Genomics & Informatics Vol. 3(1) 8-14, March 2005

Gene Expression Analysis of Megakaryocytes Derived from Human Umbilical Cord CD34$^+$ Cells by Thrombopoietin

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Abstract

Although much is known about the molecular biology of platelets, the megakaryocytes' (MKs) molecular biology was not understood so well because of their rareness. By the cloning and characterization of thrombopoietin (TPO), which is the principal regulator of the growth and development of the MKs, researches on the MKs have been growing rapidly. To understand megakaryocytopoiesis, we investigated the gene expression profile of the MKs using oligonucleotide microarray where 10,108 unique genes were spotted. Comparing the fluorescence intensities of which ratio is $\geq |2|$, 372 genes were up-regulated and 541 genes were down-regulated in MKs. For confirmatory expression, RNase protection assay (RPA) establishing abundant apoptotic gene expression was carried out. In MKs, many of the known genes, including several platelet related genes, GATA binding protein were highly expressed. Particularly, TGF beta, clusterin (complement lysis inhibitor), and thymosin beta 4 (actin-sequestering molecules) were expressed highly in MKs. As MKs specific expressed genes may regulate normal and pathologic platelet (and/or MK) functions, the transcript profiling using microarray was useful on molecular understanding of MKs,

Key words: megakaryocyte; microarray; gene expression; RNase protection assay
Abbreviations: SAGE, serial analysis of gene expression; TPO, thrombopoietin; MKs, megakaryocytes

Introduction

While megakaryocyte (MK) plays critical roles as a reservoir of platelets, the process of differentiation and platelet formation is not yet illustrated (Tao *et al.*, 1999; Falcieri *et al.*, 2000). The major limiting factor in investigating the process of megakaryocytopoiesis is the low frequency of MKs in hematopoietic tissues. The discovery and cloning of thrombopoietin (TPO), which can differentiate hematopoietic stem cells to MKs, has provided the sufficient number of cells for the further study of MKs (Piacibello *et al.*, 1997). TPO is the primary regulator of megakaryocytopoiesis and thrombopoiesis. TPO supports the differentiation and proliferation of MK progenitor cells, and has an essential role for the complete maturation of MKs. TPO also supports the formation and the function of platelets *in vivo* and *in vitro*.

For the comprehensive analysis of transcript expression, the array-based hybridization analysis and the serial analysis of gene expression (SAGE) are commonly used platforms. DNA microarray is a powerful tool for monitoring thousands of transcript levels, but it can detect only relative expression level by competitive hybridization (Schena *et al.*, 1995). SAGE analysis is a high-throughput sequencing-based technique and the relative abundance of transcripts in the sample can be estimated because it is able to scale of the transcripts by sequence tags in the SAGE data(Velculescu *et al.*, 2002). The microarray technique has some advantages. If using high-density microarray, it can profile several thousands of transcripts, simultaneously. Previously, we have performed microSAGE in MKs and non-MKs derived from the human umbilical cord blood(CB) CD34+ cells by *ex vivo* expansion using TPO (Kim *et al.*, 2002). In the present study, the gene expression profiles of MKs were analyzed by high-density oligonucleotide microarray hybridization and RNase protection assay (RPA) was used to validate the microarray result. The data present herein showed that many of the genes differentially expressed in MKs and non-MKs were involved in encoding proteins related to apoptosis and intracellular signaling pathways.

Materials and Methods

Purification of CB CD34$^+$ cells

Human umbilical CB was obtained from the full-term deliveries with the informed consent. Mononuclear cells were isolated from CB using Ficoll-Hypaque (density, 1.077; Pharmacia Biotech; Upsalla, Sweden) density

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Accepted 7 February 2005

centrifugation. The CD34+ cell fraction was positively isolated using an anti-CD34 monoclonal antibody conjugated paramagnetic bead (Miltenyi Biotech; Bergisch Gladbach, Germany) and CD34 progenitor cell isolation kit (Miltenyi Biotech), followed twice by the MidiMACS system (Miltenyi Biotech). The purity of the selected population was verified by flow cytometry with an anti-human CD34+ antibody conjugated with fluorescein isothiocyanate (FITC; HPCA-2; Becton Dickinson (BD), Mountain View, CA, USA). Aliquots of enriched cells were checked for purity and were consistently greater than 95%.

