Global analysis of G-protein-coupled receptor signaling in human tissues

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Received 28 May 2003; revised 30 June 2003; accepted 30 June 2003

First published online 31 July 2003

Edited by Robert B. Russell

Abstract The G-protein-coupled receptor (GPCR) family mediates a host of cell–cell communications upon activation by diverse ligands. Numerous GPCRs have been shown to display anatomically selective patterns of gene expression, however, our understanding of the complexity of GPCR signaling within human tissues remains unclear. In an effort to characterize global patterns of GPCR signaling in the human body, microarray analysis was performed on a large panel of tissues to monitor the gene expression levels of the receptors as well as related signaling and regulatory molecules. Analysis of the data revealed complex signaling networks in many tissue types, with tissue-specific patterns of gene expression observed for the majority of the receptors and a number of components and regulators of GPCR signaling.

Key words: GPCR; G-protein; Microarray; Gene expression

1. Introduction

G-protein-coupled receptors (GPCRs) constitute the largest family of cell-surface proteins. A wide variety of extracellular stimuli (such as hormones, chemokines, odorants, and neurotransmitters) signal through this receptor family. GPCRs for which the natural ligands have been identified are referred to as 'known' receptors, while those for which no natural ligands have yet been found are referred to as 'orphan' receptors.

Upon binding of extracellular ligands, GPCRs interact with GTP-binding proteins (G-proteins). G-proteins are heterotrimeric complexes composed of an α, β, and γ subunit, each of which has multiple isoforms [1]. Their interaction with GPCRs results in the dissociation of the Gα subunit from the Gβγ complex. The dissociated subunits may subsequently inhibit or activate effector enzymes that modulate secondary signaling pathways [2,3]. Regulation of G-proteins by RGSs (regulator of G-protein signaling) and GPCR trafficking by RAMPs (receptor activity modifying proteins) have also been described as mechanisms of modulating receptor signaling [4,5]. The various mechanisms of GPCR signaling likely allow for the range of extracellular stimuli to selectively induce cellular responses in target tissues. While the extent of diversity in GPCR signaling is unclear, results from a number of studies illustrate the tendency for GPCR gene expression to be enriched in particular tissues. Restricted tissue distribution of GPCRs has frequently reflected their physiological functions, as is the case of the role for the central nervous system (CNS)-enriched dopamine receptors in neurotransmission [6]. Assessment of the tissue distribution of GPCRs and related signaling molecules may therefore clarify the complexity of the molecular mechanisms by which receptors act to transduce extracellular stimuli.

The sequencing of the human genome has brought new avenues by which global approaches can be undertaken to investigate the breadth of GPCR signaling. It is now estimated that the GPCR superfamily consists of 600–1000 receptors [7]. The advent of microarray technology allows for a large sampling of the receptor family to be performed. This technology permits one to monitor the message levels of thousands of genes simultaneously in a given sample [8,9]. In this study, we have used a custom high-density oligonucleotide microarray containing probes designed to measure the gene expression levels of over 700 human GPCRs, along with a number of molecules involved in GPCR signaling and regulation. Evaluation of the transcriptional levels for these genes across a large panel of tissues would thus provide a global view of GPCR signaling in the human body. This custom microarray, in addition, was designed to monitor gene expression levels in the mouse, a common model organism used to study the function of human GPCRs. Comparative analysis of the gene expression levels of putative ortholog GPCR pairs across a panel of tissues may characterize the concordance in receptor signaling between the two species.

2. Materials and methods

2.1. Sample information and preparation of total RNA

Gross dissections of brain tissues from normal human donors were obtained from the Harvard Brain Tissue Resource Center (McLean Hospital, Belmont, MA, USA) and Clinomics Biosciences (Frederick, MD, USA). Selection of normal donors was based on gross and microscopic examinations of multiple brain regions that indicated unremarkable pathology. Total RNA was extracted from brain tissues with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The quality of total RNA was then assessed using an Agilent 2100 bioanalyzer (visible absence of significant 28S and 18S band degradation) and by spectrophotometry. High-quality total RNA derived from the same brain region of a minimum of three donors was pooled in an effort to avoid biological variability. Total RNA from normal human peripheral tissues of multiple donors was purchased from commercial sources. Stable cell lines were acquired from American Type Culture Collection (Manassas, VA, USA). Cells were cultured under recommended conditions to near confluence. Mouse tissues were dissected from 8–15 C57/BL6 mice ages 8–10 weeks.

