The protein expression profile of ACE2 in human tissues

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ABSTRACT

The international spread of the novel, pathogenic SARS-coronavirus 2 (SARS-CoV-2) poses a global challenge on both healthcare and society. A multitude of research efforts worldwide aim at characterizing the cellular factors involved in viral transmission in order to reveal therapeutic targets. For a full understanding of the susceptibility for SARS-CoV-2 infection, the cell type-specific expression of the host cell surface receptor is necessary. The key protein suggested to be involved in host cell entry is Angiotensin I converting enzyme 2 (ACE2), and its expression has been reported in various human organs, in some cases with inconsistent or contradictory results. Here, we aim to verify a reliable expression profile of ACE2 in all major human tissues and cell types. Based on stringently validated immunohistochemical analysis and high-throughput mRNA sequencing from several datasets, we found that ACE2 expression is mainly localized to microvilli of the intestinal tract and renal proximal tubules, gallbladder epithelium, testicular Sertoli cells and Leydig cells, glandular cells of seminal vesicle and cardiomyocytes. The expression in several other previously reported locations, including alveolar type II (AT2) cells, could not be confirmed. Furthermore, ACE2 expression was absent in the AT2 lung carcinoma cell line A549, often used as a model for viral replication studies. Our analysis suggests that the expression of ACE2 in the human respiratory system appears to be limited, and the expression of the receptor in lung or respiratory epithelia on the protein level is yet to be confirmed. This raises questions regarding the role of ACE2 for infection of human lungs and highlights the need to further explore the route of transmission during SARS-CoV-2 infection.

INTRODUCTION

Coronaviruses are enveloped RNA virions, and several family members of *Coronaviridae* belong to the most prevalent causes of common cold. Two previous coronaviruses that were transmitted from animals to humans have caused severe disease; the severe acute respiratory syndrome coronavirus (SARS-CoV) and the Middle East respiratory syndrome coronavirus (MERS-CoV). The novel coronavirus SARS-coronavirus 2 (SARS-CoV-2), which shares ~80% amino acid identity with SARS-CoV, is the agent of the coronavirus disease 19 (COVID-19), the new rapidly spreading pandemic.

When coronaviruses enter the target cell, a surface unit of the spike (S) glycoprotein binds to a cellular receptor. Upon entry, cellular proteases cleave the S protein which leads to fusion of the viral and cellular membranes. SARS-Cov has previously been shown to enter the cell via the ACE2 receptor, primed by the cellular serine protease TMPRSS2, and recent studies suggest that also SARS-CoV-2 employs ACE2 and TMPRSS2 for host cell entry [1]. The binding affinity of SARS-CoV-2 to ACE2 based on interaction between its S-protein and human ACE2 molecules using computational

modelling seems to be weaker than that of SARS-CoV [2], but the affinity is still considered to be high enough for human transmission.

For a full understanding of the susceptibility for SARS-CoV-2 infection and the role of ACE2, it is necessary to study the cell type-specific expression of ACE2 in human tissues, both on the mRNA and protein level. The respiratory system is of special interest due to its high susceptibility to inhaled viruses, and most viruses infecting humans use different airway epithelial cells for replication. Expression of ACE2 in alveolar type II (AT2) cells would therefore suggest that these cells can serve as a reservoir for viral invasion and facilitate SARS-CoV-2 replication in the lung. It is however also important to study other tissue locations that could serve as potential entry.

In the present investigation, we provide a comprehensive summary of ACE2 expression levels across all major human tissues based on multiple previous studies, both on the mRNA and protein level. We confirm the findings with cell type-specific localization of ACE2 in 78 different human cell types based on immunohistochemistry using two independent rigorously validated antibodies.

RESULTS

ACE2 expression in human tissues using transcript profiling

ACE2 is a carboxypeptidase negatively regulating the renin-angiotensin system (RAS), which can induce vasodilation by cleaving angiotensin II. The protein was identified in 2000 as a homolog to ACE [3] and due to the importance of ACE in cardiovascular disease, and the use of ACE inhibitors for treatment of high blood pressure and heart failure, there has been a large interest in understanding the function and expression of ACE2 in various human organs. In Figure 1, a summary of the expression levels in a selection of different organs is shown, based on publicly available datasets on both transcriptomic and proteomic levels.

