as a cistronic cluster at 13q14, which is deleted or down-regulated in most cases (~68%) of B-cell chronic lymphocytic leukemias *(39)*.

Cimmino et al. *(40)* found that both these miRNAs negatively regulate the expression of B-cell lymphoma 2 (BCL2), which inhibits apoptosis and is present in many types of cancers including leukemias. In fact, overexpression of miR-15 and miR-16 in the MEG-01 cell line induces apoptotic cell death.

Alterations in the gene copy number of miRNAs are detected in a variety of human cancers *(41,42,43)*. Zhang et al. *(41)* showed that miRNAs exhibited high-frequency genomic alterations in human ovarian, breast cancer, and melanoma using high-resolution array-based comparative genomic hybridization.

Hayashita et al. *(42)* found that the expression and gene copy number of the miR-17–92 cluster—composed of seven miRNAs—is increased in lung cancer cell lines, especially with small-cell lung cancer histology. Enforced expression of miRNAs included in this polycistronic cluster enhances cell proliferation in a lung cancer cell line. The increase in expression and gene copy number of miR-17–92 cluster was also found in B-cell lymphomas *(43)*. The expression of miRNAs in this cluster is upregulated by c-Myc, whose expression and/or function is one of the most common abnormalities in human cancers, and miR-17-5p and miR-20a in this miR-17–92 cluster negatively regulate the expression of the transcriptional factor E2F1 *(44)*.

Furthermore, it was indicated that miR-17–19b cluster included in miR-17–92 cluster inhibited apoptotic cell death, and accelerated c-Myc–induced lymphomagenesis in mice reconstituted with miR-17–19b cluster-over-expressed haematopoietic stem cells *(43)*. In addition, the miR-17–92 cluster has been reported to augment angiogenesis in vivo by down-regulation of anti-angiogenic thrombospondin-1 and connective tissue growth factor in Ras-transformed colonocytes *(45)*.

miR-155 was identified as a miRNA whose copy number and expression were up-regulated in several types of B-cell lymphomas *(46)*. The miR-155 gene is located in the final exon of the B-cell integration cluster (BIC) non-coding gene, which is shown to accelerate the pathogenesis of c-Myc–associated lymphomas and leukemias, suggesting potential roles of miR-155 in B-cell lymphomas *(47)*. Indeed, transgenic mice with miR-155 driven by the B-cell-specific Eμ enhancer rapidly develop a polyclonal B-cell malignancy *(48)*.

The up-regulated expression of miR-155 is also reported in breast, lung, colon, and thyroid cancer *(49,50)*. However, the actual molecular mechanism of miR-155 remains unknown, although it is reported that miR-155 down-regulates the expression of the angiotensin II type I receptor *(51)*.

As an antiapoptotic miRNA, miR-21 was recently identified to be up-regulated in human breast tumor tissues, glioblastoma tumor tissues, and malignant cholangiocytes *(52, 53, 54)*. Inhibition of miR-21 by antisense oligonucleotides causes activation of caspases and induction of apoptotic cell death in a human breast cancer cell line and glioblastoma cell line *(52,53)*. Furthermore, miR-21 inhibits gemcitabine-induced apoptotic cell death in cholangiocarcinoma cell lines by down-regulation of PTEN (phosphatase and tensin homolog deleted on chromosome 10), which positively regulates apoptosis via inhibition of PI 3-kinase signaling activation *(54)*. The expression of miR-141 and miR-200b are also up-regulated in malignant cholangiocytes. Inhibitions of these miRNAs using miRNA-specific antisense oligonucleotides decreased proliferation

of a malignant cholangiocyte cell line. The up-regulated expression of miR-21 is also detected in human colon, lung, pancreas, prostate, and stomach cancer *(49,55)*, suggesting the possibility that miR-21 inhibits apoptotic cell death in these cancers.

Interestingly, it has been reported that the expression level of let-7 is reduced in human lung cancers *(56)*. This result suggests that let-7 might act as a tumor suppressor gene in lung cancer. In fact, regardless of disease stage, lung cancer patients with down-regulation of let-7 had shortened post-operative survival *(56)*. Furthermore, Johnson et al. *(57)* found that let-7 negatively regulated the expression of human RAS family members, which possess potent oncogenic activity. Actually, RAS protein levels are inversely correlated with let-7 expression levels in human lung cancers, suggesting a possible mechanism for let-7 in lung cancer.

To identify novel miRNAs involved in cellular transformation, Voorhoeve et al. *(58)* performed functional genetic screens using a library of vectors expressing human miRNAs and in vitro neoplastic transformation assays. They showed that miR-372 and miR-373 accelerate proliferation and tumorigenic development in primary human cells that express oncogenic RAS and tumor suppressor p53, possibly through suppression of p53-mediated CDK inhibition by down-regulation of large tumor suppressor homolog 2 (LATS2) *(58,59)*. Furthermore, miR-372 was found to be exclusively over-expressed in most human testicular germ cell tumors that rarely exhibit loss of p53 function, suggesting contribution of miR-372 to the development of human testicular germ cell tumors by inhibition of the p53 pathway *(58)*.

Recent evidence indicates that polymorphisms and genetic variation in germ line as well as somatic cells have a critical role in cancer predisposition and malignancy *(60,61)*. However, in spite of comprehensive scanning of protein coding genes, the molecular basis of familial cancers remains largely unknown. Recently, a germ line mutation of the miR-16-1–miR-15a primary precursor, which impaired mature miRNA expressions, was identified in B-cell chronic lymphocytic leukemia patients *(62)*. Furthermore, germ line or somatic mutations of miRNAs were found in 11 of 75 patients with B-cell chronic lymphocytic leukemia, but none of these mutations were found in 160 persons without cancer *(62)*. These results suggest that genetic variation of miRNAs in a germ line may play important roles in cancer predisposition and malignancy. In addition, germ line mutation in miRNA-target sites of mRNA 3′ UTR were found in KIT and slit and trk-like family member 1 (SLITRK1), suggesting genetic variation of miRNA-target sites in a germ line may also play significant roles in disease predisposition *(50,63)*.

Human cytochrome P450 (CYP) 1B1, which is abundantly expressed in malignant tumor tissues, is a member of drug-metabolizing enzymes and catalyzes the metabolic activation of various procarcinogens. Recently, it was found that CYP1B1 expression was post-transcriptionally inhibited by miR-27b *(64)*. Furthermore, decrease of miR-27b expression and increase of CYP1B1 expression in most breast cancer tissues was detected *(64)*. These results indicate that miRNAs may play important roles in not only physiologic events but also drug metabolism and production of carcinogens.

