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Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis

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The Oct3/4 gene, a POU family transcription factor, has been noted as being specifically expressed in embryonic stem cells and in tumor cells but not in cells of differentiated tissues. With the ability to isolate adult human stem cells it became possible to test for the expression of Oct3/4 gene in adult stem cells and to test the stem cell theory of carcinogenesis. Using antibodies and PCR primers we tested human breast, liver, pancreas, kidney, mesenchyme and gastric stem cells, the cancer cell lines HeLa and MCF-7 and human, dog and rat tumors for Oct4 expression. The results indicate that adult human stem cells, immortalized non-tumorigenic cells and tumor cells and cell lines, but not differentiated cells, express Oct4. Oct4 is expressed in a few cells found in the basal layer of human skin epidermis. The data demonstrate that adult stem cells maintain expression of Oct4, consistent with the stem cell hypothesis of carcinogenesis.

Introduction

Stem cells exist in most adult organs, being best characterized in the bone marrow and gut. They are defined as cells that undergo symmetric and asymmetric division to give rise to daughter cells needed for self-renewal and amplification or to a daughter cell that acts as a progenitor cell for the purpose of producing specific differentiated lineages, respectively. Given the recent interest in the multiple uses of embryonic and adult stem cells for basic and applied research (i.e. reproductive cloning or regenerative tissue therapy), attempts have been made to characterize markers that would identify these stem cells. Oct3/4 or Oct4 (also referred to as Pou5f1), a transcription factor, was discovered in 1990 (1–3). It was found in ovulated oocytes, mouse pre-implantation embryos, ectoderm of the gastrula (but not in other germ layers) and primordial germ cells, as well as in embryonic stem cells but not in their differentiated daughters (4). More recently, OCT4/Pou5f1 has been shown in cells isolated from human amniotic fluid (5). Subsequent studies seemed to suggest that *Oct4* might be a specific gene marker for totipotency or a gene required for totipotency (6–8). In fact, it is thought that the success or failure of cloning depends on expression of this gene during reprogramming of the genome of a nucleus transferred to an enucleated oocyte (9).

Seemingly in contrast, the *Oct4* gene has also been shown to be expressed in some human tumor cells but not in normal somatic tissues (10–12). Since cancer cells have been characterized as having many phenotypic traits similar to undifferentiated embryonic cells (cancers as a 'disease of differentiation', a stem cell disease or as 'oncogeny as blocked or partially blocked ontogeny') (13–15), the question that still needs to be answered is 'is the tumor derived from an adult stem cell in the tissue or from some somatic progenitor or differentiated cell that has de-differentiated?' (16).

Among the 'hallmarks' of cancer cells (17) is the potential for indefinite proliferation. In addition, cancer cells do not have functional homologous or heterologous gap junctional intercellular communication (GJIC) (18), due either to non-expression of connexins (e.g. HeLa and MCF-7 cells) or to non-functioning of expressed connexins (tumor cells expressing the *ras*, *src* or *neu* oncogenes) (19). Gap junctions have been associated with both normal development (20), growth control, differentiation, wound repair, synchronization of metabolic secretion and electrotonic function in tissues (21,22). Interestingly, several isolated presumptive adult human stem cells have been characterized as being deficient in expression of connexins and GJIC [kidney epithelial cells (23), breast epithelial cells (24), pancreas cells (25), keratinocyte cells (26), corneal epithelium cells (27) and mesenchymal cells (28)].

In this study we have found that the *Oct4* gene and Oct4 protein are expressed in several adult pluripotent stem cells, as well as in several human and rat tumor cells, but not in normal differentiated daughters of these stem cells. Two major conclusions were drawn from these observations, namely that adult cells expressing the *Oct4* gene are potential pluripotent stem cells and that these cells could be the target cells for initiation of the carcinogenic process.

Materials and methods

Materials

Keratinocyte serum-free medium, Neurobasal medium with N2 supplement, RPMI-1640 medium, recombinant human epidermal growth factor (EGF), bovine pituitary extract (BPE), fetal bovine serum (FBS), penicillin, streptomycin, trypsin–EDTA and TRIZOL reagent were purchased from Invitrogen (Carlsbad, CA). *N*-acetyl-L-cysteine, DMSO and nicotinamide were obtained from Sigma Chemical Co. (St Louis, MO). L-Ascorbic acid 2-phosphate was from Wako Pure Chemical Industries Ltd (Osaka, Japan). Exendin-4 was obtained from Bachem Bioscience (Torrance, CA). LY294002 was obtained from Calbiochem (La Jolla, CA). Anti-Oct4 monoclonal antibodies were purchased from Santa Cruz (Santa Cruz, CA).

Generation of adult human tissue-derived stem cell cultures

The cell culture methods used to develop two types of normal human breast epithelial cell (HBEC) cultures from reduction mammoplasty material have been described (24). The stem cell characteristics of Type I HBEC are: (i) a

Abbreviations: BPE, bovine pituitary extract; cpdl, cumulative population doublings; DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride; EGF, epidermal growth factor; ES, embryonic stem; GJIC, gap junctional intercellular communication; HBEC, human breast epithelial cell; HPCs, human pancreatic stem cells; PBS, phosphate buffered-saline.