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Abbreviations: GPCR, G-protein-coupled receptor; G-protein, GTP-binding protein; RGS, regulator of G-protein signaling; RAMP, receptor activity modifying protein; CNS, central nervous system

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00762-2

2.2. Microarray procedure and data analysis

High-density oligonucleotide microarray analysis was performed essentially as previously described [10] using the Affymetrix (Santa Clara, CA, USA) GeneChip technology. A custom dual-species microarray was designed to contain probes that monitor the expression of over 700 known, orphan, and olfactory-like human GPCRs. Additionally, probes for a comparable number of putative mouse ortholog GPCRs were designed. Putative mouse ortholog receptors were identified by reciprocal BLAST searches. Probes for mediators and regulators of GPCR signaling described in the literature were also included. Microarray analysis of total RNA was performed in duplicate for each sample.

GeneChip software MAS 5.0 was used to analyze the relative abundance of labeled cRNA hybridized to the microarray from the scanned image. A threshold signal (Avg. Diff.) level was defined for human and mouse samples to call a gene transcript present or detectable based on polymerase chain reaction (PCR) validation of the microarray data and the expression levels of genes reported in the literature to be expressed in particular tissues. Tissues were grouped based on functional similarity and classified as primary and stable cell lines, adipose, endocrine, CNS, immune, reproductive, gastrointestinal, or other tissue types. Hierarchical cluster analysis was performed on genes that were detected in at least one tissue sample and differentially expressed (ANOVA, $P > 0.05$) by a hierarchical clustering algorithm [11] using an average linkage method [12].

2.3. Comparative analysis of human and mouse microarray data

A set of common human and mouse tissues were used for the analysis. Receptors that were detected and differentially expressed (ratio of standard deviation to mean greater than 0.5) in at least one of the common tissues from both human and mouse samples were incorporated into the analysis. Retained ortholog pairs were compared by Pearson's correlation coefficient.

2.4. PCR validation of microarray data

PCR validation was assessed for 87 receptors for which expression was detected by microarray analysis in at least one of six selected tissues. cDNA was synthesized from the total RNA samples profiled following the same protocols used for microarray analysis. Primers were designed with the nucleotide sequence used to devise the oligonucleotides synthesized on the microarrays. For each receptor, PCR was performed with 100 ng cDNA of the tissues that showed detectable receptor expression by microarray analysis (total of 120 reactions). Reactions were scored based on successful amplification of receptor fragments from each cDNA. The incidence of receptor expression as determined by microarray analysis was then assessed in each of the reactions.

3. Results

3.1. GPCR detection by microarray analysis

The gene expression levels of over 400 known and orphan receptors and approximately 300 olfactory-like receptors were assayed using a high-density oligonucleotide microarray. Microarray analysis was performed on 80 CNS and peripheral non-diseased tissues, as well as primary cells and stable cell lines. Results from the microarray experiments were validated by PCR of 87 receptors in six different tissues. GPCRs that were detected by microarray analysis were successfully amplified by PCR 85% of the time, while 15% of the reactions showed no expression (data not shown). Of the GPCRs assayed, 373 receptors had detectable transcriptional levels in at least one of the tissues profiled. The lack of detection of the other receptors represented on the microarray may, in part, be due to non-optimal probe design, inadequate profiling of other tissue types and developmental stages, or signal dilution caused by cell types present in the sample assayed that do not express the receptor.

