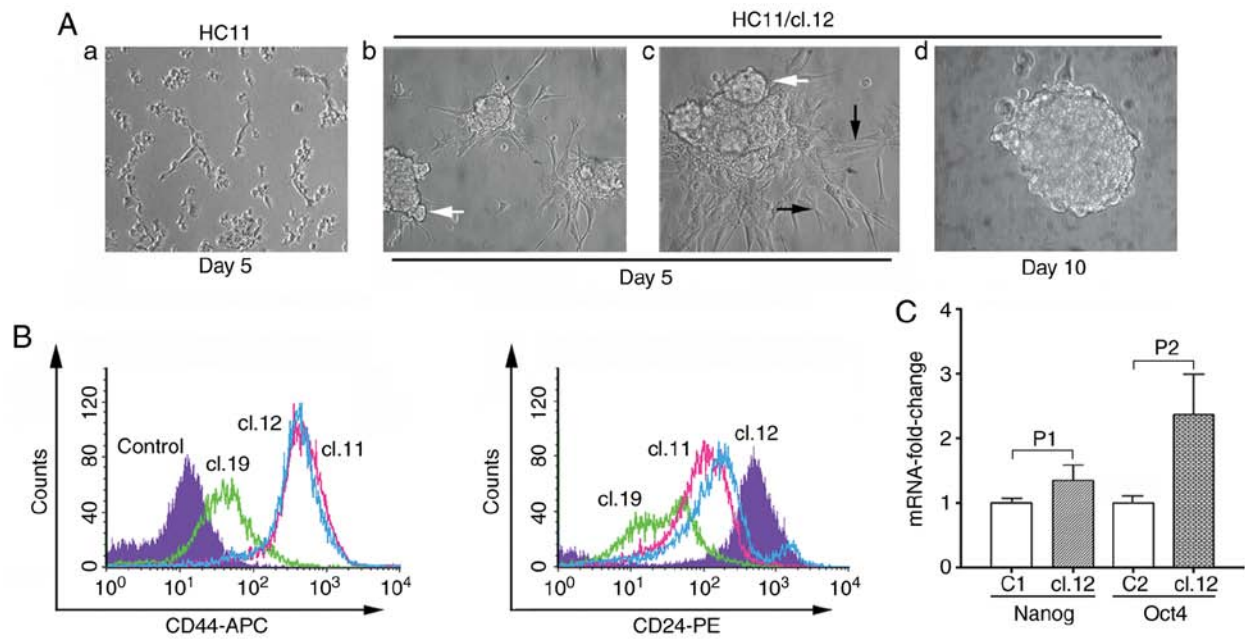


Figure 5. Mammosphere formation in anchorage-independent growth conditions, CD44/CD24 antigen analysis and RNA expression levels of Nanog and Oct4 genes in HC11 retrotransposition-positive cells. (A) 0.2×10^6 trypsinized control HC11 or HC11/cl.12 cells were seeded into non-adherent plates and cultured for 10 days. White arrows (in panels b and c) indicate mammosphere outgrowth, while black ones (in panel c) filopodia-like structures. In panel d, a fully formed-floating mammosphere is illustrated after 10 days of culture. Magnification, $\times 20$ in (a and b), and $\times 40$ in (c and d). Data shown are representative of 3 experiments. (B) 15,000 cells of clones 11, 12 and 19 stained with specific CD44 or CD24 antibodies were analyzed by FACS ($n=3$). Filled-histogram in violet represents control HC11 cells while empty color-histograms in green, light blue and pink correspond to clones 19, 12 and 11, respectively. (C) Total RNA isolated from control HC11 or mammospheres of retrotransposition-positive clone 12 cells was subjected to RT-qPCR analysis with designed Nanog or Oct4 gene primers (please see Materials and Methods). Columns represent the mean value of mRNA expression measurements with \pm SD indicated with bars ($n=3$). Filled columns indicate mammosphere mRNA expression levels of Nanog or Oct4 genes, while empty C1 and C2 columns their respective HC11 control levels. GAPDH was used as control cDNA template normalization. P1 and P2 correspond to statistically significance values of 0.0198 and 0.0119, respectively (paired sampler Student's t-test).

