Generation and network analysis of an RNA-seq transcriptional atlas for the rat

Kim M. Summers1,*,†, Stephen J. Bush2,†, Chunlei Wu3 and David A. Hume3,*,†

1Mater Research Institute—University of Queensland, Translational Research Institute, 37 Kent St, Woolloongabba, QLD 4102, Australia, 2Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, OX3 9DS, UK and 3Department of Integrative and Computational Biology, The Scripps Research Institute, La Jolla, CA 92037, USA

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ABSTRACT

The laboratory rat is an important model for biomedical research. To generate a comprehensive rat transcriptomic atlas, we curated and downloaded 7700 rat RNA-seq datasets from public repositories, down-sampled them to a common depth and quantified expression. Data from 585 rat tissues and cells, averaged from each BioProject, can be visualized and queried at http://biogps.org/ratatlas. Gene co-expression network (GCN) analysis revealed clusters of transcripts that were tissue or cell type restricted and contained transcription factors implicated in lineage determination. Other clusters were enriched for transcripts associated with biological processes. Many of these clusters overlap with previous data from analysis of other species, while some (e.g. expressed specifically in immune cells, retina/pineal gland, pituitary and germ cells) are unique to these data. GCN analysis on large subsets of the data related specifically to liver, nervous system, kidney, musculoskeletal system and cardiovascular system enabled deconvolution of cell type-specific signatures. The approach is extensible and the dataset can be used as a point of reference from which to analyse the transcriptomes of cell types and tissues that have not yet been sampled. Sets of strictly co-expressed transcripts provide a resource for critical interpretation of single-cell RNA-seq data.

INTRODUCTION

In the year of the rat (2020), the Rat Genome Database (RGD) celebrated 20 years of development (1). Those 20 years saw completion of the draft genome (2). Around 90% of protein-coding genes had an inferred 1:1 ortholog in humans. Subsequent technology advances allowed the sequencing of multiple inbred strains, including several with disease-associated alleles (3). Szpirer (4) catalogued >350 rat genes where rat lines with natural or introduced variants provide models for human disease.

Analysis of transcriptional regulation in human and mouse has been driven by large consortium projects such as GTEx (5) and FANTOM (6), and there are many online resources for these species. Multi-tissue transcriptional atlas projects have also been published for other species, including chicken, sheep, buffalo, pig and goat (7–11). Although it was once suggested that guilt by association is the exception rather than the rule in gene regulatory networks (12), the principle is now very well established. Genes associated with specific organs, cell types, organelles and pathways (e.g. the cell cycle, protein synthesis, oxidative phosphorylation/mitochondria) are co-expressed along with transcription factors that regulate them (5,6,8,13–18). An extension of the principle of co-regulated expression is that it is possible to extract signatures of specific cell types, for example the stromal component of tumours (19) or resident tissue macrophages (20), based upon analysis of a large number of samples in which their relative abundance is variable.

The functional annotation of the rat genome is still a work in progress. Many rat genes in Ensembl are described as ‘novel rat gene’ and annotated solely by a gene number. Transcriptional regulation has evolved rapidly among mammalian species (21,22). Even where there is 1:1 orthology at the level of protein-coding sequence and conservation of synteny with other mammals, the expression may not be conserved. Two substantial studies have contributed to annotation of the rat transcriptome through RNA-seq analysis of a partly overlapping set of major rat organs (23,24). Long-read RNA sequencing has also contributed to refine-ment of rat transcriptome annotation (25). Because of the extensive use of the rat as a model in biomedical research, there are thousands of RNA-seq datasets in the public domain from isolated cells and tissues in various states of activation that could provide an additional resource for functional annotation. By combining random library downsiz-
MATERIALS AND METHODS
Selecting samples for an expression atlas of the rat
To create a comprehensive expression atlas for the rat, we first downloaded the daily updated NCBI BioProject summary file from ftp://ftp.ncbi.nlm.nih.gov/bioproject/summary.txt (obtained 19 July 2021) and parsed it to obtain all BioProjects with taxonomy ID 10116 (Rattus norvegicus) and a data type of ‘transcriptome or gene expression’, supplementing this list by manually searching NCBI Geo and NCBI PubMed for the keywords ‘RNA-seq AND rat’. BioProjects were selected to extend the diversity of tissues, cells and states from two existing rat transcriptomic atlases that analyse gene expression in a subset of major rat tissues (23,24). For each BioProject, we automatically extracted the associated metadata using pyrsadb v1.0.1 (27) with parameter ‘-detailed’ or by manual review. Metadata for each BioProject, indicating (where available) the breed/strain, sex, age, tissue/cell type extracted and experimental condition (e.g. treatment or control), are detailed in Supplementary Table S1, which includes both the data downloaded via the pipeline and additional information retrieved manually from the European Nucleotide Archive record, NCBI BioProject record and cited publications. For incorporation into the expression atlas, we required that all samples have, at minimum, tissue/cell type recorded. Overall, the input to the atlas comprised 7682 samples from 363 BioProjects.

Quantifying gene expression for the atlas
For each library, expression was quantified using Kallisto (26) as described in detail in previous studies on other species (7–9,20). Kallisto quantifies expression at the transcript level, as transcripts per million (TPM), by build-}

Network analysis and functional clustering of atlas samples
To examine the expression of genes across this wide range of tissues and cell types, the expression data were analysed using the network analysis tool BioLayout (derived from BioLayout Express3D (30)), downloaded from http://biolayout.org. The same files can be uploaded into the recently developed open source package, Graphia (https://graphia.app), which supports alternative clustering approaches and dynamic modification of parameters.

The initial analysis used the values averaged by age and BioProject for each tissue. Subsequent analyses used individual values for samples of liver, musculoskeletal system, cardiovascular system, kidney and central nervous system. For each analysis, a sample-to-sample correlation matrix was initially constructed at the Pearson correlation coefficient \( r \) threshold necessary to include all samples in the analysis (shown in the ‘Results’ section and figure legends). Pearson correlations were then calculated between all pairs of genes to produce a gene-to-gene correlation matrix of all genes correlated at \( r \geq 0.75 \).

Gene co-expression networks (GCNs) were generated from the matrices, where nodes represent either samples or genes and edges represent correlations between nodes

The purpose of the integration was to include transcripts that had not already been assigned Ensembl transcript IDs and whose sequence was not already present in the Ensembl release (under any identifier). RefSeq mRNAs incorporate untranslated regions (UTRs) and so could encapsulate an Ensembl CDS. The trimmed UTRs from each mRNA were generated excluding all sequence outside the longest open reading frame. In total, the reference transcriptome comprised 71,074 transcripts, representing 25,013 genes. Using this reference, expression was quantified for 7682 publicly archived paired-end Illumina RNA-seq libraries. The BioProjects are summarized in Supplementary Table S1. Prior to expression quantification, and for the purpose of minimizing variation between samples, we randomly downsampled all libraries to 10 million reads, five times each, using seqtk v1.2 (https://github.com/lh3/seqtk, downloaded 4 June 2018). Expression level was then taken to be the median TPM across the five downsampled replicates.

Within individual BioProjects, median TPM for replicate samples of the same tissue, age or condition was averaged. The final expression atlas is based on the averaged median downsampled TPM per gene for each distinct set of replicate samples. As in previous projects for other species (7–11), the full dataset of 585 averaged expression data from cells and tissues is displayed on BioGPS (28,29) at biogps.org/ratatlas to enable comparative analysis across species. The full processed primary dataset and the averaged data are available for download at an Institutional Repository (https://doi.org/10.5287/bodleian:Am9aky72). The latter is a comma-separated text file, which can be directly loaded into the network analysis software used herein or alternatives such as Gephi (https://gephi.org) or Cytoscape (https://cytoscape.org). This file can be easily supplemented by addition of further RNA-seq data processed in the same way. All scripts for generating the atlas are available at https://github.com/sjbush/expr_atlas.
above the selected correlation threshold. For the sample-to-sample analyses (essentially analogous to a principal component analysis), an initial screen at the r value that entered all samples was performed, followed by subsequent analyses with a higher r value that removed outliers and revealed more substructure in the networks. For each gene-to-gene analysis, an r value threshold of 0.75 was used for all analyses (Supplementary Figure S1).