deficiency in GJIC; (ii) the ability to differentiate into Type II HBEC; (iii) the ability to form budding ductal structures on Matrigel similar to mammary gland lobule or terminal end buds, where mammary stem cells are primarily located; (iv) a high susceptibility to telomerase activation, immortalization and neoplastic transformation (24,29,30). Immortal SV40 large T-antigen-transfected Type I cells (clone M13SV1), X-ray-induced weakly tumorigenic cells (clone M13SV1R2) and *neu* oncogene-transfected highly tumorigenic cells (clone M13SV1R2N1) (31) were cultured in MSU-1 medium with 5% FBS at 37°C in a humidified incubator with 5% CO_2 (24). Human pancreatic islets and duct and acini remnants were obtained from the JDRF Human Islet Distribution Program. Upon arrival cells were plated and cultured in RPMI-1640 medium supplemented with 8.0 mM glucose and 10% FBS. After 24 h the culture medium was then switched to keratinocyte serum-free medium supplemented with 5 ng/ml recombinant human EGF, 50 μg/ml BPE, 2.0 mM *N*-acetyl-L-cysteine and 0.2 mM L-ascorbic acid 2-phosphate. To induce pancreatic endocrine cell differentiation from the progenitor cells, the cells were plated and cultured in neurobasal medium containing 1% N2 supplement with 10 mM nicotinamide, 0.1 μM exendin-4 and 5 μM LY 294002 for 14 days before RNA was extracted. The method to develop putative human fetal kidney epithelial stem cells has been previously reported (23). These putative kidney epithelial stem cells have been shown to be contact insensitive and to be deficient in GJIC (23). The procedure to obtain adult human liver stem/precursor cells of clonal origin has been described (32). The stem cell features of these cells include: (i) a high proliferation potential, with more than 50 cumulative population doublings (cpdl); (ii) a deficiency in GJIC; (iii) the ability for anchorage-independent growth; (iv) expression of liver 'oval cell' markers, i.e. vimentin and α-fetoprotein. The isolation of gastric stem/precursor cells and mesenchymal stem cells from adipose tissues was as described for liver stem cells (32). Although the derivation of mesenchymal stem cells from adipose tissues has been reported before (33), our method gave a greater number of cells in a shorter time (32 cpdl in 51 days compared with 22 cpdl in 165 days) (28). We have shown that these cells had a high differentiation ability to become adipocytes, osteoblasts and chondrocytes (28).

Cell preparation and immunostaining

Different cell cultures were plated in glass chamber slides for a few days until they reached the desired confluency. Cells were fixed with 4% paraformaldehyde in phosphate buffer.

After washing with phosphate buffered-saline (PBS), paraformaldehyde-fixed cells were permeablized with 0.2% Triton X-100. Cells were then blocked for 60 min with PBS containing 10% normal goat serum. Cells were then incubated with primary antibody for 2 h at room temperature or overnight at 4°C. The primary antibodies were then removed and the cells were washed with PBS and incubated for 2 h with Cyanine 3- or FITC-conjugated goat anti-mouse secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). Slides were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min before final embedding. Fluorescent images were obtained using a Nikon epifluorescent microscope equipped with a SPOT-RT digital camera (Diagnostic Instruments, Detroit, MI), interfaced with a Dell Pentium 4 PC installed with Spot Advanced analysis software (Diagnostic Instruments).

Tissue sections

Human normal tissue sections were purchased from Zymed (San Francisco, CA). Zymed's MaxArray Human Normal Tissue Arrays contain 30 human samples arrayed on microscope slides. Slides were deparaffinized, rehydrated and then processed following the protocol for immunohistochemistry. The slides were analyzed under a bright field microscope for Oct4 staining. Dog mast cell tumor sections were provided by Dr Matti Kiupel at the Department of Veterinary Medicine, Michigan State University. Dog mast tumor sections were processed by the same procedure as for Oct4 immunostaining.

Reverse transcription–PCR (RT-PCR)

Total RNA was extracted from the cells with TRIZOL following the protocol suggested by the manufacturer and treated with DNase I to remove contaminating DNA. Oligo(dT) primers (Integrated DNA Technologies, IA) were used with Superscript II reverse transcriptase (Invitrogen) for cDNA synthesis from 1 μg total RNA following the guidelines provided by the manufacturer. PCR was conducted with PlatinumTaq Polymerase (Invitrogen). We used glyceraldehyde 3-phosphate dehydrogenase as an internal standard for *Oct4* RT-PCR. The primer sequences for human Oct4 were: 5′-GAC AAC AAT GAG AAC CTT CAG GAG A-3′ and 5′-CTG GCG CCG GTT ACA GAA CCA-3′. The mixture was first heated at 94°C for 3 min in a PTC-200 DNA Engine Thermal Cycler (MJ Research, Waltham, MA). Amplification was performed for 35 cycles at 94°C for 45 s, 55°C for 30 s and 72°C for 90 s, followed by 72°C for 10 min. The PCR products were separated on 1.5% agarose gels by electrophoresis. Digital images were captured on a Kodak gel documentation system.