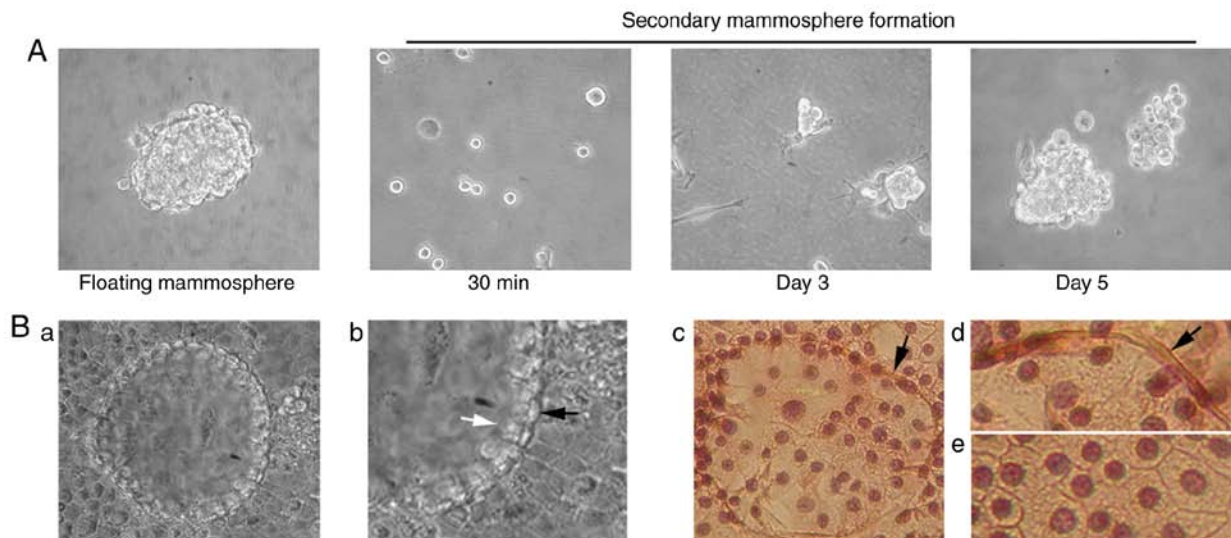


Figure 6. Self-renewal and differentiation of VL30 retrotransposition-induced mammospheres. (A) Single cells deriving from trypsinized floating mammospheres, formed in non-adherent plates, were seeded into fresh plates and cultivated up to 5 days. Formation of secondary mammospheres is shown after 3 and 5 days of cultivation (magnification, $\times 20$). (B) Mammospheres grown in normal cell-culture plates (a-e). The image in (a) shows a mammary acinus-like structure after 20 days of cultivation. White and black arrows in (b) indicate the respective inner and outer cell layer of an acinus. Images in (c-e) show acinus-like structures stained with hematoxylin/eosin: Black arrow in panels (c) and (d) indicates myofibroblast-like cells surrounding luminal epithelial-like cells shown in panel (e). Magnification, $\times 40$ in (a and c). Panel (b) is an enlarged image of panel (a). Panels (d and e) are enlarged images of acinus-like structures similar to the image in (c). Data shown in (A and B) are representative of 3 independent experiments.

rapamycin (AKT/mTOR) signaling pathways, respectively, associated with both breast cancer and EMT (45,46). A detailed list of integration data is presented in Table SI.