Global expression profiling analysis of protein coding genes is known to be useful for cancer diagnoses and prognosis predictions *(65)*. Recently, Lu et al. *(37)* indicated that miRNA expression profiles can successfully classify poorly differentiated cancers that cannot be classified by mRNA expression profiles. Accordingly, miRNA expression profiles are more accurately correlated with clinical severity of cancer malignancy than

Table 1
Canser-Associated miRNAs

miRNA	*Cancer types*	*Targets*[a]	*References*
Oncogene			
miR-17–92	BCL, lung	CTGF, E2F1,Tsp1	42–45
miR-21	breast, cholangiocyte, colon, glioblastoma, lung, pancreas, prostate, stomach	PTEN	49, 52–55
miR-155	BCL, breast, colon, lung, thyroid	AT1R	46, 49–51
miR-372/373	testicular germ cell	LATS2	58
Tumor suppressor gene			
let-7a	breast, lung	RAS	55–57
miR-15a/16	B-CLL	BCL2	39, 40

[a] Target genes identified by the biological experiments are listed.
Abbreviations: AT1R, angiotensin II type I receptor; B-CLL, B-cell chronic lymphocytic leukemia; BCL, B-cell lymphoma; BCL2, B-cell lymphoma 2; CTGF, connective tissue growth factor; LATS2, large tumor suppressor homolog 2; PTEN, phosphatase and tensin homolog deleted on chromosome 10; Tsp1, thrombospondin-1.

protein-coding gene expression profiles. This result indicates the potential of miRNA expression profiles in cancer classification and prognosis prediction *(37)*.

Because miRNAs act as oncogenes or tumor suppressor genes (Table 1), miRNAs are potential targets of therapeutic strategies. Recently, Krutzfeldt et al. *(66)* indicated that chemically engineered oligonucleotides, called antagomirs, efficiently inhibited miRNAs in vivo. Additionally, it is reported that introduction of 2′-O-methoxyethyl phosphorothioate antisense oligonucleotide of miR-122 (abundant in the liver and regulates cholesterol and fatty-acid metabolism) decreases plasma cholesterol levels and improves liver steatosis in mice with diet-induced obesity *(67)*. These findings indicate that antisense oligonucleotides are also potential targets for drug discovery, suggesting the possibility that intractable cancers may become curable by over-expression and/or inhibition of miRNAs. However, for miRNAs to be used in gene therapy, further improvement is required to make miRNAs more effective and less toxic than other cancer therapy.

4. PERSPECTIVE

It has been established that miRNAs play critical roles in cell differentiation, proliferation, and apoptosis, and the abnormalities of specific miRNA expression contribute to tumorigenesis. Additionally, recent studies show that polymorphisms or genetic variation of miRNAs and miRNA-target sites of mRNAs in a germ line may play important roles in cancer predisposition and malignancy *(50,62)*. Therefore, miRNAs are expected to be powerful tools for cancer classification, diagnosis, and prognosis prediction, as well as to be potential targets of cancer therapy.

Furthermore, identification of target mRNAs regulated by miRNAs, elucidation of the oncogenic or tumor suppressive molecular mechanisms by miRNAs, and identification

of genetic variation in miRNAs and miRNA-target sites of mRNAs may lead to the discovery of new molecular targets related to oncogenesis. Bioinformatics approaches have predicted that a single miRNA may have hundreds of target genes *(5, 6, 68, 69, 70, 71, 72, 73, 74)*, although detailed experimental validation has yet to be done. Development of a comprehensive assay to rapidly identify target mRNAs would greatly assist our understanding of miRNAs and lead to novel therapeutic approaches against cancer.

ACKNOWLEDGEMENTS

We would like to thank Dr. N. Hirota for her invaluable advice. We apologize for the incompleteness of the referencing due to space limitations and timing.

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2．最新技術/システム
2）高感度マイクロアレイ

土屋創健・清水一治・辻本豪三

DNAマイクロアレイのスポット部位に柱状構造を有する黒色樹脂の基板とその柱状構造を利用したビーズによる攪拌を用いることで，従来のガラス基板のマイクロアレイと比べて，約100倍高感度なマイクロアレイが開発された。この高感度マイクロアレイは，わずか0.01μgのtotal RNAから遺伝子増幅を行うことなく正確な遺伝子プロファイルを取得することが可能であった。今後，高感度マイクロアレイを用いることにより，臨床の場において治療前生検標本から迅速かつ簡便に正確な遺伝子プロファイルを取得できると期待される。

はじめに

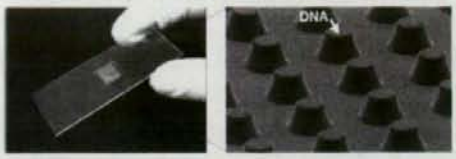
一度に大量の遺伝子の量的変動を分析するDNAマイクロアレイは極めて強力な研究ツールとしてこれまで主に基礎研究分野において使用され，ゲノム機能科学研究の発展に多大な貢献を果たしてきた。このDNAマイクロアレイを医療へ応用しようする試みは当然盛んであり，創薬ターゲット分子の同定や病態・予後診断のための疾患特異的なバイオマーカーの探索のためにDNAマイクロアレイが使用され，実際，病態の発現や予後に関連する遺伝子が数多く同定された。また，DNAマイクロアレイを用いた解析から薬物の治療効果や副作用に関与する遺伝子も同定され，DNAマイクロアレイは患者個人の体質，病態，薬剤応答性に応じて薬の種類や量を決定するなどの患者個人に最適化した医療，個別化医療を実現するためのツールとしても期待されている。

このように病態の診断や個別化医療の実現に向けてDNAマイクロアレイに大きな期待がかけられているが，個々の遺伝子の検出感度やその信頼性に関しては定量PCR法などの他の検出方法と比較しても劣っており，とりわけ低発現の遺伝子の検出においてはいまだ十分な性能が得られていなかった。そのため，低発現ながら生理作用や病態発現に重要な役割を担っていることの多い転写因子や受容体をもれなく検出できるような高感度のDNAマイクロアレイが渇望されていた。また従来，治療前生検標本などの微量なサンプルをDNAマイクロアレイで解析するためには，RNAポリメラーゼによる転写反応やPCR法などによる遺伝子増幅が必要不可欠であったが，複数の遺伝子間で増幅効率が異なることや，これらの操作は複雑かつ煩雑で時間も要し，専門的な技術の習得が必要であること，消耗品および設備の面で非常にコストがかかることから，臨床の場においてDNAマイクロアレイが使用されるには，生検標本のような微量の検体からでも遺伝子増幅を行うことなく，迅速・簡便に正確な解析を行うことができる高感度なDNAマイクロアレイが必要であった[1]。

われわれは東レより開発された立体構造を有するマイクロアレイの性能評価を共同研究で行った

key words
高感度，柱状構造，黒色樹脂，ビーズ攪拌，診断，個人化医療，臨床，生検標本，microRNA

図❶ 高感度マイクロアレイの柱状構造（文献2より改変）



結果，0.01 μgという少量のtotal RNAから遺伝子増幅を行うことなく遺伝子プロファイルを取得できることが明らかとなったので，以下にこの高感度マイクロアレイの特徴と性能評価について概説する。

Ⅰ．柱状構造によるスポット形状の安定化とバックグラウンドノイズの低下

DNAマイクロアレイの高感度化を実現するため，スポット形状の安定化とバックグラウンドノイズの軽減を目的にして，検出部に直径0.15 mmおよび高さ0.2 mm柱状構造を有する黒色樹脂のDNAマイクロアレイ基板が考案された（図❶）。その結果，柱の上部にプローブを固定化することでスポットの形状が安定化し，より高密度にプローブを固定化することが可能となった。また，従来のDNAマイクロアレイのバックグラウンドノイズは主に周囲のガラスへの非特異的な標識核酸の吸着に起因していたが，この柱状構造を有する高感度マイクロアレイはスキャン時に読み取り面を柱の上端に設定することでスポット周囲に空気のみの空間を作り出し，バックグラウンドノイズを大幅に軽減させた（表❶）[2]。さらに，バックグラウンドノイズの低下には黒色樹脂自体の自家蛍光がガラスよりも低いことも寄与していることが，底面でのバックグラウンドノイズの測定結果から明らかとなった（表❶）。

