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Oncogenic *ras*-driven cancer cell vesiculation leads to emission of double-stranded DNA capable of interacting with target cells

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ABSTRACT

Cell free DNA is often regarded as a source of genetic cancer biomarkers, but the related mechanisms of DNA release, composition and biological activity remain unclear. Here we show that rat epithelial cell transformation by the human H-*ras* oncogene leads to an increase in production of small, exosomal-like extracellular vesicles by viable cancer cells. These EVs contain chromatin-associated double-stranded DNA fragments covering the entire host genome, including full-length H-*ras*. Oncogenic N-*ras* and *SV40LT* sequences were also found in EVs emitted from spontaneous mouse brain tumor cells. Disruption of acidic sphingomyelinase and the p53/Rb pathway did not block emission of EV-related oncogenic DNA. Exposure of non-transformed RAT-1 cells to EVs containing mutant H-*ras* DNA led to the uptake and retention of this material for an extended (30 days) but transient period of time, and stimulated cell proliferation. Thus, our study suggests that H-*ras*-mediated transformation stimulates vesicular emission of this histone-bound oncogene, which may interact with non-transformed cells.

1. Introduction

Several lines of evidence suggest that mutant oncogenes are not confined to their host cancer cells, but may also exit them and interact with distant cells [1]. Indeed, insoluble oncoproteins, such as epidermal growth factor receptor (EGFR/EGFRvIII), KRAS or MET have been found to exit cancer cells as cargo of extracellular vesicles (EVs) [2–4], the production of which oncogenes stimulate [2,5]. Similarly, oncogenic transcripts and non-coding RNA are found in the cargo of EVs released by different cancer cells [6–8]. Notably, horizontal transfer of oncogene-containing EVs evokes cellular responses reminiscent of malignant transformation and impacts growth, inflammation and angiogenesis [2–4,9,10].

The mechanisms of packaging and vesicular emission of proteins and nucleic acids are poorly understood and controversial [11]. Processes of membrane blebbing and protein clustering on the cell surface have been previously implicated [11,12], including in the release of larger EVs (100–1000 nm) known as ectosomes, microvesicles or large oncosomes [5]. Alternatively, endocytosis of membrane receptors and their interactions with subcellular vesicle trafficking pathways may result in formation of small EVs (50–100 nm) known as exosomes [7,11,13]. Other vesiculation pathways may also lead to the emission of oncogenic proteins and RNA, which are thereby protected from degradation and conditioned for intercellular transfer.

Mutant oncogenes may also exit cancer cells as circulating tumor DNA (ctDNA). This property attracted considerable attention as a way to remotely and continually access driver mutations in biofluids of cancer patients [14,15]. While the nature and biogenesis of ctDNA is presently unclear, its presence is often attributed to necrosis and cellular breakdown leading to the release of free DNA, nucleosomes and chromatin into the surroundings [15]. However, the considerable stability of ctDNA [16] suggests the existence of protective mechanisms that may involve cellular vesiculation. Indeed, oncogenic DNA sequences were found in large vesicular structures known as apoptotic bodies (ABs) formed upon death of cancer cells [17]. EVs emanating from viable cancer cells were also found to contain single and double-stranded DNA [8,18–21]. However, the scope, nature and biological significance of this emission remain poorly characterized.

Here we show that oncogenic H-*ras* drives vesiculation of intestinal epithelial cells resulting in shedding of small EVs that contain chromatin-associated double-stranded DNA sequences representative of the entire host cell genome. We also document EV-mediated

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transfer of H-*ras* DNA to recipient cells and their concomitant proliferative responses. Thus, we postulate that oncogenic mutations can serve to propagate extracellular emission of mutant DNA via EVs that may interact with non-transformed cells.

2. Materials and methods

2.1. Cells and culture conditions

The following cell lines were used: IEC-18 – non-tumorigenic, immortalized rat intestinal epithelial cell line; RAS-3 – tumorigenic variant of IEC-18 cells transfected with the V12 mutant c-H-*ras* human oncogene; Spontaneous primitive glioblastoma-like brain tumor (PBT) cell lines – generated by integrating N-*ras* and *SV40LT* expression vectors into neonatal mouse brain using the sleeping beauty transposase, as described [22]. PBTs were generated in either wild type C57BL6 mice or in their strains harboring null mutation of the acidic sphingomyelinase gene (ASMase−/−), or in heterozygous littermates (ASMase+/−), from which cell lines were established; RAT-1 – immortalized, non-tumorigenic rat fibroblasts. IEC-18 and RAS-3 cells were grown as previously described [23]. PBT and RAT-1 cells were maintained in DMEM supplemented with 10% FBS and 1% Penn/Strep. For the experiments, FBS added to the media was EV-depleted by ultracentrifugation at 150,000×*g* for 5 h.

