

研究成果の刊行に関する一覧表

別紙4
雑誌

発表者氏名	論文タイトル名	発表雑誌	巻号	ページ	出版年
Masato Tamai, Ryusuke nakaoka, Kazuo Isama, Toshie Tsuchiya	Novel calcium phosphate ceramics: The remarkable promoting action on the differentiation of the normal human osteoblasts, Bioceramics	Key Material Eng	Vol. 309- 311	97-100	2005
Ryusuke Nakaoka, Toshie Tsuchiya	Enhancement of differentiation and homeostasis of human osteoblasts by interaction with hydroxyapatite in microsphere form, Bioceramics	Key Material Eng	Vol. 309- 311	1293-1296	2005
Yuping Li, Tsutomu Nagira, Toshie Tsuchiya	The effect of hyaluronic acid on insulin secretion in HIT-T15 cells through the enhancement of gap junctional intercellular communication	Biomaterial	27	1437-1443	2005
Sadami tsutsumi, Duck-Young JUNG, Yu-Bong KANG, Toshie Tsuchiya	A Novel Non - Destructive Method To Measure Elastic Mduli Of Cartilage Cell In Situ	IFMBE	in press		2005
Ryusuke Nakaoka Saifuddin Ahmed, Toshie Tsuchiya	Hydroxy apatite microspheres enhance gap junctional intercellular communication of human osteoblasts composed of connexin 43 and 45	J Biomed Mater Res	74(2)	181-186	2005
Misao Nagahata, Ryusuke Nakaoka, Akira Teramoto, Koji Abe, Toshie Tsuchiya	The response of normal human osteoblasts to anionic polysaccharide polyelectrolyte	Biomaterials	26(25)	5138-5144	2005
Kazuo Isama, Toshie Tsuchiya	Osteoblast Differentiation and Apatite Formation on Gamma- Irradiated PLLA Sheets	Key Engineering Materials	288-289	409-412	2005
土屋利江	再生医療・繊維工 学・人工臓器に使用 される医療用材料の 安全性・有効性に関 する基本的考え方	繊維学会誌	61	148-149	2005

細胞	移植方法	患者数	参加条件	追跡期間(平均) or(MA) 結果	著者	出典
自己BMC	バルーンカテーテルで ステントを入れた部位に注入	20人 (更にコントロール13人)	心筋梗塞患者*	11±5ヶ月	Francisco Fernandez-Aviles, s Circulation Research	October 1, 2004 742-748
自己骨髓細胞	バルーンカテーテルで ステントを入れた部位に注入	60人ランダム化 (うちコントロール30人)	心筋梗塞患者	6ヶ月	Kai C Wollert.	Lancet 2004; 364: 141-48
自己BMC	カテーテルによる (血管造影・EMM・SPECTで位置決め)	20人 (うち9人コントロール)	虚血性の心不全	12ヶ月	Emerson C. Perin, MD, PhD Circulation. 2004;110 [suppl]II-213-II-218	
自己筋芽細胞	バイパス術の際に注射 (染色剤で見ながら)	10名	心筋梗塞	12ヶ月	Tomasz Siminiak, MD, PhD American Heart Journal Volume 148, Number 3 531-537	
自己CPCまたは 自己BMC	バルーンカテーテルで注入	59名(ランダム化) (28名BMC30名CPC) 4名	急性心筋梗塞	4ヶ月	Volker Schächinger, MD Journal of the American College of Cardiology Vol. 44, No. 8, 2004October 19, 2004:1690-9	
自己骨髓・末梢幹細胞共に集める (CD133陽性で純化)	バイパス術の際に注射	5名	冠動脈疾患	8ヶ月(4.5ヶ月)	Giulio Pompilio, MD PhD Ann Thorac Surg 2004;78:1808 -13	
鎖骨下静脈からの CD34陽性単核球分離純化	バイパス術の際に注入	19名 (+コントロール16名)	陳旧性心筋梗塞	52週	Abel Archundia Life Sciences 78 (2005) 279 - 283	
骨髓単核球のCD133陽性純化	ステント術の際に注入	9名	急性心筋梗塞	10±3ヶ月	Jozef Bartunek, MD, PhD Circulation. 2005;112[suppl]I-178-I-183.	
骨髓単核球のうち CD34陽性を磁石で分離(放射能ラベル)	カテーテルによるステント術の際 ガイドカテーテルのガイドで Transaccessカテーテル24ゲージ 針で注射	10名	急性心筋梗塞	不明	Michael Hofmann, MD, MS Circulation. 2005;111:2198-2202. (2005) 26, 1188-1195	
骨格筋細胞	ガイドカテーテルのガイドで Transaccessカテーテル24ゲージ 針で注射	10名	急性心筋梗塞	6ヶ月	Tomasz Siminiak European Heart Journal	
骨髓細胞(CD34陽性を分離)	バイパス術の際に注射	10名	心虚血疾患	32ヶ月(17.9ヶ月)	Hiroyuki Yacota The Journal of Nuclear Medicine; Oct 2005; 46, 10; J Thorac Cardiovasc Surg 2005;130:1631-8	
骨髓細胞 (CD34陽性が70パーセント以上になるように調製)	バイパス術の際に注射	20名 (10名がコントロール)	心虚血疾患	6ヶ月	Amit N. Patel, MD, MS, 8	
BMC・骨髓前駆細胞	バイパス術においてはすべてoffが 細な参加条件が決まら ない					
CPC・循環前駆細胞						

* * LVEF、Chordae shortening、LVSP/LVESVI、LVEDVI
細胞入れた群では入れないより有意にLVEF上昇²改善、LDEDV改善
1例を除き全例でNYHAクラス改善。LVEFも3例の不変を除き上昇
1例は6か月後に心筋梗塞死。そのほかはSPECTによる機能改善見られる

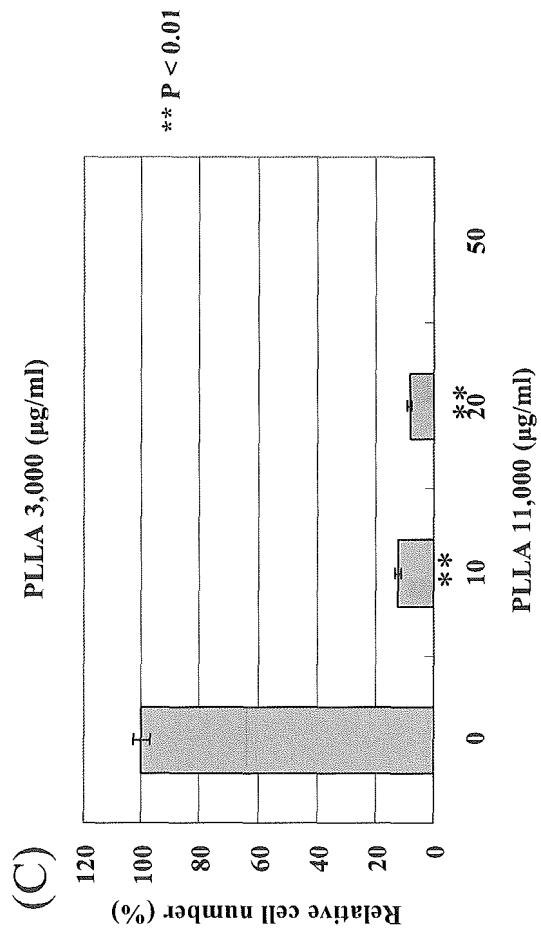
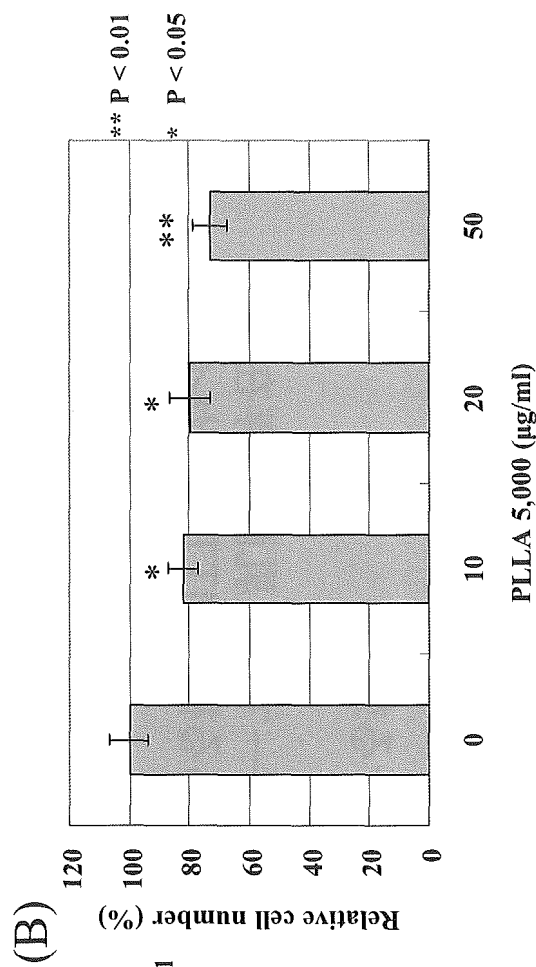
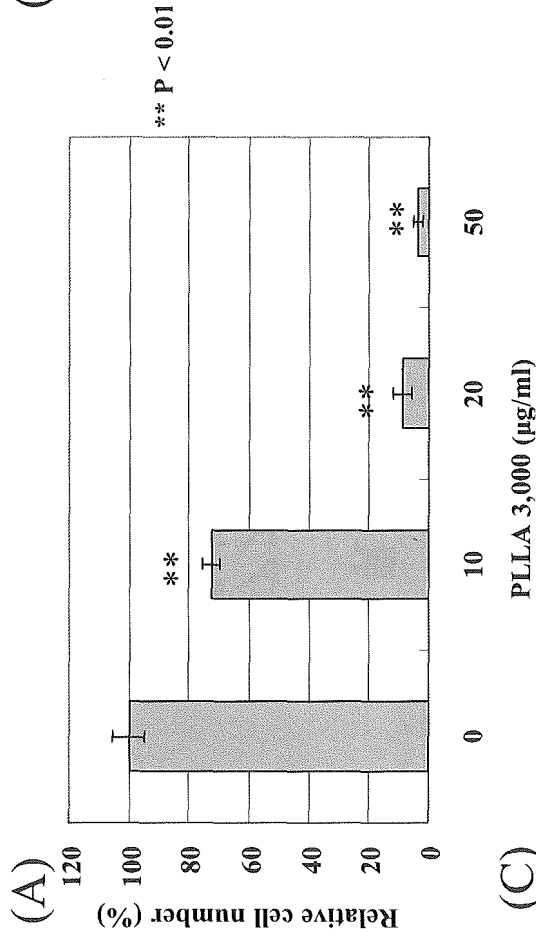