Results

Oct4 expression in human adult breast stem cells during differentiation, immortalization and neoplastic transformation

Previously we demonstrated that Type I HBECs have stem cell characteristics. These characteristics include: (i) a deficiency in GJIC; (ii) the ability to differentiate into Type II HBEC; (iii) the ability to form budding ductal structures on Matrigel, similar to mammary gland terminal end buds, where mammary stem cells are primarily located; (iv) a high susceptibility to telomerase activation, immortalization and neoplastic transformation (24,29,30). Type I HBECs showed Oct4 protein expression in the nucleus (Figure 1A). A punctate nuclear staining of Oct4 was seen homogenously in Type I HBECs. Treatment of Type I HBECs with cAMP-inducing agents or differentiation medium (24) has been shown to induce Type I HBECs to differentiate into Type II cells, characterized by a wholesale switch in gene expression (30). The transition of Type I HBECs to Type II HBECs by cAMP-inducing agents is associated with a reduction in Oct4 protein expression (Figure 1B). A reduction in Oct4 expression could be readily observed at the fringe of the cell colony, with only a few cells in the center of the colony showing no Oct4 expression. Type II HBECs with basal epithelial cell phenotypes showed very low to non-detectable levels of Oct4 expression (Figure 1C). Oct4 protein expression was also examined in SV40 large T antigen-immortalized Type I HBEC line M13SV1, X-ray-transformed weakly tumorigenic cell line M13SV1R2 and the highly tumorigenic (*neu* oncogene-transduced) cell line M13SV1R2N1 (31). Oct4 protein was clearly detected in all three of these cell lines (Figure 1D–F). As with the primary Type I HBEC cultures, Oct4 protein expression was observed as punctate staining located in the nucleus. RT-PCR was then used to detect *Oct4* transcripts in HBECs. We used monkey embryonic stem (ES) cell RNA as a positive control for *Oct4* expression (Figure 1G). *Oct4* transcripts were easily detected in Type I HBECs and in the immortalized, weakly tumorigenic and highly tumorigenic cell lines, sequentially derived from a Type I HBEC (Figure 1G). In contrast, differentiated Type II cells lacked the *Oct4* transcript.

GJIC in adult human breast epithelial stem cells and differentiated daughter cells

Scrape loading dye transfer was then used to examine GJIC in human breast epithelial stem and differentiated cells. In Type I human breast epithelial stem cells Lucifer yellow dye did not transfer from dye-containing to neighboring cells, thus indicating that Type I HBECs did not have functional gap junctions (24). In contrast, differentiated Type II HBECs had efficient transfer of Lucifer yellow dye from initially loaded to neighboring cells, thus indicating that Type II HBECs have functional gap junctions (24) (Figure 1H). These results suggest that Type I HBECs have two characteristics of stem cells, i.e. the loss of functional GJIC and expression of Oct4. These characteristics are also observed in immortalized breast epithelial cell lines derived from Type I HBEC. The fact that differentiation of Type I HBECs to Type II HBECs is associated with induction of functional GJIC and loss of Oct4 protein expression strengthens the argument that GJIC and Oct4 expression are directly linked to cell plasticity and differentiation.