Early studies based on multi-tissue Northern blotting and QRT-PCR suggested moderate to high transcript expression in kidney, testis, heart and the intestinal tract, with most prominent in small intestine and lower levels in colon and/or rectum [3, 4]. No or low expression of ACE2 was observed in human lung. By the introduction of microarray analysis, whole-body maps quantifying global gene expression patterns based on mRNA transcripts were launched, such as BioGPS [5], soon followed by several large-scale transcriptomics efforts based on next-generation sequencing in samples representing most major human organs and tissues. Three such recent initiatives include the Human Protein Atlas (HPA) consortium [6] and the genome-based tissue expression (GTEx) consortium [7] based on RNA-seq, and the FANTOM5

consortium based on cap analysis gene expression (CAGE) [8]. The HPA consortium aims at characterizing the entire human proteome in tissues, organs, cells and organelles using a combination of transcriptomics and antibody-based proteomics. In the open-access database www.proteinatlas.org, expression profiles based on both external and internal datasets are shown, together with primary data, criteria used for validation and original high-resolution images of immunohistochemically stained human tissue samples. The HPA displays mRNA expression data from HPA, GTEx and FANTOM5, and also summarizes the three datasets as normalized expression levels (NX) across 37 tissues and organs covering all major parts of the human body, including human blood [9, 10]. The consensus of these three datasets show medium or high expression levels in kidney, testis and the intestinal tract, together with low expression in heart and adipose tissue and lack of expression in most other normal organs, including lung (Figure 1).

ACE2 expression in human tissues using antibody-based profiling

In order to study the functional role of ACE2 in human tissues, it is necessary to analyze the expression at a cell type-specific level, since smaller subsets of cells may express the receptor that may be below detection limit when mixed with all other cells in a complex tissue sample. Complementing the transcriptomics data with immunohistochemistry not only gives the possibility to define protein localization in different compartments at a single-cell level, but also provides important spatial information in the context of neighboring cells in intact tissue samples. Furthermore, the combination of transcriptomics with proteomics provides an advantageous strategy for studying the functional representation of the human genome. A previous immunohistochemistry study by Hamming et al [11] analyzing ACE2 across a large set of human samples, showed staining in most tissue types examined (Figure 1). Abundant immunoreactivity was displayed in microvilli of small intestine and proximal tubules of kidney, in line with all mRNA expression datasets, but strong positivity was also found in several other organs, including the respiratory system, oral mucosa and skin. While this is partly consistent with transcriptomics data from microarray experiments [5], the findings are contradictory to all three more recent mRNA expression datasets. In the respiratory system, both alveolar epithelial AT1 and AT2 cells, and epithelial cells of nasopharynx were distinctly stained. The authors also presented a consistent abundant expression of endothelial cells, smooth muscle cells, fibroblasts and adipocytes of all examined tissues, despite the fact that several of the corresponding organs that normally contain high fractions of such stromal and mesenchymal cells had low or absent mRNA levels. The immunohistochemical analysis by Hamming et al was based on a single antibody, quality ensured using three different criteria: 1) incubation of control sections with anti-ACE2 antibody solutions pre-incubated with the synthetic peptide to which the antibody was raised, 2) incubation of control sections with unrelated rabbit polyclonal antibodies, and 3) incubation with PBS instead of primary antibody. All three control sections were negative for immunohistochemistry, however, these methods for validation still do not

prove that the antibody is specific for endogenous ACE2 in formalin-fixed, paraffin-embedded tissue samples. The antibody thus does not meet the criteria for enhanced validation proposed by the International Working Group for Antibody Validation (IWGAV), formed with representatives from several major academic institutions [12]. IWGAV proposed five different pillars to be used for antibody validation to ensure reproducibility of antibody-based studies and promote stringent strategies for validation. To ensure that an antibody binds to the intended protein target, it is necessary that the validation has been performed in an application-specific manner, using at least one of the strategies suggested by IWGAV.