図1 3種のポリ-L-乳酸(PLLA)のヒトアストロサイトの増殖における抑制効果

ヒトアストロサイトを(A) PLLA 3,000 (B) PLLA 5,000 (C) PLLA 11,000存在下で1週間培養した後に、研究方法に記述してある様にてMTTアッセイを用いて細胞数を決定した。

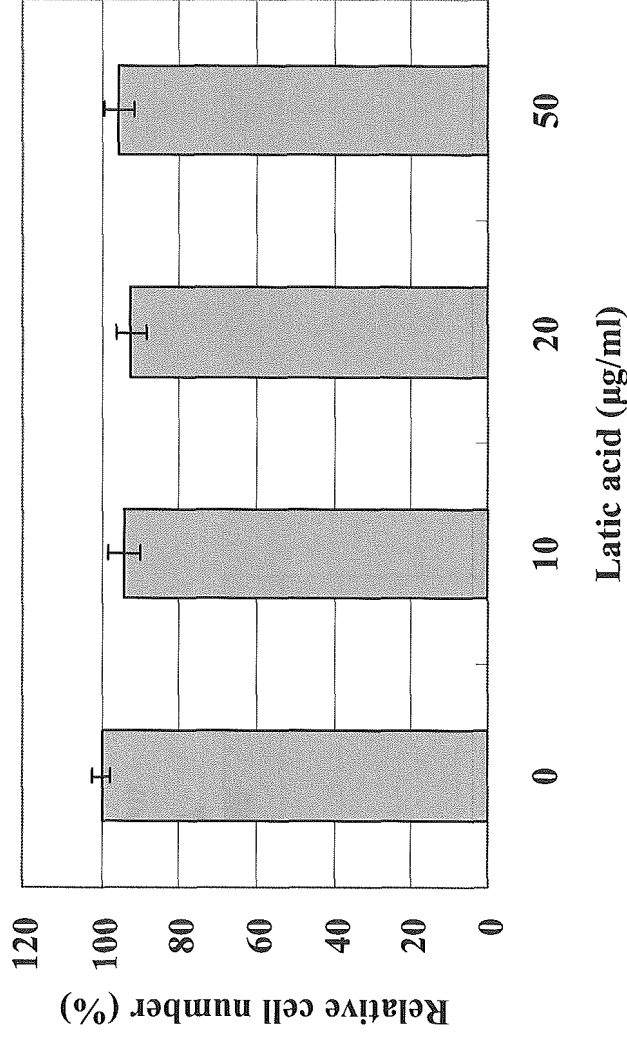


図2 乳酸のヒトアストロサイトの増殖における効果

ヒトアストロサイトを、ポリ・L・乳酸のモノマーである乳酸の存在下で1週間培養した後に、研究方法に記述してある様にMTT アッセイを用いて細胞数を決定した。

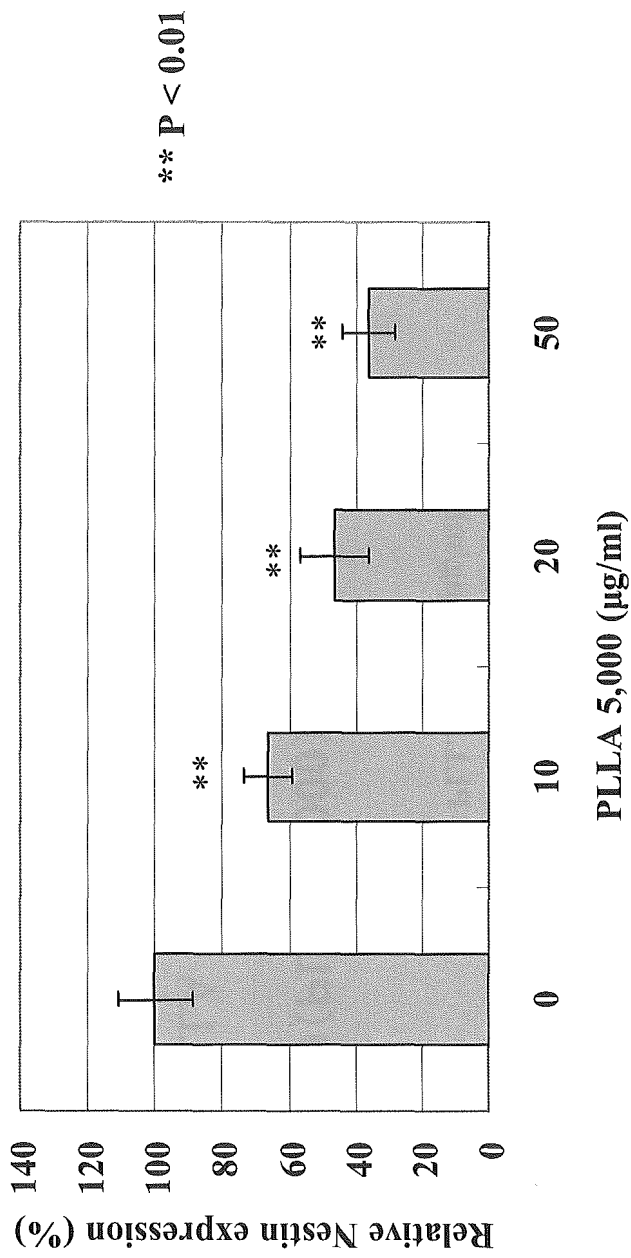


図3 ポリ-L-乳酸5000(PLLA 5,000)のNestinの発現における抑制効果

ヒトアストロサイトをPLLA 5,000存在下で1週間培養した後に、total RNAを抽出し、Nestinの発現量をリアル・タイムPCRを用いて検出した。

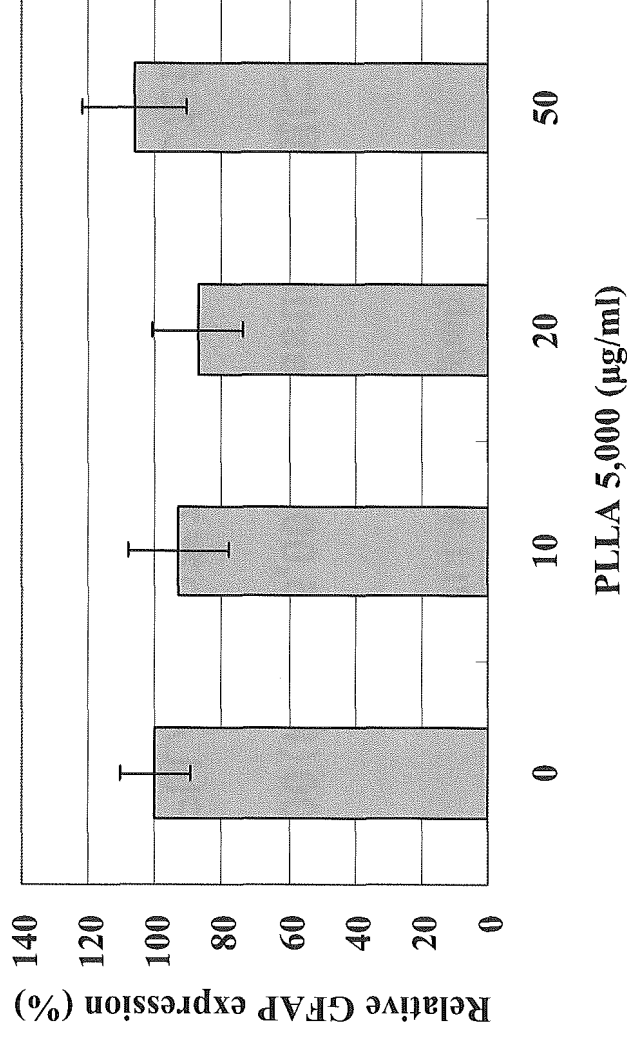
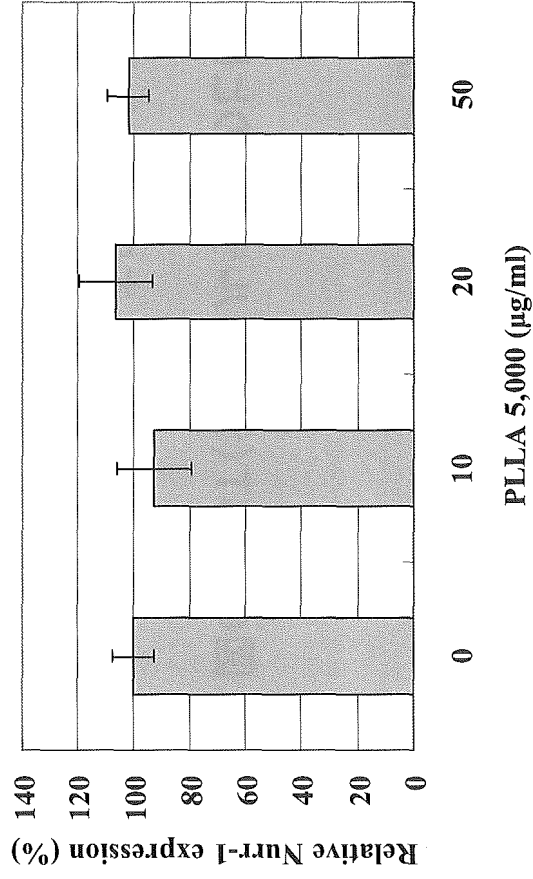


図4 ポリ・L・乳酸5000(PLLA 5,000)のGFAPの発現における効果

ヒトアストロサイトをPLLA 5,000存在下で1週間培養した後に、total RNAを抽出した。GFAP はアストロサイトの特異的マーカーである。GFAPの発現量をリアル・タイムPCRを用いて検出した。

(A)



(B)