2.2. Isolation of extracellular vesicles

EVs were obtained by ultracentrifugation as previously described [2,24]. Briefly, conditioned media was centrifuged at 400×*g* for 10 min to remove cell debris, followed by filtration through a 0.2 μm PES filter (#565-0020, Thermo Fisher Scientific). The filtrate was ultracentrifuged at 110,000×*g* for 1 h to isolate EVs. For differential centrifugation, the filtrate was first spun at 10,000×*g* for 30 min (to separate P2/P3 fraction) and then at 110,000×*g* for 1 h to isolate P4 fraction.

2.3. Transmission electron microscopy (TEM)

Isolated EVs were washed once with 0.1 M sodium cacodylate buffer (pH 7.4), fixed with 2.5% glutaraldehyde in the same buffer and processed for whole mount according to standard protocols. The Tecnai 12 BioTwin 120 kV TEM was used to capture images.

2.4. Analysis of EV profile

For quantification of the EV output, IEC-18 and RAS-3 cells were seeded overnight (5000 cells/well), washed with PBS the following day and then incubated with 5 μM FM1-43 dye (#T35356, Invitrogen) in complete media. Cells were cultured for two additional days and their conditioned media were collected by centrifugation (400×*g*). The total fluorescence present in the media was measured by Victor multiplate reader. For size distribution, conditioned media were loaded onto the nanoparticle tracking analysis system (NTA; #NS500, Nanosight) and three recordings of 30 s were obtained and processed using NTA software.

2.5. Characterization of EV-associated DNA

Isolated EVs were lysed in the presence of proteinase K. DNA was precipitated by isopropanol, amplified by PCR and resolved in 2% agarose gel. DNase (#AM1907, Invitrogen) treatment was in accordance with the manufacturer's protocol, except for replacing the provided denaturing buffer with PBS to avoid EV disruption. To assess extraluminal DNA, EVs were treated with DNase prior to lysis and examined for the total and H-*ras* DNA content. These samples were treated with RsaI to specifically digest double-stranded DNA and Bioanalyzer QC was used for their analysis (Genome Quebec). Exonuclease-I (#EN0581, Thermo Fisher Scientific) treatment was in accordance with the manufacturer's protocol. The content of nucleosomes in EVs was detected by ELISA for DNA/histone complexes (#11774425001, Roche) in unfractionated EVs, P2/P3 and P4 fractions. For long-range PCR using H-*ras* primers 3 and 4, KB extender was added to the reaction and samples were resolved in 1% agarose gel.

2.6. PCR primers

The following primers were obtained from Integrative DNA Technologies: Human H-*ras* (forward 5′-GCAGGAGACCCTGTAG GAGGACCC-3′ and reverse 5′-TGGCACCTGGACGGCGGCGCCAG-3′); H-*ras* #2 (forward 5′-TCCCTTTAGCCTTTCTGC CG-3′ and reverse 5′-CCCATCAATGACCACCTGCT-3′); H-*ras* #3 (forward 5′-GCAGGA-GACCCTGTAGGAGGACCC-3′ and reverse 5′-GGCCTGAGGTTCCGAC ATAC-3′); H-*ras* #4 (forward 5′-TGCCCTGCGCCCGCAACCCGAGCC-3′ and reverse 5′-TCAAGACCATCCAATAATTTACTG-3′); ASMase (sense 5′-AGCCGTGTCCTCTTCCTTA C-3′, antisense 5′-CGAGACTGT TGCCAGACATC-3′ and neo 5′-GGCTACCCGTGATATT GCTG-3′); N-*ras* (forward 5′-AATACATGAGGACAGGCGAAGGCT-3′ and reverse 5′-TGTCTGGTCTTGGCTGAGGTTTCA-3′); *SV40LT* (forward 5′-ATTTTG CCCTTGGA CAGGCTGAAC-3′ and reverse 5′-CACTGCGTTCCAGGC AATGCTTTA-3′). All PCR amplifications were performed using the human H-*ras* primers unless otherwise indicated.