In line with the IWGAV recommendations, the HPA has introduced and implemented this concept for enhanced validation of antibodies [13]. The antibodies used in the HPA program for the immunohistochemical analysis of ACE2 have passed several of the criteria for enhanced antibody validation. The protein expression levels of two different antibodies showed high correlations with mRNA expression levels from three different datasets (HPA, GTEx and FANTOM5), thereby validating the antibodies using an orthogonal method. Furthermore, the cell type-specific localization of the protein expression across 78 different cell types was similar between the two independent antibodies. Of all 78 cell types evaluated, reliable expression was only observed in 10 cell types: microvilli of the duodenum, small intestine, colon and rectum, renal proximal tubules, in gallbladder epithelium, testicular Sertoli cells and Leydig cells, a subset of glandular cells in seminal vesicle, and in cardiomyocytes. (Figure 2). The cardiomyocyte staining was more diffuse and mainly cytoplasmic, while all other positive locations were as expected more prominent towards the plasma membrane. One antibody also showed positivity in a subset of apical membranes in liver bile ducts, while the other antibody was consistently negative in bile ducts of all three individuals. Due to the discrepancy between the antibodies, the staining in bile ducts for one of the antibodies should therefore be interpreted with caution, as it may represent off-target binding. No distinct expression confirmed by both antibodies could be found in any of the other analyzed cell types, including epithelial cells of the respiratory system, blood vessel endothelial cells, smooth muscle cells and fibroblasts. This immunohistochemistry analysis suggests a much more limited expression of the ACE2 in human tissues than previously suggested [11], and the present body-wide expression profile shows a high correlation to different mRNA expression datasets.

ACE2 expression in human tissues using mass spectrometry-based profiling

Another method for determining protein levels in a tissue sample is mass spectrometry, and in one of the large-scale studies based on mass spectrometry of different normal human tissues [14, 15], samples from lung were included. The dataset in this Human Proteome Map [14] generally correlated well with both mRNA expression levels from HPA, GTEx and FANTOM5, and IHC data from HPA, showing the highest expression in kidney and testis, and no expression in lung (Figure 1).

ACE2 expression in human tissues using single cell transcriptomics profiling

Recently, several datasets presenting the expression of ACE2 in human lung based on single cell RNA-seq (scRNA-seq) have been published. This new platform for single cell analysis constitutes an important complement to the *in situ* localization by immunohistochemistry and thus provides an excellent tool in studies of transcript levels expected to be found in smaller subsets of cells in complex tissue samples. The data for ACE2 from multiple scRNA-seq datasets in human respiratory tract are however inconclusive. In Figure 3, a summary of ACE2 expression in human lung based on three different publicly available scRNA-seq datasets is provided [16-18] with the transcriptomics expression in single cells of ACE2 and the surfactant protein SFTPA1. The data in all three datasets reveal that only a small fraction of the cells in lung express ACE2 at a low level, in contrast to the abundant expression of the surfactant protein in clusters representing AT2 cells. This is in contrast to other studies suggesting an enrichment in AT2 cells, although it should be noted that expression was often identified only in a very small fractions of the AT2 cells, often less than 1% [19, 20]. There are also other studies concluding that the ACE2 expression is considerably higher in other cells of the respiratory tract, such as ciliated or secretory cells [21, 22], or in squamous epithelial cells of oral mucosa or esophagus [23, 24]. Similarly, in the recently published Human Cell Landscape (HCL) [16], the expression levels of ACE2 were highest in cell types supporting the data from antibody-based immunohistochemistry from the HPA program, including jejunal and duodenal enterocytes, gallbladder epithelial cells, renal proximal tubules and testicular Sertoli cells. In the three samples from adult lung, the expression level was low, and no elevated expression was observed in AT2 cells as compared to secretory cells, macrophages or fibroblasts. In summary, the data from single cell transcriptomics studies reported here (Figure 3) supports the conclusion from the antibody-based immunohistochemistry analysis described above, suggesting that there are no or very low detectable protein levels of ACE2 in the lung.

