FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 45, No. 3, 2007
pp. 245-254

The comparison of multipotential for differentiation of progenitor mesenchymal-like stem cells obtained from livers of young and old rats

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Abstract: The presence of stem cells differentiating to hepatocytes and cholangiocytes has been previously reported in livers of young rats. Here, we have isolated, cultured, and characterized mesenchymal stem cells (MSCs) from livers of young and old rats and tested their multipotential for differentiation. The mesenchymal stem cells in liver sections were identified by the presence of markers, respectively for primary stem cells Thy-1 and CD34, for differentiation to early cholangiocytes GST and CK19, and for differentiation to hepatocytes GSTα and CK18. Ki67 was detected as the cell proliferation marker. Cells isolated from livers of either age group were tested in a culture for their viability following storage and were characterized for the presence of most of the markers detected in cells in situ. The results revealed age-dependent changes in the number of recovered primary MSCs. In both age groups we have observed cells changing under differentiating conditions to liver cell lineages, such as cholangiocytes and hepatocytes, as well as to non-liver cells such as adipocytes, astrocytes, neuroblasts, and osteoblasts. Our data revealed that from the livers of rats 20 months and older the primary MSCs could be isolated and expanded; however, they were significantly fewer, even though their differentiation multipotential was preserved. The mechanism involved in the differentiation of liver MSCs seemed to depend on a constellation of signals in Notch signalling pathways. Thus, our results support the idea of potential use of liver as a source of MSCs, not only for liver reconstruction but also for cell therapy in general.

Key words: Cell differentiation - Differentiation markers - Liver stem cells - Notch signalling - Progenitors

Introduction

There are about 230 different cell types in the body. All these cells are derived from three embryonic layers. Ones form the brain and spinal cord, another form the internal organs, including the liver and the third layer gives origin to muscles and bones. Therefore, embryo-derived stem cells have been the most studied cells for therapeutic use. However, beyond embryonic stem cells (ES), several potential adult cell progenitors derived from the pancreas, liver, umbilical cord blood and bone marrow have also been extensively explored. But none of the candidate sources has been fully explored and characterized to the extent that they could yield serious clinical applications [1]. The most extensive research on non-embryonic sources of stem cells has been conducted on adult bone marrow. In adult bone marrow, resides a population of cells

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referred to as bone marrow stromal or mesenchymal stem cells. They seem to be the most attractive candidates for cell replacement and gene therapy because they possess some unique properties. Adult MSCs exhibit a marked self-renewal capacity. They are readily available from patients under local anesthesia and can differentiate into various cell lines (reviewed in [2]). Recently, genetically modified bone marrow stromal cells have shown differentiation to adipocytes and osteoblasts [3]. Thus, these cells are now even more attractive for gene therapy after acquiring exogenous gene expression in place of a damaged gene.

However, there are also other non-embryonic sources of multipotent cells. One such example is liver regenerating following certain types of its toxic injury, which requires the action of certain types of residing cells with the original phenotype of oval cells [4-7]. Similarly, reconstruction of liver mass loss due to surgical resection is accomplished by the proliferation of resident, normally quiescent hepatocytes and bile duct cells responding rapidly to injury and giving rise to a substantial number of progenies whilst maintaining their differentiated phenotype [8,9]. Regeneration of liver in response to different types of toxic damage affecting hepatocyte's replication seems to be accomplished by vigorously proliferating small hepatocyte-like progenitors expressing phenotypic characteristics of foetal hepatoblasts and adult mature hepatocytes [10,11].

Copious evidence has already been published indicating that ductal plate and bile duct cells in the human liver express hematopoietic stem cell markers, such as c-kit and CD34, in association with cytokeratin markers CAM 5.2 and CK18 [12]. Identification of such ductal plate cells as likely progenitors for both bile ducts epithelial cells and hepatocytes, and their possible reappearance as oval cells in the regenerating liver, have generated much interest in their pluripotential capacities. There is still controversy in choosing markers for mesenchymal stem cells. Some investigators report on CD34 as positive marker exclusively for hemopoietic lineages, whereas others report on CD34 as marker positive for both hemo and non-hemopoietic precursosrs for cells derived from fat, skin, muscles, and liver [12-19]. Lemmer and co-workers [19] have reported that approximately 0.9% of isolated mononuclear cells from the liver were positive for CD34. The number of isolated mononuclear cells correlated with foetal liver weight. About 3 to 8% of these $CD34^+$ cells also stained positively for non-hemopoietic marker CAM 5.2. Additionally, $CD34^+$ cells were positive for HLA-DR, but only a small fraction was positive for c-kit. On the other hand, staining for the committed hematological markers such as CD33 and CD38, were consistently negative [17].

Thus purpose of the study was to verify our hypothesis that, similar to other organs, the cellular lineage of the liver consists of true resident stem cells and progenitor cells. Another goal was to determine variability in Notch signalling pathway during proliferation and differentiation of MSCs obtained from livers of rats at different age.

Materials and Methods

Detection of MSCs in the liver. Small pieces of livers with or without perfusion were excised from healthy Sprague-Dawley rats of ages 2, 4, 20, and 28 months, and fixed in formalin or frozen at -80°C. The fixed specimens were embedded in paraffin and used for the preparation of sections for hematoxyline and eosine (H&E) staining and for immunostaining with antibodies detecting CD34 (Chemicon), Thy-1 (CD90), Ki67 (Novocastra), CK18 and 19 (Chemicon), GST π and α (Novocastra), and 5'BrdU (Roche). The paraffin sections were also used to detect the presence of Notch-1 and Notch-3 cytoplasmic domains and to determine their location in the cell types as well as within the cells.

Frozen livers were used for staining with antibodies against Thy-1 labelled fluorescently. The nuclei were stained with methylene green (BDH).