VL30 retrotransposition-positive HC11 cells are tumorigenic. The above-mentioned sequencing data showing that the two independent clones 19 and 7 had a sum of 21 new integrations,

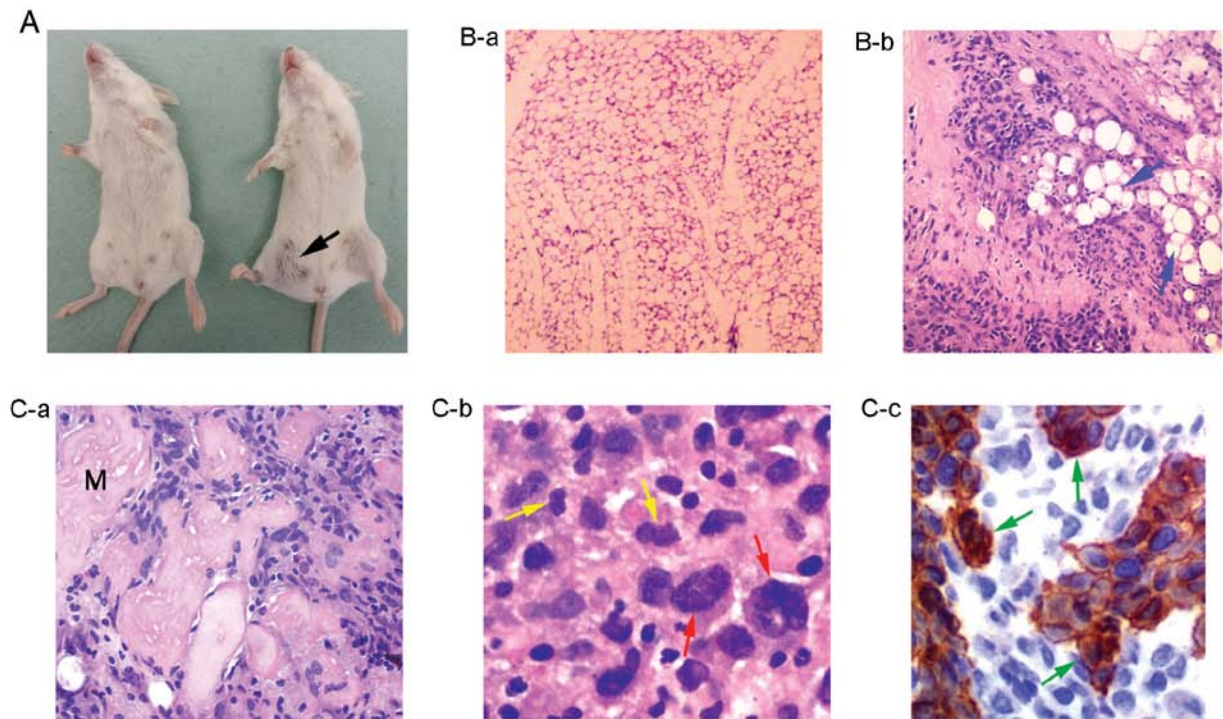


Figure 7. Tumorigenicity of massive VL30 retrotransposition-positive cells in Balb/c mice. (A) Presentation of Balb/c mice injected either with 5×10^6 normal HC11 (left mouse) or massive VL30 retrotransposition-positive clone cells (right mouse). Black arrow indicates both the location of cell injections performed and a tumor developed after injection with retrotransposition-positive cells. (B-a and -b) Adipose tissue sections from a mouse injected with HC11 cells (B-a), and developed tumor (B-b) stained with hematoxylin and eosin. Blue-stained nuclei in (B-b) correspond to invasive tumor cells into the adipose tissue, and blue arrows indicate adipocyte clusters. (C-a-c) Tumor analysis. 'M' in panel C-a denotes skeletal muscle tissue surrounded by tumor cells. A higher magnification of tumor tissue cells is shown in panel C-b where red and yellow arrows indicate enlarged polymorphic nuclei with multiple nucleoli and mitotic cells, respectively. Panel C-c shows cytokeratin-positivity of tumor cells at high magnification, in a tissue location beyond the initial injection site. Green arrows indicate clusters of cytokeratin-positive cells. Panels B-a and -b are shown at a $\times 4$ and $\times 20$ magnification, respectively. Panels in C-a-c, initially taken at $\times 20$ for C-a and $\times 40$ for C-b and -c, are presented at a higher magnification. B-b and C-a-c are representative photographs derived from the analysis of 3 tumor sections ($n=3$).