ACE2 expression in human cell lines

The most commonly used experimental models for assessing the tropism and replication of viruses in humans include *ex vivo* cultures of human respiratory tissues, primary cells from the human respiratory system, or cell lines derived from the human respiratory system. The most commonly used cell line in such studies is A549, derived from an alveolar epithelial AT2 cell lung carcinoma [25]. To explore which cell lines that may be suitable experimental models for analysis of ACE2, we analyzed the expression levels based on RNA-seq in 64 different cell lines, as part of the Cell Atlas in the HPA [26]. Interestingly, the expression levels of ACE2 were below cut-off in all three cell lines derived from human lung, including A549, the immortalized bronchial epithelial cell line HBEC-KT, and the small cell lung carcinoma cell line SCLC-21H. The expression levels were below cutoff also in most other cell lines, however, low

expression (<5 NX) was found in BEWO (metastatic choriocarcinoma), HaCaT (keratinocytes), Hep G2 (hepatocellular carcinoma), NB-4 (acute promyelocytic leukemia), RPMI-8226 (multiple myeloma), RT4 (urinary bladder) and SH-SY5Y (neuroblastoma). This suggests that none of the lung cell lines are appropriate models for studying the role of ACE2 in the human respiratory system. In the previous immunohistochemistry study by Hamming et al, the positivity of ACE2 in cells from A549 was presented as a confirmation of expression in AT2 cells. Due to the lack of expression on ACE2 in A549 on the mRNA level and the fact that the same study suggested expression in multiple other tissue locations neither confirmed by mRNA levels or by well-validated antibodies in the present investigation, it is therefore likely that the previously published positivity of ACE2 in A549 was due to off-target binding.

DISCUSSION

In the present investigation, we used two independent well-characterized antibodies for immunohistochemical analysis of ACE2. Based on stringent validation criteria and comparison of the protein expression profiles with multiple transcriptomics datasets, reliable expression could only be confirmed in microvilli of the intestinal tract and renal proximal tubules, in gallbladder epithelium, testicular Sertoli cells and Leydig cells, a subset of glandular cells in seminal vesicle and in cardiomyocytes, with no detectable expression in lung or respiratory epithelia. No reliable data confirms protein level expression of ACE2 in AT2 cells, previously shown to harbor ACE2. Limited expression of ACE2 in AT2 cells suggested in recent datasets based on scRNA-seq, however, this finding is only observed in some of the individuals, often at a low level and only in a small fraction of the cells.

All studied datasets confirm a consistent high expression in the intestinal tract, gallbladder and kidney. Further studies are needed to explore the potential involvement of ACE2 in the manifestation of symptoms related to these organs as a result of COVID-19 disease. In addition to respiratory symptoms, the closely related SARS-CoV that caused the SARS outbreak was shown to also cause diarrhea, impaired liver function and elevation of non-cardiac creatine kinase, suggesting tropism of the virus to other organs well in line with the tissues showing the highest expression levels of ACE2. Interestingly, in a recent study on pediatric COVID-19 individuals, eight out of 10 cases showed rectal swabs positive for SARS-CoV-2, suggesting that the gastrointestinal tract may shed virus and that fecal-oral transmission may be a possible route for infection [27]. It should also be noted that the prerequisites of the interplay between SARS-CoV-2 attachment pattern to human cell surface receptors and COVID-19 disease progression are multifactorial, including not only the binding of the virus to the target cells, but also other factors such as the host's immune system and the total virus load.

In summary, based on multiple datasets on the mRNA and protein level, using both single cell methods and larger tissue extracts, we here show that ACE2 expression in human tissues is mainly localized to microvilli of intestine and renal proximal tubules, gallbladder epithelium, testicular Sertoli cells and Leydig cells, glandular cells of seminal vesicle and cardiomyocytes. Several additional locations suggested by previous studies, including expression in AT2 cells, could not be confirmed. This raises questions regarding the role of ACE2 for infection of human lungs, and highlights the need to further explore the route of transmission during SARS-CoV-2 infection to understand the biology of the disease and to aid in the development of effective treatments to the viral infection.