Ex vivo expansion and separation of MK and non-MK fractions

The CD34+ cells were cultured at a density of 1.0 x 10^5 cells/㎖ in serum-free essential media supplemented with bovine serum albumin, insulin, and transferrin (Stem Cell Technologies; Vancouver, Canada). The cultures were stimulated with recombinant human TPO (50 ng/ml; Kirin Brewery, Maebashi, Japan) alone. After 10 days, MK fraction was separated from non-MK fraction using an anti-CD61 monoclonal antibody conjugated paramagnetic bead (Miltenyi Biotech) followed twice by the MidiMACS system (Miltenyi Biotech). The purity of each separated fraction was verified by flow-cytometry with a different antibody reacting with MKs (FTIC-conjugated anti-human CD41; BD).). Aliquots of enriched cells were checked for purity and were consistently greater than 95%.

Microarray

Total RNA was prepared from the separated MK and non-MK fractions using the TRIZOL (Gibco BRL; NY, USA) according to the manufacturer's instructions. Total RNA (5㎍) was converted into double stranded cDNA using the cDNA synthesis System (Roche) using T7-$(dT)_{24}$ primer. The each cDNA was purified using the Rneasy kit (Qiagen; Valencia, USA). Each Cy3- (MKs), or Cy5- (non-MKs) labeled cRNA was synthesized using the Megascript T7 kit (Ambion; Austin, USA), using Cy3-CTP and Cy5-CTP (APB; Uppsala Sweden). The cRNA was purified using the RNeasy. The purified 15㎍ of each cRNA was mixed and fragmented in the fragmentation buffer (40 mm Tris (pH 8.1), 100 mm KOAc, and 30 mm MgOAC) by heating to 94C for 15 min. The fragmented cRNA was mixed with the hybridization buffer containing 100 mm MES, 1 m NaCl, 20 mm EDTA and 0.01% Tween 20, and hybridized with MAGIC II-10 K Oligo Chip (Macrogen; Seoul, Korea) for 16 h at 42C. All preparations met Macrogen's recommended criteria for use on their expression arrays. The arrays were then washed and scanned with the Array scanner (APB). Acquired images were processed and analyzed statistically for interpretation of analyzed spot intensity results using Imagene v4.1 software (Roche). Non-biological factors that may contribute to variability of data were minimized using global normalization/scaling with data from all probes sets. Each chip contains a total of 10,368 elements of which 10,108 are unique genes/ clusters. The length of oligonucleotides was 50-mer.

RPA (RNase protection assay)

The RNase protection assay was performed using the mouse cell cycle regulator multiprobe template sets, hAPO-3, hAPO-5C and Hck-4 (BD Biosciences). [^{32}P]-UTP labeled anti-sense RNA probe was synthesized using T7 RNA polymerase, and was purified by phenol chloroform extractions and ethanol precipitation. The purified probe was mixed with 3㎍ of total RNA from MK and non-MK cells in the hybridization buffer and incubated for 16 h at 56°C. Free probe and single stranded RNA molecules were digested with a mixture of RNase A. The RNase protected molecules were purified and resolved on a denaturing polyacrylamide gel and dried. Autoradiographic signal was scanned on a Gel Doc analyzer (Bio-Rad), and the signal intensity of each band was using the Quantity ONE version 4.2 (Bio-Rad) and normalized to the corresponding GAPDH levels.

Results

Comparison of Expression Patterns Between MKs and non-MKs

To reduce individual variation of gene expression, we obtained CB from three volunteers as described our previous report (Kim *et al.*, 2002). The purified CB CD34+ cells were cultured in the serum-free liquid culture system stimulated with TPO for 10 days. Under this condition, MK fraction could be isolated using anti-CD61 antibody, and the purified CD61+ cells had the characteristic MK morphology (Ryu *et al.*, 2001). The total RNA prepared from these MK and non-MK fractions were processed to microarray analysis. The each purified RNA was used for cRNA generation and hybridization to the MAGIC II-10 K Oligo Chip. After the normalization of the fluorescence intensities, 8,889 transcripts were obtained. The scatter plot that was plotted with Cy3 and Cy5 fluorescent signal values displayed a quite dispersed pattern in distribution (data not shown). Each dot represents a gene expressed in these two fractions. Most of the spots gathered around a 45° diagonal line,

