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Establishment and characterization of a unique 1 μm diameter liver-derived progenitor cell line

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ABSTRACT

Liver-derived progenitor cells (LDPCs) are recently identified novel stem/progenitor cells from healthy, unmanipulated adult rat livers. They are distinct from other known liver stem/progenitor cells such as the oval cells. In this study, we have generated a LDPC cell line RA1 by overexpressing the simian virus 40 (SV40) large T antigen (TAg) in primary LDPCs. This cell line was propagated continuously for 55 passages in culture, after which it became senescent. Interestingly, following transformation with SV40 TAg, LDPCs decreased in size significantly and the propagating cells measured 1 μm in diameter. RA1 cells proliferated *in vitro* with a doubling time of 5–7 days, and expressed cell surface markers of LDPCs. In this report, we describe the characterization of this novel progenitor cell line that might serve as a valuable model to study liver cell functions and stem cell origin of liver cancers.

Introduction

The liver is the only organ in the human body that is capable of renewing itself following the loss of the natural tissue. Even after 70% hepatectomy, this remarkable regenerative capacity is achieved due to the proliferation of hepatocytes, and under special circumstances, stem/progenitor cells and bone marrow cells that repopulate the liver [1]. Tissue restoration is a process in which damaged cells are replaced to restore the architecture and function of the organ [2]. Stem/progenitor cells are critical for this process as they are bipotent and can differentiate into the primary cell types of the liver: hepatocytes and biliary ductal cells (cholangiocytes).

To date, a number of stem/progenitor cells have been isolated from human, rodent, hamster, and simian livers [3–15]. Among these, the most studied are the "oval cells", the rodent counterparts of human hepatic progenitor cells [16]. Oval cells are commonly isolated from liver following treatment with agents such as 3,5-diethoxycarbonyl-1,4-dihydrocollidine, or from animals fed on choline-deficient diets and treated with 2-acetylaminofluorene [17,18]. They are localized in biliary ductules (canal of Hering) in normal adult liver, and are bipotential. Recently, liver-derived progenitor cells (LDPCs) were isolated from healthy, adult rat liver that are distinct from any other stem/progenitor cell type isolated from the livers of mammals [19].

Primary cells have a finite life span and they do not divide *in vitro* due to their inability to maintain chromosomal stability caused by the shortening of telomeres [2]. When adult stem cells differentiate to form progenitor cells, they reach a replication limit of 8–10 population doublings for rodent cells [20] and 50–70 population doublings for human cells [21] before senescence and programmed cell death occurs. Over the years, efforts have been made to isolate, generate and culture cell lines from the livers with the purpose of using them for various clinical applications, biochemical and pharmacological studies, and for cell transplantation. While a few cell lines were isolated from carcinogen-treated or choline-deficient rodents as well as from liver tumors, others were obtained by immortalizing primary hepatocytes. Successful establishment of progenitor cell lines by overexpressing the simian virus 40 (SV40)-encoded large T antigen (TAg) in fetal epithelial liver cells from cynomolgus monkeys has also been reported [22].

In this study, we describe the generation of a LDPC cell line RA1 by constitutively overexpressing the SV40 TAg. Interestingly, transformation by TAg induced "reprogramming" of LDPCs, resulting in a stable, proliferating cell population with cells of 1 μm in diameter.

Abbreviations: SV40 TAg, simian virus 40 large T antigen; LDPC, liver-derived progenitor cell; Rb, retinoblastoma; TEM, transmission electron microscopy; RT-PCR, reverse transcription-polymerase chain reaction

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Materials and methods

Isolation of LDPCs from adult rat livers. Liver cells from male Fisher rats (Jackson Labs, Bar Harbor, ME) were isolated using a modified two-step *in situ* collagenase perfusion protocol as described previously [19]. Following digestion with collagenase, cells were filtered through a sterile 100 μm nylon gauge (Sigma–Aldrich, St. Louis, MO) to obtain a single cell suspension. They were then washed twice with phosphate-buffered saline and cultured at 37 °C with 5% CO_2. All animal studies were conducted under a protocol approved by the IACUC Committee of the University of Minnesota.