possibly linked with either various types of cancer or breast cancer (Table S1), prompted the investigation of whether HC11 retrotransposition-positive cells could experimentally produce tumors in syngeneic mouse models. To this end, 2 groups of 14 Balb/c syngeneic mice were injected with either 1×10^6 or 5×10^6 massive retrotransposition-positive clone cells (Fig. 1B) using as control an equal number of mice injected with a respective number of normal HC11 cells. In the case of injections either with 1×10^6 HC11 or retrotransposition-positive cells, following visual and palpation examination of the treated mice for 4 months, no tumor development was detected. However, in the case of 5×10^6 cell injections, while none of the mice injected with HC11 cells produced visible tumors, 2 out of 14 (2/14) mice injected with retrotransposition-positive cells developed ~ 1 cm-sized tumors at the same latency period (Fig. 7A). Tumor section analysis revealed a phenotype of undifferentiated solid neoplasm with a generally diffuse growth pattern, and in some sites tumor cells were arranged in cohesive clusters without an obvious glandular differentiation (Fig. 7B-b and C-a). Moreover, these cells were characterized by a relatively abundant cytoplasm; enlarged polymorphic nuclei with prominent nucleoli; a moderate mitotic activity (Fig. 7C-b), and a potential to spread into surrounding adipose (Fig. 7B-b) and skeletal muscle tissues (Fig. 7C-a). In addition, sections from surrounding tumor tissues were examined by immunohistochemical analysis for expression of the epithelial marker pan-cytokeratin (AE1/AE3) (47). This analysis

revealed the presence of pan-cytokeratin-positive tumor cells able to invade and spread beyond the initially injected tissues, into surrounding healthy ones (Fig. 7C-c).

Discussion

A principal finding of the present study was that either progenitor HC11 or differentiated C127 and J3B1A mouse mammary epithelial cells elicited VL30 retrotransposition events upon their transfection with an engineered VL30 retrotransposon (Figs. 1A and B, and S1). Notably, the retrotransposition frequency of the HC11 cells was higher than that of the C127 and J3B1A cells. This was documented by the following: i) Comparing primarily the retrotransposition value of massive HC11 clones with that of C127 and J3B1A ones being 1.56- and 1.71-fold higher, respectively (Figs. 1B and S1); and ii) the fact that 2 out of 10 single HC11 clones (clones 1 and 19) had the highest scored retrotransposition frequency of $\sim 21\%$ (Fig. 1B) among the respective C127 and J3B1A clones (Fig. S1). The authors have previously reported that a highly induced VL30 retrotransposition frequency signals activation of a p53-dependent cell death pathway (13). In reference to HC11 cells that harbor mutated p53 gene (48), it was hypothesized that their p53 mutation, rendering these cells unable to activate the retrotransposition-associated cell death pathway, could justify the very high retrotransposition frequency of these two clones.