Cells with proliferating potential in rat livers were detected obtaining 5'BrdU intraperitonally (50 mg/kg b.w.) administered for 7 days prior to sacrificing. Unincorporated 5'BrdU was removed by perfusion of the animals with buffered saline (PBS).

Isolation, culture and characterization of liver MSCs. Approximately 2 g of the liver from the same rats at the ages described in the previous section and from those with administered 5'BrdU were excised and extensively washed in PBS. Each piece of liver was separately minced and incubated in alpha-MEM supplemented with collagenase (2 mg/mL) at 37°C for 60 min. and trypsinized (10 mg/mL) for 45 min. After the digestion was complete the sample was suspended in 10 ml alpha-MEM and cells were recovered by centrifugation for 10 min at 500 g. This step was repeated twice. The cell pellet was suspended in 10 ml of alpha-MEM and centrifuged again to remove blood remains. The cell pellet was suspended in 10 ml of MesenCult specialty medium recommended for culture of rodent mesenchymal cells (STEMCELL Technologies - Canada) and maintained for selection and expansion in a Petrie dish in a 95% air, 5% CO_2 humidified atmosphere at 37°C. Every other day the culture medium was replaced with the fresh one. At confluence the cells were lifted from the plastic with trypsin/EDTA and split in a ratio of 1 to 3 for further expansion.

The cultures were fixed for 20 min with 4% paraformaldehyde and subjected to immunostaining with appropriate antibodies. Cells were stained for CD34, Thy-1, GSTπ and α, CK18 and 19 (at dilution 1:100), Notch-1, -2, and -3 and Delta1 (at dilution 1:50). For nuclei visualization the immunostained specimens were stained with 0.5% methylene green. The cells positive for a particular marker were counted in fifteen observation fields in quadruplicates. For Notch/Delta signalling pathway the antibodies detected cellular domains of the Notch1, 2, and 3 and the Notch ligand Delta1. Therefore it was possible to discriminate the staining in the cytoplasm only from the staining in the nuclei. The specificity of the antibody location within the cell compartments was verified by confocal microscopy. To determine the ratios of cells positive to negative for a particular marker, the average numbers from fifteen observation fields in at least five specimens and the standard deviations were calculated.

Differentiation of liver MSCs to different cell lines. The cells from livers of rats of different age cultured as described in the previous section were plated in chamber glass slides at number 2×10^4 and cultured for 24 hours in MesenCult medium. Subsequently, the

medium was replaced with special differentiation media for hepatocytes, cholangiocytes, adipocytes, neuroblasts, glial cells and osteoblasts (STEMCELL Technologies - Canada). All the cultures, unless differently indicated, were maintained for 10 days in the appropriate differentiating medium. The media were changed every two days.

For differentiation to osteoblast β-glycerophosphate (5 mmol/L) with ascorbic acid in the medium was added from the beginning of the differentiation. Differentiation media of MSCs to hepatocytes and cholangiocytes were based on basal MesenCult supplemented with 2% DMSO and 3.75 mmol/L sodium butyrate, respectively.

Detection of differentiation markers in liver MSCs. The cells following culture in the appropriate differentiating medium were fixed in 4% paraformaldehyde for immunostaining and Red Oil O staining or in cold 70% ethanol at -20°C for Alizarin S staining. To determine the number of $CD34^+$ cells the control culture was terminated 24 hours after initial cells seeding before the differentiation medium was applied to tested cultures. The adipocytes were detected by staining with 0.5 % Red Oil O and visualized in a light microscope. Control cells were cultured in MesenCult for the same period of time and subjected to the same procedure as for adipocyte differentiation. Neuronal precursors were detected by immunostaining with antibodies against neuron specific enolase (NSE - Sigma). Glial precursors were identified by staining with specific antibodies against glial fibrillary acidic protein (GFAP - Sigma). Osteoblasts were detected by staining for the presence of hydroxyapatite using Alizarin S.

Statistical analyses. The statistical significances were calculated using parametric two-sample Student's t-tests for normal distributions assuming homogeneity and heterogeneity of variances (Pooled and Satterthwaite approximation) and non-parametric. The U Mann-Whitney test was applied in the case of non-normality of distribution. Prior to the parametrical analyses the normal distribution was verified with the use of Kolmogorov-Smirnov test. The homogeneity of variance was analyzed using Levene test. The 0.05 or lower level of probability (p<) was accepted as significant throughout the work. All statistical analyses were conducted using SAS 9.1 computer software package.

Results

Multipotential stem cells can be isolated from adult rat livers

An analysis of rat liver sections for the presence of markers commonly recognized as specific MSCs markers such as Thy-1 and marker specific for both hematopoietic and non-hematopoietic progenitors, CD34, revealed single cells positive for both the markers in livers from young as well as old animals (Fig. 1A and B). In both age groups the 5'BrdU, $CD34^+$ and Thy-1 could be detected in cells recovered from livers, either PBS perfused or non-perfused animals, and cultured in MesenCult medium (Fig. 2A). The cells expressing CD34 obtained from both age groups were present at similar ratios of cells positive to negative for this marker (central panel in Fig. 2B). Also, cell ratios of Thy-1^+ to Thy-1^- were comparable in both investigated age groups (left panel in Fig. 2B). The staining of liver sections from rats of different age for Ki67 revealed different numbers of cells positive for this proliferation marker. The proliferation potential of the cells derived from different age groups showed a statistically significant age dependence difference as determined by detection assay based on 5'BrdU incorporation administered to living animals in liver sections and then detected in cultured cells obtained from livers of animals perfused with PBS prior to sacrificing (Fig. 2B). The ratios of 5'$BrdU^+$ cells in two- and four-month old rats were respectively three and two fold higher than that in 20-month and older animals.