Table 1. Differential expression of top 15 transcripts in MKs compared to non-MKs

Accession	Gene name	Increased	Decreased	Function
AF352582	*ATP-binding cassette transporter mrp8*	38.2		Multi drug resistance
NM_01418	hspc159 protein	33.9		Heat shock protein
NM_000782	cytochrome p450, subfamily xxiv precursor	23.7		Drug metabolism
NM_018914	protocadherin gamma subfamily a, 11, isoform 1 precursor	17.4		Trigger common cellular responses
NM_000697	arachidonate 12-lipoxygenase	16.8		Lipid metabolism
NM_016509	c-type lectin-like receptor-2	16.1		Cell adhesion
NM_001709	brain-derived neurotrophic factor	14.8		Neuronal survival
NM_003005	selectin p precursor	12.8		Cell adhesion
NM_003736	protocadherin gamma subfamily b, 4, isoform 1 precursor	11.8		Trigger common cellular responses
NM_001769	cd9 antigen (p24)	10.8		Cell migration
NM_002704	pro-platelet basic protein	10.7		Platelet-specific chemokine
NM_001879	mannan-binding lectin serine protease 1	10.6		*Activate the complement pathway*
NM_014059	rgc32 protein	9.6		N.D
NM_003246	thrombospondin 1	9.5		Stimulate platelet aggregation
AB051833.	*proacrosin binding protein sp32 precursor*	9.0		*Cancer antigen*
AF051321	sam68-like phosphotyrosine protein alpha		196	N.D
NM_020163	semaphorin sem2		140	Regulation of immune and inflammatory responses.
NM_006275	*splicing factor, arginine/serine-rich 6*		131	Splicing factor
NM_003626	protein tyrosine phosphatase, receptor type, f polypeptide (ptprf), interacting protein (liprin), alpha 1		96.9	Signaling molecule
X98307	hur 7		57.6	mRNA stabilizing factor
NM_024423	desmocollin 3, isoform dsc3b preproprotein		49.8	Desmosomal cadherins
NM_021192	homeo box d11		42.1	Transcription factor
NM_000438	paired box gene 3, isoform pax3a		41.3	Transcription factor
NM_000395	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)		34.6	Cytokine, granulocyte activation
NM_014278	heat shock protein (hsp110 family)		33.9	Heat shock protein
NM_017797	btb (poz) domain containing 2		33.1	N.D
NM_002628	profilin 2 isoform b		33.7	Actin sequestering
AL137483	hypothetical protein; dkfzp434g1930		27.8	N.D
NM_004799	smad anchor for receptor activation, *isoform 3*		22.9	*Endosomal protein*, TGF beta signaling block
NM_000922	phosphodiesterase 3b,		21.6	Activation of PI3K

significant difference in the expression level between the two fractions was found in many transcripts. Subsets of genes were selected based on the differential Cy5/Cy3 expression ratios that were |2| in response. Out of the spotted genes, 372 genes were up-regulated and 541 genes were down-regulated in MKs. As shown on the tables, we addressed each of these groups separately. We included accession numbers, a short description of the gene function and a reference for each gene to a recent review on the function of the corresponding gene limited MKs.

Table 1 through 3 show the genes differentially expressed in MKs and non-MKs. Table 1 shows the increased, or decreased top 30 transcripts in MKs compared to those in non-MKs. The most frequently increased transcript was identified to be ATP-binding cassette transporter mrp8 (38.24-fold) followed by hspc159 protein (33.9-fold), cytochrome p450, subfamily xxiv precursor (23.7-fold), protocadherin gamma subfamily a, 11, isoform 1 precursor (17.4-fold), and so on. The transcripts increased in MKs could be classified into several groups according to the functional relevance. Table 2 shows the transcripts regulation-related proteins such as GATA-binding protein 1 (globin transcription factor 1), transcription factor BMAL2, partial transcription factor sp3, gata-3 binding protein (g3b), transcription factor 19 (SC1) (TCF19), transcription factor ap-2 (activating enhancer binding protein 2 , nuclear factor i/c (ccaat-binding transcription factor). The transcripts for metabolic pathway related proteins are cytochrome p450, subfamily xxiv precursor, arachidonate 12-lipoxygenase. The gene for protease and apoptosis related genes, such as mannan-binding lectin serine protease 1, clusterin, sentrin/sumo-specific protease could be found. Table 3 showes the MAP kinase related genes such as map/erk kinase kinase 5, mitogen-activated protein