For the gene-to-gene networks, further analysis was performed to identify groups of highly connected genes within the overall topology of the network, using the Markov clustering algorithm (MCL) (31). The MCL is an algebraic bootstrapping process in which the number of clusters is not specified. A parameter called inflation effectively controls granularity. The choice of inflation value is empirical and is based in some measure on the predicted complexity of the dataset (31). The inflation value was 1.7 or 2.2 as indicated and only genes expressed at ≥10 TPM in at least one sample were included. Gene Ontology (GO) terms and Reactome pathways were derived from the Gene Ontology Resource (http://geneontology.org, release of 18 August 2021) using PANTHER overrepresentation test (PANTHER release of 24 February 2021). The reference list used was R. norvegicus (all genes in database), the Gene Ontology database was the release of 2 July 2021 (DOI: 10.5281/zenodo.5080993) and the Reactome pathway analysis used Reactome version 65, released 17 November 2020. These resources are all available at the Gene Ontology Resource (http://geneontology.org).

RESULTS

Samples in the atlas

Seven thousand six hundred eighty-two RNA-seq libraries, each with a unique SRA sample accession from 363 BioProjects, were obtained by the pipeline as described in the ‘Materials and Methods’ section and used to create a global atlas of gene expression. Metadata for the individual BioProjects are summarized in Supplementary Table S1. For comparative tissue analysis and the core atlas, expression across libraries was averaged by tissue, age and BioProject. This reduced the dataset to 585 different averaged samples of rat tissues and cells summarized in Supplementary Table S2A. For a separate analysis of liver, kidney, musculoskeletal, cardiovascular and central nervous systems to extract tissue-specific co-expression signatures, individual RNA-seq datasets from within each BioProject were used.

Network analysis of the rat transcriptome

Initially, we performed a sample-to-sample correlation to assess whether there were likely to be batch effects resulting in outlier samples that were unrelated to tissue type. To include all 585 samples, it was necessary to use a sample-to-sample r ≥ 0.21. An image of the resulting network graph is shown in Figure 1. This visualization is analogous to a principal component analysis. Since BioProjects tended to focus on one strain, age, sex and tissue/treatment, some BioProject-specific clustering was expected. However, illustrating the robustness of the sampling and downsizing approach, the same or related tissues analysed in different BioProjects generally clustered together (compare Figure 1A where nodes are coloured by organ system and Figure 1B where they are coloured by BioProject). Note, for example, the tight clustering of liver sample (olive) generated by multiple independent laboratories. At a more stringent correlation coefficient threshold of 0.7, only 15 samples of relatively low connectivity were removed, but the association of nodes by organ system rather than BioProject becomes even more clear-cut (Figure 1C and D). No clear outliers or BioProject-specific clusters (batch effects) were identified, so all averaged samples were included in the subsequent gene-centred network analysis.

The threshold correlation coefficient for the gene-to-gene network was chosen empirically to maximize the number of nodes (genes included) while minimizing the number of edges (correlations between them) (Supplementary Figure S1). At the chosen correlation coefficient of r ≥ 0.75, the graph contained 14 848 nodes (genes) connected by 1 152 325 edges. The full set of averaged expression profiles is provided as a web resource at http://biogps.org/ratatlas. On this site, a gene name query opens a display of the expression profiles across all 585 samples, links to rat genomic resources and a gene wiki connected to data related to the orthologous human gene. A click on the ‘Correlation’ button enables the user to define a correlation threshold and to generate a ranked list of correlated transcripts. This function can enable confirmation of relationships inferred from the clustering described below. It can also identify potential co-regulated transcripts for genes that were excluded from the network at the r value used for clustering.

Supplementary Table S2A shows all of the clusters detected for transcripts with a minimum expression of 10 TPM in at least one sample. In comparison to previous network analysis of mouse, human, pig, chicken, sheep and water buffalo transcriptomes (7–11) at this relatively stringent correlation coefficient, the much larger and more diverse rat transcriptomic dataset has a more fine-grained distribution with >1300 clusters having two nodes or more. In the published RNA-seq transcriptional atlas of 11 rat organs (32) that is included in the current data, around 40% of transcripts were expressed in all organs, in both sexes and at all development stages. In this larger set of averaged data, reflecting the much greater diversity of tissues and isolated cells sampled here, only 95 genes (0.38%) were detected above the 10 TPM minimal threshold in all 585 samples. These are shown in Supplementary Table S2B, with calculated maximum, minimum and variance. There is an obvious enrichment for mitochondrial and ribosomal subunit genes. There is still considerable variation among tissues and samples, but these genes have potential as controls for qRT-PCR. Commonly used controls such as Actb, Gapdh, Top and Hprt are also widely expressed but very low or absent in selected tissues that can be identified in the BioGPS site.

Significantly enriched GO terms (with associated corrected P-values) for clusters discussed later are included in Supplementary Table S2C. Consistent with previous analysis, there are clusters that show no evidence of tissue specificity but are clearly enriched for genes involved in defined biological functions. For example, clusters 11, 54 and 69 are associated with the cell cycle, DNA synthesis and repair.
Figure 1. Sample-to-sample network graph for samples averaged by BioProject, age and tissue type. The 585 averaged RNA-seq profiles generated as described in the ‘Materials and Methods’ section. A pairwise sample-to-sample Pearson correlation coefficient \( r \) was calculated. The resulting matrix is displayed as a network graph using BioLayout. The individual samples (nodes, coloured balls) are connected by edges (lines) that reflect the chosen \( r \) value threshold. For panels (A) and (B), a correlation coefficient threshold of \( r > 0.21 \) was used to include all samples. For panels (C) and (D), the threshold was increased to a more stringent \( r > 0.7 \), which removed 15 nodes that make no connection at this \( r \) threshold and increased the separation of the remaining nodes. In panels (A) and (C), the nodes are coloured by organ system: dark red, auditory system; light red, cardiovascular system; salmon, digestive system; orange, endocrine system; olive, liver; bright green, female reproductive system; teal, immune system; dark teal, integumentary system; dark green, male reproductive system; black, mixed tissues; light blue, nervous system; dark blue, primordia/early development; purple, renal system; pink, respiratory system; mauve, skeletomuscular system; and grey, whole body (embryo). In panels (B) and (D), nodes are coloured by BioProject, data being generated by different laboratories. Note that in panels (A) and (C), where nodes are coloured by organ system, nodes of the same colour cluster together, whereas there is no pattern of association when the same nodes are coloured based on BioProject in panels (B) and (D).

Cluster 41 (see also Supplementary Table S2D) is made up almost entirely of histone-encoding transcripts, likely due to incomplete removal of non-polyadenylated transcripts in some of the RNA-seq libraries. This cluster is not specific to any BioProject. The 18 transcripts within this cluster identified by LOCID also have provisional annotation as histones. Although this cluster is the product of a technical error, it also highlights the power of the clustering approach to extract signatures of co-expression.