METHODS

Data sources

ACE2 RNA expression data from Northern blot and QRT-PCR were translated into expression levels for Figure 1 by interpretation of figures and description of the results from the original publications [3, 4]. RNA expression data from BioGPS were retrieved from http://biogps.org [5], while the RNA expression data from HPA[6], GTEx[7], FANTOM5[8] as well as the normalized RNA expression dataset were retrieved from the HPA database (http://www.proteinatlas.org). The RNA expression levels from BioGPS, HPA, GTEx, and FANTOM5 were dichotomized into the groups used in Figure 1 based on the following cut-offs: High expression = 51-100% of the maximum value, Medium = 11-50% of the maximum value, Low = 3-10% of the maximum value, or Not detected (no circles are shown) = 0-2% of the maximum value. Protein expression data based on mass spectrometry (MS) was translated into levels using the summary provided for ACE2 on the Human Proteome Map database (http://www.humanproteomemap.org). Protein levels based on immunohistochemistry correspond to staining intensity based on manual annotation. Immunohistochemistry data from Hamming et al [11] was manually re-evaluated by interpretation of figures and description of the results from the original publication, while the immunohistochemistry data from HPA corresponds to images on the HPA database http://www.proteinatlas.org, generated as described below. The lung scRNA-seq data (gene raw counts) were downloaded from the Gene ExpressionOmnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) database under the series numbers: GSE130148 [18], GSE134355 [16] (samples: GSM4008628, GSM4008629, GSM4008630, GSM4008631, GSM4008632 and GSM4008633) and GSE122960 [17] (samples: GSM3489182, GSM3489185, GSM3489187, GSM3489189, GSM3489191, GSM3489193, GSM3489195 and GSM3489197).

Sample preparation and ethical approval

Human tissues samples for analysis of mRNA and protein expression in the HPA datasets were collected and handled in accordance with Swedish laws and regulation. Tissues were obtained from the Clinical Pathology department, Uppsala University

Hospital, Sweden and collected within the Uppsala Biobank organization. All samples were anonymized for personal identity by following the approval and advisory report from the Uppsala Ethical Review Board (Ref # 2002-577, 2005-388, 2007-159, 2011-473).

Antibody-based protein profiling

Formalin-fixed, paraffin-embedded (FFPE) tissue blocks from the pathology archives were selected based on normal histology using a hematoxylin-eosin stained tissue section for evaluation. Tissue microarray (TMA) generation, immunohistochemical staining and high-resolution digitalization of stained TMA slides has been described previously [28]. In short, representative 1 mm diameter cores were sampled from FFPE blocks and assembled into an array of normal tissue samples, corresponding to a toal of 144 individuals. TMA blocks were cut in 4 µm sections using a waterfall microtome (Microm H355S, ThermoFisher Scientific, Freemont, CA), placed on SuperFrost Plus slides (ThermoFisher Scientific, Freemont, CA), dried overnight at room temperature (RT), and then baked at 50°C for at least 12 h. Automated immunohistochemistry was performed by using Lab Vision Autostainer 480S Module (ThermoFisher Scientific, Freemont, CA), as described in detail previously [28]. Primary antibodies against human ACE2 were the polyclonal rabbit IgG antibody HPA000288 (Atlas Antibodies AB, Sweden) and monoclonal mouse IgG antibody CAB026174 (cat # MAB933 Human ACE-2 antibody, R&D Systems, Minneapolis, MN). The antibodies were diluted and optimized based on IWGAV criteria for antibody validation [12]. Protocol optimization was performed on a test TMA containing 20 different normal tissues. The stained slides were digitalized with ScanScope AT2 (Leica Aperio, Vista, CA) using a 20x objective. The tissue cores were manually annotated by certified pathologists (Lab SurgPath, Mumbai, India), and curated by a second observer. Annotation parameters included staining intensity, quantity of positive cells and subcellular localization in 78 different normal cell types. All high-resolution images are readily available in version 19.3 of the Human Protein Atlas (https://www.proteinatlas.org).

Analysis of scRNA-seq data

Data analysis including filtering, normalization, and clustering were performed using Seurat V3.0 (https://satijalab.org/seurat/9) in R (CRAN). Cells were removed when they had less than 500 genes and greater than 20% reads mapped to mitochondrial expression genome. The gene expression data were normalized using default settings. Cell clustering was based on the 5000 highly variable genes. All scatter plots were obtained using the UMAP method. Cluster identity was manually assigned based on well-known cell-type markers and named based on the main cell type in each cluster.

ACKNOWLEDGEMENTS

The project was funded by the Knut and Alice Wallenberg Foundation. Pathologists and staff at the Department of Clinical Pathology, Uppsala University Hospital, are acknowledged for providing the tissues used for RNA-seq and immunohistochemistry. The authors would also like to thank all staff of the Human Protein Atlas for their work.