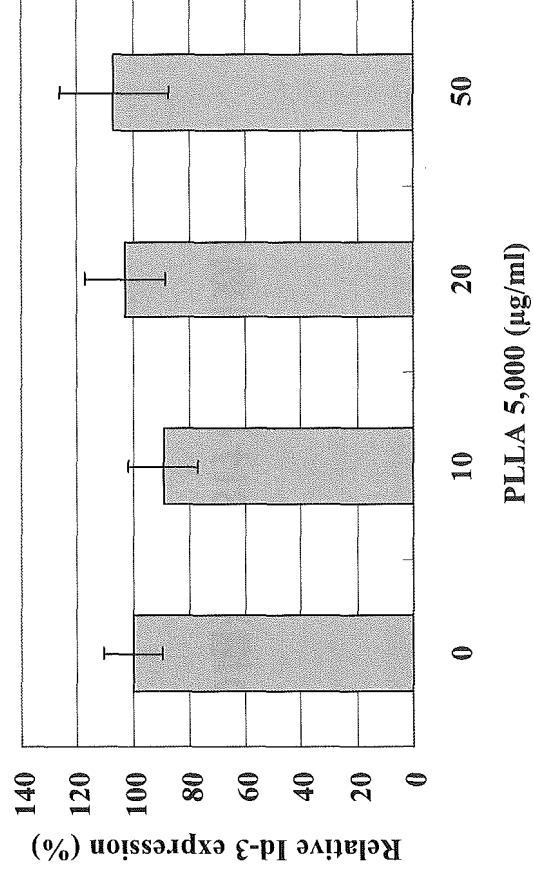


図5 ポリ-L-乳酸5000(PLLA 5,000)のNurr-1および Id-3の発現における効果

ヒトアストロサイトをPLLA 5,000存在下で1週間培養した後に、total RNAを抽出した。

(A) Nurr-1 (B) Id-3の発現量をリアル・タイムPCRを用いて検出した。

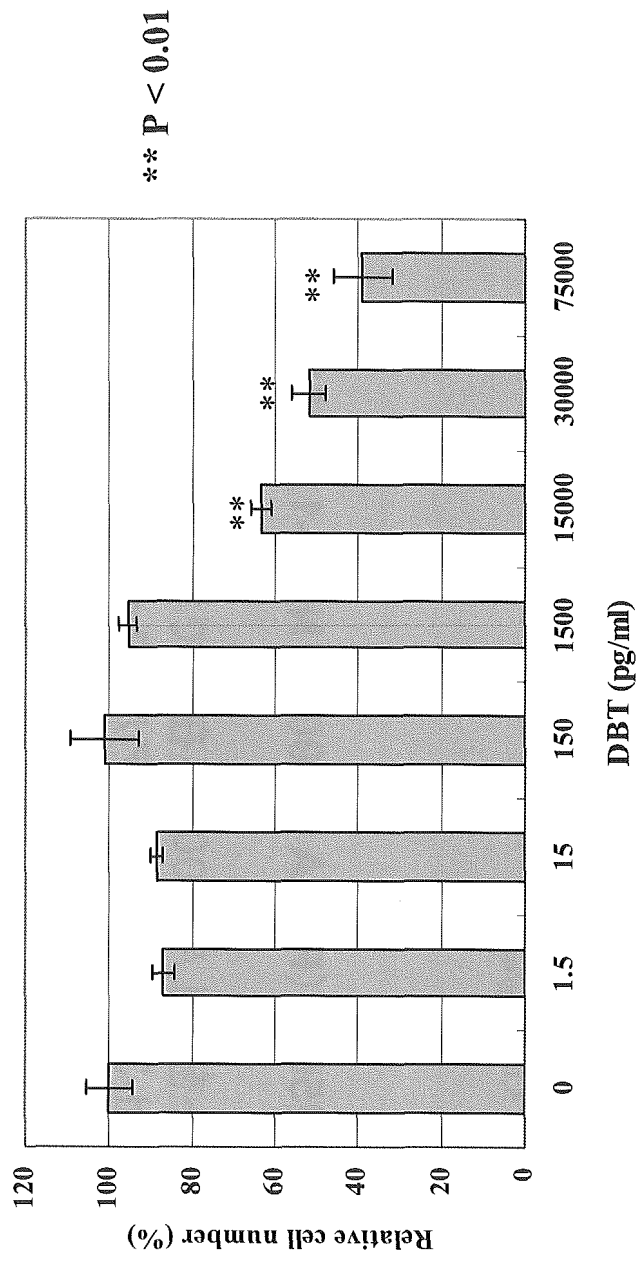


図6 DBT(ジブチルチン)のヒトアストロサイトの増殖における抑制効果

ヒトアストロサイトをDBT(ジブチルチン)存在下で1週間培養した後に、研究方法に記述してある様 にMTT アッセイを用いて細胞数を決定した。

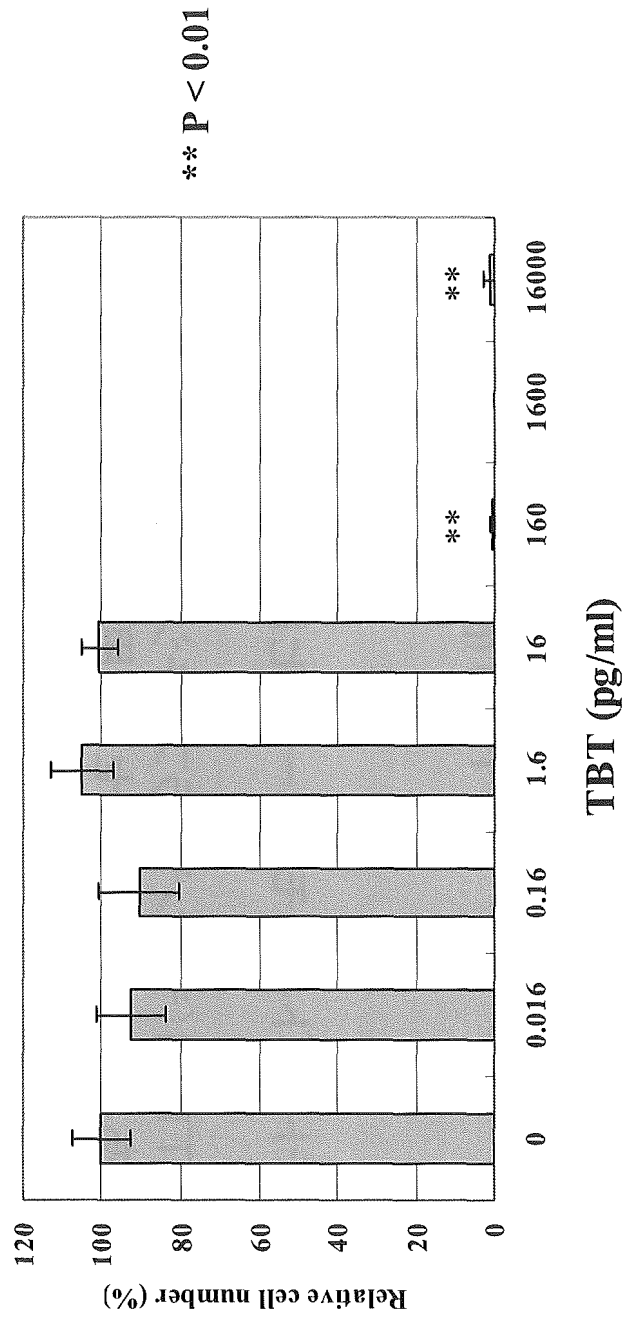


図7 TBT(トリブチルチン)のヒトアストロサイトの増殖における抑制効果