2.7. Bioinformatics

Whole genome sequencing (Next-generation; Illumina HiSeq) was conducted on EV DNA and analyzed using the following pipelines: Trimomatic (sequence trimming), BWA and Samtools (genome alignment), and BVAtools and DNACRD (CNV detection). The viral insertion pipeline approach, developed by E.A., extracts all unmapped reads (EV-derived) relative to the rn5 rat reference genome and categorizes them into 4 types: OEA, Orphan, sclip and scOEA. The unmapped reads were filtered for duplicates by using MarkDuplicate from Picard (OEA; sclip) and kmer approach (Orphan), and assembled to create scaffolds (Ray; kmer = 21; default parameter). The quality of the scaffolds was assessed by mapping unmapped reads to rn5 and by blasting to human H-*ras* sequences.

2.8. EV transfer assay

RAS-3 cells were labeled with PKH26 (#MINI26, Sigma–Aldrich) according to the manufacturer's protocol and cultured until 80–90% confluent. EVs obtained from these cultures were resuspended in complete media and incubated overnight with recipient RAT-1 cells, which were FACS-analyzed for PKH26 fluorescence. To assess EV-mediated H-*ras* DNA transfer, following incubation with EVs (2–30 days with or without starvation), RAT-1 cells were lysed and PCR-amplified. To assess the biological responses to this transfer, MTS proliferation assay (#G3580, Promega) was performed using 4500 RAT-1 cells, which were incubated with EVs and analyzed for MTS signal at days 0, 1 and 5.

2.9. Data analysis

All experiments were performed at least twice (except for sequencing) with similar results. Animal material was obtained according to the protocol approved by the Facility Animal Care Committee at our Institution and in agreement with Guidelines of the Canadian Council of Animal Care. Numerical data were

processed for significance using two-tailed *t*-test with the threshold *p*-value of 0.05.

3. Results

3.1. Oncogenic H-ras stimulates formation of extracellular vesicles

We have previously observed that expression of the oncogenic EGFRvIII drives vesiculation of cancer cells [2]. To examine whether intracellular oncogenes also exhibit a similar property, we employed the well-characterized model of rat intestinal epithelial cells (IEC-18) and their human H-*ras* transformed RAS-3 variant. Interestingly, electron microscopy (TEM) of conditioned media collected from the respective cell lines revealed the presence of exosomal-like EVs, approximately 100 nm in diameter and in vastly larger numbers for RAS-3 cells versus IEC-18 (Fig. 1A). Membranes of both cell types were also labeled with the FM1-43 dye resulting in fluorescence of their derived EVs. This emission was quantified using a fluorescence reader (Victor), once again indicating more pronounced vesiculation of RAS-3 cells (Fig. 1B). As FM1-43 measures all membranes emanating from cultured cells, we also performed a nanoparticle tracking analysis (NTA) to gain insight into the size distribution of EVs produced by IEC-18 and RAS-3 cells. We observed that RAS-3 cells emitted elevated numbers of EVs in the size intervals between 51 and 250 nm, which was consistent with TEM results (Fig. 1C). These observations suggest that transformation by mutant H-*ras* stimulates production of exosomal-like EVs.