Ⅱ．柱状構造を利用したビーズ撹拌による反応性の向上

一般にハイブリダイゼーション溶液中の核酸の拡散は遅く（～200 μm/hr），特に希薄な標識核酸濃度のハイブリダイゼーション溶液中では溶液中の標識核酸とプローブの十分な反応効率が期待できなかった[3]。そこで，柱状構造を利用して柱間にビーズを封入し，ハイブリダイゼーション時にマイクロアレイを振盪させてビーズを動かすことで物理的に溶液の撹拌を加速させたところ，感度が約3倍亢進することが明らかとなった（表❷）。この際，ビーズの直径をカバーのガラス板と柱上部の間隙より大きくすることで，ビーズは柱間のみを移動し，スポット部位を傷つけることがないように工夫を施している。

Ⅲ．高感度マイクロアレイの性能評価

上記の改良を行った高感度マイクロアレイの性能を評価するため，Cy3標識したヒト脳由来のcDNAとCy5標識したヒト肝臓由来のcDNAをハイブリダイゼーションさせた際の蛍光画像を従来のガラス基板のDNAマイクロアレイと比較した結果，高感度マイクロアレイではバックグラウンドノイズの軽減とシグナル値の上昇が観察された（図❷）。また，ハイブリダイゼーションさせる標識核酸の量を1，0.1，0.01 μgと段階的に減らしたところ，従来のガラス製チップでは0.1 μgにおいて著しくシグナルが減弱し，0.01 μgではほとんどシグナルが検出されなかったのに対し，高感度マイクロアレイでは0.1 μgにおいてシグナルの減弱

表❶ 基板底面からの焦点距離とノイズシグナル値の関連（文献2より改変）

基板底面からの焦点距離（μm）	0	50	100	200
高感度マイクロアレイ基板	330	250	190	180
従来のガラス基板	460	－	－	－

表❷ シグナル強度に対するビーズ撹拌の効果（文献2より改変）

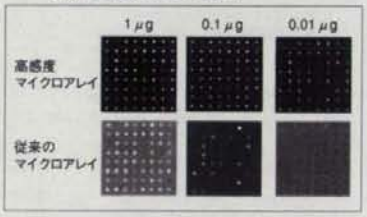
標識核酸濃度（ng/μl）	0.15	0.30	0.75
ビーズ撹拌あり	2,020	2,470	4,510
ビーズ撹拌なし	660	770	1,400
シグナル強度比（ビーズ撹拌あり/なし）	3.1	3.2	3.2

ほとんど検出されず，0.01 μgにおいても大部分のターゲットにおいて同等のシグナルが検出された（図❷，図❸）。また，0.01 μgにおいても高感度マイクロアレイではほぼ同等のダイナミックレンジが維持されていたのに対し，従来のガラス基板のDNAマイクロアレイでは0.1 μgにおいてすでにダイナミックレンジが顕著に狭まっていた（図❸）。さらに，異なるインプット量におけるCy3とCy5のシグナル比の相関係数を従来のガラス基板のDNAマイクロアレイと比較したところ，高感度マイクロアレイにおいてより高い正の相関が得られることが明らかとなり，高感度マイクロアレイの高感度性と信頼性の高さが確認された（表❸）。

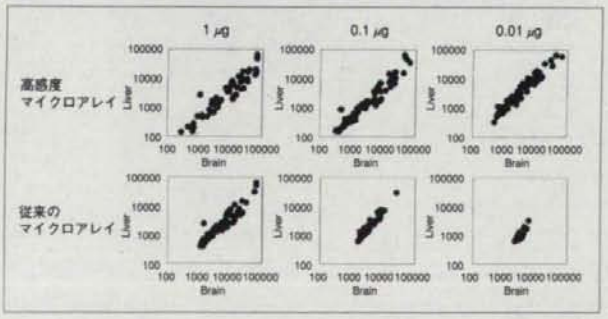
高感度マイクロアレイから得られた遺伝子の発現差の精度を調べるため，高感度マイクロアレイより得られる発現差が定量PCR法から得られる発現差と一致するかどうかを，9つの遺伝子（*HLA-F, PARVB, NR2C1, MDH1, MTI1E, RPL23AP7, BRDT, CD84* および *SERPINA1*）に関して検証を行った（図❹）。その結果，いずれの遺伝子においても高感度マイクロアレイと定量PCRから得られた発現差はよく一致し，両者の相関係数は0.94であった。これは，他の市販されているDNAマイクロアレイを用いて検討された報告結果と比べても，高感度マイクロアレイの結果が定量PCRの結果とよりよく相関していることを示している[6]。

以上の結果から，この高感度マイクロアレイが遺伝子増幅を行わずに0.01 μgのtotal RNAから高精度の遺伝子発現プロファイルを取得できることが確認され，この高感度マイクロアレイは，臨床の場において使用されるに十分な性能を有していることが明らかとなった。

図❷　高感度マイクロアレイと従来のマイクロアレイのスキャン画像（文献2より改変）



図❸　高感度マイクロアレイと従来のマイクロアレイのスキャッター図（文献2より改変）



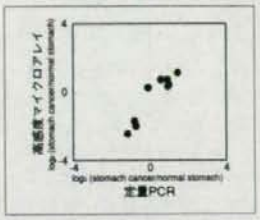
表❸　異なるインプット量におけるCy3/Cy5比の相関係数（文献2より改変）

インプット量（μg）	1 and 0.1	1 and 0.01
高感度マイクロアレイ	0.87	0.80
従来のマイクロアレイ	0.49	0.10

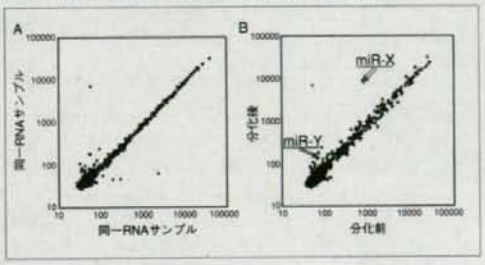
Ⅳ．microRNA高感度マイクロアレイ

近年，短鎖（約20塩基）のnon-coding RNAであるmicroRNAが細胞の発生・分化や癌の発症・悪性化などに重要な役割を果たすことが明らかとなり，これまで単なる伝達役に過ぎないと思われてきたRNAの機能性に着目した新たな生命科学の展開に大きな期待が寄せられている。しかしながら，microRNAは非常に短鎖であるがゆえに，DNAマイクロアレイでのプローブ部位の選択ができないなどの問題があり，従