Table 1 summarizes the expression patterns and biological processes associated with clusters of transcripts showing evidence of tissue or cell type enrichment. The largest cluster of transcripts (cluster 1), >1500 in total, is expressed almost exclusively in the testis. A smaller cluster 70 is also testis specific. More than 500 of the transcripts in clusters 1 and 70 are identified only by a LOCID, RGD or other uninformative annotation and many more are identified only by structural motif (e.g. 50 members of the Ccde family, 35 undefined Fams, 20 testis-expressed (Tex) and 15 Tmem protein genes). The complexity of the testis transcriptome in all mammalian species has been widely recognized [reviewed in (33)]. The set of testis-enriched transcripts with functional annotations encodes proteins associated with meiosis, sperm differentiation, structure and motility, and acrosomes. Unannotated genes are likely to be involved in male fertility. For example, mutation of Dlec1, a putative tumour suppressor gene, was recently shown to cause male infertility in mice (34). LOC498675 is a predicted 1:1 ortholog of mouse testis-specific gene 1700102P08Rik, which is expressed in spermatocytes and is essential for male fertility (35,36). Other smaller testis-enriched clusters include cluster 29, which contains Sertoli cell markers such as Aard and Tsx (37,38), cluster 72, which contains Fshr and the essential testis-specific transcription factor Taf7l (39,40), and cluster 88, which includes the male-determining transcription factor Sry.
| Cluster number | Number of transcripts | Specificity | Index genes and TFs | Functional annotation |
|----------------|-----------------------|-------------|---------------------|-----------------------|
| 1 and 70       | 1514 and 27           | Testis      | Acr, Amhr2, Ccnal, Fsh, Meico, Spata16, Tnpl/1, 2, Rec8, Stag3, Nrf6a1, Pbx4, Rfx2/8, Sox5, Sox30, Tcf5, Tafl7 |
| 2              | 1303                  | CNS neurons | Amigo1, Camk2a, Cx3cl1, Gabbr1/2, Grik1/5, Nfasc, Sncal, Atf2, Be7a, Chx16, Hdac11, Hwp2, Lmo3, Pou6f1, Rfx3, Tcf25 |
| 3              | 583                   | Non-specific variable | Atm, Birc6, Ccn1/2, Cdk12/13, Ddx5/6, Fancd, Hrc1/2, Hipk1, Arid2, Ceb1, Kdm5a, Nfl, Nfe213, Nr2c2, Smad4/5 |
| 4              | 342                   | Liver       | Aif, Alb, Apoc1–4, C3, Cfb, Cth, Cyp2a1, F2, Fetub, Gcgr, Ghr, Hps, Igf1, Plg, Serpin1a, Ceb3/3, Fox2, Mox2, Nrb2, Nrl3/12/13, Rxxa |
| 5              | 310                   | Oocyte      | Axin2, Bmp15, Bub1b, Ccnb3, Dlgap5, Esr1, Eya1/3, Gdf9, Gpr1, Zn1–4, Chx2, Dux4, Foxn4, Fox1, Gata3, Lhx8, Nobox, Sal1, Taf4b, Taf5, Tead4 |
| 6              | 213                   | Skeletal muscle | Acrl, Casq1, Ckm, Dm, Myh2, Myl1, Plkm, Ryrl1, Lhx1, Myf6, Pou6f2, S1x1, Sna1, Zip106 |
| 7              | 211                   | Kidney      | Ac01, Adm2, Cyp4a2/8/a, Klk1, Nox4, Pth1, Slc5a2 |
| 8              | 194                   | Oocyte      | Aurk2, Ccnb1, Magoh, Mnd1, Mos, Nanos2, Ooep, Brdt, Dazl, Gsc, Nrf5a2, Pegril6, Sall4, Sox15, Tcf15, Tef1a, Zip57 |
| 9              | 188                   | Variable, not tissue | Bab1, Ccna2, Cdk1/2, Cenkpi, Lig1, Mki67, Orc1, Pena, Pola1, E2F8, Foxm1 |
| 10             | 165                   | ES cells    | Dppa3/a4, Dusp10, Fgf17, Fzd6, Slc2a3, Def1, Fzd2, H1ax1, Left1, Lmo2, Mybl2, Nanog, Nlx2/8, Tbx3 |
| 11             | 124                   | Intestine   | Ace2, Cdhl17, Cldn7, Defa family, Dgat1, Heph, Il10ra, Krt20, Lgal4s, Muc13, Vili, Hnf4g |
| 12             | 111                   | Stimulated T cells | Cd2, Cdc3e, Cd69, Dock2, I2rg, Lib, Pyrre, Sla, Was, E2F2, Ets1, Gtf1, Ixzf1/3, Limd2 |
| 13             | 96                    | Pineal gland/retina | Aanat, Arr3, Asmt, Gch1, Opn1sw, Bx, Crx, Isda2, Lhx4, Mif, Neurod4, Taf1a3 |
| 14             | 95                    | Retina/pineal gland | Cngal, Gabbr1/2, Opn1lw, Pde6a/b/g/h, Rh3, Rdh8, Rpl1, Rbtdm, Blhie23, Pax4, Prdm13 |
| 15             | 94                    | Thymus      | Cc125, Cd3, Cdh8a/f, Fas, Rag1, Tap2, Thbta, Fox1, Ixzf2, Myb, Pax1, Rorc, Tcf7, Themis |
| 16             | 94                    | Liver, kidney | Cyp2c23, Dcker, Fbp1, Gopc, Gk, Hfkd, Pck1, Slc22a1, Slc37a4, Hnf1a/4a, Nr1b4 |
| 17             | 94                    | Macrophage, microglia | C1qa/b/c, Csfr1, Cts, Gpr84, Hexb, Mpegl1, P2ry12/13, Siglec5, Tgb1r, Trem2, Tyrobp, Blhie41, Irf5 |
| 18             | 90                    | Skin        | Cdsn, Csta, Klk9/10/12, Krt4/13/23, Lce3d/e, Lipk, Ppl, Trex2, Vgsl2, Barx2 |
| 19             | 87                    | T cells, NK cells | Cc1l1, Cer4/5/8, Cdagolg, Gpr183, Ifng, Il17a, Il12, Il12r/a/b, Lta, Zap70, Batf, Icos, Runx3, Stat4 |
| 20             | 85                    | Dorsal root ganglia | Ac3p, Calca/b, Grik1, Htr1d, Nfie1/2/m, Nnb, Piezo2, Prokr1, Retic, Dyrx, Hoxd1, Pou4f1/2, Smad9, Tlx3 |
| 21             | 75, 74 and 65         | Skin        | Adgrf4, Ces4a, Colla1a, keratins, Krtaps, Lce family, Lgals7, Lipm, Perp, Tp63, Tprg1 |
| 22             | 69                    | Testis      | Aard, Clec12b, Gk5, Hornd1, Incal, Shbg, Spyel1/2, Msh4, Nks3/1, Rhoxo8, Tbx22, Ttx |
| 23             | 68                    | B cell      | Bda, Cd19, Cd79a/b, Cxcr5, Fcna, Gpr174, Ighm, Ichain, Citta, Pax5, Pou2af1, Spib, Tlx1 |
| 24             | 65                    | Prostate    | Andpro2, Cys, Dach2, Eaf2, Fuf4, Lao1, Lyc2, Mcs5, Pbsn, Sbp, Seng1, Blhia15, Cebh34, Esr2 |
| 25             | 64                    | Adrenal     | Chrb1, Cyp11a1/b2/b3, Cyp1b1, Fxd1, Kcnk3/9, Mc2r, Pecs5, Pmnt, Soap1, Star, Ar, Nr5a1 |
| 26             | 60                    | Brain       | Crmp1, Ephb2, Gpc2, Gpr85, Marcks1, Mdga1, Mex3b, Dcx, Hmgbh3, Lhx6, Mycl, Neurog2, Runx11, Sox11 |
| 27             | 56                    | Variable    | Bab3, Ddx39a, Dkel1, Srsf2/3, Tripl3, Mycn |
| 28             | 52                    | Cochlea, middle ear | Cd164f2, Chrna9/10, Cldn9, Fbxo2, Grxcr1/2, Kncn, Loxhd1, Otoa/r/s |

| Functional annotation |
|-----------------------|
| Spermatogenesis, motility, meiosis |
| Neurotransmission, neural development |
| Misfolded protein/stress response, tumour suppressors |
| Hepatocyte secretory products, xenobiotic metabolism |
| Oocyte-specific transcription, zona pellucida structure, meiosis |
| Muscle contraction, calcium signalling |
| Tubule function, resorption, metabolism |
| Stem cell renewal, meiosis |
| Cell division cycle, DNA synthesis/repair, mitosis |
| Stem cell maintenance |
| Intestinal barrier function |
| T-cell function |
| Glucogenesynthesis |
| Innate immune function, microglial differentiation |
| Skin barrier function |
| Activation, cytokine secretion |
| Ganglion cell differentiation |
| Skin barrier function |
| Sertoli cell differentiation, synaptonemal complex |
| B-cell differentiation, immunoglobulin production |
| Prostate secretion, differentiation |
| Steroid hormone production, adrenalin |
| Trophoblast differentiation, secretion |
| Neurogenic progenitor cell differentiation |
| Genotoxic damage response, tumour suppressors |
| Hearing, cochlear function |
Clusters 17 and 18 contain transcripts expressed in both the retina and the pineal gland, both intimately involved in chronobiology and light sensing. Chang et al. (41) recently produced an aggregated resource describing the shared and divergent transcriptomes of these structures. Cluster 17 contains Opm1sw, the pineal-enriched transcription factor Crx and its target Aanat encoding the rate-limiting enzyme in melanatonin synthesis (42). One unexpected inclusion in cluster 17, enriched in pineal, is the transcript encoding the transcription factor MITF. MITF in humans may be driven by as many as seven distinct promoters, including one used specifically by melanocytes. A unique transcription start site is shared by retinal pigment epithelial cells and pineal gland. Mitf overexpression in mouse pineal gland relative to other tissues has been noted previously (42,43) and in humans also MITF is most highly expressed in pineal gland (http://biogps.org). However, whereas targets of MITF have been identified in melanocytes and many other cell types (44) and mutations impact many complex phenotypes in mice and humans, there appears to be no literature on its role in the pineal gland. To illustrate the utility of the data, in Supplementary Table S2D we have reviewed the annotation of transcripts in clusters 17 and 18. Several novel transcripts of unknown function [e.g. KIAA0586; also annotated as LOC361646; KIAA0586 (Taipid3), encoding a highly conserved ciliary protein associated with the human genetic disease Joubert syndrome (45); and Ltm1 (LOC102547963), a novel membrane protein] are also almost uniquely expressed in the human pineal gland (http://biogps.org).