LDPC cultures. Freshly isolated cells with at least 50% viability were cultured in Type I collagen (Upstate Biotechnology, Lake Placid, NY) coated 75 m^2 tissue culture flasks at a density of 3 x10^4 cells/ml. Growth medium for LDPCs contained 75% DMEM-LG (Cellgro) + 25% F12 Ham's medium (Sigma–Aldrich) supplemented with 10% rat serum (Equitech, TX), 1 mg/ml of BSA (Sigma), 100 μM of β-mercaptoethanol (Gibco), 25 mM of HEPES (Cellgro, Herndon, VA), 5 mM of nicotinamide (Sigma), and 100 μg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA). On day 4, 80% of the medium was replaced with the fresh medium. During the next 2–3 weeks, LDPCs were allowed to appear and proliferate, and between days 15 and 21, they were harvested for transfection studies.

Transfection of LDPCs. LDPCs were seeded at a density of 2×10^5 cells/cm^2 in 6-well plates. The transfection cocktail contained 1 μg of plasmid pRITA [23] and the reagent Fugene HD transfection reagent (Roche Applied Science, Indianapolis, IN) in the ratio of 1:3. Forty-eight hours post-transfection, cells were examined for the expression of eGFP and the growth medium was replaced with fresh medium supplemented with 50 μg/ml G418 (InvivoGen, San Diego, CA). This antibiotic selection would eliminate untransfected cells from the culture. Twenty-four hours later cells were washed twice in 1× PBS and fresh medium was added to cells together with doxycycline (Sigma–Aldrich) at a final concentration of 200 μg/ml. Cells were cultured for 2 weeks by changing the growth medium every 5 days.

Transmission electron microscopy. For TEM, cells were centrifuged at 750 rpm in a centrifuge at 4 °C. Cell pellets were resuspended in a glutaraldehyde fixative and embedded into an epoxy resin for sectioning. Cells were sectioned in 1 mm thick sections, stained and analyzed in a microscope.

Flow cytometry. FACS analysis was performed using antibodies against three surface markers of LDPCs: RT1a, CD45, and CD90. Cells were treated sequentially with the primary antibodies against either anti-rat RT1a or CD45 clone OX-33 (Pharmingen) and with PE-labeled fluorescent secondary antibody anti-rat IgG. Alexa Fluor® 488 Anti-rat CD90/mouse CD90.1 Antibody (BioLegend, San Diego, CA) was used for some assays. Cells were analyzed using FACSCalibur (BD Biosciences, San Diego, CA), and data was quantified with FlowJo software (Tree Star, Ashland, OR).

Total RNA isolation and RT-PCR. Total RNA from 1×10^5 cells was isolated with Trizol reagent (Invitrogen) following manufacturer's instructions. One microgram of total RNA was used for RT-PCR. cDNA synthesis was carried out using the SuperScript RT assay kit (Invitrogen). PCR was performed with rat-specific primers (listed in supplementary material) by using the following conditions: initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 60 °C, and elongation for 1:30 min at 72 °C.

Western blot assay. Western blot was performed using the mouse anti-SV40 TAg monoclonal antibody clone PAb101 (BD Biosciences). Ten micrograms of total protein from LDPCs and RA1 cells were isolated, and loaded onto a 10% denaturing SDS–PAGE gel. Following separation, proteins were transferred to a nitrocellulose filter and were treated with the blocking solution containing 5% milk powder. Primary antibody at a concentration of 1:500 was then added to the filter and incubated for 2 h in a rotating chamber at 4 °C. After several washes in the washing buffer, the secondary anti-mouse IgG antibody was added at a concentration of 1:1000 and the filter incubated for 2 h at room temperature. The blot was washed and probed with ECL solution to visualize bands (Thermo Fischer Scientific, Rockford, IL).