The overall number of cells recovered from livers lowered with the animal age. The young rats have had 2.4 times higher number of the MSCs than the old ones. Thus the same number of stem cells, obtained from the same amount of liver, were gained from a culture conducted for shorter time in the case of young rats than that from livers of 20-months or older animals.

Markers specific to hepatocytes and cholangiocytes were present in liver-derived cells

In both the liver specimens and in the primary cell cultures small size cells have been identified expressing markers specific for precursors of hepatocytes and cholangiocytes (upper panels in Fig. 1C). Precursors of hepatocytes expressed detectable amounts of GSTα and CK18 proteins, both detected with specific antibodies in the livers of young and old rats. The number of cells present in the liver specimens was at similar levels both from young as well as old animals (two left panels in Fig. 1C). The precursors of cholangiocytes were detected with antibodies against GSTπ and CK19 (lower panels in Fig. 1C). There were fewer of $GST\pi^+$ /$CK19^+$ cells in comparison to the precursors of hepatocytes. Additionally, larger densities of positively stained cells around the portal spaces in the liver of young rats than in old ones have been detected. In the primary cell cultures both precursors of hepatocytes as well as cholangiocytes were present as single cells in cultures of young and old animals (right column of panels in Fig. 1C).

Liver-derived stem cells gave rise to both liver and non-liver cell lineages

Primary cell cultures from young and old rats were subjected to ten day-long exposure to differentiating specialized culture media (Fig. 3). Some of the cells from both young and aged rats exposed to MesenCult enriched with 2% DMSO or 3.75 mmol/L sodium butyrate at day 10 of culture were $GST\alpha^+$/$CK18^+$, some of them were $GST\pi^+$/$CK19^+$. However, the cultures from old rats contained a lower number of differentiated cells than the cultures from young rats (Table 1).

We have, also, cultured the primary MSCs from the livers of young and old rats in the commercial specialty medium for neuroblasts/glial cells differentiation called NeuroCult (STEMCELL Technologies, Inc.) and subsequently detected precursor cells for neurons by antibody staining against neuron specific enolase (NSE) and for glial cells by staining the cell layer with antibodies against glial fibrillary acidic protein (GFAP) (first two rows, respectively in Fig. 3). In both cases MSCs from young and old rats differentiated to neuroblasts and glial cells. However, younger rats showed slightly more MSCs capable to differentiate to glial cells and neuroblasts than the old rats (Table 1).

Detection of mineral deposition following a culture of MSCs in the medium containing β-glycerophosphate and ascorbic acid revealed the presence of mineral crystals in both cultures obtained from both young and old rats (third row in Fig. 3). The content of the minerals, however, differed in the same manner with age as differentiation capacities to other cell lineages analyzed in this work. The minerals per 1,000 cells were about 1.5 times lower in MSCs from old rats (0.2 pmol/1000 cells, n=4) as compared to young rats (0.3 pmol/1000 cells, n=4).

The next cell type we have assayed was adipocytes. The MSCs were cultured for 10 days in the presence of commercial differentiating medium (STEMCELL Technologies, Inc.), and lipids were detected in the cells using Red Oil O staining (last line of panels in Fig. 3). Once again, the cells derived from old rats did differentiate almost two times less frequently than the cells obtained from livers of young rats (Table 1).

Notch signaling changes with age of MSCs donors

An analysis of distribution cytoplasmic domains of Notch-1, -2, and -3 receptors and the Delta-1 ligand revealed that they were differently distributed depending on the donor's age. Notch-1 cytoplasmic domain has been detected in single small cells in paraffin sections of both young and old rats, whereas cytoplasmic domain of Notch-3 was observed mostly in cytoplasm of single small cells in liver sections of old rats (data not shown). The data suggest slow but continuous proliferation of oval cells in the liver.

MSCs obtained from livers of young and old donors revealed different expression levels and distribution of the cytoplasmic domains of the three Notch receptors (Table 2). Changes in distribution of the cytoplasmic domain of ligand Delta-1 followed changes of the receptor domains (first two rows in Fig. 4 and Table 2). The Notch-1 cytoplasm domain was detected in 53% of nuclei of cells from young rats and in 45.1% of nuclei of cells from old donors. The cytoplasm domain of Delta-1 has been detected in lower fraction (34.0%) of nuclei of cells from older animals when compared to young donors (45.3 %). Percentiles of cells positive for cytoplasmic domains of Notch-2 and Notch-3 receptors were higher in cultures of MSCs from old donors (two bottom rows in Fig. 4), and the differences were statistically significant. The ratio of cytoplasmic domain of Notch-3 positive to negative cells from old rats was ten fold higher than in cells from young donors. However, no age-related statistical significance was obtained in percentiles of Notch-3 cytoplasmic domain presence in the nuclei and in the cytoplasm. The cellular domain of this receptor has been detected in cytoplasm at 3 to 4 fold higher levels than in nuclei (bottom row in Fig. 4) indicating that there was a presence of Notch-3, but either the receptor was not activated or the activation was through a different mechanism than by cleavage of the cellular domain and its transport to nuclei.

The specificity of the locations in the particular cell compartment of the cytoplasmic domains of the Notch receptors and Delta-1 ligand was verified by confocal microscopy.

Discussion

At least two sources of stem cells have been called on to participate in liver regeneration. One pool of cells comprising the stem cells supposed to be located in the canal of Hering, whereas the another stem cells capable of differentiating into hepatocytes and bile duct cells derive from bone marrow and require homing to the injured liver [20,21]. In this work we have presented evidence that in the liver reside cells Thy1^{+}, CD34^{+} capable to differentiate into at least four additional cell types beyond hepatocytes and cholangiocytes.