Table 2. Differential expression of transcription factors in MKs compared to non-MKs

Accession	Gene name	Increased	Decreased	Function
NM_002049	GATA-binding protein 1	4.11		Globin transcription factor 1
NM_020183	transcription factor BMAL2	3.95		Regulation of circadian rhythm
AJ310752	partial transcription factor sp3	3.13		Regulation of many hematopoietic-specific genes
U23736	GATA-3 binding protein	2.63		Regulation of many hematopoietic-specific genes
NM_007109	transcription factor 19 (SC1)	2.52		N.D
NM_003220	transcription factor ap-2 alpha (activating enhancer binding protein 2 alpha)	2.46		General transcription factor
NM_005597	nuclear factor i/c (ccaat-binding transcription factor)	2.41		N.D
NM_000438	paired box gene 3, isoform pax3a		41.3	Transcription factor
NM_002916	replication factor C (activator 1) 4 (37kD)		13.5	N.D
S74703	histone h1 transcription factor large subunit 2a=glutamine-rich ccaat-binding factor		8.50	N.D
NM_004348	runt-related transcription factor 2 (RUNX2)		3.30	Bone formation related transcription factor
NM_002097	general transcription factor iiia		2.83	General transcription factor
NM_005526	heat shock transcription factor 1		2.46	Stress response transcription factor
NM_002504	nuclear transcription factor, x-box binding 1		2.45	Hepatocarcinogenesis-related transcription factor

kinase 13, mitogen-activated protein kinase 6, protein kinase c, 1 and . The surface marker proteins, such as CD9, and CD62P (P selectin) were observed (not shown).

RNase protection assay (RPA) for confirming genes showing different expression level in MKS and non-MKs

The RPA was carried out wheather the expression profile from the microarray is consistent with the conventional RNA analysis strategies. We used probe set (BD), which were expressed differentially in MKs and non-MKs. Among the RPA probe sets (hAPO-5C, hAPO-3), cluserin, TRADD and caspase 8 are matched with transcripts on microarray. Using RPA assay, selected genes were evaluated in MKs and non-MKs derived from CBs of two other volunteers (Fig. 1). These results validated our expression profiles from the microarray for MKs and established the general expression profile of the identified genes.

Discussions

In the present study, the microarray technique was employed to understand comprehensively the properties of transcripts in MKs derived from the CB CD 34+ cells. The isolated CD 34+ cells were expanded with TPO. Then, these expanded cells were separated using anti-CD61 antibody, which is known as a marker of MKs. Approximately 10 thousands of genes expressed in the MKs were analyzed using high density oligonucleotide microarray. Previously, we reported global gene expression of MKs using SAGE technique (Kim *et al.*, 2002). When comparing to SAGE technology, the microarray method has some advantages, such as simultaneity, detection limit of low expressed genes, etc. But, it is not easy to compare the transcription profiles of the microarray with that of expression level of SAGE, because of the different bases of the underlying technology; the competitive hybridization of probes vs. the sequencing of ditags.