ヒトアストロサイトをTBT(トリブチルチン)存在下で1週間培養した後に、研究方法に記述してある様様にMTT アッセイを用いて細胞数を決定した。

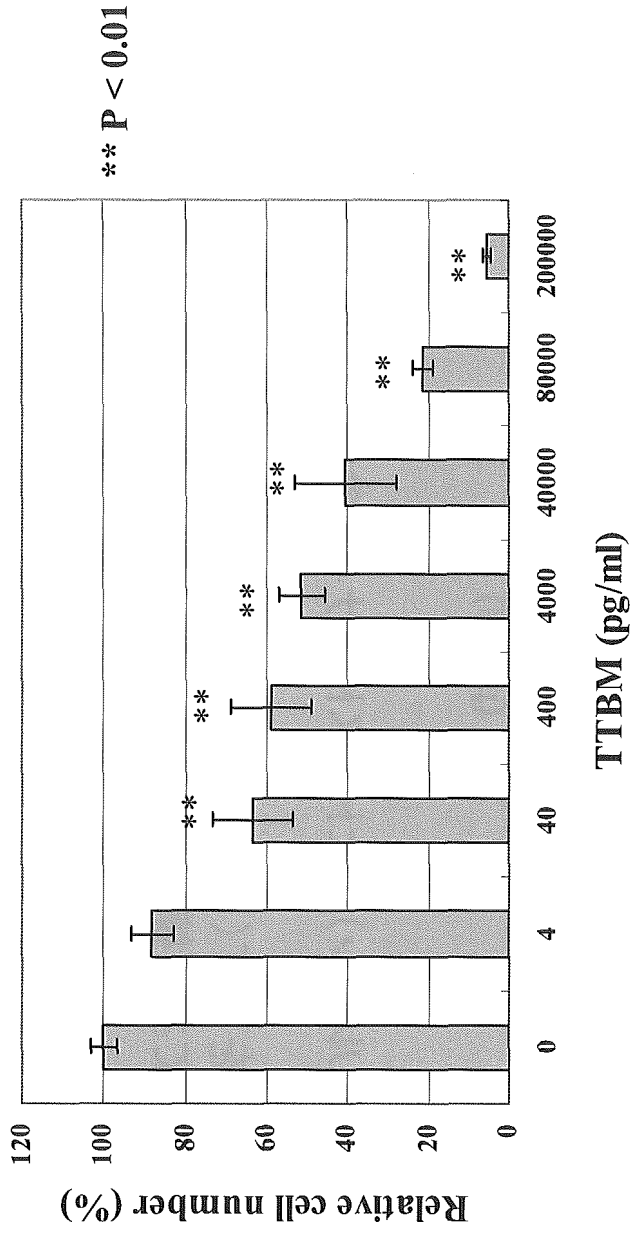


図8 TTBM(チタンテトラブトキシモノマー)のヒトアストロサイトの増殖における抑制効果

ヒトアストロサイトをTTBM(チタンテトラブトキシモノマー)存在下で1週間培養した後に、研究方法に記述してある様様にMTTアッセイを用いて細胞数を決定した。

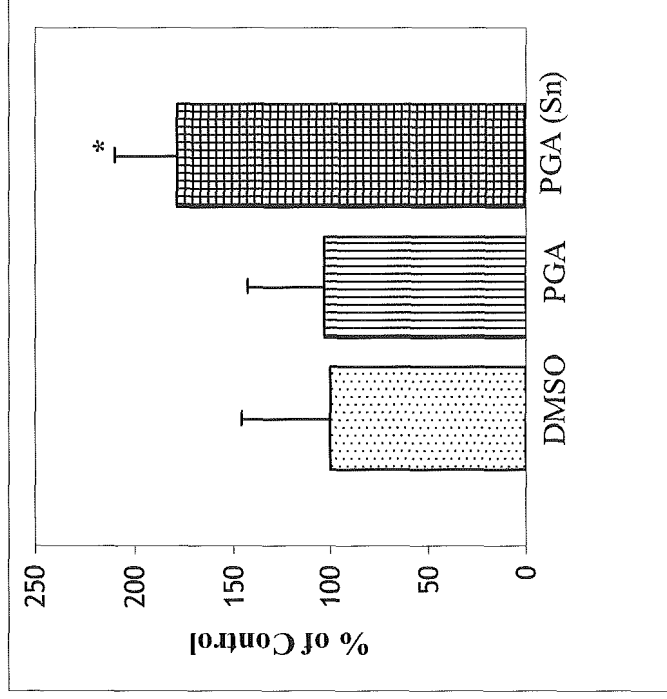


図9 クリスタルバイオレット染色評価によるヒト関節軟骨細胞の増殖
細胞増殖はPGA(Sn)培養軟骨細胞において増加している* $p < 0.05$.

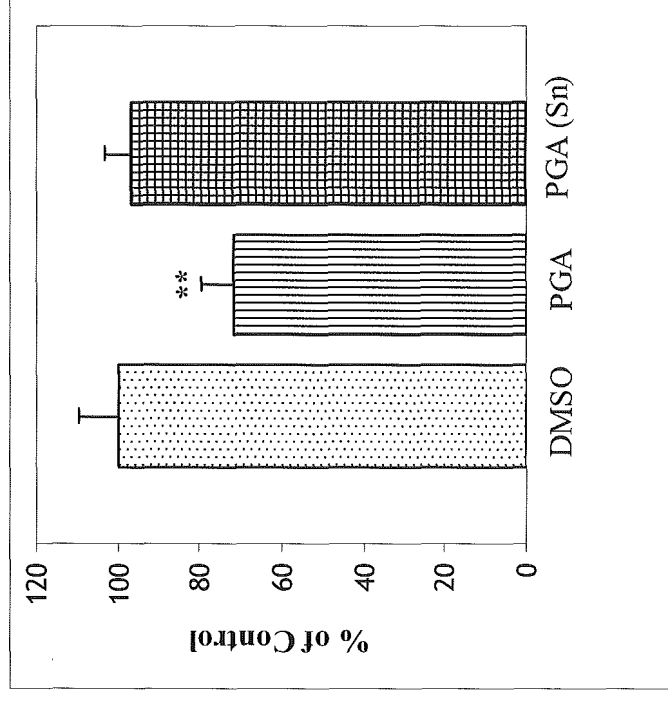


図10. アルシアンブルー染色法によるヒト関節軟骨細胞の分化

細胞の分化は、PGA培養軟骨細胞で阻害されていた。 $**p < 0.01$.