3.2. Hierarchical cluster analysis of expression data

Hierarchical cluster analysis of the 373 detected receptors was performed to identify tissue-specific patterns of GPCR expression (Fig. 1A). Dendrogram representation of the sample clustering partitioned the tissues into neuronal and peripheral groupings. These data, not surprisingly, show a greater similarity in GPCR expression patterns among neuronal tissues relative to peripheral tissues. Most notably expressed within the neuronal tissues was a cluster of 67 GPCRs. Among these receptors were the dopamine, GABA, and brain-specific angiogenesis inhibitor receptors (Fig. 1B). Tissue-specific clusters of GPCR expression were also present in a number of peripheral tissues. Immune-related sample profiles revealed a cluster of 65 enriched GPCRs, with a number of selectively expressed receptors in eosinophils, neutrophils, and natural killer cells. Prominent clusters of GPCRs were additionally found in the profiles of the pancreas, liver, testes, and adipose tissues. The specificity in receptor tissue distribution indicates that functionally related tissues share subsets of GPCRs with overlapping expression, while other tissues having specialized physiological functions express clusters of GPCRs with more distinct expression patterns. Additionally, a set of cultured primary and stable cell lines derived from unrelated tissues (including neural progenitors and PC-3) showed similar patterns of GPCR expression. Clustering of these samples in the same dendrogram branch may indicate that in vitro culturing environments may significantly modulate GPCR expression.

Cellular responses induced by GPCR activation are mediated by a number of signaling and regulatory molecules. The tissue distribution for over 100 expressed genes was therefore examined by hierarchical cluster analysis to determine whether particular mechanisms of GPCR signaling and regulation are predominant in certain tissue types. Three prominent gene clusters were identified in neuronal tissues, immune-related cell and tissue types, and a set of peripheral tissues. Among the gene clusters found were a set of 10 G-proteins and eight RGSs enriched in neuronal tissues and five G-proteins and three RGSs enriched in immune cell types (Fig. 1C, upper and middle panels). Another set of genes found to be enriched in the peripheral tissues contained RAMPs 1–3 as well as two RGSs and two G-proteins (Fig. 1C, lower panel). Approximately half of these peripheral tissues are adipose-related samples, implying a significant regulatory role for the genes in GPCR signaling within adipose tissues. Genes that displayed ubiquitous expression with nominal tissue specificity included dynamin2, the heavy and light polypeptide chains of clathrin, and β-arrestin 1 and 2. β-arrestin 2, however, was more highly expressed in monocytes, eosinophils, and neutrophils in comparison to the other tissues (data not shown). Such patterns in tissue distribution imply broad employment of receptor internalization mediated by these genes.

3.3. Expression and enrichment of GPCRs

Further examination of the microarray data revealed that nearly all tissues profiled expressed between 40 and 120 receptors (Fig. 2A). The pancreas, followed by CNS tissues, had the greatest number of expressed receptors, suggesting a high degree of cellular regulation by GPCR signal transduction. Approximately 210 of the detected receptors were expressed in CNS tissues, alluding to the complexity of regulating neuronal networks. Interestingly, a number of peripheral tissues