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FIGURES

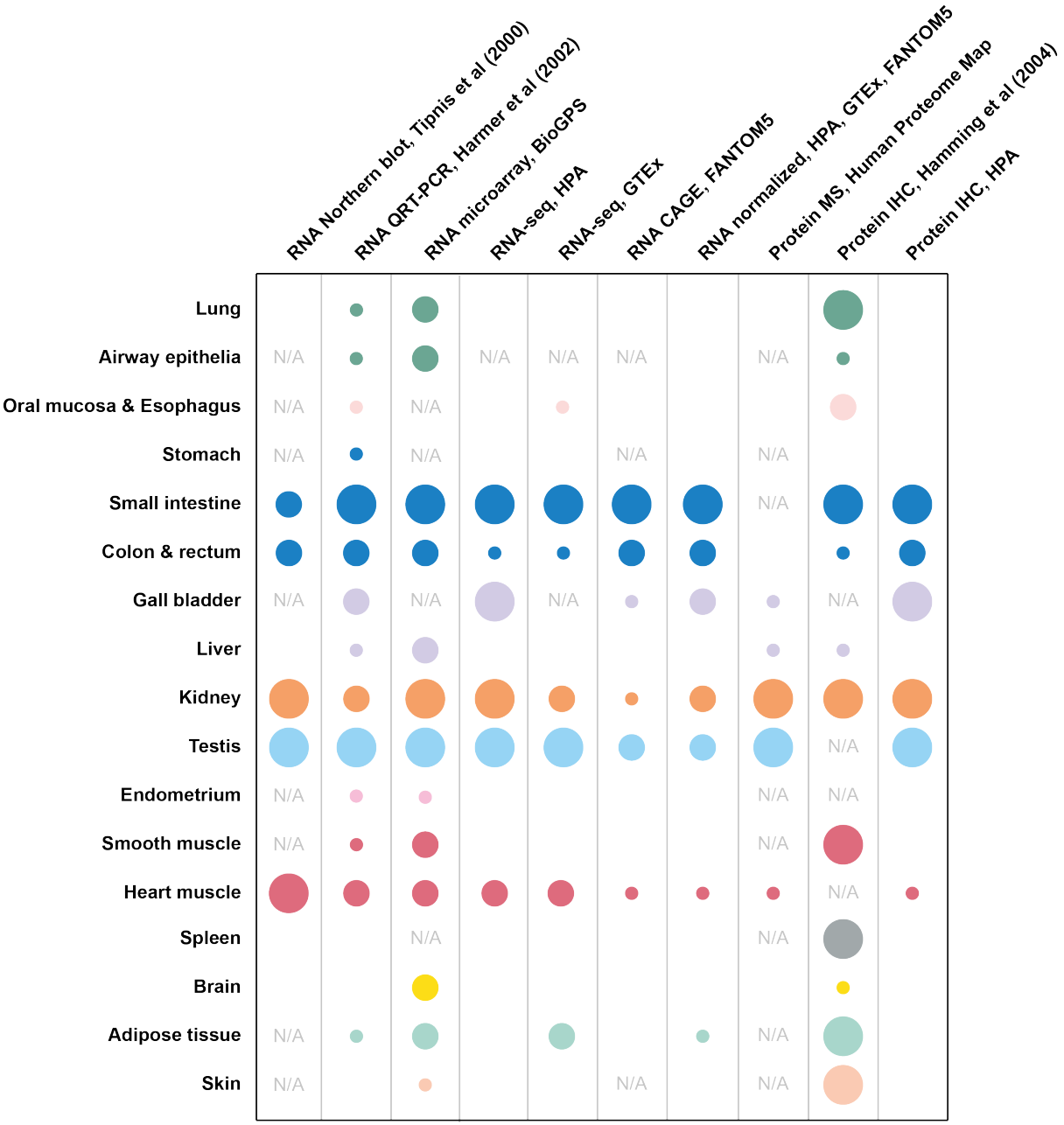

Figure 1. A summary of ACE2 expression in human tissues based on publicly available transcriptomics and proteomics datasets. Three different sizes of circles represent high, medium or low expression levels, and each circle is color-coded based on organ system. A consistent expression in the intestinal tract, gallbladder, kidney, testis and heart muscle was observed across all datasets. The broadest expression profile was reported from Hamming et al, where also lung, oral mucosa, and esophagus, spleen, adipose tissue, smooth muscle, brain and skin showed significant staining. N/A = no data available.