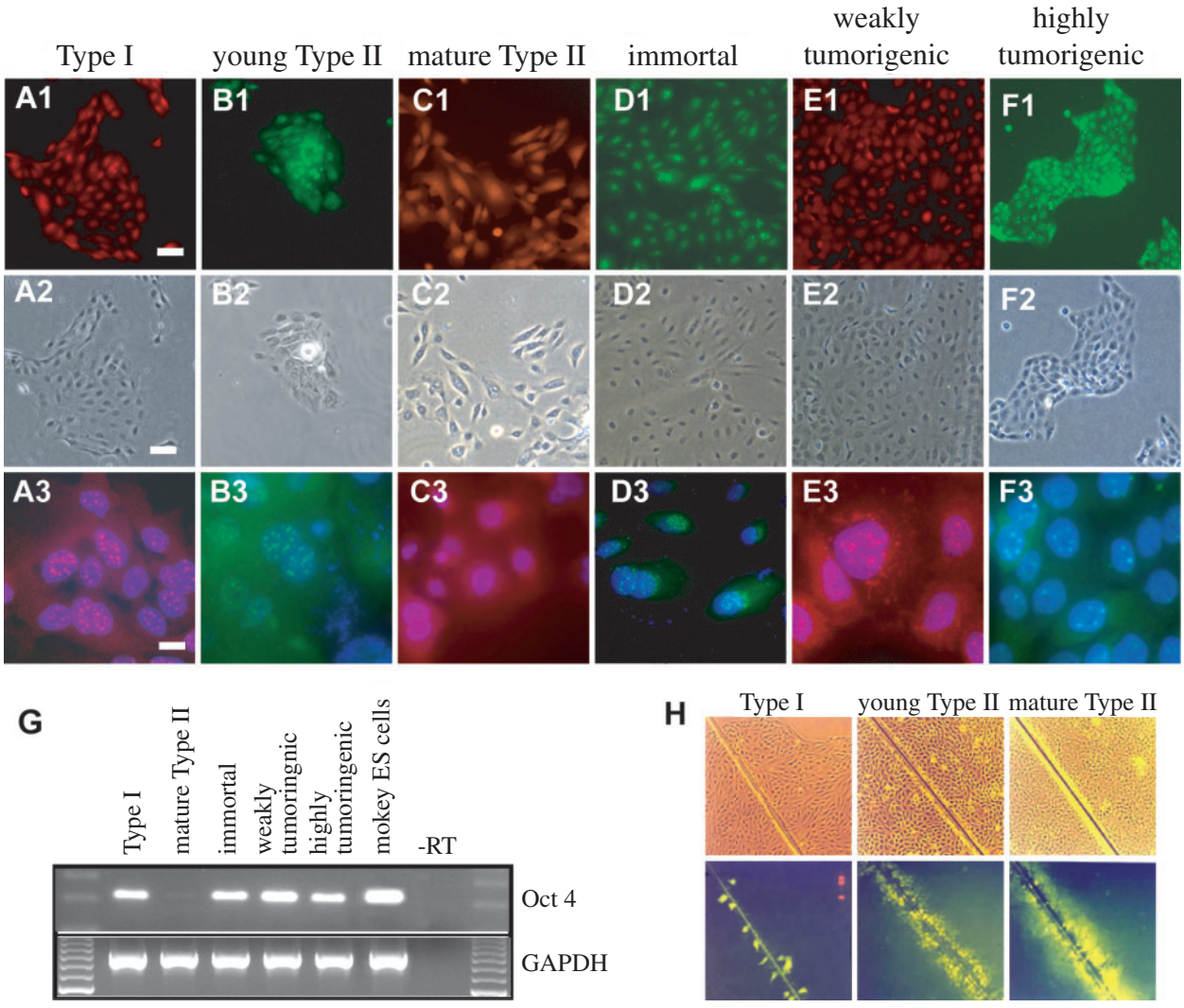

Fig. 1. Oct4 protein expression in human breast epithelial cells. Human breast stem cells (Type I) (**A**), differentiated daughter cells (young and mature Type II) (**B** and **C**), an immortal cell line (SV40-transfected Type I cells) (**D**), a weakly tumorigenic cell line (X-ray-transformed) (**E**) and a highly tumorigenic cell line (*neu* oncogene-transduced) (**F**) were immunostained, with Oct4 showing in red or green (A1–F1). (A2)–(F2) are phase contrast images showing the morphology of the cells. (A3)–(F3) are higher magnification images of A1–F1 superimposed on 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) blue nuclear stain. Punctate staining of Oct4 was seen in most cell types, located in the nucleus, except for mature Type II cells (C3). In (B) Oct4 staining was heterogeneously distributed in young Type II cells, only the cells in the center of the colony staining positive. Scale bars: (A1)–(F2) 60 μm; (A3)–(F3) 20 μm. (**G**) RT-PCR analysis of *Oct4* expression in Type I human breast stem cells (lane 1) and human breast immortal (lane 3), weakly tumorigenic (lane 4) and highly tumorigenic cell lines (lane 5). No expression of *Oct4* was seen in Type II differentiated cells (lane 2). Monkey ES cells were used as a positive control (lane 6). Lane 7 is a no template control. (**H**) Scrape loading dye transfer assay (SL/DT) to examine GJIC in human breast epithelial cells. Type I cells were deficient in GJIC. Young and mature Type II cells were efficient in GJIC.

Oct4 expression in adult human pancreatic stem cells during differentiation and in pancreatic cancer cells

Human pancreatic stem cells (HPCs) were cultured under conditions that favor proliferation. HPCs are characterized by the unique expression of amylase, albumin, vimentin and nestin, but can be differentiated into pancreatic endocrine hormone expressing cells by culture in differentiation medium (34). In proliferation medium Oct4 protein was localized in discrete regions within the nucleus (Figure 2A). Expression of *Oct4* mRNA in HPCs cultivated in proliferation medium was confirmed by RT-PCR (Figures 2F and 3D). In contrast, there was diminished Oct4 protein expression in HPCs cultivated in differentiation medium (Figure 2B). Consistent with this observation, HPCs cultured in differentiation medium for 2 weeks expressed insulin mRNA but showed down-regulation of *Oct4* mRNA (Figure 2F). These results indicate that under differentiation conditions HPCs lost the stem cell marker Oct4 and differentiated into endocrine-positive cells. Oct4 expression was also examined in two human pancreatic cancer cell lines, Capan-2 and Pan-1 (Figure 2D and E). Oct4 expression was shown to have punctate staining in the nuclei of Capan-2 cells. In contrast to Capan-2 cells, Pan-1 cells showed variable cell shapes and Oct4 nuclear expression was shown to be heterogeneous. RT-PCR confirmed that *Oct4* mRNA was present in both Capan-2 and Pan-1 cells (Figure 3D).

Oct4 expression in adult human liver stem cells, in vitro immortalized liver stem cells and a hepatoma cell line

When human liver stem cells were cultured under propagation conditions (KNC medium) they expressed vimentin and α-fetoprotein, whereas under differentiation conditions (D medium) vimentin and α-fetoprotein expression was reduced and albumin expression was stimulated (32). Oct4 expression