図❹ 高感度マイクロアレイより得られた発現差の定量PCRによる検証（文献2より改変）

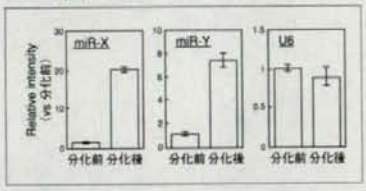


図❺ microRNA高感度マイクロアレイのスキャッター図



来のガラス基板のDNAマイクロアレイでは精度の高いマイクロアレイ解析を行うのは困難であった。そこで，この高感度マイクロアレイにヒトmicroRNAのプローブ471種類（miRBase：Release 9.1）をduplicateに固定化し，マイクロアレイ間における再現性の確認を行ったところ，相関係数が0.99という非常に高い再現性が得られること，3桁を超える幅広いダイナミックレンジが得られることが明らかとなった（図❺A）。さらに，あるヒト由来細胞株に分化を誘導し，その前後におけるmicroRNAの変動をこの高感度マイクロアレイで解析したところ，スポットしたduplicateがそろって2倍以上変動したmicroRNAが2種類（ともに仮名：miR-X，miR-Y）検出された（図❺B）。これらのmicroRNAの発現変動を定量PCR法で検証したところ，高感度マイクロアレイから得られた結果が確認され，コントロールとして用いたU6の発現には差はみられなかった（図❻）。以上のことから，この高感度マイクロアレイがmicroRNAのような短鎖のRNAの検出においても優れた性能を有していることが確認された。

図❻ 定量PCRによる高感度microRNAマイクロアレイ結果の検証



おわりに

2006年9月，米国食品医薬品局（FDA）によって進められていたMicroArray Quality Control（MAQC）プロジェクトのフェーズIが終了し，マイクロアレイ解析による遺伝子発現プロファイルの互換性・再現性が確認され，遺伝子発現プロファイル診断薬の実用化への展望が開けた。さらに2007年2月には，最初のマイクロアレイ体外診断法として，FDAにより遺伝子発現プロファイルから乳癌の再発リスクを評価するDNAマイクロアレイMammaPrint®が認可され，個々の患者の体質・病状を加味した個人化医療がいよいよ現実のものになろうしている。

高感度マイクロアレイは，微量の検体からでも遺伝子増幅なしに正確な遺伝子プロファイルをすることができる臨床の場でのニーズに応えたマイクロアレイであり，今後，病態の診断や個別化医療に活用されることが期待される。

謝辞
本研究の一部はNEDO（新エネルギー・産業技術総合開発機構）の「バイオ・IT融合機器開発プロジェクト」および「機能性RNAプロジェクト」の助成を受けて取り組んだものです。

Technical Tips

DNAマイクロアレイはこれまで主に医療および基礎研究分野においてその威力を発揮してきたが，最近では土壌汚染浄化などの環境や食品といった分野においても脚光を浴びてきている。

安価で安全な土壌や地下水の浄化方法として，微生物を用いて汚染物質の分解を行う方法があるが，これには汚染物質を分解するために有効な微生物を事前に特定する必要がある。この微生物を用いた浄化方法の有効性を事前検証するための方法として，マイクロアレイが注目されている。従来までの方法と比較して，短時間かつ低コストで検証を行うことができ，さらに多種類の微生物を同時に検出できることから，汚染物質の異なる分解過程に携わる微生物を組み合わせた浄化方法の検討が可能となる。

また，マイクロアレイは食品や飼料中にどのような生物種由来のものが含まれているのかを迅速かつ網羅的に同定することが可能であり，昨今の食品の品質・安全性に対する関心の高まりを受け，食品の品質鑑定におけるマイクロアレイの使用増大が見込まれている。

今後，さらに様々な分野においてマイクロアレイの適用が拡大することが期待される。

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BMC Genomics

BioMed Central

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Characterization of gene expression profiles for different types of mast cells pooled from mouse stomach subregions by an RNA amplification method

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Published: 20 January 2009

BMC Genomics 2009, **10**:35 doi:10.1186/1471-2164-10-35

Received: 9 July 2008
Accepted: 20 January 2009

This article is available from: http://www.biomedcentral.com/1471-2164/10/35

Abstract

Background: Mast cells (MCs) play pivotal roles in allergy and innate immunity and consist of heterogenous subclasses. However, the molecular basis determining the different characteristics of these multiple MC subclasses remains unclear.

Results: To approach this, we developed a method of RNA extraction/amplification for intact *in vivo* MCs pooled from frozen tissue sections, which enabled us to obtain the global gene expression pattern of pooled MCs belonging to the same subclass. MCs were isolated from the submucosa (sMCs) and mucosa (mMCs) of mouse stomach sections, respectively. 15 cells were pooled, and their RNA was extracted, amplified and subjected to microarray analysis. Known marker genes specific for mMCs and sMCs showed expected expression trends, indicating accuracy of the analysis.

We identified 1,272 genes showing significantly different expression levels between sMCs and mMCs, and classified them into clusters on the basis of similarity of their expression profiles compared with bone marrow-derived MCs, which are the cultured MCs with so-called 'immature' properties. Among them, we found that several key genes such as *Notch4* had sMC-biased expression and *Ptgr1* had mMC-biased expression. Furthermore, there is a difference in the expression of several genes including extracellular matrix protein components, adhesion molecules, and cytoskeletal proteins between the two MC subclasses, which may reflect functional adaptation of each MC to the mucosal or submucosal environment in the stomach.

Conclusion: By using the method of RNA amplification from pooled intact MCs, we characterized the distinct gene expression profiles of sMCs and mMCs in the mouse stomach. Our findings offer insight into possible unidentified properties specific for each MC subclass.

Background

Mast cells (MCs) are derived from hematopoietic stem cells and play important roles in allergic responses, innate immunity and defense against parasite infection. Unlike other blood cells, MCs migrate into peripheral tissues as immature progenitors and differentiate into mature mast cells. One of the unique features of MCs is that they show a variety of phenotypes depending on the different tissue microenvironment of their maturation [1]. In MCs, various MC-specific serine proteases are stored in the secretory granules, and their gene and protein expressions are dramatically altered when their cell environment is altered. For example, Reynolds *et al.* have shown that at least six distinct members of mouse MC-specific serine proteases are expressed in different combinations in different mast cell populations [2]. In addition, recent studies have shown that mature MCs vary in terms of what surface receptors and lipid mediators they express [3,4]. Because each mast cell population *in vivo* must play a specific role in the body, it is important to determine the character of each population of MCs.

Comprehensive gene expression analysis is a powerful approach to understand the characterization of various MC subpopulations. To date, several studies on microarray analysis of MCs have been conducted [5-7], but most of them dealt with MCs cultured *in vitro*. Alternatively, gene expression profiles of MCs isolated from skin and lung have been analyzed [3,8-10]. However, the numbers of MCs analyzed as one sample were relatively high and they were exposed to physical forces, enzymes and the anti-Kit antibody for purification, during which the original properties of the MCs may have been affected.

In the gastrointestinal tract, there are MCs that are mainly classified into two subclasses; mucosal MCs (mMCs) and submucosal MCs (sMCs) on the basis of their location, morphology (size and shape) and granule contents [11,12]. mMCs are mainly found in the mucosa of the gastrointestinal system, having chondroitin sulfate-containing granules, which are stained with toluidine blue but not safranin, and their activation occurs during parasite infection [13], while sMCs are localized in the submucosa of the gastrointestinal tract and their granules are rich in heparin and stained with both toluidine blue and safranin [1,11]. However, the molecular basis determining the differences in biochemical properties of these two MC subclasses remains uncertain, partially due to the difficulty of their isolation.