The mechanism of the retrotransposition process requires both a retrotransposon transcript and an active reverse transcriptase, while the methylation of repetitive DNA (49,50), inhibiting retrotransposon RNA expression, is a cell-defense mechanism against retrotransposition-derived mutation/deleterious effects. The authors have previously reported VL30 retrotransposition in normal NIH3T3 fibroblasts solely following treatment with agents, such as vanadium (3), H₂O₂ (51) or arsenic (52), indicating that normal NIH3T3 cells do not provide the minimum RNA expression of endogenous VL30s and enRT enzymes, both required for the generation of a retrotransposition event (4,12). Of note, by applying RT-qPCR analysis in this study, higher levels of enRTs and endogenous VL30 transcripts were found in HC11 cells than in NIH3T3 cells (Fig. S5). This demonstrates that the HC11 progenitor state (53) is endowed with an increased expression, independent of an external stimulus, of both factors required for a retrotransposition event. Given that the HC11 cells have stem-like properties, their higher retrotransposition values could be explained by their hypomethylation status as has been suggested for human stem cells (54). Apart from the nominal retrotransposition frequencies found, e.g., in HC11 clones (Fig. 1B), it was hypothesized that their true values are higher. This could be due to: Either the initial VL30 plasmid integration occurred into a relatively low methylated genomic site, permitting thus the emergence of a respective low retrotransposition frequency, or the ensuing new VL30 retrotransposed copies integrated into genomic sites with a locally higher methylation status. Thus, it can be considered that the retrotransposition frequencies presented here, particularly of low retrotransposition-positive clones, are at the lower threshold that can be reliably measured by FACS. Overall, these data suggest that both the hypomethylation and p53-mutated states of HC11 cells, being first permissive for the occurrence and then accumulation of retrotransposition events, provide an amenable cellular environment for the emergence of a high retrotransposition frequency. In addition, the fact that normal HC11 cells do not exhibit an EMT-cellular phenotype and induced expression of EMT markers (Fig. 2) implies that the level of endogenous VL30 transcripts (Fig. S5) rather consists a cut-off for endogenously or self-derived retrotransposition events, at least, at a high frequency. Plausibly, this barrier was exceeded following RNA expression of our transfected non-autonomous VL30 retrotransposon (less methylated as plasmid DNA) while an enRT, presumably that of MLV (10), acted as a *trans*-complementation factor for generation of retrotransposition events.

Three linked lines of evidence document the specificity of the retrotransposition events found. First, our VL30 recombinant was constructed so that the EGFP is solely expressed after a retrotransposition event (12,13). Second, the retrotransposition-positive cells were observed as such through the EGFP fluorescence (Fig. 1C) further used for making the measurement of a retrotransposition frequency feasible by FACS analysis (Fig. 1A). Third, the existence of retrotransposition events was confirmed both as genomic integrations through the diagnostic 342bp PCR band (Fig. 1D), and target sequencing (Fig. S4 and Table SI). Generally, the natural retrotransposition frequency is extremely low and for a non-autonomous or defective endogenous retrovirus/retrotransposon, such as VL30, it

has been estimated to be up to 10⁻⁶ retrotransposition events per cell (55). In comparison, HC11 retrotransposition frequencies ranging from 0.3 to 21.2% were scored (Fig. 1A and B), which correspond to 30,000- and 212,000-fold higher values, respectively. Alternatively, assuming a 24-h replication rate of retrotransposition-positive clone cells and given that their retrotransposition frequencies were measured 18 days following antibiotic selection, these values correspond to an increase between ~1,666- and 11,777-fold per cell/generation, respectively. Therefore, it is suggested that these unusually high retrotransposition frequencies, mirroring new VL30 genomic integrations, contributed to the induced EMT, CSCs generation and tumorigenesis found.

In an approach to obtain genomic information about novel VL30 integrations, targeted sequencing analysis was performed with DNA of clones 7 and 19, and only 41 high-confidence specific integrations were performed (Table SI). Notably, the mouse genome contains 458 endogenous LTRs (1) and their number is expected to highly increase in retrotransposition-positive cells. Thus, it was hypothesized that a technical difficulty was responsible for being unable to identify a much larger number of new integrations, given that the sequencing design was also based on 3' LTR sequences (Fig. S4). Accordingly, this limited number of integrations cannot support a chromosome preference of new VL30 integrations. Regarding the location of integration relative to a gene, the distance of integrations was not immediately near to genes found (Table SI). Nevertheless, the expression of these genes could be influenced by enhancers of the VL30 LTRs (1) even at a long distance, as an enhancer residing 1 Mb upstream or downstream of a gene can affect the transcription of a gene in favor of a DNA-looping model, whereby the enhancer and core gene promoter are brought into close proximity (reviewed in ref. 56).