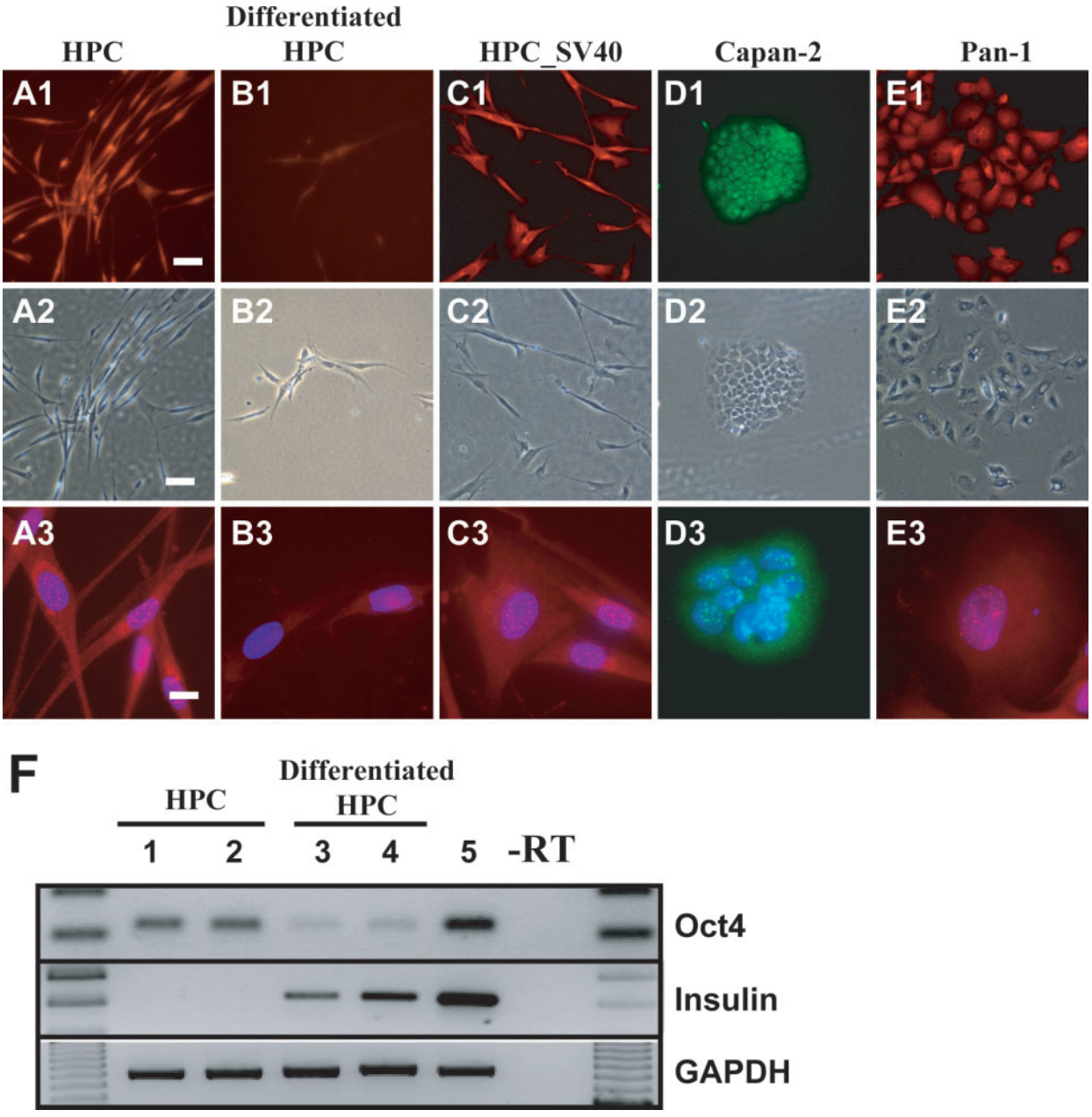

Fig. 2. Human pancreatic stem cells (**A**), differentiated pancreatic cells (**B**), SV40-transfected cells (**C**) and the pancreatic cancer cell lines Capan-2 (**D**) and Pan-1 (**E**) were double labeled with Oct4 (red or green) and DAPI. Punctate red or green staining indicated Oct4 protein expression in the nucleus of cells. Scale bars: (A1)–(E2) 60 μm; (A3)–(E3) 20 μm. (**F**) *Oct4* and insulin mRNA expression in human pancreatic stem cells and differentiated cells. Human pancreatic stem cells cultured in the proliferation medium (KNC) showing Oct4 expression and no insulin message (lanes 1 and 2). Culture in differentiation medium for 2 weeks produced a decrease in Oct4 message and an increase in insulin message (lanes 3 and 4). Lane 5, 80% purified human islets cultured for 2 days in KNC (positive control); lane 6, no template (negative control).

in human liver stem cells was observed using immunostaining and RT-PCR. Oct4 protein showed clear punctate staining in the nuclei of human liver stem cells cultivated under propagation conditions (Figure 3A). When the cells were cultured in differentiation medium Oct4 expression was decreased (data not shown). Oct4 expression was also measured in a SV40 large T antigen-immortalized liver cell line derived from the same liver stem cells just described and a liver tumor cell line (Mahlava cells) (Figure 3B and C). RT-PCR analysis also demonstrated that *Oct4* mRNA was present in the liver stem cells, SV40 large T antigen-immortalized liver cell line and Mahlava cells (Figure 3D).

Oct4 expression in adult human kidney, mesenchymal, gastric stem, HeLa and MCF-7 cells

We examined Oct4 expression in human kidney, mesenchymal and gastric stem cells. Punctate nuclear staining of Oct4 was clearly shown in these cells (Figure 4A–C). *Oct4* mRNA expression in the human kidney, mesenchymal and gastric stem cells was also confirmed (Figure 3D). Two cancer cell lines, HeLa and MCF-7 cells, were also examined for Oct4 protein expression. HeLa cells showed heterogeneous expression of Oct4 when cultured in D medium, previously shown to cause some of these cells to differentiate (Figure 4D). Some HeLa cell colonies had intense Oct4 nuclear expression