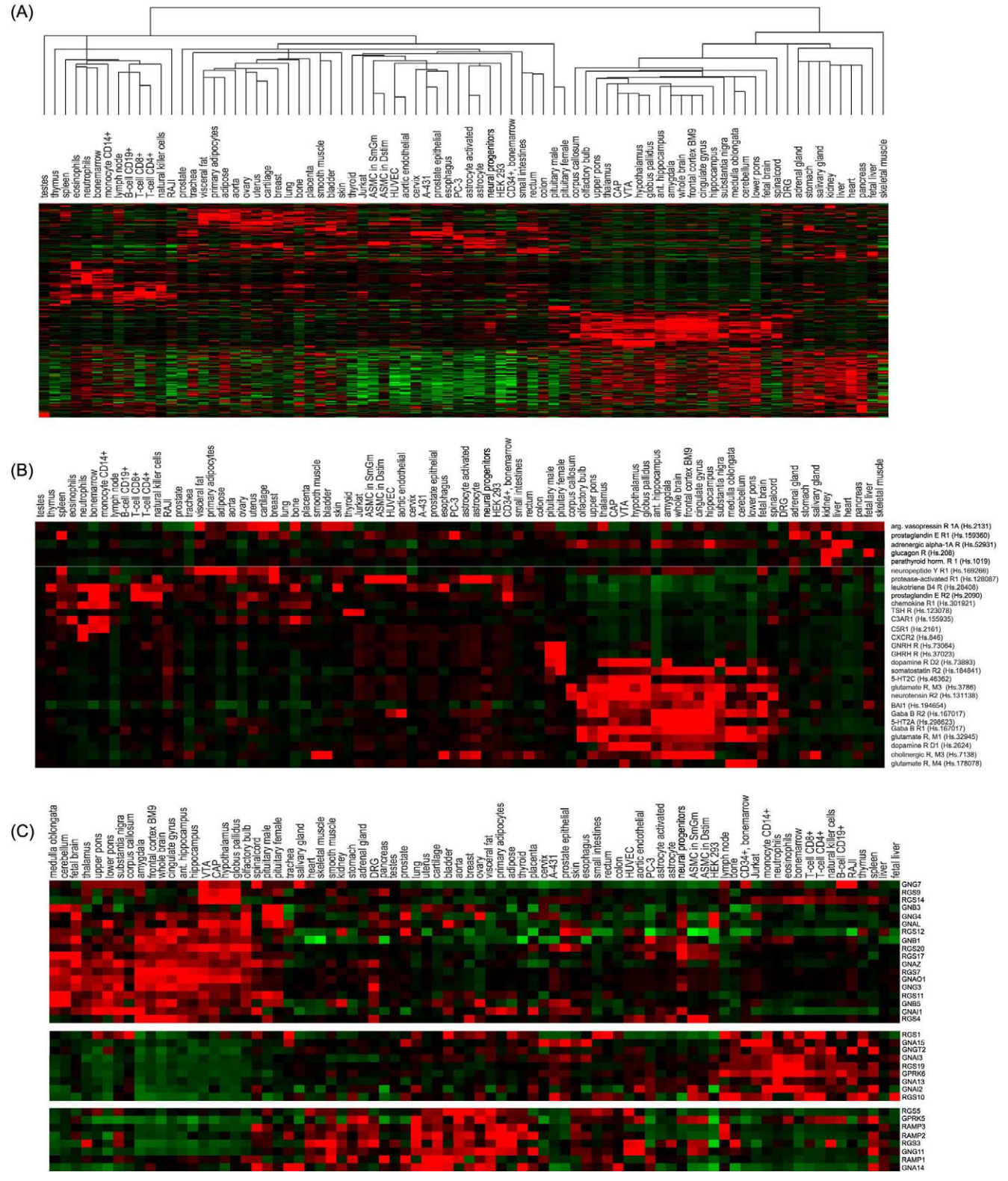

Fig. 1. Hierarchical cluster analysis of gene expression in human tissues. Expression data for genes detected in at least one tissue were used for hierarchical cluster analysis. Expression levels were normalized for each gene across all tissue samples (red, above median expression; black, median expression; green, below median expression). Genes were clustered based on correlations in tissue distributions. Tissues were clustered based on correlations in gene expression profiles. A: Dendrogram of tissue relations is generated using the expression profiles of 373 GPCRs. Expression data from hierarchical cluster analysis of (B) a select set of GPCRs with respective UniGene numbers and (C) select clusters of signaling and regulatory molecules are shown.

