

# Subtypes and proliferation patterns of small intestine neuroendocrine tumors revealed by single cell RNA sequencing

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## eLife Assessment

This is a **valuable** study that uses single-cell RNA sequencing to define tumor-intrinsic transcriptional programs that characterize distinct types of small intestine neuroendocrine tumors. The evidence supporting the claims of the authors is **solid**, but would benefit from a larger sample size. The work will be of interest to cancer biologists studying neuroendocrine tumors, as well as those studying tumor heterogeneity more broadly.

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## Abstract

Neuroendocrine tumors (NETs) occur primarily in the small intestine, lung and pancreas. Due to their rarity compared to other malignancies in these organs, their complex biology remains poorly understood, including their oncogenesis, tumor composition and the intriguing phenomena of mixed neuroendocrine non-neuroendocrine neoplasms (MiNEN). Here we profiled ten low-grade small intestine NET (SiNET) samples as well as one mixed lung tumor by single-cell or single-nuclei RNA-seq. We find that SiNETs are largely separated into two distinct subtypes, in which the neuroendocrine cells upregulate epithelial or neuronal markers, respectively. Surprisingly, in both subtypes the neuroendocrine cells are largely non-proliferative while higher proliferation is observed in multiple non-malignant cell types. Specifically, B and plasma cells are highly proliferative in the epithelial-like SiNET subtype, potentially reflecting the outcome of high Migration Inhibitory Factor (MIF) expression in those tumors, which may constitute a relevant target. Finally, our analysis of a mixed lung neuroendocrine tumor identifies a population of putative progenitor cells that may give rise to both neuroendocrine and non-neuroendocrine (squamous) cells, potentially explaining the origin of the mixed histology. Taken together, our results provide important insights and hypotheses regarding the biology of neuroendocrine neoplasms.

## Introduction

Neuroendocrine cells are present throughout the body and are thought to give rise to neuroendocrine tumors (NETs) in multiple organs. Small intestine NETs (SiNETs) have the second highest incidence, after lung NETs [1]. Histopathological grading of SiNET is based on mitoses rate and on the Ki67 proliferation index, with low-grade comprising G1 and G2, and high-grade comprising G3. In addition, neuroendocrine carcinomas (NECs) are included in the G3 category, and have poorly differentiated histopathological appearance [2].

The majority of SiNETs are low grade well-differentiated tumors, usually diagnosed at advanced stages and often present with metastases and stage IV tumor, highlighting their high metastatic potential [3]. Interestingly, SiNETs are often multifocal, with a common finding of synchronous multiple primaries along the small intestine [4, 5]. Two recent studies demonstrated that synchronous SiNETs primaries within the same individual usually do not share somatic alterations, indicating that they arise from different cell clones [6, 7].

SiNETs are usually sporadic, with a surprisingly low mutational burden or recurrent mutations, and show mainly chromosomal alterations [8-10]. The most frequent chromosomal aberration found in SiNETs is the loss of chromosome 18 as seen in 61-89% of patients [11]. In a cohort of 180 SiNETs, the most frequent mutation (found in 8% of tumors) were heterozygous frameshift mutations of *CDKN1B*, encoding the p27 cyclin regulator, suggesting a role of cell cycle deregulation in the progression of SiNETs [12]. Further studies reported heterogeneity of the *CDKN1B* mutations even among lesions of the same patient [13]. Overall, there is a limited frequency and consistency of genetic aberrations in SiNETs, of which mechanisms of initiation and progression remain poorly understood.

Another intriguing phenomenon with respect to the large group of neuroendocrine neoplasms (NENs) is that they are sometimes accompanied by a carcinoma component, resulting in “mixed” neuroendocrine non-neuroendocrine neoplasms (MiNEN) [14]. For a NEN to be termed mixed, it is necessary for each component to comprise at least 30% of the tumor, suggesting that many additional tumors may have both components but are not recognized as MiNEN. The origin of MiNENs remains unknown, and their complexity hinders conventional treatment approaches.

In this study, we sought to investigate the molecular characteristics of SiNETs to uncover novel insights into their oncogenic transformation, the resulting tumor ecosystems, and potential vulnerabilities. To this end, we applied single-cell transcriptomics to SiNETs as well as to one MiNEN.

## Results

### Single cell and single nuclei RNA-seq profiling of SiNETs

To understand the tumor ecosystem of low-grade SiNETs, we generated scRNA-seq profiles for ten primary tumor samples from eight patients using 10x Chromium (Table S1). We profiled single cells from three fresh surgical samples (SiNET1-3) and single nuclei from the remaining seven frozen samples (SiNET4-10). After initial quality controls, we retained 29,198 cells from the ten samples (see Methods). For comparison, we also profiled one fresh small intestine adenocarcinoma (SiAdeno) and included it in our analyses.