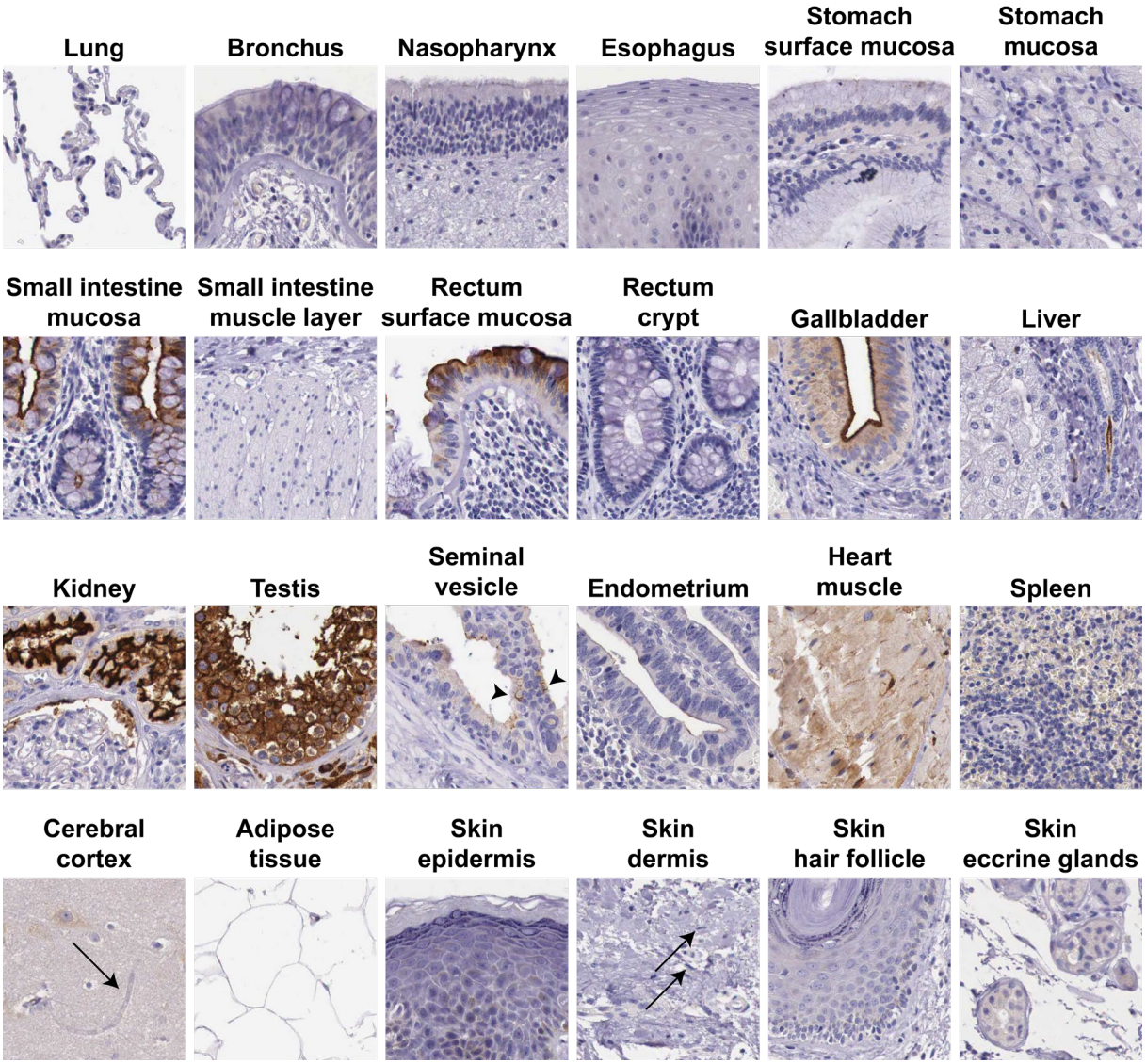

Figure 2. Cell type-specific localization of ACE2 in human tissues based on immunohistochemistry. Representative images of 18 tissue types and histological structures stained with immunohistochemistry using the polyclonal rabbit antibody HPA000288 (Atlas Antibodies AB), targeting human ACE2 protein (brown), and counterstained with hematoxylin (blue). Highest ACE2 expression was observed in microvilli of the intestinal tract and renal proximal tubules, in membranes of gallbladder epithelium, testicular Sertoli cells and Leydig cells, a subset of glandular cells in seminal vesicle (arrowheads), and in cytoplasm cardiomyocytes. Note that ACE2 protein expression was not detected in the lower parts of the mucosal intestinal layer. No protein expression was observed in lung, bronchus, nasopharynx, esophagus, stomach, endometrium, smooth