Although, some investigators reported that CD34 is rather marker specific for hemopoietic precursors, recently large body of reports indicated that this marker is present on both hemo- and non-hemopoietic precursors isolated from various tissues [12-16] but also from livers of rodents [18,19].

The animals investigated in this work were healthy ones, thus, it seems that the cells giving rise to non-hepatic and non-hemopoietic precursors are present all the time in the liver rather than they would have been released from the liver in the process of recruitment following liver damage. Also, the cells divided at a relatively low rate as 5'BrdU still could be detected, although it was administered to living rats and subsequently removed by whole animal perfusion.

Comparison of percentile of particular cell types indicates that cell which differentiated to the certain lineage derived rather from MSCs positive for CD34 and Thy-1. The larger number of differentiated cells than original CD34^{+}/Thy-1^{+} cells could be explained by the fact that the cell were cultivate in culture medium specially composed for expansion of MSCs and follow-

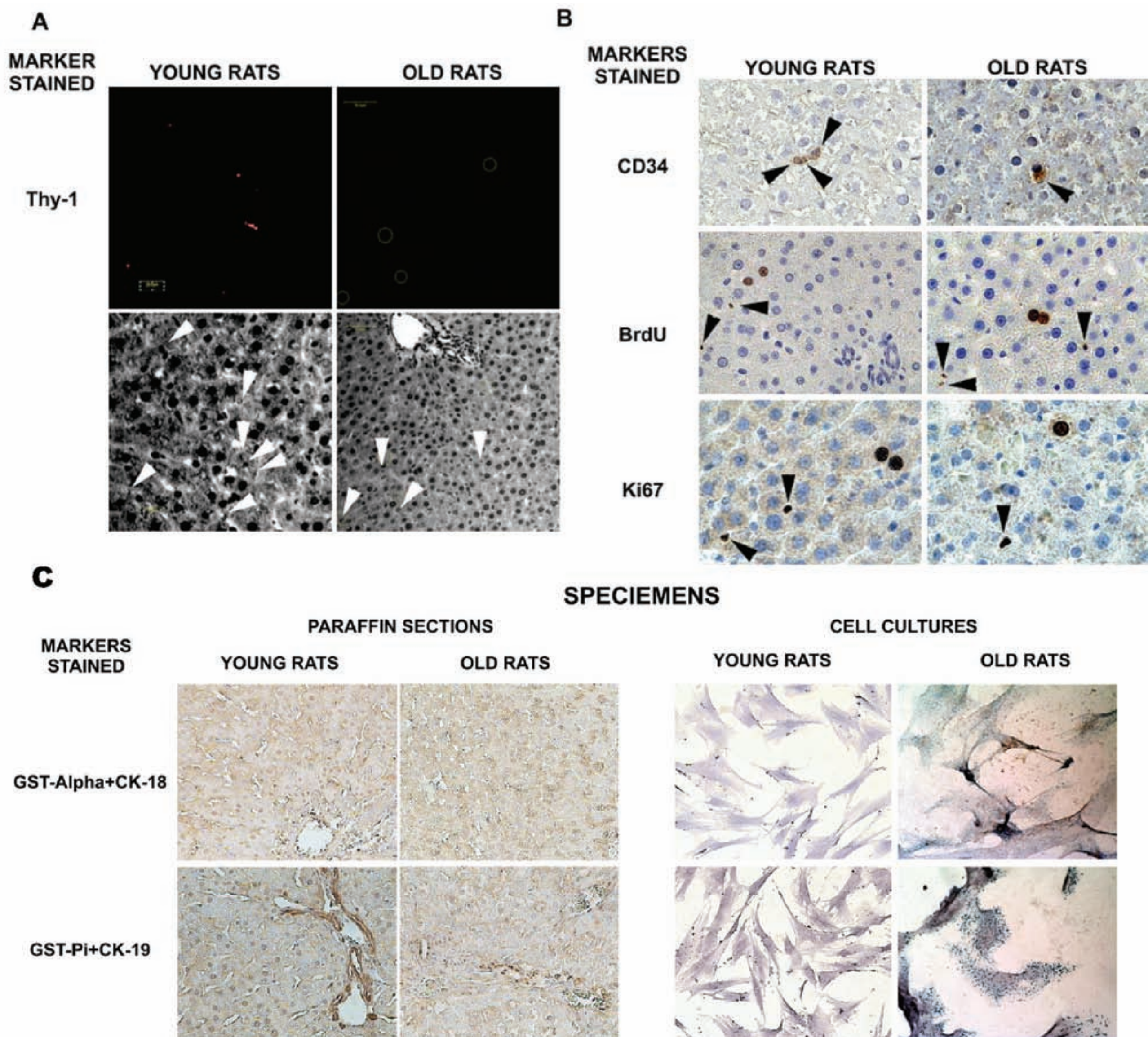

Fig. 1. Detection of markers specific for MSCs, proliferating cells, and liver cell lineages. **A**. Thy-1 - positive cells were detected following labelling with fluorescence tagged antibodies against human Thy-1 marker. The location of the marker was visualized using confocal microscope Olympus (magnification ×200 for the left column and ×100 for the right column). **B**. Cells positive for CD34, BrdU, and Ki67 were detected with specific primary antibodies and visualized using substrates for enzymes conjugated with secondary antibodies - DAB for HRP and BCIP/NBT for alkaline phosphatase (magnification ×400). **C**. Cell cultures were treated with specific antibodies against GSTα and CK18 or GSTπ and CK19. Secondary antibodies to detect positive reaction with either GSTα or π were conjugated with alkaline phosphatase and the colour was obtained from conversion of BCIP/NBT as a substrate. Secondary antibodies to detect positive reaction with either CK18 or 19 were conjugated with horseradish peroxidase and colour was developed after adding DAB as a substrate (magnification ×100 for paraffin sections and ×400 for cell cultures).