Table 1 shows the each top 15 transcripts that of expression levels were increased or decreased in MKs and non-MKs. Many transcription factors related MKs

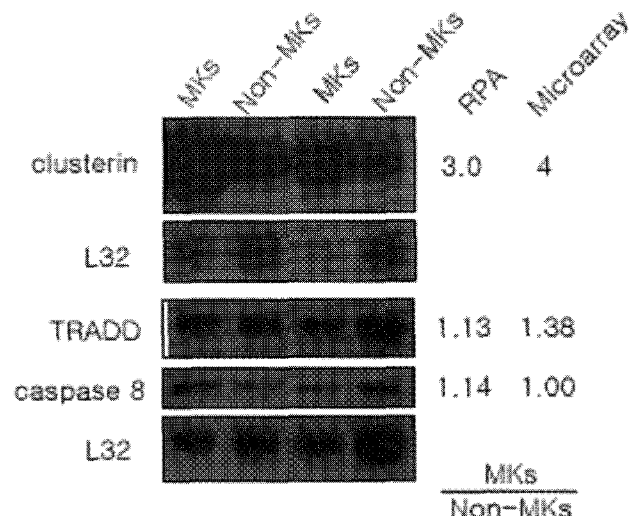

Fig. 1. RPA analysis of genes expressed differentially in the MK and non-MK fractions. The RNase protection assay was performed for two different cord bloods of volunteers, using the mouse cell cycle regulator multiprobe template sets, by following the manufcturer's instruction (BD Biosciences). Among the RPA probe sets (hAPO-5C, hAPO-3), cluserin, TRADD and caspase 8 are matched with transcripts on microarray. The values are the relative ratio of autoradiographic band intensities.

Table 3. Differential expression of MAP kinases, signaling molecules in MKs compared to non-MKs (2)

Accession	Gene name	Increased	Decreased	Function
NM_000627	latent transforming growth factor beta binding protein 1 precursor	4.16		Regulates cell growth, differentiation
NM_000660	transforming growth factor, beta 1	2.43		Regulates cell growth, differentiation
AJ000185	vascular endothelial growth factor-d	2.32		Regulates cell growth, differentiation
NM_003242	transforming growth factor, beta receptor ii (70-80kd)			2.25Regulates cell growth, differentiation
AF108756	fibroblast growth factor 13 isoform 1y1v	2.26		Regulates cell growth, differentiation
NM_005923	map/erk kinase kinase 5	3.53		Ser/Tre MAP kinase
NM_002754	mitogen-activated protein kinase 13	3.19		Ser/Tre MAP kinase
NM_002748	mitogen-activated protein kinase 6	2.22		Ser/Tre MAP kinase
NM_002738	protein kinase c, beta 1	2.53		Calcium dependent kinase
NM_002739	protein kinase c, gamma	2.02		Calcium dependent kinase
AJ317956	hngrc3 neurogranin	1.90		Neuron-specific protein kinase C substrate
NM_004202	thymosin, beta 4, y chromosome;	11.2		Actin-sequestering
NM_005354	jun d proto-oncogene	3.54		Immediate early gene
NM_021103	thymosin, beta 10		7.28	Actin-sequestering
L16464	ets oncogene		4.93	T cell specific immediate early gene
NM_005417	v-src sarcoma (schmidt-ruppin a-2) viral oncogene homolog (avian)		2.61	Signal transduction

specific gene expression were reported several articles (Terui *et al.*, 2000; Goodwin *et al.*, 2001; Matsumura *et al.*, 1991); nuclear factor (erythroid derived) 45 kD, cAMP responsive element binding protein-like 1, erythroid differentiation-related factor 1, nuclear factor (erythroid-derived 2)-like 1, GATA 1 binding protein, etc. In this study, the genes encoding GATA binding protein 1 and GATA 3 binding protein is highly expressed in MKs (4.11 and 2.63 fold change; Table 2). GATA binding protein 1 is essential for the development of megakaryocytic and erythroid lineages (Pevny *et al.*, 1991). However, some MKs specific transcription factors, such as Kruppel-like factor 1 (erythroid) known as GATA binding protein 1 binding partner, erythroid differentiation-related factor 1, Nuclear factor (erythroid-derived 2)-like 1, sp1 like transcription factor were not detected on microarray. But these genes of MKs, known as specific transcription factors, were detected in MKs SAGE library. This phenomenon induced from limitation of microarray analysis because it could analyze about finite number of gene sequences localized to the chip. BMAL 2 was expressed highly in MKs (3.95 fold change). The BMAL2 gene encodes a member of the basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) family of transcription factors, which act as transcription factor with CLOCK protein (BMAL2:CLOCK). Its role in MKs was not reported yet, but it is known as the regulator of circadian rhythm in brain (Cohen *et al.*, 1997). These up-regulated transcription factors, such as GATA related genes and BMAL2, AP-2, nuclear factor i/c may influence MK-specific gene expression directly.