To overcome these problems, here we established a method of RNA amplification from intact MCs isolated from frozen tissue sections, which enables us to conveniently obtain the global gene expression pattern of MCs in various tissues. To validate this method, we first determined the minimum cell number required to achieve reproducible RNA amplification. We then compared the gene expression profiles obtained from small numbers of mMCs and sMCs in the mouse stomach, and found several key genes to be specifically expressed in one subclass of MCs, which may reflect some aspects of the distinct properties between the two MC subclasses in the gastrointestinal tract.

Results and discussion

Development of an RNA amplification protocol to obtain gene expression profiles from a small amount of RNA

To gain insight into the functional differences between the different subclasses of MCs, we employed three rounds of the T7-based RNA amplification method. Based on the preliminary experiments using peritoneal MCs and bone marrow-derived MCs (BMMCs), we estimated that a single MC yields 2 pg of RNA. Before we performed comparative analysis of MCs from different tissues, we first evaluated the accuracy and reproducibility of three rounds of the T7-based RNA amplification method, starting with the amount of RNA that can be obtained from a single MC. To assess this, we first compared the microarray results obtained from 5 μg of BMMC RNA prepared by the standard protocol with those obtained from the same RNA diluted 10^5- or 10^6-fold (30 pg, 10 pg and 2 pg) and subjected to three rounds of T7-based amplification (Figure 1a–c). Although three rounds of amplification yielded enough quantity of RNA for microarray analysis (>20 μg) even in the case of 2 pg RNA, scatter plot analysis revealed that the qualities of the obtained results were quite different between the samples from 5 μg and 2 pg RNA. The genes judged as 'Presence' in both 30 pg and 5 μg of RNA were 8,149 genes, which corresponded to 72% of genes judged as 'Presence' in the 5 μg of RNA (11,344 genes; Figure 1a), while only 4,116 genes were judged as 'Presence' in both 2 pg and 5 μg of RNA, which corresponded to only 36% of genes judged as 'Presence' in the 5 μg RNA (Figure 1c). The decrease in the number of genes judged as 'Presence' in the diluted samples (30 pg, 10 pg and 2 pg) may be due to the loss of low copy number RNA species during amplification.

We next examined the reproducibility of the microarray results obtained from two sets of 30 pg BMMC RNA samples (30 pg-1 and 30 pg-2) or two sets of 2 pg samples (2 pg-1 and 2 pg-2) (Figure 1d and 1e). In the 30 pg RNA samples, 7,537 (30 pg-1) and 8,777 (30 pg-2) genes were judged as 'Presence'. However, only 4,324 (2 pg-1) and 4,460 (2 pg-2) genes were judged as 'Presence' in each 2 pg RNA sample, again suggesting the loss of low copy number RNAs during amplification from a small amount of RNA. As to the reproducibility, 86% of the 'Presence' genes in the 30 pg-1 and 74% of 'Presence' genes in the 30 pg-2 sample were judged as 'Presence' in both 30 pg RNA

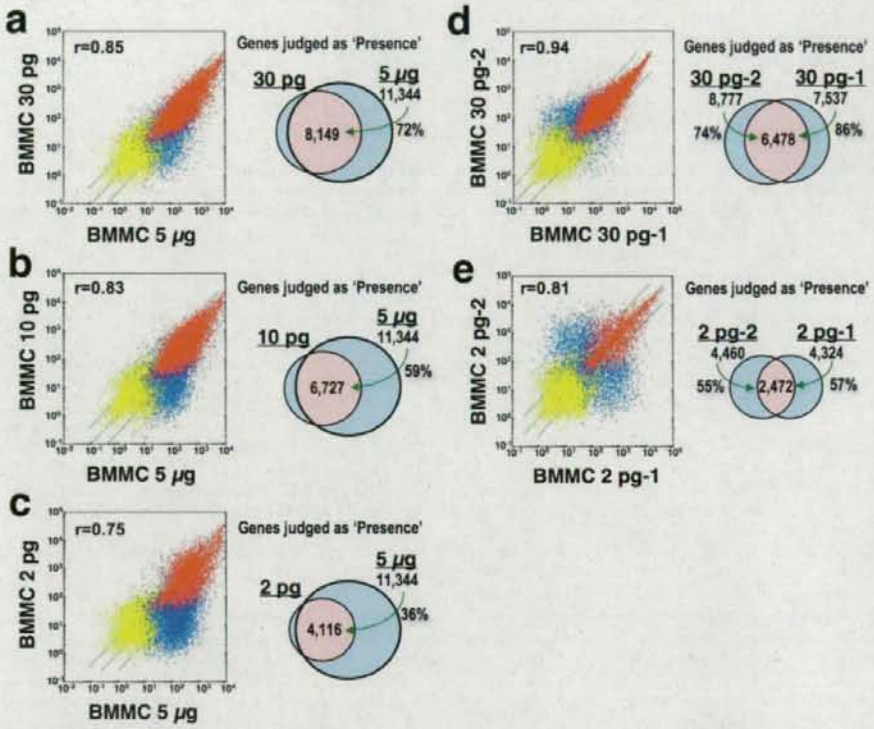

Figure 1

Comparisons of three round-amplified products starting with very small quantities of RNA. (**a-c**) Amplification biases in the products starting from a small quantity of RNA. Scatter plots of signal intensity obtained from 5 μg of BMMC RNA prepared by the standard protocol and from 30 pg (*a*), 10 pg (*b*) and 2 pg (*c*) of BMMC RNA prepared by three rounds of amplification are shown. (**d, e**) Reproducibility of the three-round amplification of a small quantity of RNA. Scatter plots of signal intensity between two independent products from 30 pg of BMMC RNA (BMMC 30 pg-1 and BMMC 30 pg-2) (*d*) or from 2 pg of BMMC RNA (BMMC 2 pg-1 and BMMC 2 pg-2) (*e*), are shown. Red dots show probe sets judged as "Presence", and yellow dots represent probe sets judged as "Absence" in both arrays. Blue dots show probe sets judged as "Presence" only in either array. The correlation coefficients (r) are presented. The same, four-fold induction and suppression thresholds are indicated as diagonal lines. Genes judged as "Presence" are placed in groups corresponding to pairwise overlaps shown in the accompanying Venn diagrams.

samples, while only 57% of 'Presence' genes in the 2 pg-1 and 55% of 'Presence' genes in the 2 pg-2 sample were judged as 'Presence' in both 2 pg RNA samples. These results suggested that the amplified products from the RNA from a single MC (about 2 pg) by the current method may include considerable amplification artifacts causing problems in accuracy and reproducibility. On the other hand, because of the higher reproducibility (>74%), we concluded that amplification from 30 pg RNA collected from 15 MCs would be suitable for the practical analysis of tissue MCs. Based on these results, we set our goal in this study to acquire gene expression profiles of MCs

pooled from different regions. To minimize the influence of cell-to-cell variations within the same class and potential amplification artifacts, we prepared three sets of 15 MCs for each region and compared genes with significantly different expression between MCs from the different regions (Figure 2b). We chose stomach as the source organ, since we can isolate two kinds of MCs from the mucosa (mMC) and the submucosa (sMC) regions of the same sections, and mMCs and sMCs have been suspected to be different in several MC properties such as protease expression profile and sensitivity to safranin staining [1,11].