ing application of specialty differentiation media they change their phenotype. More detail comparison of the percentage of CD34$^+$/Thy-1$^+$ in livers from both young and old rats with percentage of differentiated cells in these groups 40% and 20% respectively revealed that the ratios were consistent. Noticeably difference are even more evident when the values obtained for cells CD34$^+$/Thy-1$^+$ are compared with numbers obtained for differentiation of the liver derived cells to cholangiocytes or to hepatocytes, respectively 15% for cells from livers from young rats and 2 to 10% for cells from livers of old rats. This evidence might indicate that in our studies rather CD34-/Thy-1$^-$ not CD34$^+$/Thy-1$^+$ gave rise to cholangiocytes or hepatocytes and/or the remaining cells could be an oval cells. However, this conclusion is just another hypothesis to verify in a different set of experiments where the CD34$^+$/Thy-1$^+$ or CD34$^-$/Thy-1$^-$ liver-derived cells will be separated and subsequently tested for their ability to differentiate toward particular cell lineages.

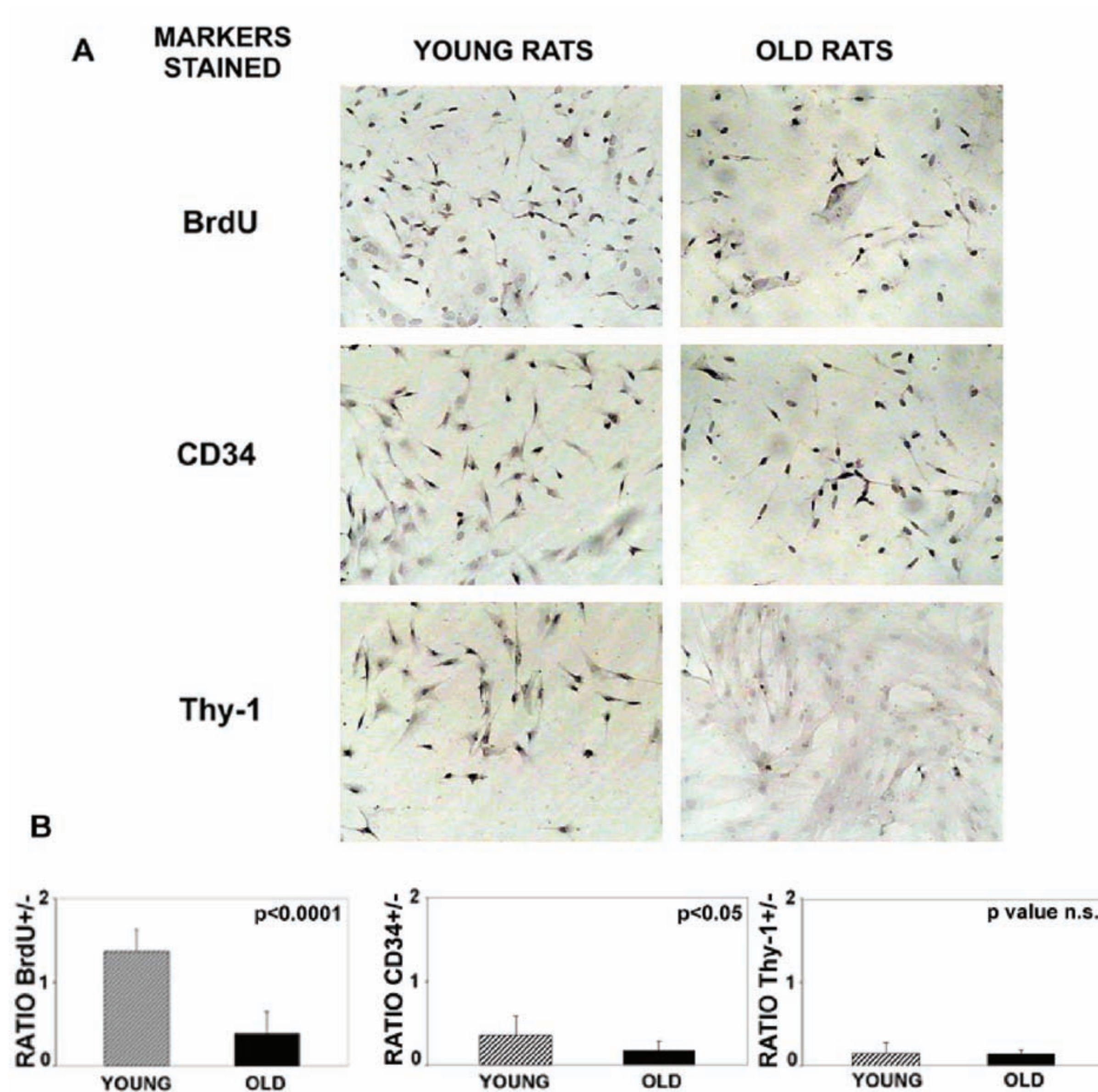

Fig. 2 . Cells isolated from livers of young and old rats proliferate in cultures at different rate and numbers. **A**: Cells positive for CD34, Thy-1 and 5'BrdU were identified following labelling of primary cultures at confluence in glass culture chambers with specific primary antibodies and visualization by the colour reaction resulting from activity of HRP or alkaline phosphatase conjugated with secondary antibodies. CD34+ and Thy-1+ cells were stained with DAB and BrdU+ cells were stained with BCIP/NBT (magnification x100). **B**: Total number of cells was counted and the content of cells positive for 5'BrdU (top panel), CD34 (central panel), and Thy-1 (lower panel) was expressed as the ratio of positive to negative cells for the particular marker. Number of analyzed specimens of young rats for 5'BrdU and CD34 was n=12 and for Thy-1 was n=6. For old animals the number of analyzed specimens for 5'BrdU was n=12, for CD34 was n=6 and for Thy-1 was n=5.