muscle tissue (visualized in muscle layer of small intestine), spleen, cerebral cortex, adipose tissue or different structures of the skin. Protein expression was neither detected in endothelial cells, here shown in cerebral cortex (arrow), nor in fibroblasts, here sown in dermis layer of skin (arrows). For details, see https://www.proteinatlas.org/ENSG00000130234-ACE2/tissue.

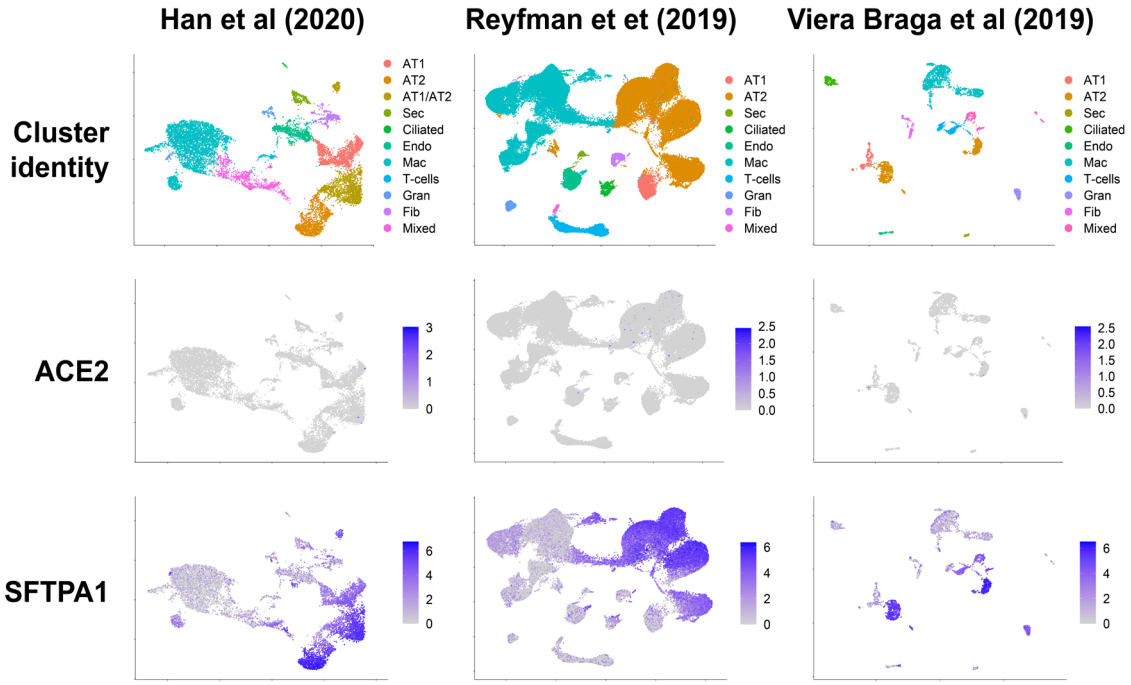

Figure 3. Single cell RNA-seq analysis of human adult lung. Uniform manifold approximation and projection (UMAP) analysis of lung cells based on three publicly available datasets. Each dot represents a single cell colored according to its cluster identity. No high cluster-specific expression of ACE2 could be identified in any of the datasets. The expression pattern of the AT2 cell marker SFTPA1 is shown as a reference.