Many smaller clusters detailed in Supplementary Table S2A are enriched in tissues, cell types or activation states that were not analysed in the existing rat atlases or indeed in any previous atlas project in other species. They can be annotated based upon known markers. For example, cluster 145 with 12 nodes contains transcripts encoding major secreted products of the pituitary (Cga, Gh1, Fshb, Lhb, Tshb) and the transcription factors that regulate their expression (Pitx1, Six6, Tbx19). Cluster 180 contains a subset of known immediate early genes (Egr1, Fos, Jun) mostly associated with isolated primary cells, and likely reflects cell

Clusters were generated at r ≥ 0.75 and MCL inflation value 2.2. Selected transcripts encoding transcription factors are highlighted in bold. The full lists of transcripts in these clusters and the average expression profiles are provided in Supplementary Table S2. Index genes were chosen for illustrative purpose based upon known function in the indicated tissue confirmed by a PubMed search on gene name AND tissue. Where two cluster numbers are shown, the two clusters are in the same region of the network graph and show closely related expression profiles.

### Table 1. Continued

| Cluster number | Number of transcripts | Specificity | Index genes and TFs | Functional annotation |
|----------------|-----------------------|-------------|---------------------|-----------------------|
| 44             | 51                    | Blood       | Cxc2r, Gp9, Gypa, Kd, Pf6, S100a9, Tpt1, Tspo2 | Platelets, granulocytes |
| 46             | 49                    | Lung        | Ager, Arg5, Clec4a, Cyp2a3, Dna1l, Fmo2, Lamp3, Ly2, Segr1a1/31a/3a2, Sftp1a/b/c/d, Wnt3a, Hoxp, Nkx2-1, Smad6, Tbx4 | Alveolus type 1 and type II cell function and secretion |
| 47 and 83      | 48 and 24             | Heart       | Actc1, Cav3, Fglf6, Mhy7, Myl2, Palld, Ryr2, Tnncl, Ehdo, Lrx4, Nkx2-5, Pdlim5, Tbx20 | Cardiac-specific muscle contraction. |
| 48             | 48                    | Monocyte, macrophage | Cs4r1, Cer1, Cdl4, Csf2r1, Cyba, Fgr1a, Igam, Msr1, Nclf2/4, Nfr2, Sc11a1 | Innate immune function, free radical production |
| 49             | 49                    | Kidney      | Aac2, Aap2/3, Cldn8, Inscr, Knc1, Ogr1, Foxi1, Hox2, Hoxd3 | Distal tubule, collecting duct, water resorption |
| 51             | 45                    | ES cells    | Fgf4, Fgf19, Gdf3, Nodal, Pou5f1, Prdm14 | Regulation of pluripotency |
| 55             | 38                    | Granulocytes | Camp, Ctsg, Elane, Fnce, Mpo, Prg2/3, S100a8 | Neutrophil granule proteins |
| 63             | 33                    | Brain       | Aqp4, Edil3, Gpr37/62 Mag, Mbp, Mobp, Opalin, Pipl, Sema4d, Nkx6-2 | Myelination, oligodendrocytes |
| 64             | 33                    | Pancreas    | Amy2a3, Cel, Cels1a/2/3a/b, Cpa1/2, Ctrc, Lpl, Snip1, Tomil1, Pnplp1/2 | Pancreatic enzymes, secretion |
| 66             | 29                    | Stomach     | Atp4a/b, Chia, Ctse, Cym, Ghrl, Gkn1/2, Pgc | Acidification, digestive enzymes |
| 68             | 27                    | Brain, PC12 cells | Prx2r, Prph, Th, Vgf, Gata2, Hand2, Phox2a | Sympathetic neurons? |
| 77             | 26                    | Mast cell?, lymphatic | Adgrg5, Cma1, Cpa3, Lilr3a, Lyve1, Selp, Sirpd, Sl9, Tim4, Cebp | Fat storage, lipolysis, adipokines |
| 82             | 24                    | Adipose     | Adiponq, Fabp4, Lepy, Lip, Lox, Oxr1, Plin1, Pnpla2, Retn, Suen1, Thdr, Pparg | Lipoprotein-associated protein |
| 87             | 21                    | Lens        | Cbfy family, Crgy family, Lim2, Opn4 | Lens structural proteins |
| 88             | 20                    | Macrophage  | Adam8, Cd68, Ctsb, Ctx1, Gpmnb, Pbrx4 | Endosome/lysosome |
| 90             | 19                    | Colon       | Krt19, Lypd8, Pghr1, Pla2g10, Tspan1a, Cd2x | Colon epithelium differentiation, secretion |
| 92             | 19                    | Cerebellum  | Ca9, Cbn1/3, Cha2, Fat2, Gabra6, Gpm4, En2, Hex3 | Purkinje cell differentiation, granule proteins |
| 95             | 19                    | Variable in many tissues | Adgrd4, Cd93, Chd5, Dll4, Egfl7, Kdr, Pcdh12, Pecam1, Tiel, Erg, Myet1 | Endothelial cell differentiation |
| 97             | 19                    | Cartilage growth plate | Acan, Clec11a, Cofb1/2/3, Loxl3, Rflna, Aix1, Nkx3-2, Nkx3-3 | Cartilage structural proteins |
| 98             | 18                    | Activated T cells, thymus | Ccr7, Cd7, Cdh9, Hecl, Fopx3 | Immune cell activation |
| 101            | 18                    | Macrophage  | Acod1, Ccel10, Il1a/b, Nos2 | Response to LPS |
| 106            | 16                    | Cartilage, tendon | Col2a1, Col10a1, Coll1a1/2, Myh3, Ptx4, Zfp648, Zmp1 | Cartilage structural proteins |
activation during isolation or tissue processing (20). Other known genes in the immediate early class cluster separately, or not at all, because they are constitutively expressed by specific cell types. Similarly, groups of inducible genes in innate immune cells are all expressed by LPS-stimulated macrophages but divide into at least three clusters (cluster 101, including Il1a; cluster 112, including Ifit2 and other interferon targets; and cluster 126, including Ifn) because of expression by non-immune cells.

Other smaller clusters in Supplementary Table S2A group genes that share functions. The large protocadherin family of cell adhesion molecules is broadly divided into the clustered (α, β, γ) and non-clustered (δ) subgroups (46). The 6 protocadherins are predominantly expressed in the nervous system and indeed Pedh1, Pedhδ, Pedhδ and Pedh20 are brain restricted and part of the second largest cluster (cluster 2). However, cluster 81 includes Pedh22 and 16 members of Pedh (A and B) families, which are collectively enriched in the CNS but also widely expressed in other tissues. In addition, LOC108353166 within this cluster is annotated as protocadherin gamma-B2-like. Further members are more brain restricted and grouped together in cluster 250.

Nine of the 13 known mitochondrially encoded peptides group together in cluster 212, whereas clusters 61 and 76 group nuclear-encoded mitochondrial genes involved in the TCA cycle and oxidative phosphorylation (as expected, most highly expressed in heart and kidney). Cluster 102 groups 18 transcripts encoding proteins involved in mitochondrial β-oxidation of fatty acids. Several of the genes in this cluster are mutated in multiple acyl-CoA dehydrogenase deficiency (also known as glutaric aciduria type II) and related metabolic disorders (47). One additional gene involved in this pathway, Etfb, does not form part of a cluster. The web server (http://biogps.org/ratatlas) shows that Etfb is significantly correlated with many other genes associated with mitochondrial β-oxidation (e.g. with Etfa at \( r = 0.59 \) and with Etfdh at \( r = 0.54 \)) but is expressed at lower levels in certain tissues, including the pineal gland.