The presence of liver-specific lineages as well as the ability to induce stem cells from the liver toward hepatocytes or cholangiocytes could, also, be explained by the mechanism involving a distinct expression of Delta-like Protein/Preadipocyte Factor 1/Fetal Antigen 1 axis [22]. Between 20 and 30% of the progenitor cells derived from young rats expressed cytoplasmic domains of the three Notch receptors and the Delta-1 ligand. The older donors provided cells with a higher percentile of cells showing the presence of the Notch and Delta molecules, but the overall number of MSCs obtained from them was several times lower. The exceptionally high ratio of the Notch-$3^{+/-}$ cells in the culture of MSCs from old rats could be because only the cells with the Notch-3 active signalling pathway survived and thus only these cells contributed to the MSCs pool in older individuals.

The important new observation is that the MSCs detected in livers of both young and aged rats could give rise to lineages other than hepatic lineages. They

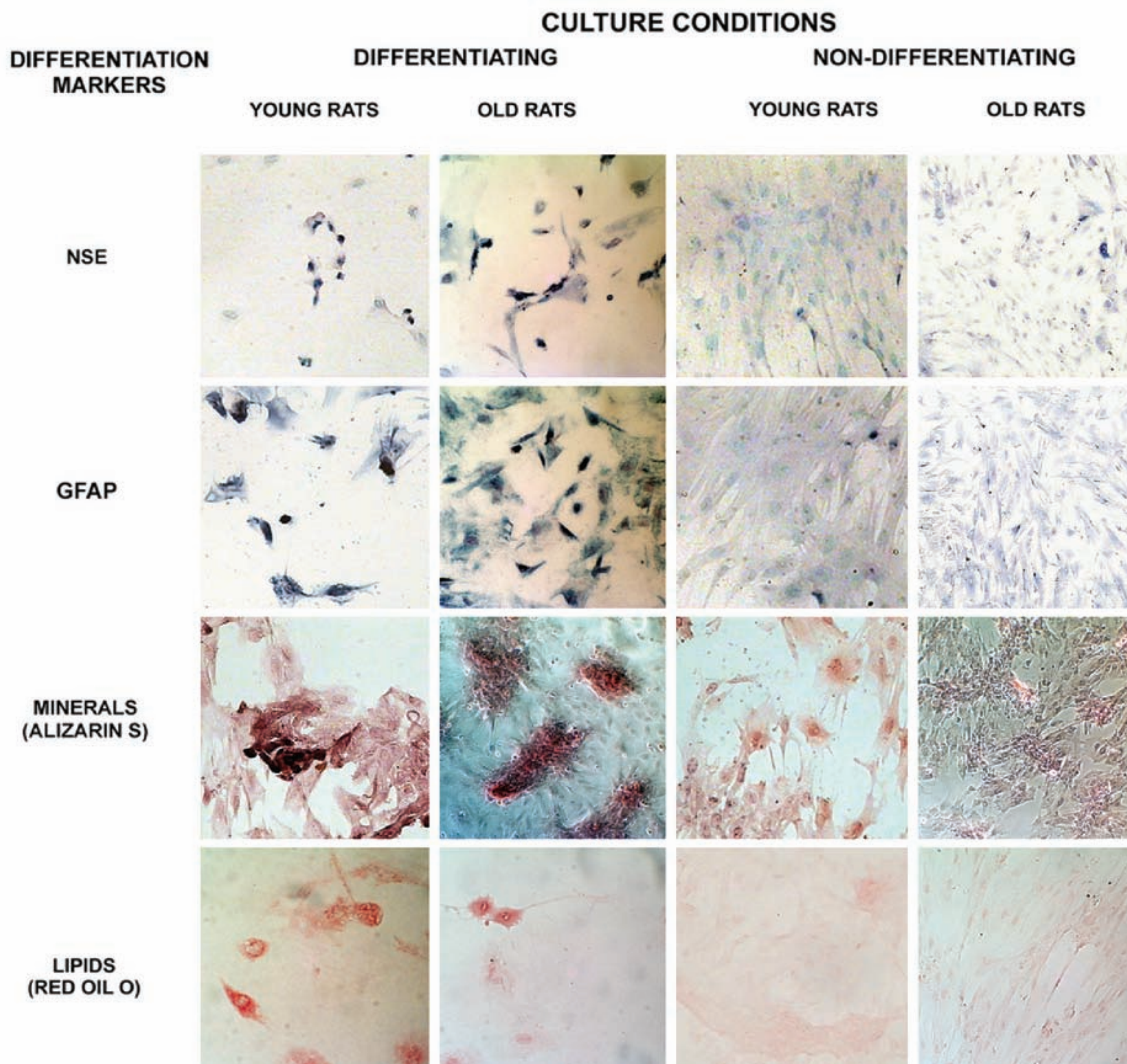

Fig. 3. Liver-derived MSCs both from young and old rats revealed the multi-potency to differentiate toward different cell lineages. Liver MSCs from both young and old rats isolated in MesenCult medium were cultured under either differentiating conditions (two left columns) using specialty culture media or under non-differentiating conditions using MesenCult medium (magnification ×100 for second column in third row and entire last column, ×200 for first column, second column except third row and third column except for the last row).

Table 1. Differentiation in culture of multi-potential stem cells isolated from livers of young and old rats.