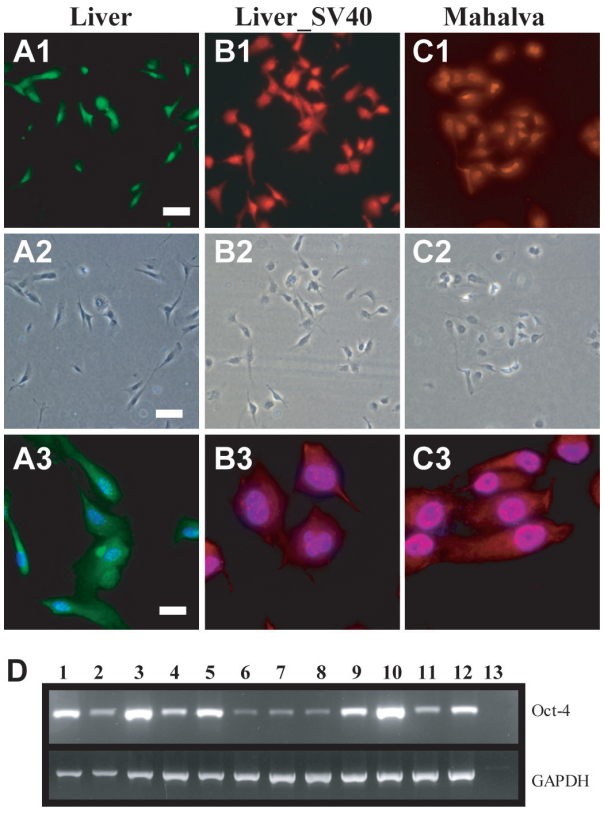

Fig. 3. Oct4 protein expression in human liver cells. (**A–C**) Images showing Oct4 immunostaining in human liver stem cells, SV40-transfected liver cells and a liver cancer cell line (Mahlava cells). Punctate red or green staining indicates Oct4 protein expression in the nuclei of the cells. Scale bars: (A1)–(C2) 60 μm; (A3)–(C3) 20 μm. (**D**) RT-PCR analysis of *Oct4* gene expression in human pancreatic stem cells (lane 1), SV40-transfected human pancreatic cells (lane 2), pancreatic cancer cell lines Capan-2 (lane 3) and Pan-1 (lane 4), human liver stem cells (lane 5), liver cancer cell line Mahlava (lane 6), human mesenchymal stem cells (lane 7), human gastric stem cells (lane 8), SV40-transfected human gastric cell line (lane 9), human kidney stem cells (lane 10) and cell lines HeLa (lane 11) and MCF7 (lane 12). Lane 13, no template (negative control).

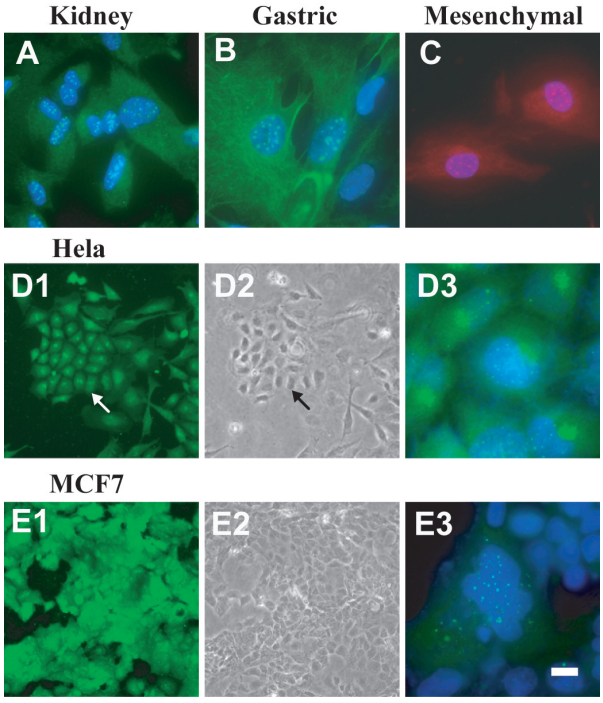

Fig. 4. Oct4 protein expression in human kidney (**A**), gastric (**B**) and mesenchymal (**C**) stem cells and HeLa (**D**) and MCF-7 (**E**) cells. Punctate red or green staining indicates Oct4 protein expression in the nuclei of the cells. The HeLa cell culture shows heterogeneous expression of Oct4 when cultured in differentiation medium D. A colony in the center (arrow) shows very intense Oct4 expression in the cell nuclei compared with no expression of Oct4 in cells at the side of the image. Few MCF-7 cells show Oct4 expression when cultured in differentiation medium D. Scale bars: (A)–(C), (D3) and (E3) 20 μm; (D1)–(E2) 60 μm.

(Figure 4D, arrows), whereas other cell colonies had relatively little Oct4 protein expression (Figure 4D, edge of the image). In MCF-7 cultures only discrete cells within the population expressed Oct4. The presence of *Oct4* mRNA in HeLa and MCF-7 cell lines was confirmed by RT-PCR (Figure 3D).

Oct4 expression in normal human skin and dog mast tumor tissue sections

To examine whether Oct4 protein was expressed in normal human tissues a Human Normal Tissue MaxArray from Zymed was immunostained for Oct4. Among the 30 different normal tissue sections present on the tissue array, only a few cells within the skin section showed positive staining for Oct4 (Figure 5A). Interestingly, these Oct4-positive cells were scattered in the basal layer of the skin epidermis, previously shown to be where skin stem cells reside (Figure 5A). Oct4 protein expression was also examined in mast tumor cells found within a section of dog skin (Figure 5B). As with normal human skin tissue, Oct4-positive cells were found within the basal layer of skin epidermis, where epidermal stem cells reside (35).

Discussion

Given acceptance of the expression of Oct4 as a marker of embryonic stem cells, our study was to test the hypothesis that adult stem cells might also express the *Oct4* gene. This hypothesis was generated by the observation that several cancer cells expressed *Oct4*, whereas cells in normal tissues did not (10). In addition, the hypothesis that stem cells serve as the target cell for carcinogenesis (13–15) implied the existence of adult stem cells and a possible association with Oct4 expression. With the demonstration of several adult stem cell types, characterized by the absence of GJIC or non-expression of connexin genes (36), it became possible to determine if these adult stem cells expressed the *Oct4* gene. There are two main results of this study: (i) several human adult stem cells express the *Oct4* gene; (ii) Oct4 is expressed in normal adult stem cells, their 'immortalized' but non-tumorigenic derivatives and tumorigenic cells. These results are consistent with the stem cell hypothesis of carcinogenesis.