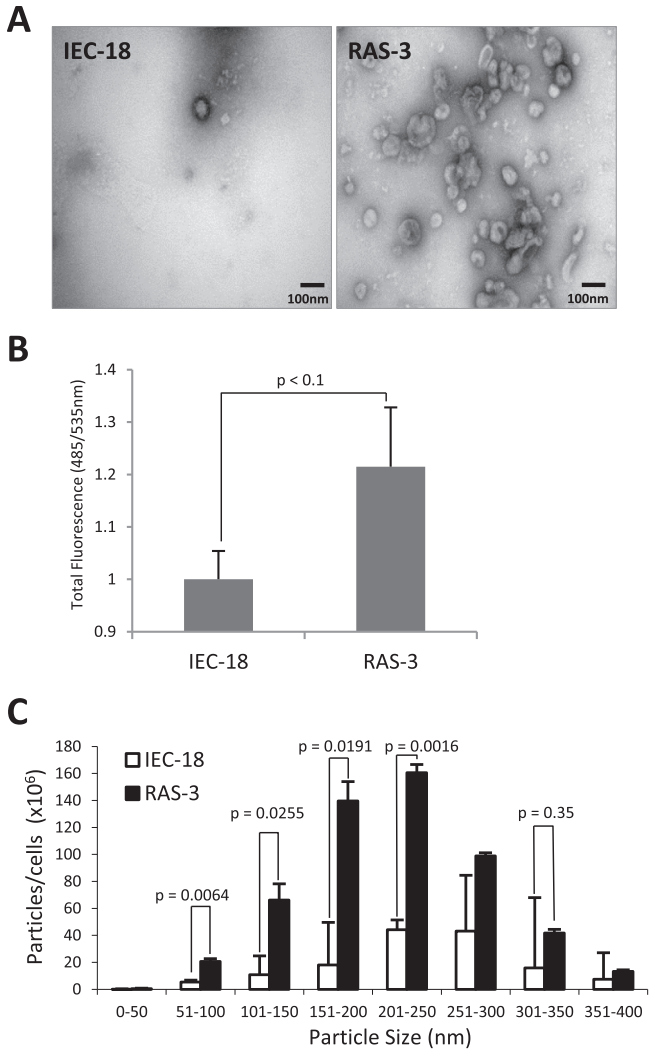

Fig. 1. Stimulation of extracellular vesicle formation by oncogenic H-*ras*. (A) EVs were isolated from IEC-18 (left) and RAS-3 (right) cells conditioned media by ultracentrifugation. Their images were taken by TEM at 49k magnification. (B) IEC-18 and RAS-3 cells were stained with FM1-43. The fluorescence in the conditioned media was measured by Victor reader. (C) Conditioned media of IEC-18 and RAS-3 cells were collected to obtain their nanoparticle size distribution. The analyses were performed by NTA (*p*-values as indicated).

3.2. Extracellular vesicles contain double-stranded genomic DNA sequences including oncogenic H-ras

While oncogenic mutations lead to EV-mediated emission of corresponding oncoproteins and transcripts [2,6,9], it is unclear whether this is also true for DNA. To address this question, we isolated DNA from RAS-3 cells as well as their EVs and performed PCR amplification of the human H-*ras* gene, which was found to be present in both entities (Fig. 2A). H-*ras* DNA was located in the EV lumen, as DNase treatment of intact EVs (in PBS) did not eliminate the signal, while disruption of EVs in the DNase buffer resulted in the disappearance of the band (Fig. 2B). One mechanism whereby genomic DNA may be encapsulated within the cargo of EVs is the case of apoptosis where dying cells break up into large ABs (>1000 nm), containing nuclear DNA fragments [25]. However, this was not the case for cultured RAS-3 cells, which were highly viable (>95% trypan blue exclusion) and the EVs they emitted were of small, near-exosomal sizes (Fig. 1). Therefore, we reasoned that H-*ras* sequences in this material may represent the previously reported single-stranded [8], or double-stranded [18–21] vesicular DNA. To discriminate between these possibilities, DNA isolates from RAS-3 EVs were treated with the restriction enzyme RsaI (specific for double-stranded DNA), which indeed resulted in a significant leftward shift in the Bioanalyzer QC profile. EV-associated DNA was therefore found to be largely double-stranded (Fig. 2C). This is consistent with our observation that Exonuclease-I, which only cleaves single-stranded DNA, had no effect on the amount of total DNA present in RAS-3 EVs (data not shown) or detection of H-*ras* sequences by PCR amplification (Fig. 2D). Collectively, these results suggest that H-*ras*-triggered vesiculation leads to the emission of double-stranded genomic DNA including H-*ras* sequences.

3.3. Subsets of extracellular vesicles preferentially carry chromatin and mutant H-ras sequences

Among multiple subsets of EVs [11], those emanating from the plasma membrane are often larger, sedimented at lower centrifugal forces (P2/P3) and dependent on ASMase activity [24]. In contrast, smaller exosomal-like EVs are formed within endosomes, sediment at higher centrifugal forces (P4) and may be regulated by p53 [26]. To further investigate the role of these variables in the emission of EV-related DNA, we isolated P2/P3 and P4 fractions of RAS-3 media and assessed for the human H-*ras* DNA content by PCR. Interestingly, H-*ras* DNA was preferentially (though not exclusively) concentrated in the P4 fraction (Fig. 2E). We also used ELISA that quantifies DNA/histone complexes to test whether, and which, EVs may contain chromatin. Once again, the DNA/histone signal was concentrated in P4 and not in the P2/P3 fractions (Fig. 2F). These results suggest that extracellular DNA, including H-*ras* nucleosomes, exit cells via small, exosomal-like EVs (Fig. 1).