Results

Generation of a LDPC cell line

Primary LDPCs were isolated from healthy adult rat livers as described under 'Materials and methods', and were transfected with the plasmid pRITA that contains the TAg ORF under the control of CMV IE promoter, an enhanced GFP (eGFP) and the blasticidin resistance gene as a selectable marker. Several transfections were carried out simultaneously, and 48 h post-transfection, cells were visualized under the fluorescent microscope to detect GFP expression, which indicated successful transfection of LDPCs (Fig. 1). Transfection efficiencies typically ranged between 1% and 5%. At this point, blasticidin was added to the cultures to select for transfected cells. Untransfected cells did not survive in culture and were discarded 24 h after adding the antibiotic. Growth medium was replaced every 2 weeks and the antibiotic was not added after 2–3 passages of cell culturing. Of note, cells transformed with TAg underwent a significant change in their size under blasticidin selection and were typically around 1 μm in diameter, when compared with 10 μm size of the parental LDPCs (Fig. 1). These small cells multiplied continuously and, after passage 36, they started to increase in size and reached a maximum size of 10–12 μm by passage 42. After 55 passages in culture, they underwent senescence. Interestingly, transformed cells grew individually and did not form any visible colonies. All the transformed cells from a set of transfections were pooled together and were considered as a single cell line RA1. Growth kinetics of RA1 cells were determined by constructing a growth curve (Suppl. Fig. 1A), and their ability to form colonies was tested using the soft agar assay (Suppl. Fig. 1B).

Morphological analysis of RA1 cells

RA1 cells up to passage 35 were around 1 μm in diameter and were globular in shape. They did not display any distinct morphological features and did not resemble LDPCs. To determine whether 1 μm cellular structures were indeed mammalian cells, they were stained with DAPI and were visualized under a fluorescent microscope. As shown in Fig. 2, chromosomal DNA in LDPCs, RA1 cells, and primary rat hepatocytes was stained positively with DAPI demonstrating that the 1 μm cells are indeed normal eukaryotic cells.

To examine the intracellular morphology and to visualize various organelles inside these small cells, transmission electron microscopic (TEM) analysis was performed. Primary LDPCs contained a large number of vacuoles and clear cytoplasmic and nuclear membranes (Fig. 3). The vacuoles varied in size, density, and shape. In addition, LDPCs had cellular organelles such as mitochondria and the endoplasmic reticulum (ER). Remarkably, RA1 cells had a small cytoplasm and most of their size is comprised of the nucleus. Very few mitochondria were detectable and the cells had no other organelles, such as the ER, Golgi apparatus, ribosomal structures, and vacuoles. These findings collectively show that RA1 cells constitute a novel cell type that possesses sufficient cellular