We have demonstrated that adult human kidney, breast epithelial, pancreatic, mesenchymal, liver and gastric stem cells exhibit Oct4 expression. When the human breast epithelial, pancreatic and liver stem cells were induced to differentiate, Oct4 expression markedly diminished. In the case of our human breast epithelial stem cell series we have previously characterized the differences between stem cells and their differentiated daughters (30). In addition, we were able to isolate non-tumorigenic but immortalized clones that exhibited phenotypic markers similar to the original stem cell. After X-ray irradiation we were also able to isolate weakly tumorigenic

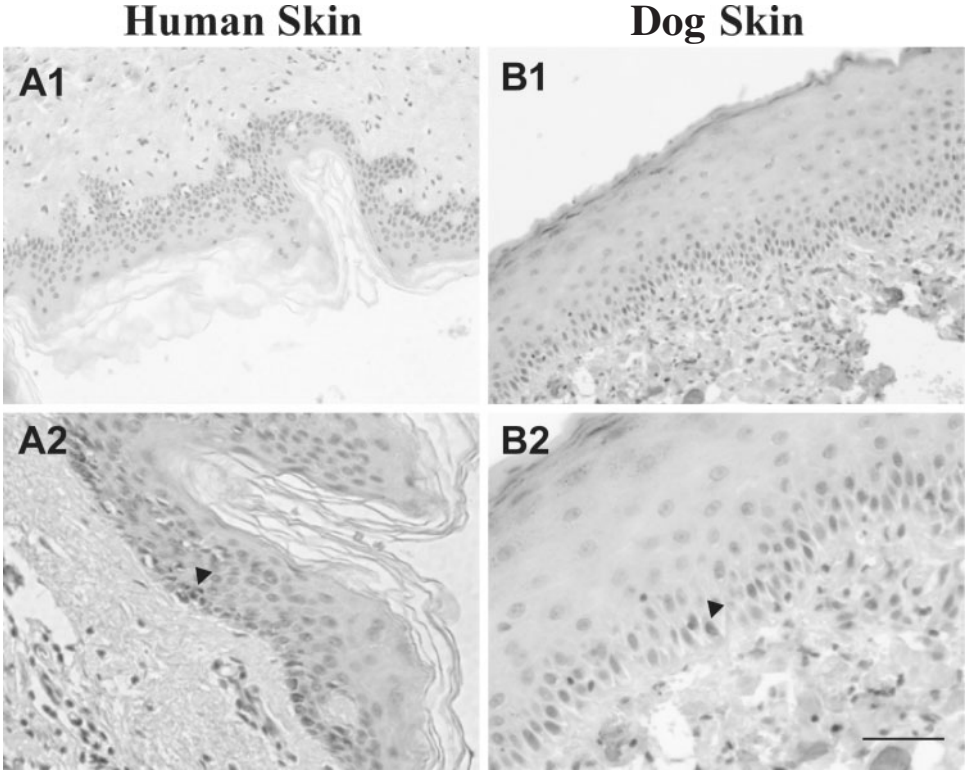

Fig. 5. Oct4 protein expression in stem cells of human and dog skin basal layers. Human (**A**) and dog (**B**) skin tissue sections were deparaffinized and subsequently stained with Oct4 primary antibody and avidin–HRP and finally visualized with DAB (dark brown color). Oct4 expression, shown in brown, is located in the nuclei of the cells, suggesting that the few Oct4-positive cells are stem cells (arrowheads). Scale bars: (A1) and (B1) 100 μm; (A2) and (B2) 50 μm.

clones which could be rendered highly tumorigenic after transfection with the c-*erb* B-2/*neu* oncogene. These cells maintained many of the original phenotypic characteristics of the adult stem cells (e.g. non-expression of the connexin genes and estrogen receptor-positivity) (30). These results, the constant expression of Oct4 from the adult stem cell through the immortalized, non-tumorigenic cell, to both the weakly and highly induced tumorigenic clones, provides strong evidence consistent with the stem cell hypothesis of carcinogenesis. In the literature the *Oct4* gene was claimed not to be expressed in adult human tissue (37). As we have demonstrated here, normal human adult breast epithelial cells (Type II) lack *Oct4* gene expression. Adult human stem cells, which in the adult tissue are few in number, do express the *Oct4* gene. On isolating and enriching Type I stem cells from the tissue and enriching them *in vitro*, *Oct4* gene expression was clearly seen by immunofluorescence and by RT-PCR.

The presence of Oct4 in the other adult human stem cells from kidney, pancreas, liver, adipose (mesenchymal) tissue and gastric tissue, which are all characterized by not having functional GJIC or by a lack of expression of connexins, supports the general conclusion that adult stem cells are characterized by the expression of Oct4 and by non-functional GJIC. Since our preliminary report that adult stem cells expressed Oct4, Young and Black (38), Young *et al*. (39) and Dyce *et al*. (40) have reported expression of Oct4 in clonal cells isolated from post-natal rats and pigs. In the case of post-natal rodent mesenchymal stem cells (41), although Oct4 was detected by quantitative RT-PCR, Oct4 expression was 1000-fold lower than in ES cells. Other reports suggesting that Oct4 is not expressed in normal tissues (37) are probably misleading. Normal tissue consists of mostly terminally differentiated cells, as well as progenitor cells that have a finite lifespan. These would not be expected to exhibit Oct4. Normal tissues, however, will contain a few adult stem cells. Unless one is looking for these few stem cells, one could conclude that normal tissue has no cells expressing Oct4.