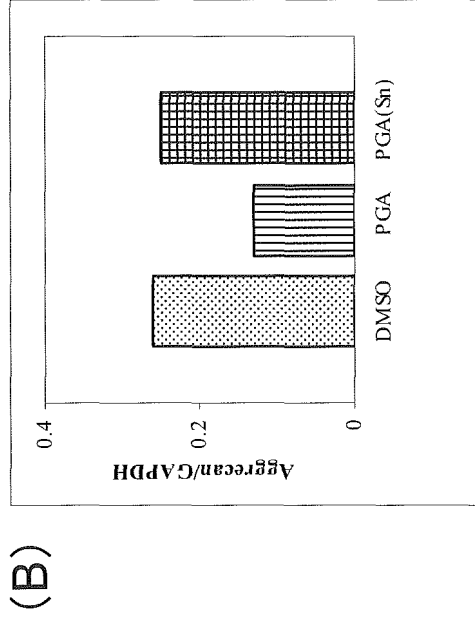
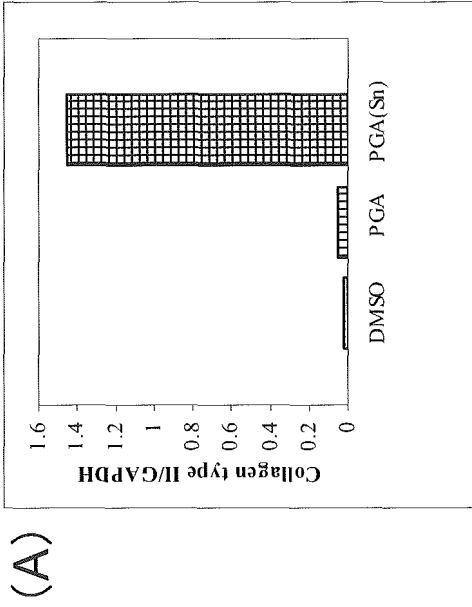


図11. real-time PCR.による細胞外マトリックス遺伝子発現

(A) PGA(Sn)で処理された軟骨細胞がPGA処理された軟骨細胞よりもコラーゲンタイプII 遺伝子を強く発現していた。

(B) アグリカン遺伝子発現はPGA,により阻害されたが、PGA(Sn)とコントロールの間に差は見出せなかった。



p57^{Kip2} is expressed in quiescent mouse bone marrow side population cells[☆]

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Abstract

Hematopoietic stem cells can be accurately identified by the side population (SP) phenotype. It has been previously shown that hematopoietic stem cells are cell cycle arrested, but the mechanisms involved are currently poorly understood. In the present study, results from quantitative real-time RT-PCR show that while SP cells have increased expression of various cyclins and cyclin-dependent kinases, the increased expression of cyclin-dependent kinase inhibitors, in particular p57^{Kip2}, is responsible for the observed cell cycle arrest. In addition, gene expression analysis of c-kit⁺/Sca-1⁺/Lineage⁻ SP (KSL-SP) cells demonstrates that only p57^{Kip2} shows both higher expression compared to both SP and non-SP cells. Furthermore, immunostaining also demonstrates significantly higher protein expression in KSL-SP cells. These results demonstrate that the maintenance of bone marrow SP cells in G0/G1 may be carefully controlled by p57^{Kip2}.
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Keywords: Side population; Hematopoietic stem cell; Cell cycle arrest; Cyclin-dependent kinase inhibitor; p57^{Kip2}

Hematopoietic stem cells (HSCs) are capable of self-renewal and govern the long-term repopulation of all cell types of the blood forming system [1]. A recently developed technique has employed the differential Hoechst 33342 staining of HSCs to identify a small fraction of bone marrow cells termed “side population” (SP) cells [2]. SP cells have been shown to be highly enriched for HSCs and represent approximately 0.1% of adult nucleated bone marrow cells in mice [3]. This unique ability of SP cells in effluxing Hoechst 33342 is mediated by the ATP-binding cassette

transporter G2 (ABCG2), a member of the multiple drug resistance (MDR) family of cell surface transporters [4–6]. SP cells have been found in the hematopoietic compartment of various species [7–10], as well as in other tissues [11–18], demonstrating that the SP phenotype may be a representative characteristic of adult tissue-specific stem cells [19].

A defining feature of adult HSCs is that they are known to reside in the G0/G1 phases of the cell cycle, representing the quiescent state [20], a characteristic which is evidenced by their relatively strong resistance to retroviral infection and cell cycle-specific chemotherapeutic drugs. In somatic cells, the initiation of DNA replication and the associated progression through the cell cycle is known to be controlled by the activity of several classes of cyclin-dependent kinases (Cdks), which are activated by the binding of their respective cyclins. In addition, Cdk activity is carefully regulated by the expression of Cdk inhibitors which can bind to cyclin/Cdk complexes, to prevent cell cycle progression. These Cdk inhibitors are separated into the INK4 family,

[☆] Abbreviations: HSC, hematopoietic stem cell; SP, side population; ABCG2, ATP binding cassette transporter G2; MDR, multiple drug resistance; Cdk, cyclin-dependent kinase; INK4, inhibitor of cyclin-dependent kinase 4; FACS, fluorescence activated cell sorting; NSP, non-side population; KSL-SP, c-kit⁺/Sca-1⁺/Lineage⁻ side population; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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which prevents transfer from G1 to S by inhibiting cyclin D/Cdk 4 complex formation; and the Cip/Kip family which inhibits the activities of various cyclin/Cdk complexes.

While the mechanisms governing HSC quiescence are currently not well understood, intuitively it seems that differential expression of proteins involved in mitotic checkpoints may account for the observed cell cycle arrest. It has previously been shown that the induction of the Cdk inhibitors p15^{INK4b} and p21^{Cip1} has an important role in the transforming growth factor (TGF)- β mediated cell cycle arrest in melanoma cells [21]. It was also recently reported that the TGF- β induced cell cycle arrest in hematopoietic progenitor cells was correlated to an increase in p57^{Kip2} expression [22]. p57^{Kip2} has similarly been shown to have an inhibitory effect in the hematopoietic family, by preventing cell cycle progression in human T-lymphocytes [23].

In the present study, we hypothesized that differential expression of various molecules involved in cell cycle checkpoints that regulate progression from G1 to S phase was responsible for the cell cycle arrest observed in bone marrow SP cells. Our findings show that SP cells have increased expression of various cyclins and Cdks, and should therefore have high proliferative potential, but that the increased expression of Cdk inhibitors, in particular p57^{Kip2}, blocks the activity of these cyclin/Cdk complexes resulting in the observed quiescent state of HSCs.

Materials and methods

Cell preparation. C57BL/6 mouse (Sankyo Lab Service, Tokyo, Japan) bone marrow cells were obtained by flushing of excised femurs with Hanks' Balanced Salt Solution (Sigma, St. Louis, MO) containing 5% fetal bovine serum (FBS; Moredgate Biotech, Queensland, Australia). Cells were collected by centrifugation, followed by disruption of red cells with 0.2% sodium chloride solution. Cells were then washed two times with Dulbecco's phosphate-buffered saline (PBS; Sigma) prior to staining and cell sorting.