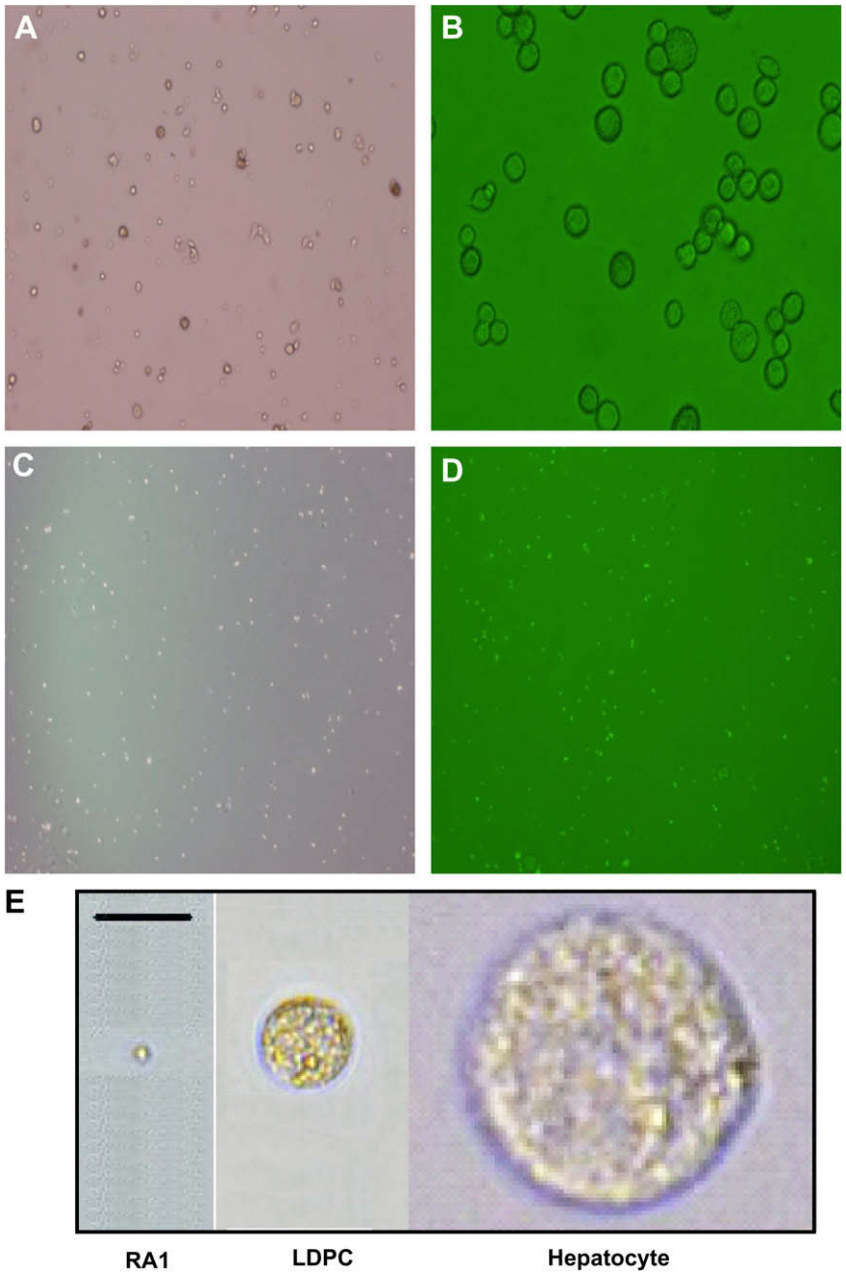

Fig. 1. Transfection of LDPCs and expression of GFP. Freshly isolated LDPCs were grown on collagen-coated flasks under normal culture conditions (see Materials and methods). (A,B) Primary LDPCs at lower and higher magnifications, respectively, (C) LDPC cell line RA1, and (D) RA1 cells expressing enhanced GFP 48-h post-transfection. Although a few cells are clustered together, most of them remain in cell culture as single cells and are loosely adhering to the flasks. (E) Size comparison between a primary hepatocyte, LDPC and a RA1 cell. Bar scale corresponds to 10 μm.

processes to allow them to proliferate in culture for a prolonged period of time.

Characterization of the LDPC cell line RA1

In order to understand the differences between RA1 cells and their parental cells, a comprehensive characterization was performed based upon known features of primary LDPCs that included immunocytochemical staining (Suppl. Fig. 2), FACS analysis and RT-PCR.

Expression of cell surface markers

Primary LDPCs express several markers on their surface. Among these, RT1a, CD45 and CD90 were prominent [19]. FACS analysis was performed to test whether the RA1 cell line also expresses these markers using rat-specific antibodies for these proteins. As shown in Fig. 4A-C, both LDPCs as well as RA1 cells expressed all three markers demonstrating that RA1 cells were phenotypically very similar to primary LDPCs.

Expression of cellular genes of p53 and pRb pathways

It has been well established that the overexpression of SV40 TAg leads to the inhibition of p53 and Rb cellular signaling pathways [24]. To test whether the LDPC cell line expresses proteins of these two pathways as well as other cellular genes, we selected a panel of cell cycle genes (cdk2), pro-apoptotic gene Bax, and genes of the Rb pathway (E2F1), and p53 pathway (ATM and MDM2). RT-PCR was performed with primers specific to each of