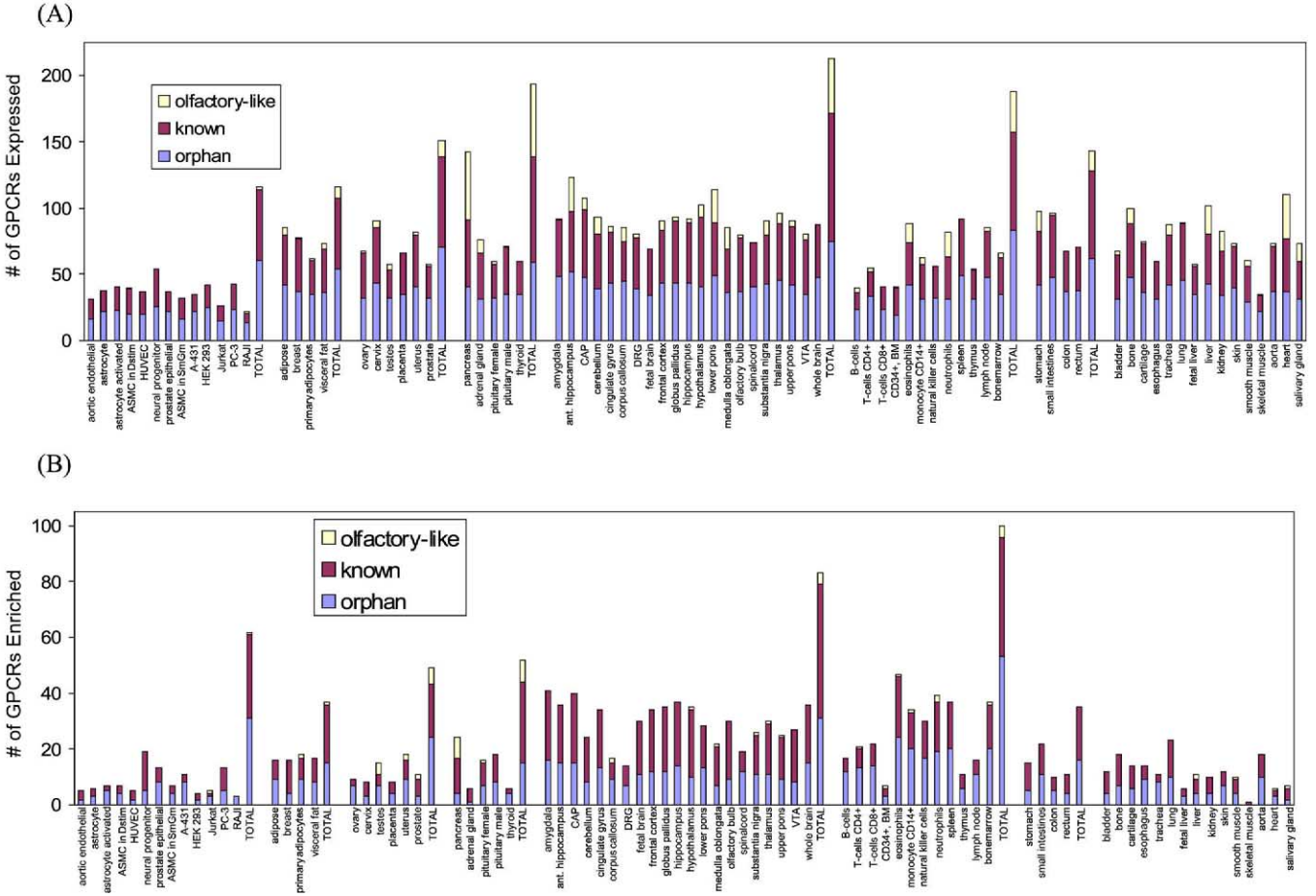

Fig. 2. Expression and enrichment of GPCRs. A: Identification of the number of GPCRs expressed in tissue samples. GPCRs that were detected in each tissue were classified as known (ligand identified), orphan (no ligand identified), or olfactory-like. B: Identification of the number of GPCRs enriched in individual tissues. GPCRs detected within each tissue that had greater than five-fold above-median expression levels across all tissues were classified as described above. The total number of non-overlapping receptors found within related tissue types is represented for each tissue group as a total.

likewise expressed a large subset of GPCRs. In contrast, cultured primary and stable cell lines trended towards having the least number of expressed receptors. Classification of all expressed GPCRs revealed that 281 of these receptors were known or orphan and 92 were olfactory-like. The majority of the samples profiled displayed comparable percentages of known and orphan receptors. Olfactory-like receptors generally composed 0–20% of the expressed GPCRs.