Hoechst 33342 exclusion assay using fluorescence activated cell sorting (FACS). Analysis and sorting of SP cells were performed as described previously [17]. Briefly, isolated bone marrow cells were stained with 5 μ g/ml Hoechst 33342 (Sigma) at a concentration of 10⁶ cells/ml in staining medium (Dulbecco's modified eagle's medium (DMEM) containing 2% FBS and 10 mM Hepes) for 90 min at 37 °C. For inhibition experiments, 50 μ M of R (+)-verapamil (Sigma) was added to the staining medium 30 min before the addition of Hoechst 33342. After staining, cells were resuspended in PBS containing 2% FBS and 1 mM Hepes. Prior to analysis and cell sorting, propidium iodide (Sigma) was added at a final concentration of 2 μ g/ml, to distinguish between live and non-viable cells. Analysis and cell sorting were then performed using a dual laser fluorescence-activated cell sorter (EPICS ALTRA FACS analysis system, Beckman Coulter, Fullerton, CA).

For the isolation of c-kit⁺, Sca-1⁺, Lineage⁻ SP (KSL-SP) cells, lineage marker-positive cells were first eliminated by magnetic cell sorting (Auto MACS system, Miltenyi Biotec, Bergisch Gladbach, Germany) using the Lineage cell Depletion Kit (Miltenyi Biotec), prior to staining with Hoechst 33342. Lineage⁻ cells were stained with Hoechst 33342 and then incubated with FITC-conjugated anti-Sca-1 antibody (E13-161.7, BD Biosciences Pharmingen, San Jose, CA) and PE-conjugated anti c-kit antibody (2B8, BD Biosciences Pharmingen) for 30 min on ice. Stained cells were then subjected to sorting by FACS.

Cell cycle analysis. Bone marrow SP cells and total bone marrow cells were pelleted by centrifugation and resuspended in a solution containing 4 mM sodium citrate (pH 7.6), 0.2% Nonidet P-40, and 50 μ g/ml propidium iodide. After incubation on ice for 30 min, cell suspensions were treated with 0.25 mg/ml RNase A for 15 min at 37 °C to remove double-stranded RNA. Cells were finally analyzed by flow cytometry at an excitation wavelength of 488 nm.

Gene expression analysis. For gene expression assays, total RNA was obtained from 10,000 SP, non-side population (NSP), or KSL-SP cells using Isogen (Nippongene, Tokyo, Japan) according to the manufacturer's suggested protocol. Single-stranded cDNA was created with the Superscript First-strand System for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (Invitrogen, Carlsbad, CA), and used as PCR templates. Primer pairs and Taqman MGB probes labeled with 6-carboxy-fluorescein (FAM) at the 5'-end and non-fluorescent quencher at the 3'-end were designed with the Taqman gene expression assay (Applied Biosystems). Quantitative PCR was performed with 7300 Real-Time PCR System (Applied Biosystems). Thermocycling programs consisted of an initial cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. All assays were run in duplicate for more than four individual samples. mRNA expression levels were normalized with the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To compare mRNA expression between SP, NSP, and KSL-SP cells, the Mann-Whitney rank sum test was applied. Statistics were calculated using SigmaStat 2.0 (SPSS, Chicago, IL).

Immunofluorescence analysis. Cells isolated by FACS were fixed with Methacarn fixation solution, followed by centrifugation onto glass slides. After incubation with 1% bovine serum albumin (Sigma) to block non-specific reactions, cells were incubated with a 1/50 dilution of anti-p57^{Kip2} antibody (E-17, Santa Cruz Biotechnology, Santa Cruz, CA), for 1 h at room temperature, followed by washing three times with PBS. Cells incubated identically with normal goat IgG were used as negative controls. After incubation with a 1/200 dilution of Alexa Fluor 546-conjugated secondary antibodies (Molecular Probes, Eugene, OR) for 1 h at room temperature, cells were again washed three times with PBS. Stained cells were finally counter-stained with 10 μ g/ml Hoechst 33342 to visualize cell nuclei, and observed using confocal laser scanning microscopy (TCS-SP, Leica Microsystems AG, Wetzlar, Germany).

Results

The SP phenotype is characterized by low blue and red fluorescence intensity on a density plot due to the efflux of Hoechst 33342 via ABCG2. Mouse bone marrow cells show a typical SP staining pattern, comparable to previously reported results [24,25] (Fig. 1A). Additionally, the SP phenotype could not be detected when bone marrow cells were incubated with verapamil, an MDR inhibitor, prior to staining with Hoechst 33342 (Fig. 1B). Quantitative RT-PCR confirmed that SP cells with low Hoechst-derived fluorescence (Fig. 1A, gated cells) had significantly higher expression of ABCG2, the mediator of the SP phenotype (Fig. 1C), as well as Bmi-1, an important factor in the self-renewal of HSCs [26] (Fig. 1D), compared to NSP cells. These results confirmed that cells with low fluorescence intensity were indeed an SP cell population that is enriched for HSCs. To verify that SP cells represented a quiescent HSC population, cell cycle analysis was performed. Although the cell cycle of total bone marrow cells was promoted with active cell division (Fig. 1E), SP cells were nearly all growth arrested in G0/G1, verifying their quiescent state (Fig. 1F).

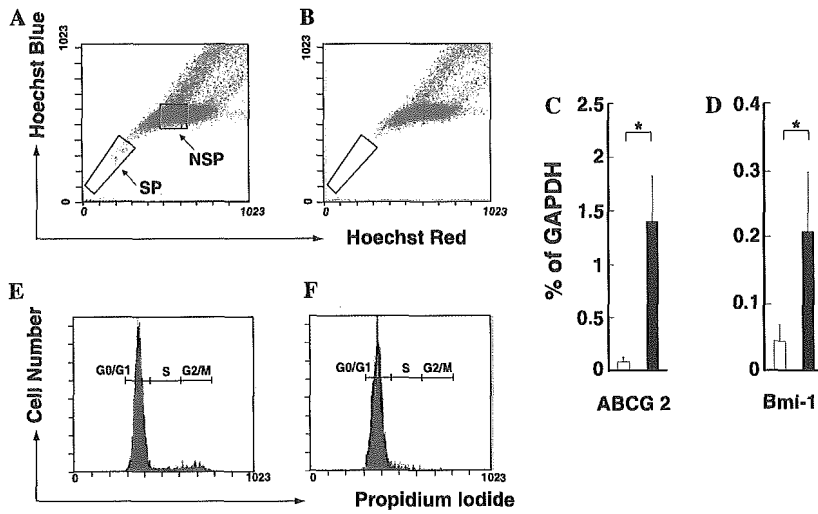


Fig. 1. Mouse bone marrow side population (SP) cells. Bone marrow cells were analyzed for Hoechst 33342 efflux by FACS (A). In the dot plot of (A), cells denoted by each enclosed area were regarded as SP cells or NSP cells for further characterization. When treated with Verapamil, an inhibitor of MDR, Hoechst 33342 efflux was antagonized (B). The expression of the stem cell markers, ABCG2 (C) and Bmi-1 (D), was quantified by real-time RT-PCR. Expression levels were determined from NSP cells: white bar and SP cells: black bar, for each individual mRNA. Data represent mean values from five samples, all performed in duplicate. Error bars indicate the SD ($*p < 0.05$). Additionally, the cell cycle phase in which each individual cell resided from either all viable bone marrow cells (E) or SP cells (F) was detected by flow cytometry.

As SP cells were confirmed to be growth arrested the G1 phase of the cell cycle, we next examined the mRNA expression of cyclin D, cyclin E, Cdk 2, Cdk 4, and Cdk 6, which are required for progression from G1 to S in the cell cycle. Interestingly, all genes examined were more highly expressed in SP cells compared to NSP cells, with significantly greater expression observed for cyclin D1, cyclin D2, and Cdk 4. Therefore, our results indicate that SP cells have high proliferative potential, but that other regulatory mechanisms are likely present to ensure the growth arrest and quiescent state of HSCs (Fig. 2).

As expression of G1- and S-phase Cdk and cyclin mRNAs was higher in SP cells compared to NSP cells,

we examined the mRNA expression of the INK4 and Cip/Kip families of Cdk inhibitors. In the INK4 family, both p15^{INK4b} and p16^{INK4a} were barely expressed in either cell fraction (Figs. 3A and B). Additionally, while expression of p18^{INK4c} was higher in SP cells (Fig. 3C), p19^{INK4d} mRNA levels were actually slightly higher in the NSP cell fraction (Fig. 3D). In the Cip/Kip family, both p21^{Cip1} and p27^{Kip2} demonstrated significantly higher expression in SP cells compared to NSP cells, while p27^{Kip1} mRNA expression was higher in NSP cells (Figs. 3E–G).