3.4. DNA-containing extracellular vesicles are generated independently of ASMase

To explore the mechanisms of vesicular DNA emission, we generated primitive brain tumor (PBT) cell lines harboring N-*ras* and *SV40LT* oncogenes in the wild type or ASMase-deficient

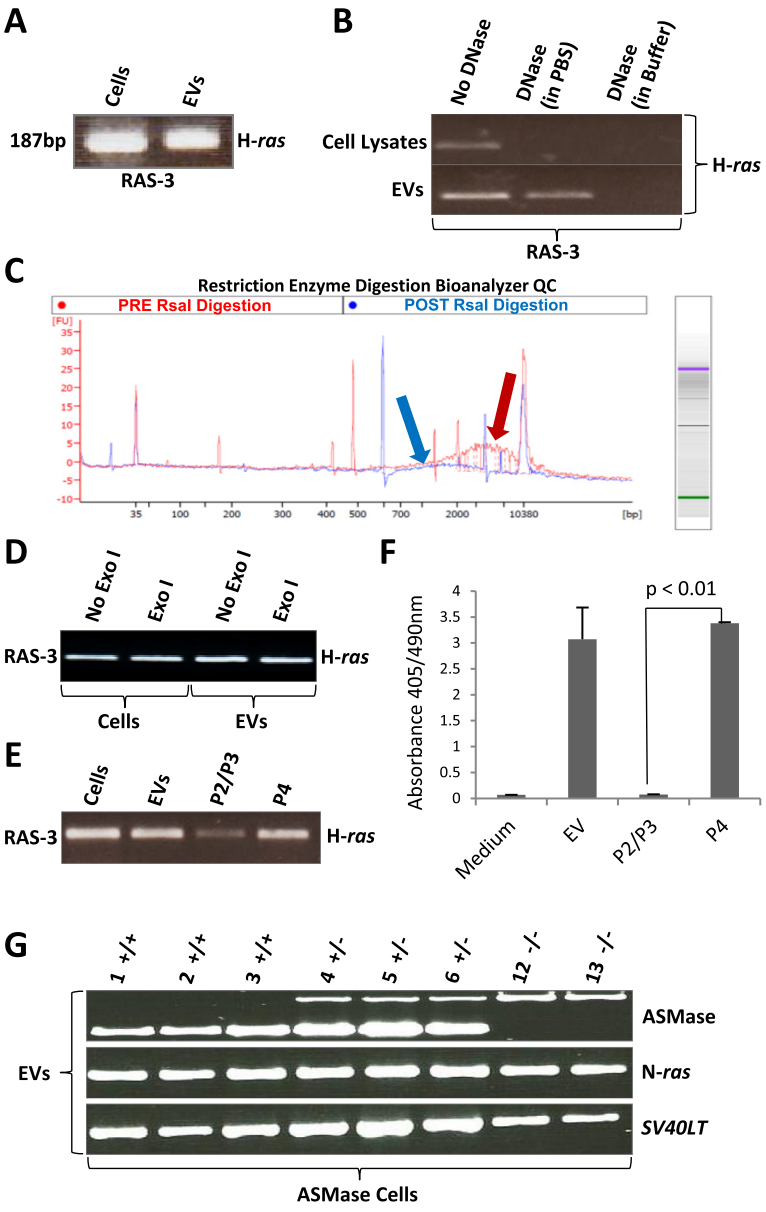

Fig. 2. Diverse vesiculation processes lead to differential emission of oncogenic, double-stranded and histone-bound DNA sequences. (A) EVs were isolated from RAS-3 cells conditioned media by ultracentrifugation. Isolated DNA was then PCR-amplified. (B) DNA from RAS-3 cells and EVs were treated with DNase, in either PBS or denaturing buffer, prior to isolation and PCR amplification. (C) Restriction enzyme RsaI was used to specifically digest double-stranded DNA. Digested samples were then subjected to Bioanalyzer QC. (D) DNA isolates from RAS-3 cells and EVs were treated with Exonuclease-I, followed by PCR amplification. (E) To capture different vesiculation pathways EV, P2/P3 and P4 fractions were isolated from RAS-3 cells by differential centrifugation. Isolated DNA was then PCR-amplified. (F) EV, P2/P3 and P4 fractions were isolated from RAS-3 cells by differential centrifugation. Presence of DNA-Histone complexes in the different fractions was then determined by ELISA. (G) EVs were collected from conditioned media of well-characterized ASMase cell lines. Isolated DNA was PCR-amplified using ASMase, N-*ras* and *SV40LT* primers.