Cluster 127, with 14 nodes, contains two markers of neurogenic cells [Str2, Mpped1 (48,49)] and a candidate regulator, Tiam2 (50), and is otherwise made up of 11 brain-specific transcriptional regulators, each of which has been shown to be essential for neurogenesis and likely interacts with the others. Clusters 125 and 332 contain 20 genes encoding proteins that have all been implicated as molecular chaperones, including multiple components of the TRIC chaperone complex (Tcp1, Cct2, Cct3, Cct4, Cct5). Cluster 557 with only four nodes contains the oligodendrocyte transcription factors, Olig1 and Olig2, as well as Sox 8, which has non-redundant function in oligodendrocyte differentiation (51). The fourth node in this cluster, LOC103692025, is predicted by the RGD to be an ortholog of Lhfp3, which in mouse is a marker of oligodendrocyte lineage commitment (52). The two calmodulin-encoding genes (Calm1 and Calm2) are co-expressed (cluster 673) as are three genes involved in cholesterol synthesis (Fdif1, Hmger, Hmgcs1) (cluster 742). Ins1 and Ins2, encoding insulin, are co-expressed with pancreatic polypeptide (Ppy) (cluster 751) but not with glucagon (Gcg). Although Ppy is normally expressed by rare gamma cells in pancreatic islets, a recent study indicated that gamma cells can produce insulin following beta-cell injury (53).

Each of the clusters contains genes that are identified only as LOCID or other numerical designation. These are obviously the subject of ongoing curation and in some cases LOCID transcripts duplicate named transcripts in the same cluster. In Supplementary Table S2, we have included an update on candidate annotations from the RGD and the http://biogps.org/ratatlas server provides a link to RGD besides the expression profile. Clearly, the co-expression information can provide additional assurance that putative orthology relationships with known mouse or human genes are likely to be correct.

Transcripts that do not form clusters

The first step in network analysis is the generation of a pairwise correlation matrix, and for any gene of interest one can immediately identify others with the most similar expression patterns. By lowering the inclusion threshold (\( r \) value), it is possible to include a larger proportion of transcripts, but the associations may become less informative biologically. For each gene of interest, the correlation function of the BioGPS site enables extraction of transcripts that are correlated at lower \( r \) values, which may provide some insight into function. Genes with unique expression profiles across the samples will not correlate with any other and therefore will not be included in the network graph. In many cases, the unique expression profile of a gene of interest arises because the gene product is ‘multi-tasking’ in different locations. Figure 2 shows the individual profiles of selected examples discussed later.

Mutations in FBN1, encoding the extracellular matrix protein fibrillin-1, are associated with Marfan syndrome that has complex impacts on musculoskeletal development, adiposity, vascular function and the eye. Distinct 3’ truncation mutations are associated with a neonatal progeroid lipodystrophy syndrome (54). Consistent with these phenotypes, Fbn1 mRNA is highly expressed uniquely in the rat eye, aorta and cardiovascular tissues and cartilage/tendons and to a lesser extent in fibroblasts and adipose. There is also moderate expression in spinal cord and dorsal root ganglia, lung and testis. Dural ectasia, enlargement of the neural canal, is a common feature of Marfan syndrome (55). Expression in the lung may underlie the pulmonary emphysema observed in mouse models of fibrillinopathy (56); patients with Marfan syndrome frequently show apical blebs in the lung and are prone to pneumothorax (collapsed lung). Although Fbn1 does not form part of a cluster at \( r > 0.75 \), the BioGPS correlation function reveals 52 genes correlated at \( r > 0.6 \), mostly associated with mesenchyme and extracellular matrix biology (e.g. Adams2, Bgn, Col5al, Loxl1, Pdgfrb, Tgfb3) (57).

The gene encoding dystrophin (DMD) associated in humans with mutations causing Duchenne muscular dystrophy is also not clustered. As expected, it is expressed in rat cardiac, skeletal and uterine muscle, but is also expressed in multiple brain regions at similar levels. This expression may be related to the neuropsychiatric impacts of the disease in both affected individuals and mouse models (58). In this case, FANTOM5 data indicate that DMD has at least
two independent promoters (6). Nevertheless, several other genes associated with muscular dystrophy (Sgcd, Lama2, Dst) are correlated with Dmd at \( r > 0.6 \) (see BioGPS site).

RGD1359108 is a clear 1:1 ortholog of human C9orf72, associated with amyotrophic lateral sclerosis and frontal temporal dementia. O’Rourke et al. (59) reported that loss of function mutation in the orthologous gene in mice did not produce motor neuron dysfunction, but did lead to macrophage dysfunction, splenomegaly and lymphadenopathy. In rat, the ortholog of C9orf72 is expressed widely in all CNS-associated tissues, most highly in spinal cord, but not enriched in any isolated CNS cell population. Outside the CNS, it is most highly expressed in stimulated macrophages and in testis.

A significant cohort of transcripts is excluded from co-expression clusters because they have alternative promoters, each with a distinct expression profile. One such gene is Acp5, encoding the widely used osteoclast (OCL) marker, tartrate-resistant acid phosphatase. Acp5 forms part of a small cluster (cluster 179, 10 nodes) that is most highly expressed in the femoral diaphysis, and includes another OCL marker Ctsk, osteoblast-associated transcripts (Bglap, Dmp1 and Sp7) and Ifitm5, mutated in a human bone-related genetic disease, osteogenesis imperfecta type V. It is surprising that so few transcripts are stringently associated with OCL; another small cluster (cluster 174, 11 nodes) that contains Dcstamp, Oestamp (Zfp334) and Mmp9 is enriched in the diaphysis sample but more widely expressed.

Figure 2. Gene expression profiles for genes that did not fall within a cluster. Y-axis shows the expression level in TPM. X-axis shows the organ system, coloured as in Supplementary Table S2. Reading from left to right: light red, nervous system; blue, auditory system; light green, respiratory system; yellow, cardiovascular system; pink, digestive system; turquoise, endocrine system; salmon, liver; grey, renal system; dark red, skeletomuscular system; dark blue, integumentary system; dark green, immune system; olive, male reproductive system; dark pink, female reproductive system; dark turquoise, primordia/early development; black, whole body (embryo); and red, mixed tissues.
The transcriptome of the rat liver

The downloaded datasets included around 1900 individual RNA-seq libraries of liver, including whole liver from various ages, sexes, inbred and outbred rat strains, disease models, liver slice cultures and isolated cells. In principle, clustering of such diverse data could identify sets of co-expressed transcripts that are associated with cell types, locations or disease processes that are hidden in the averaged data of the complete sample set. To test that view, we clustered the entire liver-related dataset without averaging the replicates. As in the main atlas, the correlation threshold was chosen empirically at 0.75. The cluster list and the average profile of transcripts in each cluster are provided in Supplementary Table S3A and informative clusters are summarized in Table 2.

It is immediately evident that not all of the samples are pure liver. Liver cluster 31 contains a set of pancreas-specific genes, including Cpa1 that overlaps with cluster 64 in the main atlas. This cluster arises because of random contamination with pancreatic tissue of liver samples in the large BodyMap project (32). Liver cluster 73 contains transcripts encoding all of the major secretory products of pancreatic islets (e.g. Ins1 and Ccp). This cluster was detected only in liver from a study of enforced activity and sleep deprivation (63). It is not clear from the paper how these samples could have been selectively contaminated with islet mRNA unless they are mislabelled. Liver cluster 5 is detected in a rather random subset of samples from multiple Bioprojects likely also indicating contamination. It includes the progenitor marker, Lgr5, but also various adhesion molecules (Cldn10/18) and neuroendocrine markers (Chga/b). There is little evidence of expression of these genes in normal liver in other species, and at least some of the genes (e.g. Cckar and Cldn10/18) are highly expressed in pancreas and/or stomach (e.g. see http://biogps.org). Liver cluster 21 is detected in a single sample, and contains smooth muscle-associated transcripts (Actg2, Tpm2).

The disadvantage of analysing a single tissue is that most transcripts do not vary greatly between datasets. In one sense, this provides a quality control for the efficacy of the random sampling approach we have used. In this dataset, the largest cluster by far (liver cluster 1) is relatively consistent with the exception of increased detection in all samples from a Bioproject that profiled liver slices from a bile duct ligation model, cultured for 48 h in vitro and treated with various agents (64). It is not clear why this gene set would be expanded in that cellular system. Liver cluster 1 includes many transcripts expressed constitutively by hepatocytes. The most abundant hepatocyte-specific transcript encoding albumin (Alb) is not strictly correlated with any other transcript presumably reflecting its specific regulation (65).