To investigate the relationship between the observed increase in expression of specific CDK inhibitors and SP cell cycle arrest, bone marrow SP cells were further enriched for

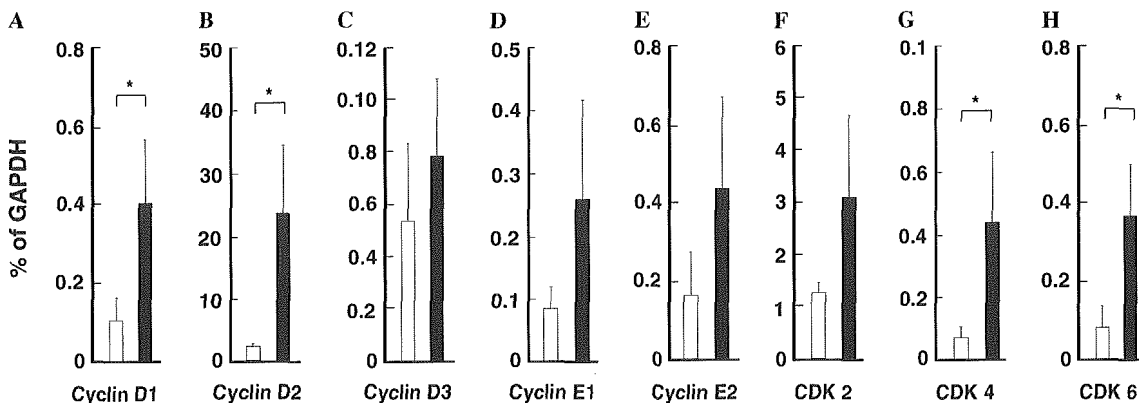


Fig. 2. Expression of cyclin and cyclin-dependent kinase (Cdk) mRNAs in SP and NSP cells. Total RNA was extracted from bone marrow SP cells and NSP cells after FACS, and subjected to real-time quantitative RT-PCR. Relative expression of the selected genes was normalized to that of GAPDH for each sample. mRNA expression of Cyclin D1 (A), Cyclin D2 (B), Cyclin D3 (C), Cyclin E1 (D), Cyclin E2 (E), Cdk 2 (F), Cdk 4 (G), and Cdk 6 (H) is shown. Expression levels were determined from NSP cells: white bar and SP cells: black bar, for each individual mRNA. Data represent mean values from five samples, all performed in duplicate. Error bars indicate the SD ($*p < 0.05$).

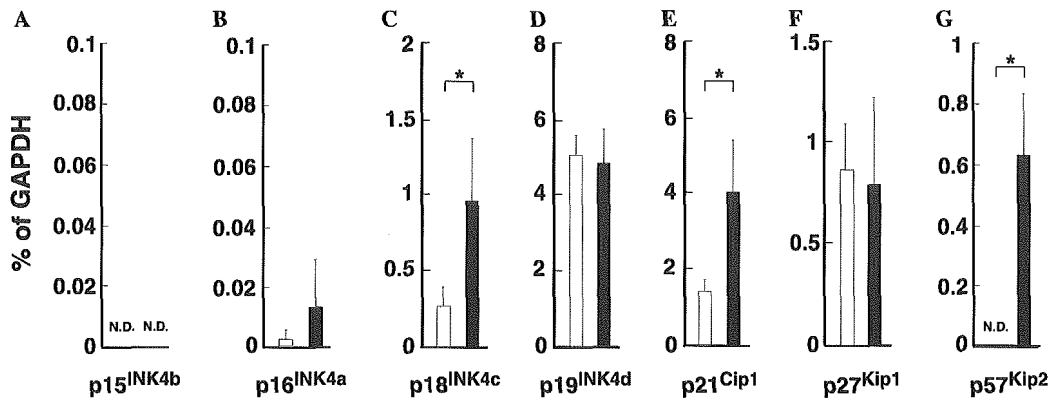


Fig. 3. Expression of cyclin-dependent kinase (Cdk) inhibitor mRNAs in SP and NSP cells. Total RNA was extracted from bone marrow SP cells and NSP cells after FACS, and subjected to real-time quantitative RT-PCR. Relative expression of the selected genes was normalized to that of GAPDH for each sample. mRNA expression of p15^{INK4b} (A), p16^{INK4a} (B), p18^{INK4c} (C), p19^{INK4d} (D), p21^{Cip1} (E), p27^{Kip1} (F), and p57^{Kip2} (G) is shown. Expression levels were determined from NSP cells: white bar and SP cells: black bar, for each individual mRNA. Data represent the mean value from five samples, all performed in duplicate. Error bars indicate the SD (* $p < 0.05$).

quiescent hematopoietic stem cells by the depletion of lineage-positive cells (Fig. 4A), followed by isolation of c-kit⁺ and Sca-1⁺ cells (Figs. 4B and C). mRNA expression of the SP cell marker ABCG2, as well as those CDK inhibitors that showed significantly higher expression in SP cells (p18^{INK4c}, p21^{Cip1}, and p57^{Kip2}), was then examined in the resulting KSL-SP cell population.

Our results demonstrated that KSL-SP cells had much higher expression of ABCG2 and p57^{Kip2} compared to both SP and NSP cells (Figs. 4D and G), but that the expression of both p18^{INK4c} and p21^{Cip1} showed insignificant differences between SP and KSL-SP cells (Figs. 4E and F). To examine p57^{Kip2} protein expression, immunofluorescence was performed with anti-p57^{Kip2} antibodies. Staining visualized with confocal laser scanning microscopy demonstrated that KSL-SP cells had higher expression of p57^{Kip2} protein, compared to NSP cells (Figs. 4H–M). In addition, while KSL-SP cells demonstrated p57^{Kip2} expression that was nearly completely localized to cell nuclei (Figs. 4I, K, and M), the observed low level of p57^{Kip2} in NSP cells showed almost completely cytoplasmic staining (Fig. 4H, J, and L).

These results indicated the possibility that the cell cycle arrest of quiescent bone marrow SP cells was correlated to increased expression of specific Cdk inhibitors, in particular p57^{Kip2}. Therefore, although SP cells may have a strong potential for proliferation due to their increased expression of G1- and S-phase cyclins and Cdks, increased expression of p57^{Kip2} and other Cdk inhibitors was able to induce cell cycle arrest via inhibition of cyclin/Cdk complexes.

Discussion

In the present study, we showed the possibility that the cell cycle arrest observed in bone marrow SP cells, in particular KSL-SP cells, is induced by the Cdk inhibitor p57^{Kip2}. p57^{Kip2} is a well-known member of the Cip/Kip

family of Cdk inhibitors that function by binding to cyclin/Cdk complexes to inhibit progression through the cell cycle. Overexpression of p57^{Kip2} has been shown to strongly inhibit somatic cell proliferation [27–29] and p57^{Kip2} has also been shown to be involved in the cell cycle arrest of immature cells in other tissues, such as the retina [30,31] and brain [13]. It was recently reported that TGF- β induced cell cycle arrest of human cord blood derived progenitors required the increased expression of p57^{Kip2} [22]. p57^{Kip2} has also been shown to have genomic imprinting [32,33], which suggests a role in fetal development, as a substantial amount of imprinted genes have critical functions in the control of fetal growth. Therefore, a loss of heterozygosity induces down-regulation and can result in tumor formation [32,34]. It seems likely that p57^{Kip2} is intimately involved in the supervision of cell cycle progression and carefully regulates cell proliferation.

While expression of both p18^{INK4c} and p21^{Cip1} was also significantly higher in SP cells, the difference in the expression of p57^{Kip2} between SP and NSP cells was greatest, with no p57^{Kip2} mRNA detected in NSP cells. Moreover, KSL-SP cells, a cell population more highly enriched for quiescent hematopoietic stem cells, demonstrate a much higher expression of both ABCG2 and p57^{Kip2} than both SP and NSP cells. Hematopoietic stem cells are known to be extremely resistant to various external stresses [35], and the demonstration that the SP cell marker ABCG2 has significantly higher expression in KSL-SP cells compared to both SP and NSP cells further emphasizes that KSL-SP cells are highly enriched for quiescent stem cells.