Three sets of data document the VL30 retrotransposition-induced EMT. First, the HC11 retrotransposition-positive cells characterized by the acquisition of a mesenchyme phenotype (Fig. 2A) and filopodia-cytoskeletal changes (Fig. 5A-c), as reported in induced EMT (57). Second, these EMT morphological features were endorsed by a modulated expression of EMT critical markers (43), such as the loss of E-cadherin, and a marked increase in N-cadherin, vimentin (Fig. 2B) and fibronectin at the protein level (Fig. 2C), as well as a potent RNA induction of Slug, Snail-1, TGF- β 1 and, mainly, Twist (Fig. 3). These data are in agreement with findings indicating that the signal transducer TGF- β 1 coordinating the transcriptional induction of transcription repressors Slug and Snail-1, as well as Twist acting by binding to the E-cadherin promoter (58) repress the expression of E-cadherin and induce EMT [reviewed in (59)], provide primarily a strong explanation for the retrotransposition-induced EMT observed. Third, at the genomic level, new VL30 integrations were identified at the vicinity of: Two zinc finger protein Zfp808 and Gm13151 genes, which may be involved in EMT; two genes Akap2 and Fbxo33 associated with cancer/EMT; as well as two genes Fam49a and Rragc involved in the EGFR/PI3K/PTEN/Akt/mTOR and AKT/mTOR signaling pathways associated with breast cancer/EMT activation (Table SI). It was thus considered that these genomic data are indirectly supportive of a VL30 integration-dependent induced EMT. As, to the best of our knowledge,

this is the first study on VL30 retrotransposition-induced EMT, the above integrations at the vicinity of 6 EMT-related genes provide a hint for a future detailed investigation on the specific EMT mechanism(s) triggered by VL30 retrotransposition.

Of note, a concomitant feature of retrotransposition-positive HC11 cells, exhibited an induced EMT phenotype, was their cancer stem cell properties documented primarily by the fact that these cells were able to actively proliferate in low-serum medium (Fig. 4) and generate mammospheres in non-adherent culture plates (Fig. 5A). Furthermore, the generated mammospheres: i) exhibited a CD44⁺/CD24^{-low} phenotype (Fig. 5B), (34) acquired self-renewal (Fig. 6A) and cell differentiation properties, which characterize mammospheres (60), forming acini-like mammary gland structures consisted of two distinct cell layers likely corresponding to myoepithelial and luminal cells (Fig. 6B-a-e) and ii) characterized by an induced RNA expression of the Oct4 gene (Fig. 5C), which is a marker of stem cells (61). These particular mammosphere properties are interrelated with the induced expression of TGF- β 1 (Fig. 3), known to be associated with signaling pathways in induction and maintenance of stem cells (62), as well as Snail-1, Twist and factors which promote EMT with the CD44^{high}/CD24^{low} phenotype of CSCs (24). Collectively, these data demonstrate that VL30 retrotransposition in HC11 cells is associated with CSCs generation confirming that induced EMT, mammospheres and CSCs are likely to be linked (24).