background [22,32]. These cells are deficient for both Rb/TP53 activity (due to *SV40LT* expression [27]) and ASMase (due to targeted insertion of neomycin expressing cassette into the gene [28]), the changes affecting both known vesiculation pathways of exosomal and ectosomal biogenesis, respectively [24,26]. Analysis of this unique panel of cell lines revealed the emission of EVs containing oncogenic N-*ras* and *SV40LT* sequences, suggesting that EV biogenesis may be independent of these two pathways (Fig. 2G).

3.5. DNA contained in extracellular vesicles represents the entire genome

To better understand the nature of DNA present in RAS-3 vesicles, EV DNA sequences were obtained by whole genome sequencing (WGS). The presence of human H-*ras* oncogene in EVs was validated by blasting the human sequence onto two scaffolds, which were generated by assembling unmapped reads that do not correspond to the rat genome (Fig. 3A; Orphan; Green). PCR amplification also indicated that large fragments (777 bp; 2200 bp) were present in EVs including the full-length H-*ras* oncogene (3308 bp; Fig. 3B), which is consistent with Bioanalyzer QC data (700–10,000 bp; Fig. 2C). Moreover, we investigated the copy number variation (CNV) of RAS-3 EV-associated DNA. While the CNV plot suggests some increase in contribution of certain loci (chromosome 11 – upward shift), we have not detected any genomic regions that would be selectively included in EVs (Fig. 3C), as suggested earlier by us [18] and others [20]. Indeed, over 90% of the cellular genome was found in EVs with at least 3 times the coverage of sequences (Fig. 3D). Collectively, these results suggest that EV-associated DNA represents the entire genome of donor cells and contains full-length H-*ras* oncogene.