GPCRs whose expression is enriched in particular tissues are postulated to play a specific regulatory role related to those tissues. Analysis of the relative expression levels of GPCRs revealed that 66.5% of the detected receptors were enriched five-fold or greater above median levels across all tissues profiled (Fig. 2B). These enriched receptors were largely comprised of known as well as orphan GPCRs. Few of the enriched receptors were olfactory-like, most likely due to their generally low gene expression levels. Tissues having the greatest occurrence of enriched receptors included various brain regions and immune cell types. The large number of enriched receptors suggests the necessity for complex and selective regulatory mechanisms in these tissues.

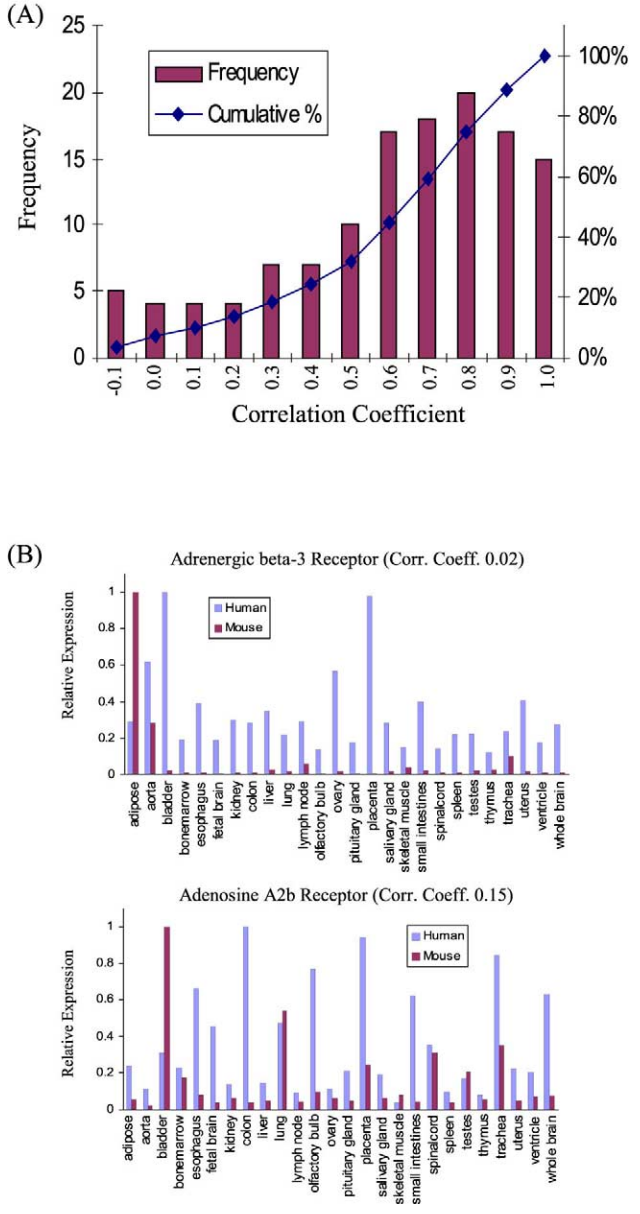

Fig. 3. Comparison of GPCR expression levels in human and mouse tissues. A: Pearson's correlation analysis was performed on 128 GPCRs across 26 common human and mouse tissues. The frequency of correlation coefficients is plotted. B: The expression levels of two receptors, adrenergic β-3 receptor and adenosine A2b receptor, with low concordance between mouse and human tissues are shown.