Gene expression profiles of submucosal and mucosal MCs from the stomach

To visualize two kinds of MCs in the stomach without causing RNA degradation, the sections were fixed with carnoy's fixative and metachromatically stained with toluidine blue for a few seconds. sMCs and mMCs were microdissected using a patch pipette (Figure 2a and 2b). We prepared three sets of 15 MCs for each region, extracted their RNA and individually amplified them (sMC_1, sMC_2, sMC_3, and mMC_1, mMC_2, mMC_3). To improve the recovery of the extraction of as little as 30 pg of RNA, we used 'poly G' as a carrier, which does not interfere with the following RNA amplification or hybridization of the amplified product to the array (data not shown). To examine the effects of nonspecifically amplified artifact products, we performed the RNA extraction/amplification procedure without adding microdissected cells ("no cell") as a negative control (described in "*Materials and methods*"). The amplified RNAs of sMCs, mMCs and the "no cell" control were separately hybridized to a murine microarray. The signal values in the "no cell" sample were low in general and similar to the background levels (Figure 2c). The scatter plots of the samples independently prepared within the same group (e.g. sMC_1 vs sMC_2) showed a similar expression pattern; the average correlation coefficient for all probe-sets was 0.945 ± 0.004 and 0.893 ± 0.019 in sMCs and mMCs, respectively (n = 3). In contrast, the average correlation coefficient between sMCs and mMCs was 0.752 ± 0.034 (n = 3), which was much lower than those within the same group, suggesting that their gene expression patterns are different.

We further evaluated the accuracy and reproducibility of our method by other comprehensive analyses (hierarchical clustering analysis and principal component analysis [PCA]) using all probe sets. Microarray data obtained from sMCs, mMCs, skin-derived MCs, peritoneal MCs, BMMCs and non-MCs (macrophages and fibroblasts) were applied to these analyses. We first checked whether the amplification process in our method affects the global expression profile due to non-linear amplification. The results from the BMMC samples using RNA prepared by the standard protocol (BMMC-std) or the amplification method (BMMC-amp) were subjected to these analyses. Both hierarchical clustering analysis and PCA revealed that microarray data from BMMC-std and BMMC-amp were clustered in the same group (Figure 3a and 3b), suggesting that the global similarity in gene expression profiles is maintained during the amplification process. We next examined the similarity of expression patterns in three independent sMC or mMC samples. Upon clustering analysis and PCA, sMC_{1-3} and mMC_{1-3} were clustered in the same group, respectively. PCA also showed that the expression profiles of sMCs, mMCs and BMMCs are mutually different (Figure 3b).

We then compared the stomach-derived MCs (sMCs and mMCs) with skin-derived MCs, peritoneal MCs, BMMCs and non-MCs (macrophages and fibroblasts) by clustering analysis. The tissue-derived MCs (stomach MCs and skin MCs) were clustered separately from peritoneal MCs and BMMCs. These results may reflect different properties between tissue-derived MCs with firm adhesion to the neighboring cells and floating MCs without a tight contact. As to the similarity of MCs with fibroblasts and macrophages, it is reasonable that fibroblasts are most distant from MCs and macrophages are closer to MCs as a leukocyte family.

Validation of microarray results by real time RT-PCR analysis

We next investigated whether the hybridization signals of known marker genes specific for sMCs and mMCs showed the expected expression trends [12,14]. The mMC-specific genes, mast cell protease 1 (*Mcpt1*) and 2 (*Mcpt2*) showed higher values in mMCs, while the sMC-specific marker genes, mast cell protease 4 (*Mcpt4*) and chymase 2 (*Cma2*), showed higher signal values in sMCs (Table 1 and Figure 4a) [15-29]. On the other hand, MC-common markers such as kit oncogene (*Kit*) and Fcε receptor (*Fcer1a*) showed significant signal values with no bias between mMCs and sMCs. To further evaluate the results, we measured the expression levels of these marker genes by real-time RT-PCR using RNA from the independently isolated MCs (Figure 4b). Moreover, we randomly selected three genes showing 'mMC-biased' expression and another three genes showing 'sMC-biased' expression; expression of these genes in MCs has not been reported previously (Figure 4a). There were no significant differences in the expression levels of *Kit* and *Fcer1a* between mMCs and sMCs. In contrast, the mMC-specific markers *Mcpt1* and *Mcpt2* and the 'mMC-biased' genes, *Anxa10*, *Ctse*, and *Fos* showed higher expression in mMCs, and the sMC-specific markers *Mcpt4* and *Cma2* and the 'sMC-biased' genes, *Cnn1*, *Ces3*, and *Cpe* showed higher expression in sMCs. These results indicate that the microarray