Cell Type Precursors	Young rats			Old rats		
	Differentiated [%] (n=4)	Calculated number of differentiated cells per g of liver [x10^6]	Ratio of differentiated /non-differentiated	Differentiated [%] (n=4)	Calculated number of differentiated cells per g of liver [x10^6]	Ratio of differentiated /non-differentiated
Hepatocytes	15.0±0.1	0.270	0.18±0.010	11.0±0.3	0.198	0.12±0.010
Cholangiocytes	14.0±0.2	0.252	0.16±0.005	2.0±0.01	0.036	0.02±0.001
Neuroblasts	37.0±1.5	0.800	0.60±0.060	32.0±5.0	0.700	0.52±0.090
Glioblasts	46.0±5.5	1.080	0.80±0.120	42.0±1.2	0.990	0.75±0.070
Adipocytes	42.0±3.0	0.900	0.70±0.140	23.4±9.0	0.550	0.38±0.120

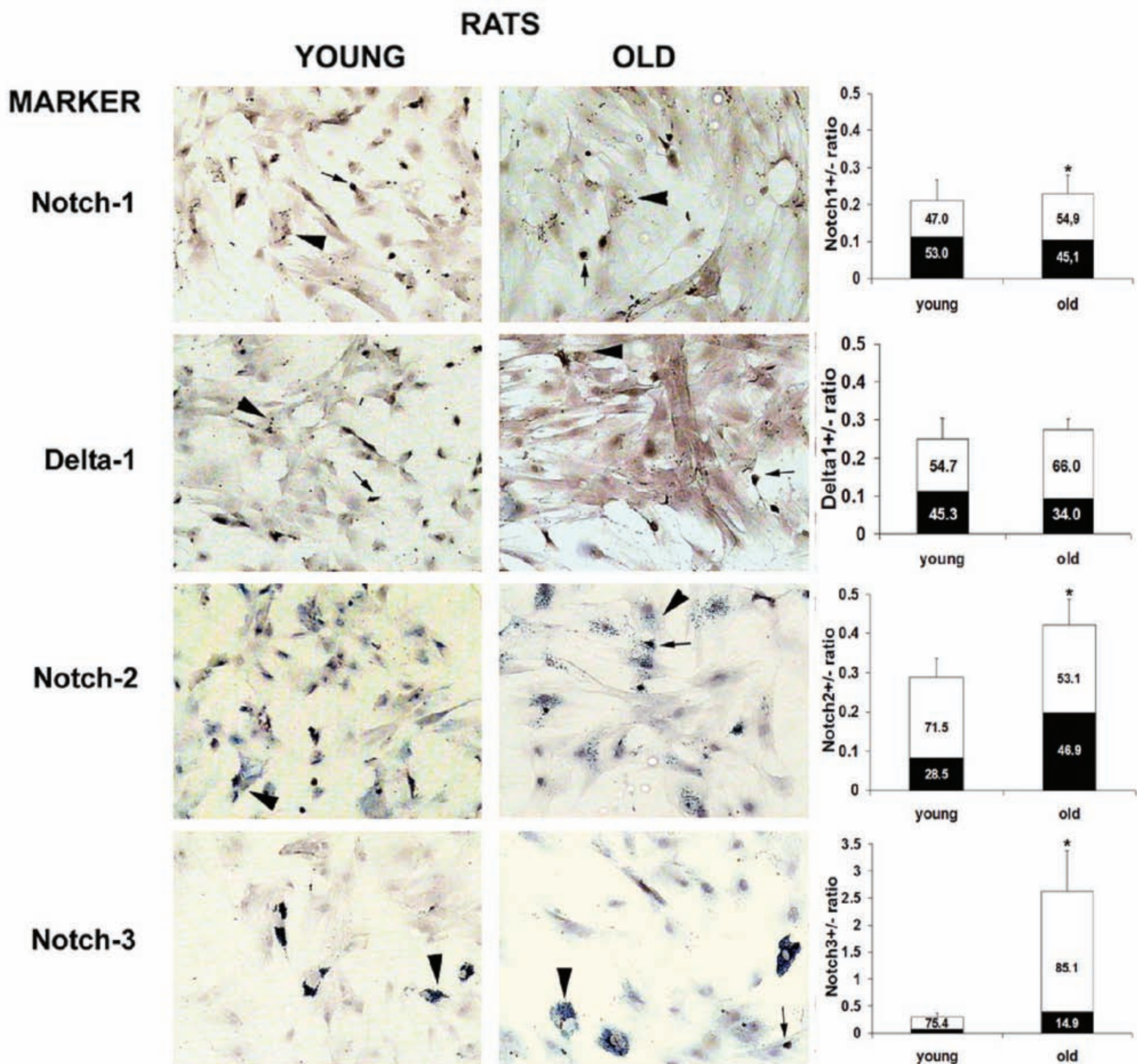

Fig. 4. Notch/Delta pathways activation depending on donor's age. Arrows point to the cells with nuclei and cytoplasm stained with antibodies. Arrowheads indicate cells with cytoplasm only immunostaining (magnification ×200). Open parts of boxes represent percentage of cells with immunostaining in cytoplasm alone. Black parts of boxes represent percentage of cells with immunostaining in both, nuclei and cytoplasm. (n=15). Asterisk in graphs indicates statistical significance of $p<0.05$.

could be differentiated toward precursors of glial cells, neuroblasts, adipocytes and osteoblasts. Details of the mechanism involved in maintenance of MSCs and their subsequent differentiation to additional cell lineages have yet to be investigated. However, some previous reports indicated involvement of the Notch/Notch receptor ligand signalling pathway in relation to different precursors [23]. The exposure of satellite cells from old mice to young serum enhanced the presence of the Notch ligand, increased Notch activation, and enhanced proliferation *in vitro*.