Tumor cell lines from the pancreas and liver, as well as the classic cancer cell lines HeLa and MCF-7, all exhibited Oct4 expression. Most interestingly, both HeLa and MCF-7 cells exhibited heterogeneous expression of Oct4. This is not surprising in that cultures of these cells have often been characterized by their morphological/genetic/epigenetic heterogeneity. It was noticed that when these two classic cancer cell lines were grown in low calcium medium the heterogeneity decreased, suggesting that the high calcium level in the medium induced some differentiation of the cells, thereby reducing expression of Oct4. It must be pointed out that one of the major factors allowing us to isolate and propagate these adult human stem cells was the culture conditions under which they were grown. The culture medium was deliberately prepared as a serum-free, low calcium medium supplemented with antioxidants (conditions favoring cell proliferation and preventing differentiation). Under these conditions Oct4 was expressed and no functional GJIC was observed. With the recent demonstration of the existence of ‘cancer stem cells’ (42), whether Oct4 expression in tumor tissues is heterogeneous and whether Oct4 is expressed in the ‘cancer stem cells’ identified by those reported studies (43–47) needs to be investigated.

Out of 30 normal human tissues screened for Oct4 protein expression, only a few Oct4-positive cells were observed, in and near the basal layer of the skin epidermis. As the cells differentiate away from the basal layer, no Oct4-expressing

cells, except in one case, were seen above an Oct4 expressing cell in the basal layer. One might speculate that this represents an adult skin stem cell having divided symmetrically to form the two neighboring Oct4 cells, with one of the stem cells dividing asymmetrically, giving rise to one Oct4 daughter no longer tied to the basal layer. This cell might then lose its ability to express Oct4, differentiate and become a terminally differentiated keratinocyte. This observation could also result from previously reported classical micro-environmental or stromal-epithelial events (48–50).

Placing these observations in the context of the multi-stage, multi-mechanism ('initiation/promotion/progression') process of carcinogenesis (51), a new paradigm could be emerging. The prevailing paradigm of carcinogenesis has a normal, 'mortal' cell first being 'immortalized', in order that the initiated cell can survive long enough to acquire the 'hallmarks of cancer' (17). If our hypothesis is correct and if it is a stem cell or its early derivative daughter cell that has not yet lost Oct4 expression and telomerase activity (52), then this adult stem cell would be naturally 'immortal' until it was induced to become 'mortalized', i.e. differentiate, and would be a target cell for initiation of the carcinogenic process. If an Oct4-positive stem cell is the target for carcinogenesis initiation, this has implications for the identification of premalignant and malignant cells, as well as for the prevention and treatment of cancers.

Initiation of carcinogenesis has been defined as the irreversible alteration of a single normal cell. If that single normal cell is an adult stem cell expressing Oct4 and without functional expression of connexins, then initiation can be defined as an irreversible change in some gene that prevents terminal differentiation. It could be prevention of the suppression of the Oct4 transcription factor. These cells would be capable of symmetrical cell division when stimulated to proliferate and resistant to apoptosis (i.e. the promotion process) (19). In addition, since tumor cells exhibit a wide range of phenotypes from very embryonic to nearly differentiated, a second cell type could be the target, namely an early derivative of the adult stem cell that still maintains Oct4 expression but which has started to express connexins. This cell could start to partially differentiate because of its having functional GJIC. Tumor promoting chemicals and activated oncogenes, such as *src*, *ras* and *neu*, would transiently or stably down-regulate gap junction function, respectively (19). The progressive steps of carcinogenesis would include acquisition of all the other hallmarks of cancer during clonal expansion of the initiated stem or early progenitor cell, both of which still express the *Oct4* gene.

In conclusion, our study has revealed two important findings: (i) expression of Oct4 in several human adult stem cells; (ii) evidence that is consistent with the stem cell theory of carcinogenesis. Identification of Oct4-expressing cells in human tissues could be used to identify the stem cells, their immortalized, pre-malignant clones and malignant cells. Equally important is the challenge to the prevailing paradigm that the first step of the carcinogenic process is 'immortalization' of a normal, mortal cell. Rather, it seems that the first step of carcinogenesis, 'initiation', is the blockage of 'mortalization' of a naturally immortal stem cell. In other words, instead of stating that embryonic-like genes are 're-expressed' during the carcinogenic process, the carcinogenic process actually prevents the down-regulation of genes such as *Oct4*, which would normally start the terminal differentiation process in the adult stem cell. If this hypothesis is correct, much of the current gene expression profiling using DNA microarray technology, in comparing normal tissue against cancer tissues, will miss the critical gene profile differences between the few target adult stem cells in normal tissues and the few tumor stem cells in the heterogeneous mass of partially differentiated and genetically unstable tumor cells.

Using RNA interference technology, as has been done in human embryonic stem and human embryonal carcinoma cells (53), as well in mouse embryonic stem cells (54), it has been shown that Oct4 plays a critical role in regulating stem cell identity. In addition, on down-regulating *Oct4* gene activity, differentiation of both stem cells and embryonal carcinoma cells occurred. To test if our hypothesis that adult stem cells express Oct4 as a fundamental property and that these stem cells are the target cells for carcinogenesis is correct, additional experiments using interference RNA will be necessary.

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