3.4. Comparative analysis of human and mouse GPCR expression

Efforts to identify the functional roles of GPCRs often rely on the mouse as a model organism. A study of the concordance in GPCR expression between mouse and human tissues was conducted by microarray analysis. The dual-species custom microarray was used to monitor the gene expression levels of over 600 putative orthologs to human GPCRs in a panel of 71 mouse tissues. Of the mouse GPCRs assessed, 365 receptors were detectable in at least one tissue, with most tissues expressing 50–70 GPCRs (data not shown). A comparison of human and mouse receptor profiles was performed across 26 common tissues for 128 putative ortholog GPCR pairs having detectable and differential expression levels within those tissues. Analysis of the expression data revealed that approximately half of the GPCRs had correlation coefficients of 0.6 or greater (Fig. 3A). A number of GPCRs (including parathyroid hormone receptor 1, formyl peptide receptor 1, dopamine receptor D2, and frizzled homolog 4) displayed highly correlative expression patterns between mouse and human tissues. Likewise, several receptors, such as adrenergic β-3 receptor and adenosine A2B receptor, had poor correlation coefficients between samples (Fig. 3B). These results indicate that while many GPCRs have relatively conserved expression distributions in mice and humans, some receptors may have divergent physiological functions in particular tissues.

4. Discussion

In this study, microarray analysis was employed to map the tissue distribution of GPCR family members, effector pathway components, and regulator molecules. Analysis of a diverse panel of tissues was essential in obtaining an accurate global perspective of GPCR signaling. Interrogation of the expression data set has provided insights into the complexity of cell–cell communication and responsiveness to environmental stimuli within various tissue types.

Data analysis revealed that of the receptors assayed, 373 were detected in at least one tissue or cell type. Among these GPCRs, an unexpectedly large number of olfactory-like receptors were found to have broad patterns of tissue distribution. These results suggest a functional role for the receptors outside the olfactory tract. A few other studies have likewise reported the expression of olfactory-like receptors in the brain, testes, and prostate [13–15]. It remains to be determined whether the messages detected for olfactory-like receptors in CNS and peripheral tissues are transcribed into functional receptors. In fact, some reports approximate that 70% of olfactory receptors are pseudogenes [16,17]. However, Spehr et al. has recently identified a functional role for an odorant receptor in mediating human sperm chemotaxis [18]. Our results add to the growing body of evidence that olfactory-like

receptors have potentially broad functionality as opposed to limited roles in olfaction detection.

Overall, the greatest number of expressed and enriched receptors was found among CNS tissues. The cellular complexity of the CNS has long been viewed as requiring specific and tight regulation of neuronal activity, perhaps more so than other tissue types. Indeed, many neurotransmitters have been shown to modulate neuronal responses via GPCR signaling mechanisms [19,20]. While individual CNS tissues expressed a large number of GPCRs, analysis of the microarray data revealed detection of comparable receptor numbers in many other tissue types. Likewise, the number of enriched receptors, which presumably have specific functional roles, was similar in neuronal tissues, the pancreas, and immune-related cell types. It is possible, however, that the number of GPCRs expressed in neuronal cell types is underestimated due to signal dilution caused by the heterogeneous nature of neuronal tissues. This issue may be clarified by current approaches of gene expression analysis at the cellular level using laser-dissected neuronal cell types [21]. Our data therefore suggest that much like CNS tissues, various other tissue types may utilize highly complex networks of GPCR signaling to regulate cellular activity.

Hierarchical cluster analysis of the GPCR expression data also identified tissue types with similar receptor profiles as well as those having unique patterns of GPCR expression. This analysis revealed clusters of GPCRs enriched in many of the tissues profiled. The most notable examples of such GPCR clusters were found in the CNS and immune cell types, indicating overlapping expression of receptor subsets within similar tissue types. Additionally, profiles of the testis, pituitary gland, stomach, and endothelial cell populations each contained highly selective GPCR clusters. It may be inferred that GPCRs in these clusters function to specifically modulate the activity of the specialized cell types within the tissues. Interestingly, a cluster of GPCRs was found within a set of cultured primary and stable cell lines. These receptors may be up-regulated as a cellular response to in vitro culturing conditions and thus possibly involved in proliferative or anti-apoptotic signaling pathways. This assertion supports previous reports of G-proteins harboring transformation and cell proliferation potential [22].