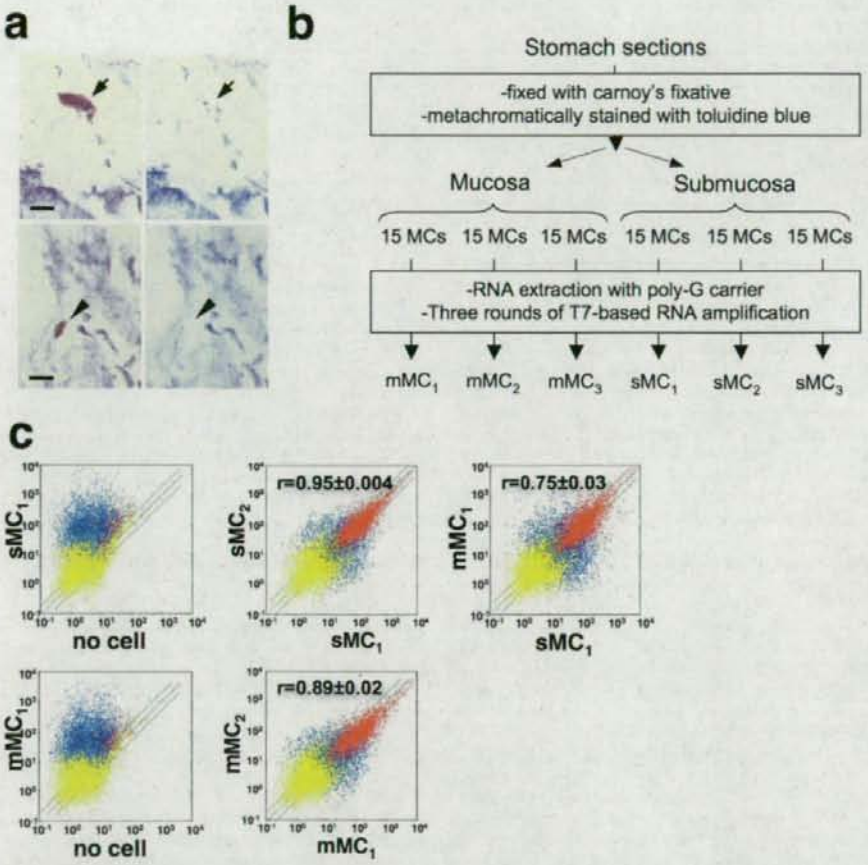

Figure 2

Gene expression profiles of sMCs and mMCs from stomach tissue. (**a**) Isolation of toluidine blue-stained MCs in the submucosa (sMC: *upper panels*) and the mucosa (mMC; *lower panels*) of stomach sections. A sMC (*arrow*) and mMC (*arrowhead*) that was metachromatically stained with toluidine blue before microdissection (*left panels*) disappeared after microdissection with a patch pipette (*right panels*). *Bars*, 10 μm. (**b**) Outline of the experimental strategy. (**c**) Labeled and fragmented antisense RNAs of three individual sMC samples, three individual mMC samples and the 'no cell' samples were hybridized to a Murine Array. Scatter plots for 'no cell' (x axis) and sMC_1 (y axis) (*upper left*), 'no cell' (x axis) and mMC_1 (y axis) (*lower left*), sMC_1 (x axis) and sMC_2 (y axis) (*upper center*), mMC_1 (x axis) and mMC_2 (y axis) (*lower center*), sMC_1 (x axis) and mMC_1 (y axis) (*upper right*) are shown. The correlation coefficients (r) for comparison within sMC_{1-3}, within mMC_{1-3} and between sMCs and mMCs are presented as means ± S.D. Red dots show probe sets judged as "Presence", and yellow dots represent probe sets judged as "Absence" in both arrays. Blue dots show probe sets judged as "Presence" only in either array. The same, two-fold induction and suppression thresholds are indicated as diagonal lines.

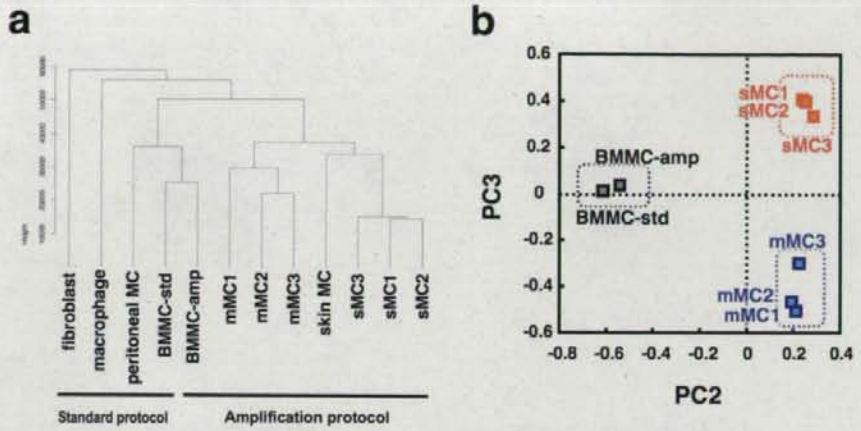

Figure 3
Global gene expression analysis of sMC_{1-3} and mMC_{1-3}. (**a**) Hierarchical clustering of global gene expression of various preparations of MCs and non-MCs. Three-round amplified products of sMC_{1-3}, mMC_{1-3}, skin MCs and BMMCs, and the standard products of BMMCs, peritoneal MCs, macrophages and fibroblasts were analyzed. (**b**) The principal component analysis (PCA) reveals different gene expression profiles of sMC_{1-3}, mMC_{1-3}, and two preparations of BMMCs. The blue dotted square indicates mMCs, the red dotted square indicates sMCs, and the black dotted square indicates BMMCs.

results are reliable and reflect the gene expression profiles of intact sMCs and mMCs in the stomach.

Clustering analysis of the gene expression profiles and functional categorization between sMCs and mMCs

Of the ~12,000 genes represented on the oligonucleotide array, we selected 1,272 genes whose expression levels between sMC_{1-3} and mMC_{1-3} were significantly different ($p < 0.05$, Limma's t test). The expression level of each gene was normalized by its level in BMMCs, which are cultured MCs with so-called 'immature' properties, and the selected genes were classified into seven clusters using the k-means clustering algorithm (CL1-7; Figure 5a and Additional file 1). We also classified the genes into functional categories, and the representative genes are listed (Figure 5b). Among them, 666 genes (52.4%) showed sMC-biased expression (*CL1-3*); in 78% (519 genes) of sMC-rich genes, the expression levels were relatively low in BMMCs and augmented in sMC (*CL1&2*). For example, the expression level of *Mcpt4* was relatively low in BMMCs, and if the expression profile of BMMCs reflects the immature properties of MC progenitors, *Mcpt4* can be concluded to be induced during the final maturation into sMCs. Interestingly, the sMC marker genes *Mcpt5* and *Mcpt6* were classified into *CL2/3*, suggesting that these genes were expressed to some extent in 'immature' BMMCs, but their expression was suppressed during maturation into mMCs. On the other hand, 606 genes (47.6%) showed mMC-biased expression (*CL4-7*); in 51% (334 genes) of mMC-rich genes, their expression levels in BMMCs were low but were augmented in mMCs (*CL4&5*). For example, expression of *Mcpt1* was low in 'immature' BMMCs but was drastically induced during maturation into mMCs.

Protein expression of Notch4 in sMCs and Ptgr1 in mMCs in stomach tissue

Among the genes showing differential expression (Figure 5b), we further focused on the expression of *Notch4* in sMCs and *Ptgr1* in mMCs, both of which have never been previously characterized in MCs. The *Notch4* gene product is a member of the Notch family, consisting of transmembrane receptors which are activated by cell surface ligands on adjacent cells. Recent studies have suggested that Notch signaling is involved in lymphocyte and mast cell differentiation [30,31]. We first confirmed that *Notch4* expression is significantly higher in the separately pooled sMCs than mMCs by real-time RT-PCR (data not shown).