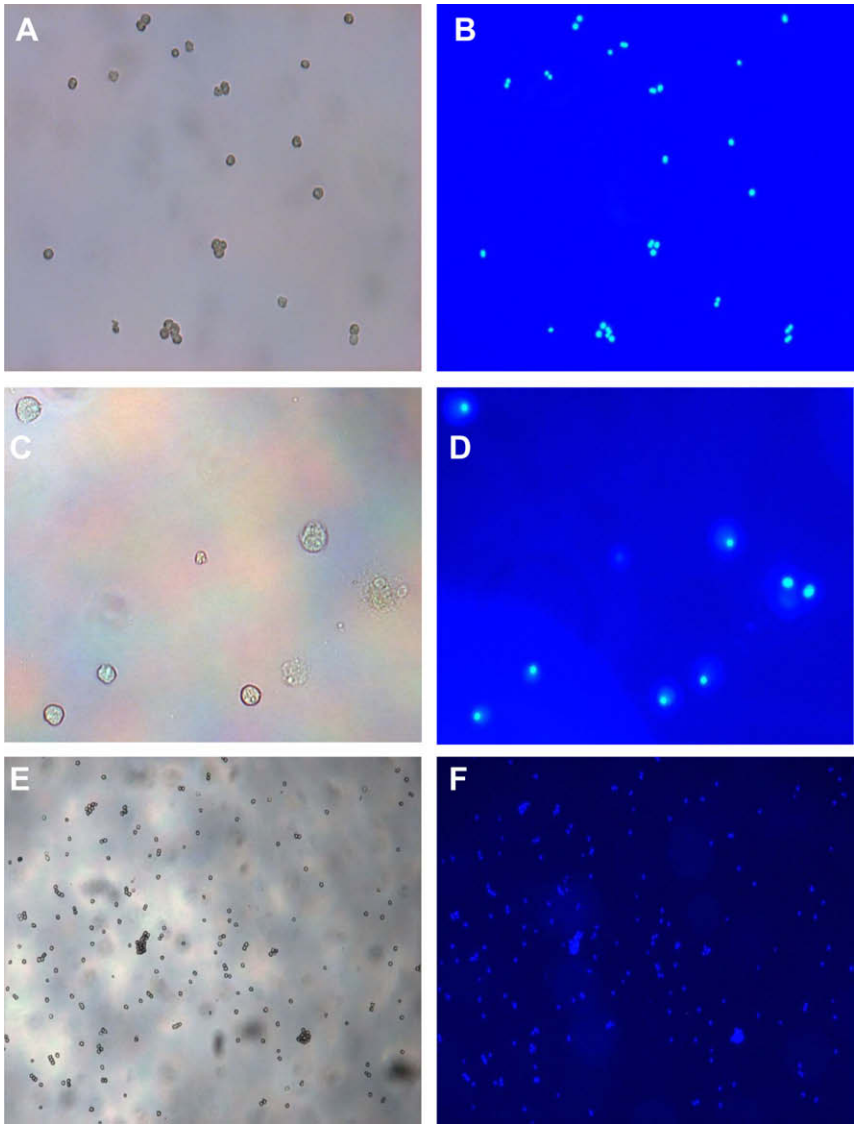

Fig. 2. Staining of RA1 cells, LDPCs, and hepatocytes with DAPI. Cells were grown in 6-well plates, washed with PBS and were fixed with 4% formaldehyde. They were then stained with 1 μg/ml DAPI for 20 min, washed and visualized using a fluorescent microscope. Unstained controls and DAPI-stained cells are shown in left and right panels, respectively. LDPCs are in panels A and B, primary hepatocytes in C and D, and RA1 cells in panels E and F.

these proteins using total RNA from RA1 cells and primary LDPCs. Positive control was total RNA from the human hepatoma cell line HuH-7. All cells showed the expression of housekeeping gene β-actin, but there were variations in the expression of other genes tested. HuH-7 cells and primary LDPCs expressed E2F1 and ATM, whereas RA1 did not show their expression suggesting that both Rb and p53 pathways were disrupted in these cells (Fig. 4D). On the other hand, RA1 cells, LDPCs, and Huh-7 cells expressed c-Met and cdk2. The expression of the pro-apoptotic gene Bax was not detectable in any of the three cell types. These results clearly indicate that TAg was able to target Rb and p53 signaling pathways in transfected LDPCs that resulted in the generation of the stable cell line RA1.