Hierarchical cluster analysis of mediator and regulator molecules of GPCR signaling has also identified clusters in gene expression patterns. Signaling molecules enriched in CNS- and immune-associated clusters included a number of RGS proteins. RGS proteins rapidly turn off GPCR signaling pathways by inactivating G-proteins [23]. Enrichment of RGS proteins in neuronal and immune tissues therefore suggests a need for tight regulation of cellular signaling. While neuronal expression of RGS proteins has previously been reported [24,25], the mechanism by which they inhibit GPCR signaling is poorly understood. Extrapolation from the cluster analysis implies that the G-proteins enriched in the CNS tissues may be candidate substrates of these RGS proteins. For instance, the concordance in gene expression suggests that GNAZ and GNAO1 are likely substrates of RGS7. Similarly, in the immune-related cluster, RGS19 may modulate the activity of GNAI3. Within the cluster of genes found in peripheral tissues were RAMPs 1–3. These recently identified GPCR-interacting proteins have been shown to modulate receptor trafficking, glycosylation, and ligand-binding pocket [5]. Though the range of receptors that interact with RAMPs remains unknown, the high gene expression levels point to their functional significance in these tissues, in particular adipose tissues. Within each of the clusters, various isoforms of the α, β, and γ G-protein subunits were also present. These results suggest that the three subunits may each contribute to tissue-specific signaling mechanisms. Further examination of the regulation of the above-mentioned signaling and regulatory molecules under physiological conditions is warranted. Given their tissue selectivity and contributions to GPCR signaling, some of these molecules may develop into appealing therapeutic targets.

Common mechanisms of GPCR regulation were also observed across the panel of tissues. Dynamin2 as well as the heavy and light polypeptide chains of clathrin and the β-arrestins were ubiquitously expressed in the profiled tissues. These molecules are involved in the desensitization of activated GPCRs via receptor internalization [26–28]. The expression data therefore suggest that clathrin-dependent endocytosis is a general feedback mechanism used to protect against acute and chronic over-stimulation of GPCRs upon ligand binding. Noteworthy are the enhanced expression levels of β-arrestin 2 in monocytes, eosinophils, and neutrophils. Studies examining the roles of β-arrestin 1 and 2 in receptor internalization have indicated their varying affinities for different GPCRs [28]. The enriched gene expression of β-arrestin 2 may indicate a prominent role for it in the internalization of receptors specifically expressed in these immune cell types.

Finally, an assessment of the concordance in GPCR expression patterns between human and mouse tissues was performed. Mouse models of human physiology and disease are increasingly being employed to characterize the functional roles of GPCRs and their natural ligands. The suitability of a model organism for the study of human genes often, in part, relies on the sequence homology between species. Our study indicates that expression data should complement sequence information when evaluating model organisms. Although many receptors had good concordance in their expression levels across mouse and human tissues, some GPCRs did not show comparable patterns of expression. For example, the adrenergic β-3 receptor displayed high concordance in adipose tissue where it is known to stimulate lypolysis. Yet, poor concordance in gene expression levels was seen in the bladder, placenta, and ovary. The microarray data indicate that, with respect to gene expression, the mouse may be a suitable model for the study of many GPCRs. However, the functional roles of certain receptors may not be conserved between human and mouse in some tissue types.

In conclusion, results from this study reveal vast GPCR signaling networks in many tissue types. Expression of numerous GPCRs in most tissues suggests broad cellular sensitivity to a host of extracellular stimuli. Furthermore, the expression data support the notion of tissue-specific mechanisms of GPCR signaling and regulation. These results are in accordance with a recent study by Vassilatis et al. [29], which similarly detected unique GPCR expression profiles within various tissues. Utilization of diverse mechanisms to transduce GPCR signals likely allows for distinct rates and durations of cellular responses required by various tissues. Understanding of global patterns of GPCR signaling provides a step towards elucidating receptor function and how their dysregulation may result in human disorders. Such information offers

important insights into the identification of novel GPCRs as therapeutic targets.

Acknowledgements: We thank J. Leonard, D. Bagnol, and L. Croner for scientific contributions and J. Richman for critical reading of the manuscript. Brain tissues were provided by the Harvard Brain Tissue Resource Center, which is supported in part by PHS Grant number MH/NS 31862.

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