Previously, Cheng et al. [36] demonstrated that p21^{Cip1} was involved in the maintenance of hematopoietic stem cell quiescence. In the present study, our results also showed that p21^{Cip1} had slightly higher expression in KSL-SP cells. While we cannot exclude the possibility that p21^{Cip1} plays a role in governing the quiescent state of KSL-SP cells, in the previous report by Cheng and colleagues, the authors did

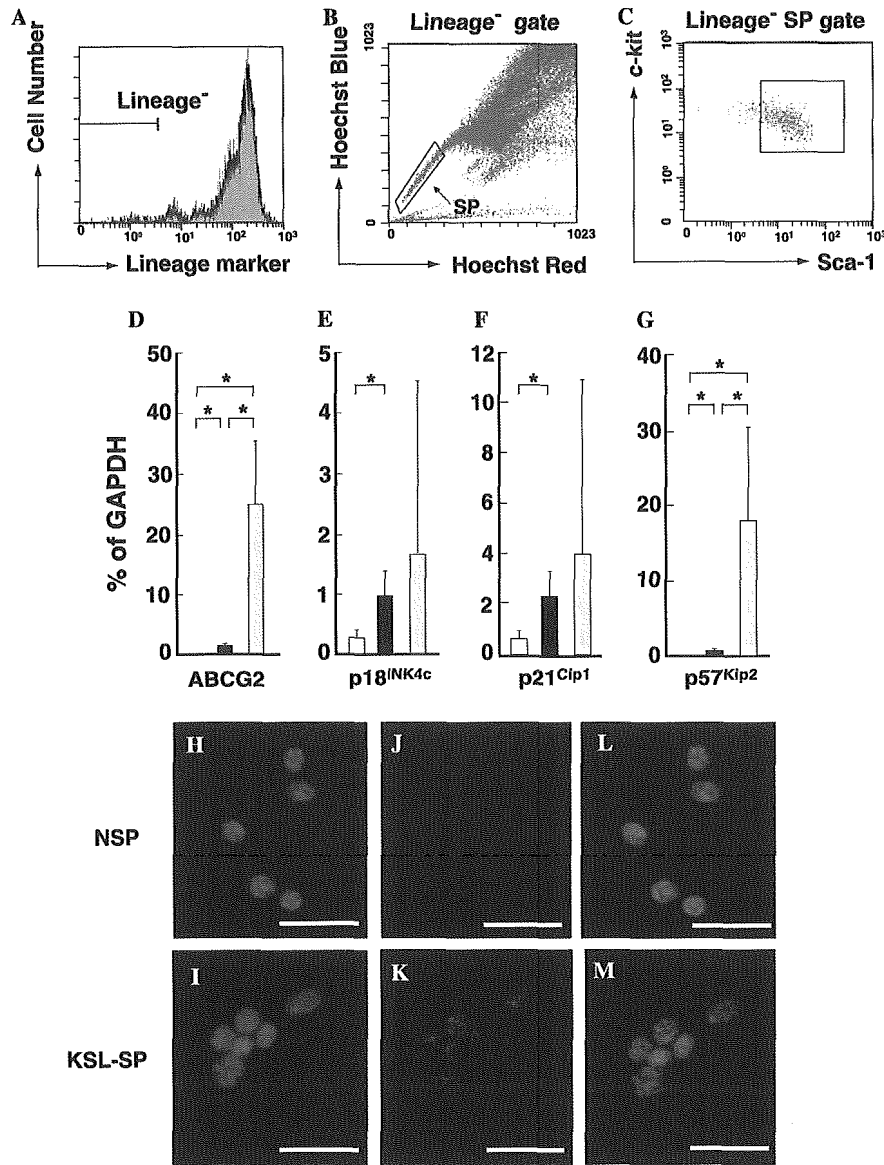


Fig. 4. KSL-SP cells show a high expression of p57^{Kip2}. KSL-SP cells were defined by the Lineage⁻ gate in (A). Lineage⁻-SP cells (B) were then selected as Sca-1⁺ and c-kit⁺ (C) for the isolation of KSL-SP cells. mRNA expression of ABCG2 (D), p18^{INK4c} (E), p21^{Cip1} (F), and p57^{Kip2} (G) was then examined using real-time quantitative RT-PCR. Expression levels were determined from NSP cells: white bars, SP cells: black bars, and KSL-SP cells: gray bars, for each individual mRNA. Data represent mean values from four to five samples, all performed in duplicate. Error bars indicate the SD (**p* < 0.05). Immunofluorescence analysis was performed on NSP cells and KSL-SP cells. Panels represent NSP (H,I) and KSL-SP (J,K,M) cells, stained with Hoechst 33342 (H,I), anti-p57^{Kip2} antibody (J,K). (L,M) show merged images. Scale bars represent 20 μm.

not examine the effects of p57^{Kip2}. In addition, our findings that p57^{Kip2} expression is specific to SP cells, and in particular KSL-SP cells lead us to believe that p57^{Kip2} is likely the major factor involved in maintaining cell cycle arrest of KSL-SP cells.

Similarly, previous reports have also demonstrated the importance of p57^{Kip2} in the control of the cell cycle. p57^{Kip2} knockout mice often die before birth or else immediately afterwards, due to abnormal apoptosis and morphogenesis [37,38]. In contrast, mice having knockouts for the other Cdk inhibitors examined here, such as

p18^{INK4c} [39,40] and p21^{Cip1} [41,42], can survive post-natally. Therefore, p57^{Kip2} seems to be the Cdk inhibitor that is most critically involved in the proper maintenance of cell cycle kinetics.

In many ways, the cancer phenotype resembles a stem cell phenotype, as several cancers are known to express ABCG2, multi-drug resistance protein (MRP), and MDR, and improper regulation of stem cell dynamics is also thought to result in malignant transformation. Loss of function and altered imprinting resulting in altered expression of p57^{Kip2} are known to cause several types of

cancers [43–47] and p57^{Kip2} has been shown to have the strongest tumor suppressor activity in the Cip/Kip family [48]. Therefore, p57^{Kip2} likely has a prominent role in inhibiting cell division.

Interestingly, we also found that SP cells may have a high potential for proliferation due to the increased expression of cyclin D and cyclin E as well as Cdk 2, Cdk 4, and Cdk 6 that are known to promote DNA synthesis and therefore cell cycle progression. Similarly, it has previously been shown that the marker of proliferating cells, Bmi-1, is required for the self-renewal of HSCs, but is also known to decrease the expression of p16^{INK4a} and p19^{INK4d} [26,49,50], and increase telomerase activity [51], which is believed to be indicative of actively proliferating cells. Therefore, it seems that some other regulatory factor, likely p57^{Kip2}, is necessary for SP cells to maintain their quiescent state despite their high proliferative potential from increased expression of Bmi-1 as well as, G1- and S-phase cyclins and Cdks.

It has previously been shown that the single characteristic that most accurately identifies a quiescent stem cell population in the bone marrow is the SP phenotype [35]. Bone marrow SP cells have indeed been shown to have the capability for the long-term multi-lineage reconstitution of the hematopoietic system and to reside in a quiescent state in the bone marrow niche [2,35]. It is believed that SP cells from the isolated from the bone marrow by Hoechst 33342 exclusion assays more accurately represent quiescent stem cells than HSCs characterized by other means [35]. Therefore, differences in gene expression between SP and NSP cells isolated from the bone marrow should accurately represent differences between quiescent stem cells and more highly differentiated cells. Because HSCs must govern the long-term maintenance of the entire hematopoietic system, it seems likely that these stem cells require a high proliferative potential to be able to sustain hematopoiesis throughout the host lifetime. However, HSCs must also remain cell cycle arrested in order to carefully regulate this proliferation over time and thus prevent dysfunctions of the hematopoietic system such as various cancers. We therefore hypothesize that the increased expression of the cyclins and Cdks involved in S-phase promotion demonstrates the high proliferative potential of bone marrow SP cells to ensure successful long-term re-population, but that p57^{Kip2} acts to inhibit these cyclin/Cdk complexes and therefore maintain the quiescent state of HSCs and carefully regulate the generation of differentiated progeny.

Previous results have demonstrated that the bone marrow niche in which HSCs reside is critically involved in the maintenance of the quiescent state of the HSCs due to signaling from HSC surface molecules such as Tie-2 [35,52]. It seems likely that the stem cell niche in the bone marrow may be critically involved in inducing p57^{Kip2} expression in order to preserve cell cycle arrest. Further study is needed to identify the system and mechanisms that regulate the expression of p57^{Kip2} such that the proliferation of SP cells, isolated not only from bone marrow, but

also other tissues, can be carefully controlled by modifications of gene expression. In the present study, we demonstrate the likely involvement of p57^{Kip2} in inhibiting cell cycle progression of bone marrow SP cells, and thus present a novel mechanism for the maintenance of the quiescent state of HSCs. The careful management of the proliferation of SP cells, which can be found in various tissues, may thus allow for improved applications of cell-based therapies, such as regenerative medicine.

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