Discussion

Cell lines are critical to our understanding of various basic cellular phenomena such as signaling pathways, for pharmacological testing such as metabolism studies, and for clinically relevant purposes such as artificial liver support and transplantation. In this study, we report the generation of a liver stem/progenitor cell line RA1 by a constitutive immortalization process using the SV40 TAg. This cell line could serve as a valuable tool to study the cell biology of LDPCs and their role in liver regeneration. The characterization of the RA1 cell line showed that, while it retained certain characteristics of primary LDPCs, it is different from parental LDPCs both in size and in proliferation potential. As we show here, TAg was able to induce primary LDPCs to divide and proliferate under normal culture conditions. Furthermore, our data also indicate that the forced cell cycle entry by TAg might have been the trigger for the "reprogramming" of cells causing a change in their cell size, possibly via the process of 'de-differentiation'. This feature of LDPCs conforms to the notion of de-differentiation seen in other stem cells and cancer stem cells (CSCs), including liver cells [25,26].

Studies with model organisms have shown that cell division and the cell cycle, although coordinated, are two distinct processes and are separable (reviewed in Ref. [27]). Yeast cells divide when they have attained a critical cell size prior to initiating cell cycle progression and cell division. A 'cell-size checkpoint' operates to ensure this transition. In contrast, mammalian cells grow linearly,

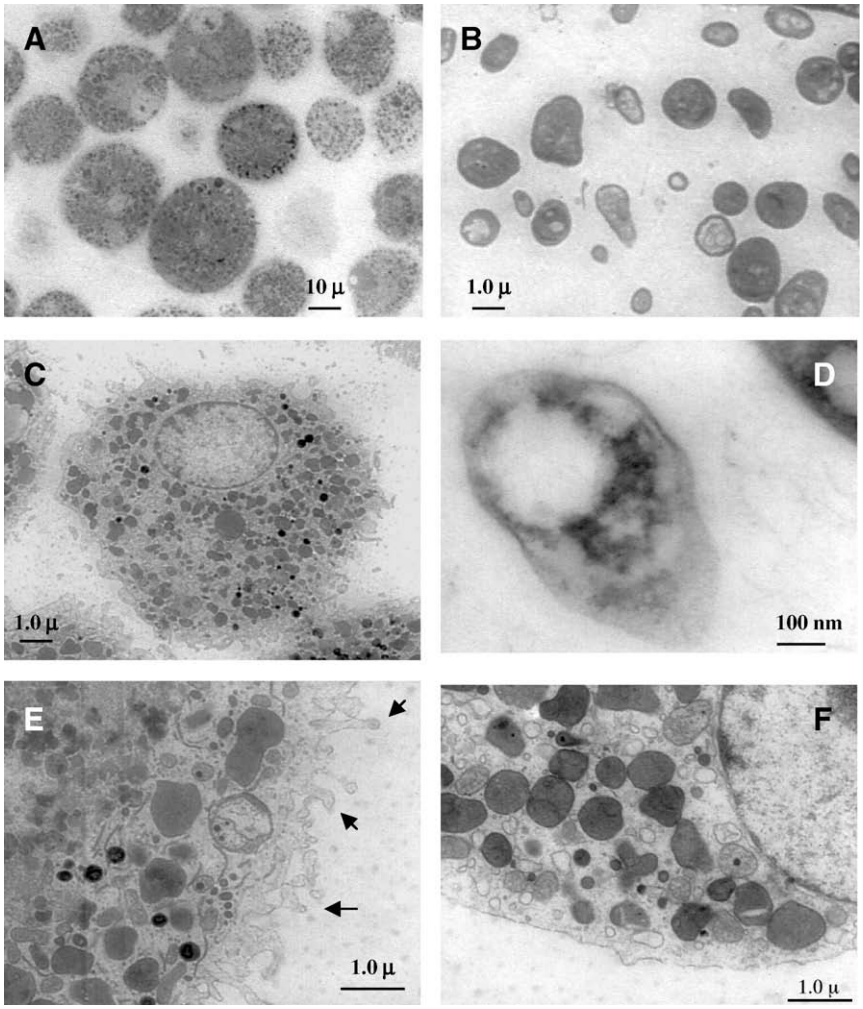

Fig. 3. Transmission electron microscopy. TEM analysis was conducted with primary LDPCs (A, C, and E) and with the RA1 cell line (B, D, and F) as described under Materials and methods. Arrows in panel E refer to the 'vesicle-like structures' that are budding out of LDPCs. In contrast, the cell membranes of the RA1-derived LDPC cell line are smooth and do not have any protrusions (F).

adding a constant amount of volume each day, independent of their size and their cell size does not increase with each doubling [28–30]. In agreement with these findings, RA1 cells multiplied every 5 days and grew linearly maintaining their small sizes (Fig. 2; Suppl. Fig. 1).