The Notch signalling is also involved in augmentation of NF-κB activity by facilitating its nuclear retention [24]. NF-κB is a transcription factor regulating the expression of numerous genes involved in various cell functions. It has been known for a long time that NF-κB regulates expression of genes encoding adhesion molecules such as ICAM1, ICAM2, ICAM3, ICAM4, ICAM5, NCAM1, NOS2A (iNOS), SELE (ELAM-1 / E-selectin), SELL (L-Selectin), SELPLG (P-selectin), VCAM1 [24]. Different NF-κB responsive genes encode such cytokines as CSF2 (GM-CSF), CSF3 (G-CSF), IFNA1, IFNB1, IFNG, IL2, IL6, IL8, IL12A, IL12B, IRF1, LTA (TNF-β), SCYA2 (MCP-1), TNF (TNFα) [24]. Also some genes for Acute Phase Response Proteins (APRP), such as angiotensinogen (AGT), BF,

Table 2. Notch receptors and Delta ligand cellular distribution in MSCs from liver depending on donor's age. The p values calculated with Student`s t-tests and *U* Mann-Whitney test.

Marker	Nuclei [% positive]			Cytoplasm [% positive]		
	Young	Old	p<	Young	Old	p<
Notch 1	53.0 ± 5.2 (n=7)	45.1 ± 4.4 (n=5)	0.00001	47.0 ± 6.2 (n=7)	54.9 ± 5.0 (n=5)	0.00001
Notch 2	28.5 ± 3.7 (n=7)	46.9 ± 4.4 (n=5)	0.0001	71.5 ± 7.4 (n=7)	53.1 ± 2.5 (n=5)	0.00001
Notch 3	24.6 ± 3.0 (n=7)	14.9 ± 3.0 (n=5)	0.00003	75.4 ± 6.0 (n=7)	85.1 ± 9.0 (n=5)	n.s.
Delta 1	45.3 ± 6.0 (n=7)	34.0 ± 8.0 (n=5)	n.s.	54.7 ± 6.0 (n=7)	66.0 ± 8.0 (n=5)	n.s.

C3 complement, ORM1 (AGP1), ORM2 (AGP2), serum amyloid A1 (SAA1), SRF, respond to NF-κB [24].

Additionally, the same mechanism could explain the phenomenon of the presence, although, at lower content of MSCs in livers of aged rats that could change their genetic programs from quiescent to proliferation and differentiation *in vitro*. Results from studies on young and old parabiotic mice suggest that the age-related decline in activity of progenitor cells can be modulated by systemic factors that change with age [23].

Recent reports on sensory fate induction in murine bone marrow SCs revealed genes that were up-regulated over 70-fold when compared with expression levels in MSCs untreated with sonic hedgehog (Shh) and retinoic acid (RA) including Irx1, Irx2, Sox10, GATA3, GluR4, P2X3, VGLUT1, and calretinin [25, 26]. All the genes are expressed in vestibule-cochlear ganglia (VCG), trigeminal ganglia (TG), or dorsal root ganglia during normal embryonic development. The commercial neuronal differentiation specialty medium used in our studies, according to the information provided by the manufacturer, contained factors inducing neuronal precursors both for neuroblasts as well as for glial cells. Thus, we speculate here, that the mechanism involving Shh/RA pathway could be activated, as well, in our neuronal/glial differentiation assay. The exact mechanism of Shh and RA action is still unclear, although several lines of evidence suggest that signalling by both factors communicates during embryonic development [27,28]. Some reports provide evidence that Tlx3 and Tlx1, Tlx-class homeobox genes, promote glutamatergic differentiation in the spinal cord's neural progenitors [29].

Fusion of donor bone MSCs to neuronal cells in the host tissues has been suggested to account for neuronal differentiation from bone marrow-derived stem cells [30,31]. However, evidences against the cell fusion phenomenon have been also reported [26,31]. Here we showed that liver-derived MSCs could differentiate without evidence of fusion (data not shown), therefore, it makes the cells suitable for cell therapy of neurodegenerative disorders.

Peister *et al.* previously reported differentiation of MSCs to different cell lineages including adipocytes and osteoblasts [32]. For adipogenic differentiation, the MSCs were grown in the basal medium supplemented with serum, 0.5 mmol/L hydrocortisone, 0.5 mmol/L IBMX, and 60 mmol/L indomethane [32]. In our studies we have used the commercial differentiation supplement recommended by the manufacturer for this type of differentiation. For osteogenic differentiation Peister and co-workers induced MSCs using medium supplemented with serum, 1×10^{-8} mol/L dexamethasone, 10 mmol/L β-glycerol phosphate, and 50 mmol/L ascorbic acid-2 phosphate. The medium was changed every 3 to 4 days. We have applied the same culture conditions except that the differentiation medium was commercial, but the supplement added to the basal medium contained all the compounds used by Peister *et al.* [32]. Thus, using comparable conditions for differentiation the mesenchymal stem cells derived from rat liver revealed the same adipocytic and osteoblastic phenotypes as MSCs isolated from bone marrow and subjected to the same type of differentiation.

Summary

In this work we have expanded the sources of adult stem cells capable of differentiating to different cell lineages on liver. Our results also proved that 20 to 28 months old rats still maintained in the liver MSCs with not only bi-potential but also multi-potential for differentiation. The commonly observed lower ability for regeneration is rather related to the significantly lower number of these cells they contained. However, more effort needs to be made in developing strategies for expansion of MSCs from aged individuals *ex vivo* in order to use them for repair or treatment of various disorders. But some hope stems from our observation that Notch signalling pathways might play a crucial role in the process

of expansion of MSCs in liver therefore, it should be possible to utilize those regulatory pathways to maintain the MSCs from an aged individual as long as needed to obtain a sufficient number of cells for clinical treatment.

Acknowledgements: The work was supported in part from KBN grant No. 3 P05D 036 23 (to PC), 3 P05D 048 25 (to RW) and Institutional grants NN-4-092/02, NN-1-007/03, and NN-4-044/04 (to ALS).

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Submitted: 3 January 2007
Accepted after reviews: 15 April, 2007