In differentiation and maturation, TPO primed signaling pathway is the major process (Miyazakiet *et al.*, 2001). Binding of TPO to its receptor, c-Mpl, results in the activation of a variety of signaling molecules, which included components of the Ras/MAPK pathway (Fichelson *et al.*, 2001), and PKC pathway (Jiang *et al.*, 2002; Minamiguchi *et al.*, 2001). In the present study, the genes encoding proteins related to Ras/MAPK and PKC pathway, such as protein map/erk kinase kinase 5, mitogen-activated protein kinase 6 and 13, protein kinase c beta 1 and gamma etc were expressed highly in MKs (Table 3). Tumor growth factor- (TGF-) related protein, such as TGF-, its receptor and binding partner, were up-regulated in MKs. TGF- regulates cell growth, differentiation, and extracellular matrix production. The intracellular signaling of TGF- is initiated following ligand binding to the TGF- type II receptor, which activates TGF- type I receptor (Kalina *et al.*, 2001; Massague, 1998). The activated type I receptor phosphorylates Smad 2 and Smad 3, resulting in their translocation into the nucleus where they can bind to DNA in the promoters of TGF- target genes included vascular endothelial growth factor (VEGF) (Table 3) (Heldin *et al.*, 1997; Tokuda *et al.*, 2003). But in our system, Smad family was not detected on microarray. In addition to the Smad signaling pathway, other signaling pathways such as the mitogen-activated protein (MAP) kinase superfamily have recently been shown to mediate the TGF signaling pathway (Hatakeyama *et al.*, 2002). MAPK pathway was mediated in TGF- signaling with Smad pathway, and PKC and MAPK pathway might influence each other in platelet formation.

Interestingly, thymosin beta 4 (Th_4) was up-regulated

(11.2-fold) and thymosin beta 10 (Th_{10}) was down-regulated (7.28-fold) in MKs. Thymosin (Th) acts as the major actin-sequestering molecule in mammalian cells, and is present ubiquitously in most tissues and cell lines and is found in particularly high concentrations in blood platelets, neutrophils, macrophages, and other lymphoid cells (Hannappel *et al.*, 1982; Huff *et al.*, 2001). Thymosin beta 4 has been reported to prevent the cycling of hematopoietic stem cells (Safer *et al.*, 1991), to reduce the toxicity of cytosine arabinoside, to enhance the attachment and migration of endothelium (Bonnet *et al.*, 1996), and to induce production of metalloproteinases (Malinda *et al.*, 1997). Clusterin was expressed highly in MKs (4 fold change; Fig. 1). Clusterin was detected highly in SAGE analysis in MKs and platelets (Gnatenko *et al.*, 2003). Clusterin, also known as apolipoprotein J (Rosenberg *et al.*, 1995), TRPM-2 (Wong *et al.*, 1993), or SGP-2 (Jenne *et al.*, 1992), is an enigmatic glycoprotein. Its expression is widely distributed in various tissues (Trougakos and Gonos., 2002; Jones and Jomary, 2002). Previous reports have correlated clusterin expression with cell responses to stress (Poon *et al.*, 2000), cell damage recovery (Bach *et al.*, 2001), senescence (Wright and Shay, 2001), tumorigenesis and apoptosis (Marinelli *et al.*, 1994). Clusterin was contained in alpha granule which is the largest and most abundant secretary granules in platelets (Tschopp *et al.*, 1993).

In summary, we identified the genes expressed differentially in MKs and non-MKs derived from human CB CD34+ cells by *ex vivo* expansion using TPO. The expression of several genes in signaling molecule such as, RAS/MAPK, PKC and TGF- related genes, was higher in MKs. Interestingly, the genes coding thymosin and clusterin were highly expressed in MKs. These gene expression data from the microarray analysis may provide useful information on MKs differentiation, maturation and platelet production.

Acknowledgements

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (HMP-00-CH-04-0004).

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