### Table 2. Gene expression clusters from rat liver

| Liver cluster number | Number of nodes | Description and index genes |
|----------------------|-----------------|----------------------------|
| 1                    | 6292            | Widely expressed, high in bile duct ligation model; growth, protein synthesis, inflammation, fibrosis, connective tissue |
| 2                    | 752             | High in foetal liver; cell cycle, haematopoiesis, embryonic liver; cyclins, Cdk1, Pena, Igf2, Hhb, S100a8/9, E2f2, Kif1, Myb |
| 3                    | 414             | General expression, metabolic regulation; Be2/2, Cdk5, Cdkr, Esraa, Foxk1, Hdac6, Nfe2l1, Nkx2, Pias3, Rara, Sle5, Tfe3, Tfeb |
| 4                    | 278             | General expression, control of lipid metabolism; Arid1a, Bcl2, Canta2, Crel1/2, Fastk, Foxj1, Foxp4, Hf1, Me3d2, Relu, Rfx1, Rxb, Tjp3 |
| 5                    | 206             | Isolated samples, gall bladder, neuroendocrine; Ceda, Chgp4, Cldn10/18, Inba, Ketapi1–3, Lgr5, Scgf3, Nub, Nis |
| 6                    | 166             | E14 liver, fibrosis model; Acta2, Cdh11, Epha4/7, Fbn2, Gpc2, Myh6/7, Sfrp1/2, Ak, Citrd, Foxk1, Gata5, Sho2, Thx15/18, Tgff2, Twist1/2, Wnt |
| 7                    | 148             | Foetal liver, fibrosis, Zucker rats: myeloid infiltration; As1l, Cd4, Cdh6, Cleo4a1, Fcgr1a, Hk3, Lyz2, Ptpc, Ifit5, Ifi13, Spal |
| 10                   | 98              | Variable expression: proteasome complex, proteolysis; Anxa7, Ctds1, Fbox22, Prdx1/6, Pon1, Pomp2, Pmp1, Pmp2d, Tm1x2, Usp5, Cbeh3 |
| 11                   | 76              | Variable, low in foetal liver, periportal hepatocytes, urea synthesis; Aegnat, Asxl1, Cesla, Cyp2el, Gl2, Ggpr, Gpi, Hsd17b11, Pink1, Slc2a5/22, Mx5pi, Nrl12 |
| 13                   | 67              | Variable, low in foetal liver, fibrosis model, fatty acid β-oxidation; Acatal, Acot1, Crt, Cyp4a14, Efaced, Hdb, Pank1, Pdk4, Slc22a5, Vnul1 |
| 14                   | 64              | Variable, cholesterol and fatty acid synthases; Acac, Acaec, Aeloy, Dho7, Fabp1/2, Fas, Flg, Hmgcr, Hmgcr1, Lss, Mvd, Nf2, Srebfl/2 |
| 15                   | 54              | Fibrosis; Angpitf, Col1a2/1, Col6a1/6, Gp1c, Lgals1, Loxl1, Lum, S100a8, Sfpl4, Tm1, Osr2 |
| 24                   | 41              | Variable, mast cells; Cpa3, Cpc, Mcpt2, Prss8 |
| 25                   | 41              | Variable, interferon response; Ddx58, Gbp1/4, If44, Ifi1l, Igf5, Mx1/2, Oas1/2, If7f |
| 26                   | 41              | Variable, mitochondrial; Atp5meff/g, Cox7ab, Ndufa2/4/5 |
| 31                   | 34              | One BioProject, pancreas contamination; Celfr, Cpa1, Klk1, Polip. Prs1l |
| 33                   | 32              | One BioProject, NK cells; Cd96, Gzma, Khla1, Ly49, Pchl1, Psvf1 |
| 34                   | 31              | Highly variable, hepatic stellate cell activation; Acvr1c, Apob, Egfr, Fcgr2b, Klb, Mve1, Stat6, Tgfj2, Tnfl |
| 43                   | 21              | Variable, interferon response; Adar, Ifih1, Parp9/10/12/14, If9g |
| 56                   | 12              | Kupffer cell; Cds1, Csf1r, Sdc3, Siglec1, Vsig4 |
| 63                   | 10              | Endothelial cell; Cdh3, Cdh5, Fct1, Nrp1, Pecam1, Tgbfr3, Tie1, Ets1, Tbx20 |
| 65                   | 10              | Class II MHC; Aif1, Basf, Cd74, Rk1-Ba/b, Rk1-Da/b, Ifj8, Citua |
| 67                   | 10              | Male-specific; Akrl1/c2, Cyp2a2, Hsd3b5, Sulz1/c3 |
| 69                   | 10              | Xenobiotic-induced; Ces2a, Gstm2, Ugt1a5 |
| 84                   | 9               | Female-specific; Akrl1/c2, Cyp2c12, Sfdn1, Sulz2a1/c6, Cux2, Tm5c4 |

Clusters were generated at r ≥ 0.75 and MCL inflation value 1.7. The full gene lists for each of the clusters are provided in Supplementary Table S3A. Transcription factors are highlighted in bold. Index genes were chosen for illustrative purpose based upon known function.
Liver cluster 1 also contains transcripts encoding markers of hepatic stellate cells (e.g. Pdgfra/b) and the corresponding growth factors (Pdgfa/b/d) as well as more general mesenchyme markers (e.g. Vim) and markers of cholangiocytes (e.g. Krt17) suggesting that their relative abundance is not highly variable among the samples.

The remaining liver clusters analyse differential development and activation states that distinguish the samples and BioProjects. These clusters are informative and consistent with prior knowledge. Liver cluster 2 is expressed specifically in embryonic liver and is a complex mix of transcripts reflecting both differentiation of hepatocytes and the function of the liver as a haematopoietic organ. Accordingly, it contains the cell cycle genes, the foetal growth factor Igf2, and markers of erythroid (e.g. Hbb) and myeloid (S100a8/a9) haematopoietic lineages. Liver clusters 3 and 4 are both expressed in almost all liver samples and the level of expression is not highly variable. Expression of each of the smaller clusters is much more variable between samples and BioProjects and known genes within those clusters indicate an association with specific cell types and processes as summarized in Table 2 and discussed later.

One signature that was not detected is that of the specialized centrilobular population that is adapted to clear ammonia generated by the urea cycle. In mice, the rate-limiting enzyme, glutamate ammonia lyase (also known as glutamine synthetase, Glul gene), is expressed exclusively in a band of cells surrounding the central vein. Liver-specific deletion of Glul leads to pathological hyperammonaemia (66). In mice, this population of cells co-expressed Rhgb (encoding an ammonia transporter) and ornithine amino-transferase (Oat) and was enriched for a number of Cyp genes (e.g. Cyp2el and Cyp1a2). However, in the diverse rat liver dataset, there was only marginal correlation with other centrilobular-enriched transcripts.

The transcriptome of central nervous, renal, musculoskeletal and cardiovascular systems

Each of these systems also contributes hundreds of RNA-seq datasets including isolated cells and specific regions or structures. To further examine the utility of these large datasets for the analysis of cell type- and process-specific signatures, the data from each of these biological systems were clustered separately in Supplementary Table S4 (nervous), Supplementary Table S5 (renal), Supplementary Table S6 (cardiovascular) and Supplementary Table S7 (musculoskeletal). The clusters are annotated in the tables and to avoid confusion with multiple cluster numbers, each system is discussed separately in Supplementary Text. Broadly speaking, as in the liver, network analysis of individual organ systems enables a more fine-grained extraction of cell type-, region- and process-specific expression signatures.