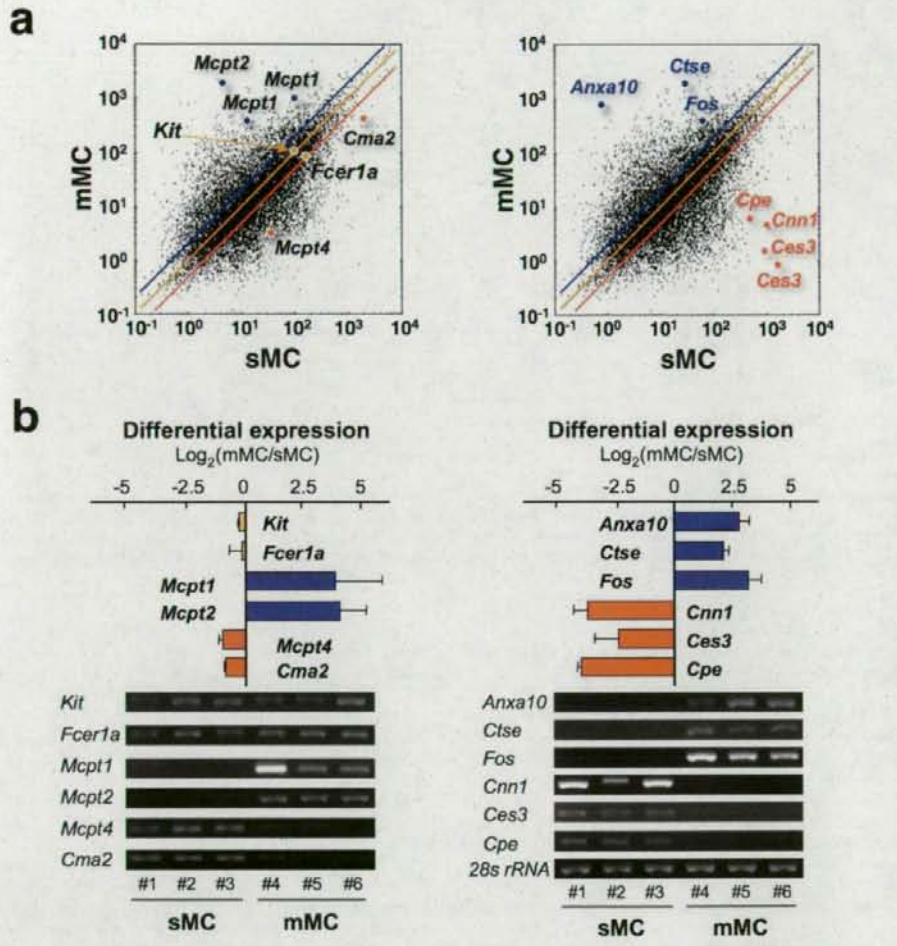

Figure 4
Validation of the differentially expressed genes between sMCs and mMCs. (**a**) sMC-specific (*Cma2*, *Mcpt4*), mMC-specific (*Mcpt1*, *Mcpt2*) and MC-common markers (*Fcer1a* and *Kit*) (*left panel*) and six randomly selected genes (*Ces3*, *Cnn1*, *Cpe*, *Anxa10*, *Ctse* and *Fos*) (*right panel*) are indicated in the representative scatter correlation graphs between sMC_1 and mMC_1. The same, two-fold induction and suppression thresholds are indicated as a yellow, blue and red line, respectively. (**b**) The expression levels of the genes in (a) were verified by real-time RT-PCR. The values represent the ratio of relative expression levels of mMCs to sMCs, and are shown as mean ± S.D. (n = 3). The specificity of the PCR product was confirmed by gel electrophoresis and analysis of the melting temperature. The expression level of each gene was normalized to 28S ribosomal RNA.

Table 1: Summary of genes examined by real-time PCR analysis.

Gene Symbol	Gene Name	RefSeq Transcript ID	Reference
Kit	kit oncogene	NM_021099	15
Fcer1a	Fc fragment of IgE, high affinity I, receptor for α polypeptide	NM_010184	16
Mcpt1	mast cell protease 1	NM_008570	17, 18
Mcpt2	mast cell protease 2	NM_008571	19
Mcpt4	mast cell protease 4	NM_010779	2, 20
Cma2	chymase 2, mast cell (mast cell protease 10)	NM_001024714	14*
Anxa10	annexin A10	NM_011922	21
Ctse	cathepsin E	NM_007799	22
Fos	FBJ osteosarcoma oncogene	NM_010234	23
Ptgr1	Prostaglandin reductase 1 (leukotriene B_4 12-hydroxydehydrogenase)	NM_025968	24 (porcine)
Cnn1	calponin 1	NM_009922	25
Ces3	carboxylesterase 3	NM_053200	26
Cpe	carboxypeptidase E	NM_013494	27 (bovine)
Notch4	Notch gene homolog 4	NM_010929	28
28S rRNA	28S ribosomal RNA	NR_003279	29

*, The coding sequence presented in this paper is the N-terminus truncated-form of *Cma2*, while the RefSeq "NM_001024714" is the complete sequence of *Cma2*.

We next investigated whether the Notch4 protein is exclusively present in sMCs by immunostaining of stomach tissue (Figure 6a). Notch4 signals were detected in the nucleus-like structures of sMCs but not in those of mMCs. Furthermore, Notch4 signals were also found in the skin MCs, which were adjacently clustered with sMCs (Figure 3a). These results show that Notch4 is present in sMCs but not in mMCs, and suggest that Notch4 participates in sMC-specific transcription of Notch-target genes, which may be required for some sMC functions. In hematopoietic cells, it has been reported that constitutively active Notch4 promotes the expansion of progenitor cells and inhibits myeloid differentiation [32]. Since Notch ligands have been shown to exist in connective tissues such as skin dermis [33], it will be interesting to explore whether Notch4 plays a role in the differentiation of sMCs and the maintenance of sMC functions.

The *Ptgr1* product, 15-oxo-prostaglandin 13-reductase/leukotriene (LT) B_4 12-hydroxydehydrogenase is an essential enzyme for inactivation of eicosanoids such as prostaglandin E_2 (PGE_2) and LTB_4 [34]. Although it has been reported that the pathways of eicosanoid synthesis differ among the different MC subclasses [1,4], our results suggest that the inactivation system of eicosanoids also varies among the MC subclasses. *Ptgr1* expression was found to be significantly higher in the separately pooled mMCs by real-time RT-PCR (data not shown). We also examined Ptgr1 expression in stomach sections by immunostaining. Signals for the Ptgr1 protein were found in granule-like structures of mMCs in the stomach mucosa but not in sMCs (Figure 6b), suggesting that the Ptgr1 enzyme may be released from mMCs upon degranulation. Since PGE_2 plays critical roles in the maintenance of gut homeostasis through mucosal protection and inhibition of acid secretion, it is possible that when activated, mMCs negatively regulate the cytoprotective actions of PGE_2 through rapid inactivation by Ptgr1.

Gene expression pattern of extracellular matrix components, adhesion molecules, and cytoskeletal proteins in sMCs and mMCs

MC phenotypes have been shown to depend on their interactions with the surrounding extracellular matrices (ECMs) and neighboring cells [1]. One of the most remarkable findings in this study is the difference in gene expression of ECM protein components, adhesion molecules, and cytoskeletal proteins, which may reflect functional adaptation of each type of MC to the mucosal or submucosal environment in the stomach (Figure 5b). mMCs express genes for mucosa-specific ECM proteins such as *Muc1* (Mucin) and *Tff1* (Trefoil factor), while sMCs express genes for conventional ECM proteins such as *Col4a* (procollagen) and *Lama2* (laminin). Moreover, sMCs express genes for adhesion molecules such as *Alcam* and *Vcam1*, and genes for ordinary cytoskeletal proteins such as *Acta2* (actin), while mMCs express desmosome-component genes such as *Dsc2* (desmocollin) and *Dsg2* (desmoglein), and genes for keratin intermediate filaments such as *Krt8* and *Krt19*. Desmosomes were reported to be present in the stomach epithelia [35], and it was found that desmosome-like structures are detected in a particular type of MC [36]. It is thus possible that mMCs interact with adjacent epithelia through desmosomal adhesion in the stomach. In contrast, sMCs appear to interact with neighboring cells via adhesion molecules such as VCAM-1, ALCAM and VE-cadherin (*Vcam1*, *Alcam1* and *Cdh5*). Since these adhesion molecules have been shown to be involved in dynamic regulation of the actin cytoskeleton [37,38], such molecule-mediated inter-