Primary cells have a limited lifespan before reaching permanent growth arrest termed 'replicative senescence' [21]. Human cells are able to divide between 500 and 5000 times in their lifetime *in vivo* whereas they double only 20–70 times *in vitro* [31]. Intracellular content of RNA and proteins increase with age, even though the synthesis of macromolecules decreases. These elevations contribute to the increase in the cell size, numbers of inclusion bodies, and other cellular components [32]. Another factor that is of critical importance is the nucleocytoplasmic (N:C) ratio. It is conserved throughout unicellular and multicellular organisms and could constitute a critical constraint of cell size. In culture conditions that stimulate cell growth, primary mammalian cells proliferate at sizes 300–500% larger than their counterparts *in vivo*, and larger cells had lower N:C ratio [33]. In this study, we found that RA1 cells also had a much larger N:C ratio with very small cytoplasm. Our efforts to isolate total RNA from RA1 cells were unsuccessful from earlier passages. This is due to the fact that, although they may possess ribosomal RNA and other proteins, mRNA for a large repertoire of cellular proteins may not have formed or may simply be present in low copy numbers. This might explain the reason for the absence of various cellular genes in our RT-PCR studies with RA1 cells. Therefore, characterization of RA1 cells for the expression of cellular genes was performed with total RNA from cells of passage 38. As the cell size of RA1 cells increased in latter passages, they started accumulating vacuoles and expressed various cellular genes (Fig. 4B). However, the genes expressed by RA1 cells were distinct from those of primary LDPCs. Furthermore, this result has demonstrated that the SV40 TAg had the expected effect of disrupting the two cellular signaling pathways, Rb and p53, in RA1 cells.

To date, RA1 cells are the smallest mammalian cells to be reported in the literature. The formation and growth of 1 µm RA1 cells raises an interesting question: how small can a mammalian cell be? In a recent study, Hung et al. used 3 µm pores to isolate a population of bone marrow mesenchymal stem cells by sieving out other contaminating cells in the mesenchymal tissues [34]. Similarly, 'spore-like' cells of less than 5 µm have been reported to be present in all tissues in adult mammals [35]. The identification of small pluripotent epiblastic-like cells of 8–10 µm from the rat skeletal muscle [36], and very small embryonic-like stem cells of 3 µm from the bone marrow [37] has also been reported. Perturbation of the Rb pathway has been shown to affect cell size and proliferation, and cells lacking proteins of this pathway were small [38,39]. As TAg directly binds to Rb and inactivates it [24], disruption of Rb signaling might be a cause of reduced size of in RA1 cells.