The transcriptome of rat macrophages

The transcriptome of rat macrophages has been analysed previously based upon microarrays (67) and the RNA-seq data included here (68). Macrophages adapt to perform specific functions in specific tissues (20). Cluster 21 (Table 1 and Supplementary Table S2), which includes Csf1r, is most highly expressed in brain and brain-derived cells and includes transcripts that are enriched in microglia compared to macrophages from other tissues (e.g. P2ry12). Around two-thirds of these transcripts are contained within a set of 119 transcripts depleted in all brain regions of Csf1r-knockout rats (69). Cluster 47 (Supplementary Table S2) contains transcripts that may be shared with microglia (e.g. Itgam, encoding CD11b) but are common to monocytes and many tissue macrophage populations. Cell surface markers of other macrophage populations cluster idiosyncratically as shown in Supplementary Table S2, indirectly supporting tissue macrophage heterogeneity; Clec4f, the Kupffer cell marker, is within the liver cluster, Vsig4 and Marco (cluster 1239), Clecl0a, Mrcl (CD206) and Stab1 (cluster 168), Lyvel and Timd4 (cluster 79), and AdgreI and Clecs4a1/3 (cluster 286) are correlated with each other, while others (e.g. Cdl63, Tnfrsf11a, Siglec1) do not cluster at all at this threshold because each has a unique pattern of expression in tissue macrophages. Figure 3 shows the profiles of Csf1r, Adgre1, Cidl63, Vsig4 and Mrcl in the averaged data.

The network analysis of such a diverse set of cells and tissues also dissociates known macrophage transcriptional regulators (e.g. Spil, Spic, Nrl3h3, Mafb, Irf8, Cebpab, Tfec) (20) from macrophage expression clusters because none of these regulators is entirely macrophage restricted. For example, transcription factor SPIC in mice is required for splenic red pulp macrophage and splenic iron homeostasis (70). In the rat, Spic mRNA is most highly expressed in spleen as expected, but also detected in ES cells and germ cells (see profile on http://biogps.org/ratatlas). Macrophage differentiation and adaptation likely involve combinatorial interactions among multiple transcription factors as exemplified by the complex regulation of the transcription of the Csf1r gene (71).

Whereas macrophages express a diversity of endocytic receptors, there is not a corresponding large cluster of transcripts encoding endosome–lysosome components including the vacuolar ATPase (ATP6v) subunits and lysosomal hydrolases. Transcripts encoding endosome-associated CD68 and GPNMB proteins are co-expressed with Ctsb and Ctsd. Although CD68 is often used as a macrophage marker, it is clearly not macrophage restricted. Most transcripts encoding lysosomal acid hydrolases (e.g. Acp1, LIPA) are widely expressed and each varies independently.

Csf1r is strongly correlated with other macrophage-specific markers in cluster 21, consistent with strong evidence that expression is entirely restricted to the macrophage lineage in rats as it is in mice (72). It is also detected at relatively high levels in all tissues (around 5–10% of the level in isolated macrophages) consistent with the abundance of tissue macrophages detectable with a Csf1r reporter transgene (72) and with a study of tissue development in mice (73). However, expression was also detected in many isolated primary cell samples that are not meant to contain macrophages. For example, BioProjects PRJNA556360 and PRJNA552875 contain RNA-seq data derived from oligodendrocyte progenitors purified using the A2B5 marker, but this population has Csf1r expression at similar levels to purified macrophages. Another BioProject, PRJNA355082, describes expression profiling of isolated
Figure 3. Gene expression profiles for macrophage-related genes. Y-axis shows the expression level in TPM. X-axis shows the organ system, coloured as in Supplementary Table S2. Reading from left to right: light red, nervous system; blue, auditory system; light green, respiratory system; yellow, cardiovascular system; pink, digestive system; turquoise, endocrine system; salmon, liver; grey, renal system; dark red, skeletomuscular system; dark blue, integumentary system; dark green, immune system; olive, male reproductive system; dark pink, female reproductive system; dark turquoise, primordia/early development; black, whole body (embryo); and red, mixed tissues.
astrocytes, but this dataset also has a similar level of Csf1r mRNA to pure macrophages. Other datasets from various ganglion cell populations, neuronal progenitor cells, cardiac fibroblasts and cardiomyocytes and hepatic stellate cells are clearly highly enriched in Csf1r and other macrophage-associated transcripts.

Csf1r has two ligands, CSF1 and IL34. In mice and rats, mutation of the Csf1 gene leads to a global reduction in many tissue macrophage populations, whereas mutation of Il34 in mice leads to selective reduction of microglia and Langerhans cells. Based upon the difference in phenotype between Csf1 and Csf1r mutations in rats, we speculated that Il34 could be more widely expressed and functional in rat macrophage homeostasis compared to mouse (68). Neither growth factor forms part of a cluster. Figure 3 also shows the profiles of Csf1 and Il34. As expected, Csf1 mRNA is widely expressed and enriched in isolated mesenchymal cells. Il34 is expressed in all brain regions and isolated cells at similar levels and also in skin. However, in contrast to mouse, Il34 is expressed at similar levels in many other tissues, notably aorta, adipose, kidney, lung and testis.

The tissue-specific analysis in Supplementary Tables S4–S7 enables the extraction of macrophage-specific signatures from resident populations that have not been isolated and characterized previously. For example, in the cardiovascular analysis, a cluster of 184 transcripts containing Csf1r as well as a smaller cluster containing Adgre1 extracts a signature of cardiac resident macrophages distinct from blood leukocytes that form a separate cluster (see Supplementary Text).

DISCUSSION

Overview

The extraction and normalization of published RNA-seq data have enabled the generation of a comprehensive rat expression atlas that samples transcriptional diversity on a comparable scale to the FANTOM5 data for human and mouse (6) and massively extends the BodyMap generated from 11 rat tissues (32). The user-friendly display at http://biogps.org/ratatlas enables a gene-specific query to visualize the expression of any gene of interest across the full dataset and use of the correlation function allows the identification of transcripts with similar expression profiles. BioGPS also hosts large expression datasets for mouse, human, sheep and pig for comparative analysis. The validity of the downsampling normalization and the utility and information content of the atlas have been exemplified by gene-centred network analysis of the averaged core dataset. The primary data are available for download by users in a form that enables local regeneration of the networks and addition of user-generated datasets. In comparison to rat, there are orders of magnitude more total RNA-seq datasets from mouse and human cells and tissues in public repositories. We previously identified and analysed 470 RNA-seq datasets from mouse resident tissue macrophages alone, excluding data from cells stimulated in vitro or in disease models (20). The approach we have used is extensible to even larger datasets in mouse and human.

Analysis of liver-specific transcriptional network

The assembled dataset includes multiple BioProjects and thousands of RNA-seq datasets related to the liver, central nervous system, heart and cardiovascular system, and kidney. Each has been analysed independently to identify signatures of individual cell types and processes (Supplementary Tables S3–S7). To illustrate the ability of network analysis to extract biologically informative expression signatures, we analysed the liver data in greater detail and considered other tissue-specific analysis in Supplementary Text.

Liver gene expression is regulated in response to numerous physiological stimuli and chronic disease processes, including fatty liver disease. Aside from hepatic parenchymal cells, the liver contains several non-parenchymal populations. To identify co-regulated clusters within the liver transcriptome, we analysed the liver samples separately using the same GCN approach used for the overall atlas. The liver is the major source of plasma protein and performs many functions in energy homeostasis, lipid and protein synthesis, and biotransformation of xenobiotics and endogenous by-products. The function of the liver depends on its structure, which comprises small units called lobules, each composed of concentric layers of hepatocytes expanding from the central vein towards the periportal vein. The metabolic function of hepatocytes varies along the periportal–central axis, a phenomenon referred to as metabolic zonation (74). In principle, if there was significant heterogeneity in metabolic state or development among the liver samples, a gene-to-gene clustering might reveal sets of genes associated with portal versus centrilobular regions of liver lobules. Halpern et al. (75) performed single-cell RNA-seq analysis of mouse hepatocyte diversity and concluded that zonation impacts as many as 50% of transcripts. However, this analysis was limited to 8-week-old fasted male C57BL/6 mice and does not necessarily capture coordinated regulation of the metabolic domains, including diurnal oscillations and response to feeding (76).

Broadly speaking, the single-cell analysis indicated a periportal bias for major secretory products of hepatocytes and a pericentral concentration of expression of genes involved in xenobiotic metabolism.

Network analysis shown in Supplementary Table S3A and summarized in Table 2 revealed a large co-regulated cluster (liver cluster 11) that includes Glis2, an archetypal periportal marker in mice, other enzymes and transporters associated with the urea cycle (Ass1, Acy3, Agmat, Cbs, Gpt, Slc25a22, Nags) and the glucagon receptor, Gcgr. Cheng et al. showed that glucagon is a regulator of zonation in mouse liver, in that glucagon deficiency led to reduced expression of periportal-enriched transcripts (77). There are candidate transcriptional regulators within this cluster with known functions in hepatic transcriptional regulation: the xenobiotic sensor Nr1i2 and the glucose-sensing transcription factor Mlzip1 (78,79). A smaller liver cluster 88 contains additional key enzymes of urea synthesis, Arg1, Cps1 and Gpt2, as well as the amino acid transporter, Slc38a4.