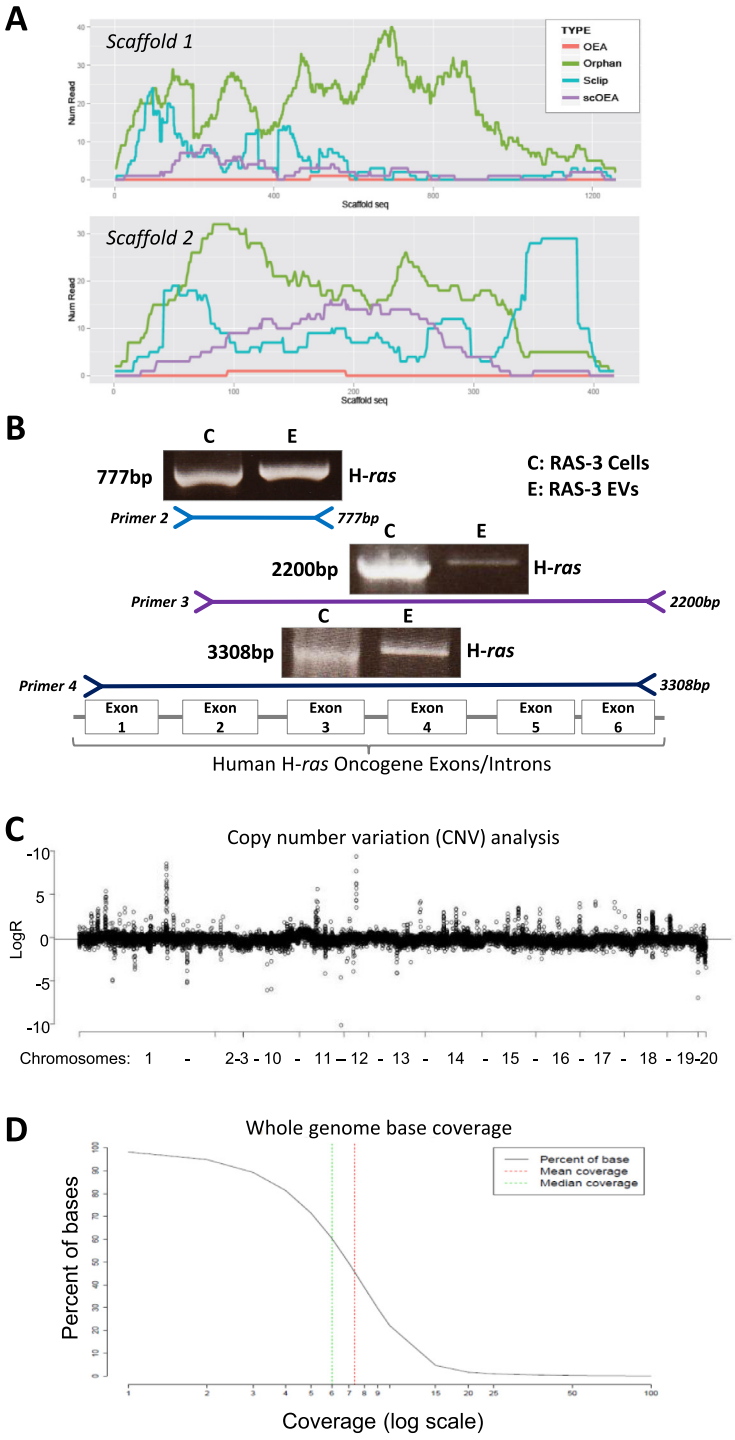

Fig. 3. Extracellular vesicles contain DNA representative of the whole genome. (A) Unmapped reads OEA (larger than blasted H-*ras* sequence; only one end unmapped), Orphan (unmapped ends), sclip (ends partially unmapped) and scOEA (smaller than blasted H-*ras* sequence; OEA close to sclip) relative to the rn5 reference genome are shown for two scaffolds that match onto the human H-*ras* sequences. (B) PCR amplification of RAS-3 cells and EVs DNA using different H-*ras* primers generate products of varying length including full-length H-*ras*. (C) CNV calls of whole EV sequences were analyzed and their corresponding genomic ratio was plotted. (D) Analysis of whole EV sequences based on the percentage of non-ambiguous genomic bases covered at different thresholds.

3.6. Extracellular vesicle-mediated intercellular transfer of oncogenic H-ras DNA

Intercellular transfer of oncogenic DNA has thus far been attributed to the uptake of ABs [17,25] and cell free DNA (cfDNA) [29]. We wished to determine whether DNA-containing EVs emitted by viable RAS-3 cells could also mediate the transfer of oncogenic H-*ras*. Thus, RAS-3 cells were surface labeled with PKH26, which binds and fluorescently tags plasma membrane and EVs. We observed that addition of such EVs to cultured RAT-1 cells leads to their fluorescent labeling (Fig. 4A), a finding indicative of an avid uptake of EVs. To assess whether this interaction led to a transfer of H-*ras* DNA, recipient RAT-1 cells were lysed at different time points and assayed by PCR for the presence of human-specific (RAS-3-derived) H-*ras* signals. Remarkably, such signal was readily detected within 2 days of EV exposure, and was still present as