It has been reported that the binding of VL30 RNA to polypyrimidine tract-binding protein-associated splicing factor (PSF), a proto-oncogene transcription repressor, regulates tumorigenesis in mice (7). Though, such a case does not apply to the observed tumor growth (Fig. 7) as the NVL-3*/EGFP-INT recombinant lacks this particular VL30 binding nucleotide sequence (12). Notably, this study found that massive HC11 retrotransposition-positive clone cells (Fig. 1B) produced tumors in syngeneic models using Balb/c mice (Fig. 7A). In principle, this finding confirms the observed EMT and CSCs features of representative clones, such as a mesenchymal phenotype (Fig. 2A), the formation of mammospheres characterized by a CD44⁺/CD24^{-low} phenotype (Fig. 5A and B), and active cell growth in low-serum culture conditions (Fig. 4). Notably, this study intentionally used massive clone cells characterized by a medium 5.5% retrotransposition frequency (Fig. 1B) to avoid: i) a bias against clones with a higher retrotransposition frequency such as clones 1 or 19 (Fig. 1B); and ii) any particular clone as its clonality per se would be possibly expected to promote tumorigenesis due to a distinctive number of integrations/cell. Accordingly, a cell population characterized by a various retrotransposition frequency/cell is adequate to produce tumors. Since 2 tumors were found per 14 injected animals, this ~14.3% tumor rate was considered as rather low, and that this was probably attributed to the normal Balb/c immune system.

Tumor analysis revealed mitotically active cells, as well as cells with polymorphic enlarged nuclei and multiple nucleoli (Fig. 7C-b), features of cancer genome instability (63), tending to invade the adipose and skeletal muscle tissues (Fig. 7B-b and C-a). In addition, distinctive cell clusters were strongly positive for pan-cytokeratin (Fig. 7C-c), a tumor marker associated with epithelial cell carcinomas and breast

cancer metastasis (reviewed in ref. 47). It was found that each of the independent clones 19 or 7 had a respective sum of twelve or nine integrations, but all different in each clone, at the vicinity of genes related to various types of cancer and breast cancer (Table SI). This suggests that each of these clones has a potentially critical number of integrations for the emergence of tumors. Overall, while the above morphological tumor features justify the observed tumor malignancy, we believe that the sum of integrations/cell acted as a triggering factor for the production of tumor-initiating cells. Without excluding the case of specific integration(s) this matter remains for a future detailed investigation, as this study is a first approach associating LTR retrotransposon-integrations and tumorigenesis.

In conclusion, the present study links, for the first time to the best of our knowledge, the retrotransposition-active LTR retrotransposon VL30 with cells of the stem-like/progenitor state. To date a direct synergy between LTR retrotransposition and cancer remains an unexplored question (16), while an association between EMT and CSCs (24) and their co-operation in the establishment of breast cancer has been proposed (23). In addition, three theories support that CSCs derive from either mutated stem (64) or progenitor (65) or de-differentiated cells (66). Based on the findings of the present study, it can be conclude that: First, the progenitor mouse epithelial mammary cell, such as stem cells endowed by a lower methylation, provides an amenable cellular environment for a high occurrence of VL30 retrotransposition events; second, LTR retrotransposition renders progenitor cells 'vulnerable' to retrotransposition-induced cancer; third, a number of new retrotransposon-copy integrations associated with cancer-related genes and activation of regulatory networks, orchestrated by key transcription factors, is a critical factor for induced EMT and CSCs generation; fourth, indeed, there is a synergy between LTR retrotransposition and cancer as in a retrotransposition-positive progenitor cell induced EMT and CSCs co-exist; and fifth, selected cells in a bulk HC11 cell population, being more permissive for elevated VL30 retrotransposition levels, have a greater tendency to acquire mesenchymal, CSCs and tumorigenesis properties which mirror a marker of such cells.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors' contributions

ST performed the laboratory experiments. GV, GM and DN performed and analyzed the FACS data. SM and FG assisted with the EMT experiments. PP performed the *in vivo* experiments. PK designed and directed the CSC analysis. AC and PP assisted with the analysis of the tumor sections. AIC and TT conceived the experiments and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Statement on the welfare of animals: Balb/c mice were housed in the animal facility of the University of Cyprus in a pathogen-free environment according to the European Commission Recommendations 2007/526 and the European Directive 2010/63. The experimental procedures were conducted in agreement with the animal welfare regulations and guidelines of the Republic of Cyprus and the European Union under a project license acquired by the Cyprus Veterinary Services (No CY/EXP/PR.L6/2011), the Cyprus national authority for monitoring animal research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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