The analysis of the liver samples does not reveal a corresponding pericentral expression cluster. Ghl2, which appears strictly restricted to a single layer of cells surrounding
the central vein in mice, rats and humans (74), showed limited heterogeneity among the liver datasets and did not form part of this cluster. This suggests that Glut is not highly regulated, whereas other centrilobular-enriched transcripts alter their expression in response to external stimulus. Another putative landmark pericentral gene, Cyp2e1, is actually part of liver cluster 11, redistributed in at least some of the experimental models sampled herein, as observed in a model of paracetamol exposure that forms part of this dataset. Other transcripts that are biased to centrilobular also form separate clusters because of their independent regulation in response to stimulation. For example, Cyp4a2 was identified as a pericentral marker (74). Liver cluster 54 (Supplementary Table S3A) is elevated in a dataset from a BioProject studying the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin, a potent aryl hydrocarbon receptor (AhR). It includes the detoxifying enzymes Cyp1a1, Cyp1a2 and Cyp1b1, the AHR repressor gene (Ahr) and transcription factor Cdx2, a known AHR target gene (80). A distinct set of xenobiotic metabolizing genes, Cest2a, Gstm2 and Ugt1a5, is coregulated in liver cluster 69, and Ephx1, Gsta2, Gsta4, Gsta5 and Gstm1 are co-regulated in liver cluster 146. The proteasome subunit Psmd4 was also pericentral in mice (75), but it is found in liver cluster 10 stringently co-regulated as one might expect with numerous other components of the proteasome complex. Liver cluster 10 contains the transcription factor Creb3 and likely reflects the activation of the Golgi stress response in a subset of samples or BioProjects (81).

The regulation of lipid metabolism is of particular interest given the current epidemic of non-alcoholic fatty liver disease. There is some evidence of zonation of fatty acid metabolism in the liver, fatty acid β-oxidation being enriched in periportal hepatocytes and lipogenesis in pericentral hepatocytes (75), but these pathways are independently regulated in this dataset. Liver cluster 13 is highly enriched for genes involved in lipolysis and fatty acid β-oxidation. It overlaps the smaller cluster in the full atlas (cluster 101) but includes many additional genes that have tissue-specific enrichment (e.g. Aco7 in CNS). Conversely, liver clusters 16 and 70 comprise enzymes of cholesterol and fatty acid synthases and the known transcriptional regulators, Nfe2 and Srebfl/2. Liver cluster 26 contains multiple genes involved more generally in mitochondrial oxidative phosphorylation, including multiple genes encoding NADH-ubiquinone oxidoreductase subunits. We are not aware of any heterogeneity in mitochondrial distribution in the liver.

The various metabolic and inflammatory disease models, with distinct effects on non-parenchymal cells, enable deconvolution of signatures of specific cell types and disease processes. Liver cluster 6, which includes the classical fibrosis marker, Acta2 (smooth muscle alpha actin), is elevated in fibrosis models, but highest in E14 liver, which may indicate that myofibroblast activation in fibrosis recapitulates the phenotype of embryonic mesenchyme. Liver cluster 18 captures transcripts associated with more advanced fibrotic disease and includes multiple collagen genes and two candidate transcriptional regulators, Etv1 and Osr2. This cluster also contains the mesenchymal gene Olfm5, which is also expressed in microglia in the mouse (see http://biogps.org) and human (82) but is not associated with microglia in the rat (69). This highlights the problems with assuming that genes have similar expression patterns and functions across species.

The fibrosis-associated clusters are clearly separated from liver cluster 7 that captures the phenotype of infiltrating CD45+ (Ptprc) myeloid cells in various models. Supplementary Table S3B summarizes distinct GO term enrichment for liver clusters 7 and 18. Two sets of interferon-responsive transcripts including key regulators Ifng and Ifi19 cluster separately (liver clusters 25 and 43) as do transcripts associated with expression of class II MHC (liver cluster 65). These clusters are separated also from the signatures of endothelial cells (liver cluster 63) and of Kupffer cells, the resident macrophages (liver cluster 56) (Supplementary Table S3A). The latter cluster includes the transcript encoding the macrophage growth factor receptor, Csf1r, and many transcripts that were also downregulated in livers of Csf1r-knockout rats (83). Clec4f, which is expressed exclusively by Kupffer cells in mice, and is in the liver-specific cluster in the extended atlas, is in a separate cluster (liver cluster 95) with the three C1q subunits (C1qa/b/c), Clp, Ctps, Pld4 and Tijfab. There is emerging interest in the last gene, a forkhead-associated domain protein, in immune cell function and inflammation (84).

Finally, in rodents, there is a set of transcripts that is expressed in the liver in a sex-specific manner in part under the influence of growth hormone (85,86). The male- and female-specific liver transcriptomes are regulated by differential expression of specific transcription factors, CUX2 and ONECUT2 in females and BCL6 in males. The majority of samples are from males, but nevertheless liver cluster 66 is excluded from female livers, and liver cluster 84 contains Cux2, Trim 24 and known female-specific transcripts.

The relationship between network analysis and scRNA-seq for the definition of cell types in tissues

As in the liver, the network analysis of other major organ systems enabled robust extraction of clusters of co-regulated transcripts often including the transcription factors that regulate them. In this case, the issue of tissue-specific promoters becomes less of an issue and genes that have multiple promoters (e.g. Mitf, Acp5) may form part of tissue-specific networks highlighting local functions. The deconvolution of large datasets by network analysis complements scRNA-seq analysis that has rapidly become a dominant approach to analysis of cellular heterogeneity. scRNA-seq is not quantitative. Typically, expression of <1000 genes is detected in each cell and even the most highly expressed transcripts are not detected in every cell (87). The output of scRNA-seq conflates two distinct types of zero values: those where a gene is expressed but not detected by the sequencing technology (stochastic sampling) and those that reflect genuine expression heterogeneity. Whereas we can readily separate entirely unrelated cells that share few markers in scRNA-seq, such as epithelial and haematopoietic cells, the identification of numerous subpopulations within individual lineages is tenuous at best (20). A second disadvantage of analysis of isolated cells by scRNA-seq or total RNA-seq is that cells are inevitably ac-
attivated during isolation and single cells can have attached remnants of other cells that contribute RNA (20,88).

Suo et al. (89) described computational analysis of mouse cell atlas to identify 202 regulons whose activities are highly variable across different cell types and predicted a small set of essential regulators for each major cell type in mouse. We have achieved the same outcome for the rat without the use of scRNA-seq. The advantage of network deconvolution as performed here is that one can explore a much wider diversity of states than can be contemplated with scRNA-seq and identify more robust co-regulatory modules. Any proposed pair of markers of a specific cell population defined by scRNA-seq should be strongly correlated with each other if both are detectable in whole tissue. The prediction was tested in a meta-analysis of mouse tissue macrophage populations that failed to support the existence of a specialized macrophage subset defined from scRNA-seq data by reciprocal expression of Lyve1 and Mrc1 (20). Herein, the detailed analysis of the liver data indicates that zonation of the liver is dynamic and individual pathways are regulated to a large extent independently of each other. So, the definition of subpopulations of hepatocytes is state dependent. The discussion of other systems in Supplementary Text casts doubt on the fine-grained definition of subsets of tissue-specific parenchymal/epithelial cells and more generic glial cells, fibroblasts, endothelial cells, parenchymal cells and macrophages in many published scRNA-seq analyses. Network analysis reveals regulons that may, or may not, be restricted to a defined cell population, but which are clearly linked to function. In that respect, one might reasonably question the value of defining cell types as an approach to understanding biology.

SUPPLEMENTARY DATA

Supplementary Data are available at NARGAB Online.

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Author contributions: S.J.B. developed the informatics pipeline and generated the primary expression data. K.M.S. performed the network and enrichment analyses and manual annotation of metadata. C.W. developed BioGPS and established the BioGPS viewer of the atlas. D.A.H. wrote the initial manuscript, reviewed all genes and clusters, and contributed to network analysis. S.J.B. and K.M.S. contributed to manuscript editing.

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