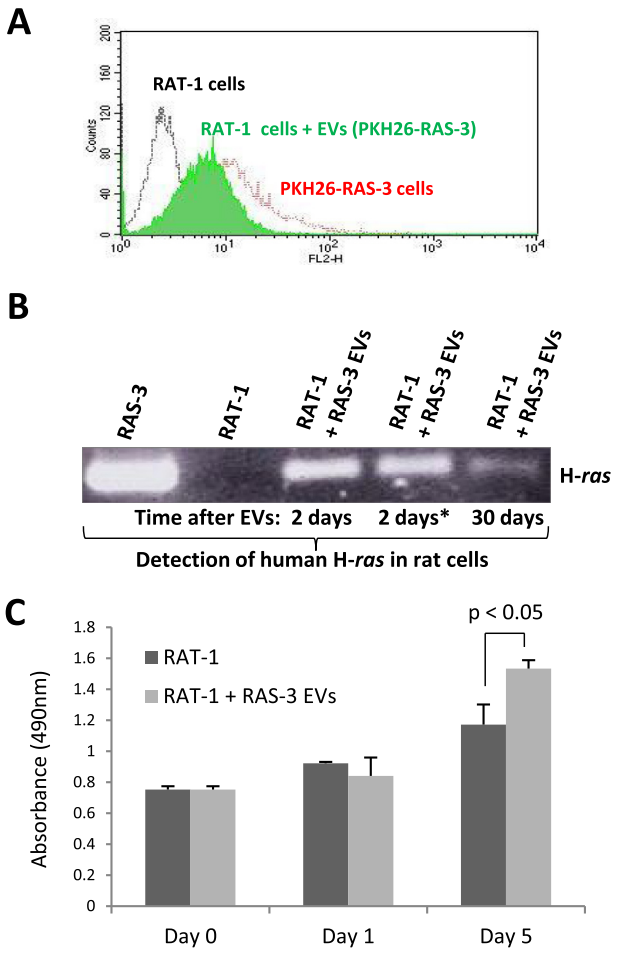

Fig. 4. Extracellular vesicles mediate the lasting transfer of oncogenic H-*ras* DNA and affect growth of recipient cells. (A) EVs collected from PKH26-stained-RAS-3 cells conditioned media were transferred to RAT-1 fibroblasts. PKH26 fluorescence was measured by FACS. (B) EVs from RAS-3 cells were transferred to RAT-1, followed by DNA isolation and PCR amplification (**with overnight starvation*). (C) EVs from RAS-3 cells were transferred to RAT-1. Changes in growth rate were measured by MTS proliferation assay.

long as 30 days later, albeit at diminished levels (Fig. 4B). We have not observed a time-dependent enrichment or permanent presence of H-*ras* DNA in the recipient cells, which suggests that the effects of this transfer do not amount to permanent transformation in spite of the fact that RAT-1 cells can readily be transformed using H-*ras* expression vectors (data not shown). However, we noticed morphological changes in RAT-1 cells immediately after their exposure to RAS-3-derived EVs. To interrogate more closely the underlying biological effects, RAT-1 cells were treated with RAS-3-derived EVs and tested for time-dependent proliferative response using the MTS assay. We observed a higher proliferation rate of these cells 5 days post-treatment (Fig. 4C). Overall, these results suggest that formation of EVs serves to emit genomic DNA from ostensibly viable cancer cells. This material includes full-length H-*ras* oncogene sequences which can be transferred to non-tumorigenic cells and remain there for extended periods of time, while these cells exhibit increased proliferation.

4. Discussion

Our present study extends prior findings suggesting the extracellular emission of oncogenes [2,6,17,25]. In particular, we provide evidence that oncogenic H-*ras* drives emission of small EVs containing H-*ras* DNA, which may enter and remain within recipient, non-transformed cells for extended periods of time, with concomitant mitogenic response. While the nature of this proliferative effect is presently unclear, it is unlikely a sign of permanent transformation due to the decline of EV-related H-*ras* DNA in recipient cells. While the entry of exogenous DNA into stromal cells was reported to trigger DNA repair [30], we did not detect such responses in RAT-1 assays (data not shown), suggesting a role of cellular background.

We also observed that EVs emitted from viable cancer cells contain DNA covering the entire (rat) genome, including the exogenously introduced human H-*ras* oncogene. Moreover, these EV-associated DNA are long fragments, double-stranded and contains histones, thereby suggesting their derivation from cellular chromatin. This differs from the results of Balaj et al. who reported the presence of single-stranded DNA in tumor-derived EVs [8]. The disparity could be related to a different cellular source, or to the diversity of vesiculation processes involved in various systems. However, the ability of cancer cells to emit double-stranded DNA is consistent with several recent observations [18–21].

It is unclear how DNA becomes incorporated into exosomal-like EVs. While EVs we describe possess some features of exosomes, their biogenesis and nomenclature of EVs are complex and controversial [11]. Thus far, we ruled out the involvement of TP53 and ASMase in vesicular emission of oncogenic DNA [24,26], but additional mechanisms are being investigated.

Our study also points to several general implications. First, we suggest that certain types of oncogenic transformation events may result in the increased emission of EVs containing mutant DNA into biofluids, and this may represent a significant proportion of cfDNA [20]. Second, EVs could be a source of extracellular histones, nucleosomes and chromatin, and may trigger both localized and systemic biological effects [31] by affecting cellular viability and proliferation. Third, while we detect double-stranded DNA in EVs that possess exosomal-like characteristics and may be present in standard exosomal preparations, formation of DNA-containing EVs likely represents a distinct process [11]. Thus, further studies are warranted to elucidate the biogenesis, activity and clinical utility of DNA-containing EVs in cancer.

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