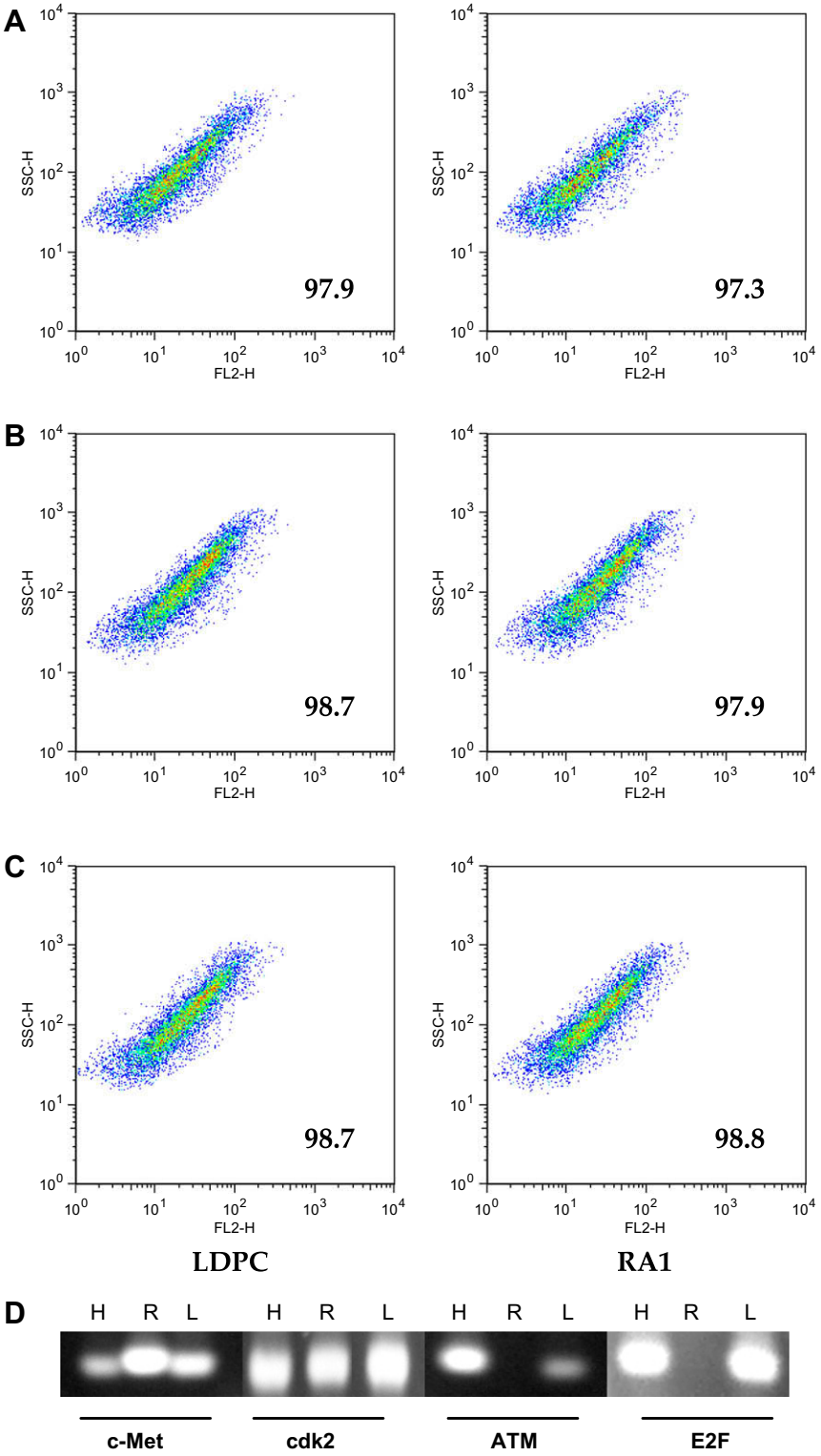

Fig. 4. (A) Expression of cell surface markers by LDPCs and RA1 cells. Flow cytometry was performed using antibodies against three cell surface markers expressed by LDPCs: RT1a, CD45, and CD90. Live cells expressing these markers were gated and counted as a proportion of the total cell population. Data presented are a representative of three independent experiments. (B) Expression of cellular genes by RA1 cells. RT-PCR was performed with total RNA from primary LDPCs (L), and RA1 cells (R). HuH-7 (H) cells were used as positive control (see Materials and methods). The expression of four cellular genes c-met, cdk2, ATM, and E2F was shown here. (C) Immunohistochemical staining of RA1 cells and LDPCs. Expression of HNF-3β and CD90 in primary LDPCs was tested in RA1 cells (A,C) and LDPCs (B,D), respectively, by immunocytochemical staining.

As discussed earlier, the process of immortalization with TAg led to the possible de-differentiation of primary LDPCs resulting in the formation of small 1 μm cells that possessed the proliferation potential. After 36 passages, cells slowly began to grow in size and resemble LDPCs morphologically suggesting that the RA1 cells might be precursors of LDPCs. Eventually between 43 and 55 they slowly moved towards senescence. It is very interesting that cellular 'de-differentiation' into smaller, dividing cells occurred

following their transformation with the TAg. Further studies with RA1 cells might help us to understand these and other phenomena that govern regulation of cell size in stem/progenitor cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.001.

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