We identified clusters of cells in each tumor and annotated them based on the expression of known marker genes as neuroendocrine (NE) cells, and as tumor microenvironment cells (TME) cells including T cells, B/plasma cells, macrophages, fibroblasts, endothelial cells, epithelial cells and natural killer (NK) cells (Table S2). While NK cells were detected only in one sample, the other six types of TME cells were each detected in multiple samples, and these exactly correspond to the TME cell types that are commonly detected in scRNA-seq analyses of other cancer types [15]. The Clustering and marker expression of two exemplary SiNETs are shown in **Fig. 1A-D**, and the remaining tumors are shown in **Fig. S1**. Cell type proportions varied considerably between tumors (**Fig. 1E**). In five tumors NE cells were the most frequent, while other tumors had the highest frequency of other cell types, such as endothelial, fibroblast, epithelial or T cells. The unique composition of each tumor sample likely reflects a combination of tumor-specific biology with spatial sampling within the tumor. Technical effects (e.g. single cell analysis of fresh samples vs. frozen single nuclei analysis of frozen samples) could also impact the capture of distinct cell types, although we did not observe a clear pattern of such bias.

## Shared NE-specific genes define an SiNET transcriptomic signature

We next focused on the NE cells and defined both their common and their variable expression profiles across patients. To this end, we examined the eight samples (one per patient) in which we identified NE cells. We first compared NE cells from each tumor to non-NE cells from the same tumor and identified all genes that are highly upregulated in NE cells. Twenty-six genes were consistently upregulated in NE cells from most tumors ( $n > 5$ ) and hence were defined as common NE-specific genes (**Fig. 2A**).

The common NE-specific genes could potentially serve as a transcriptomic signature for SiNETs. However, they may also include generic markers of NETs that are not unique to SiNETs. To examine the specificity of this signature, we examined their relative expression in a bulk RNA-seq dataset that contains NETs of the small intestine, pancreas and rectum [16]. Almost all signature genes were preferentially upregulated in NETs of the small intestine compared to the other NETs, suggesting that they reflect an efficient SiNET signature (**Fig. 2B**, Table S3). The signature genes include known markers of SiNET [17] as well as of enterochromaffin cells [18], such as *CHGA*, *CHGB*, and *TPH1*. While some of these markers are not unique to SiNETs, the bulk RNA-seq data suggests that, at least at the mRNA level, they are higher expressed in SiNETs than in other NETs. These genes were most significantly enriched with gene-sets associated with neuroendocrine-related functions such as exocytosis, Neurosecretory vesicle, and serotonergic synapse (all with adjusted  $P < 0.001$ ).

## Heterogeneity of NE cells reveals two SiNET subtypes

However, many more genes were found as upregulated in NE cells of only few of the SiNETs, and these were separated into tumor specific ( $n=1$ , see **Fig. S2A**) and subset-specific (between 2 and 5 tumors). Subset-specific genes may help to uncover functionally distinct subtypes of SiNETs and therefore we investigated them further. These genes exhibited nine distinct expression patterns across eight SiNET samples from eight distinct patients (**Fig. 2C**, Table S3). Notably, pattern #1, which was associated with the largest number of genes ( $n=26$ ), was highly expressed in three tumors that had low expression of all other eight patterns. In contrast, the other five tumors highly expressed genes from multiple patterns and had low expression of pattern #1 genes. This analysis highlighted a division of the eight SiNETs into two primary subtypes based on pattern #1 vs. all other patterns. Notably, the same subtype division was clearly detected by clustering of the SiNETs based on the global expression profiles of NE cells in each tumor (**Fig. 2D**).

The first subtype (SiNETs 1, 2 and 9) was associated with upregulation of 129 genes, including the epithelial markers *EPCAM* and *KRT8* (Table S4). While this subtype included the two fresh samples (SiNETs 1 and 2) it also included one frozen sample (SiNET9) and therefore is unlikely to reflect

**Figure 1.**

### Cellular composition of SiNETs as determined by single cell and single nuclei sequencing.

(A,B) UMAP plots showing the diversity of single cells from SiNET2 (A) and SiNET5 (B), colored by their cluster assignment. (C,D) Cluster annotations (top bar) in SiNET2 (C) and SiNET5 (D) are supported by the expression of canonical cell type markers (rows). Also shown are three cell cycle markers (bottom rows) (E) Cell type frequencies in each of the 10 SiNETs that we profiled, along with one SiAdeno sample.

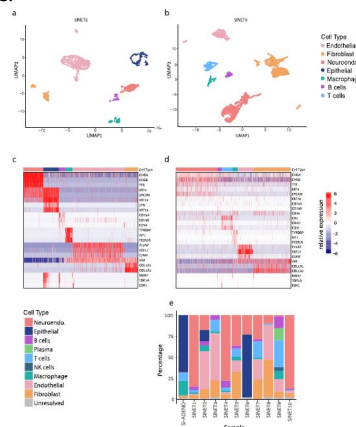
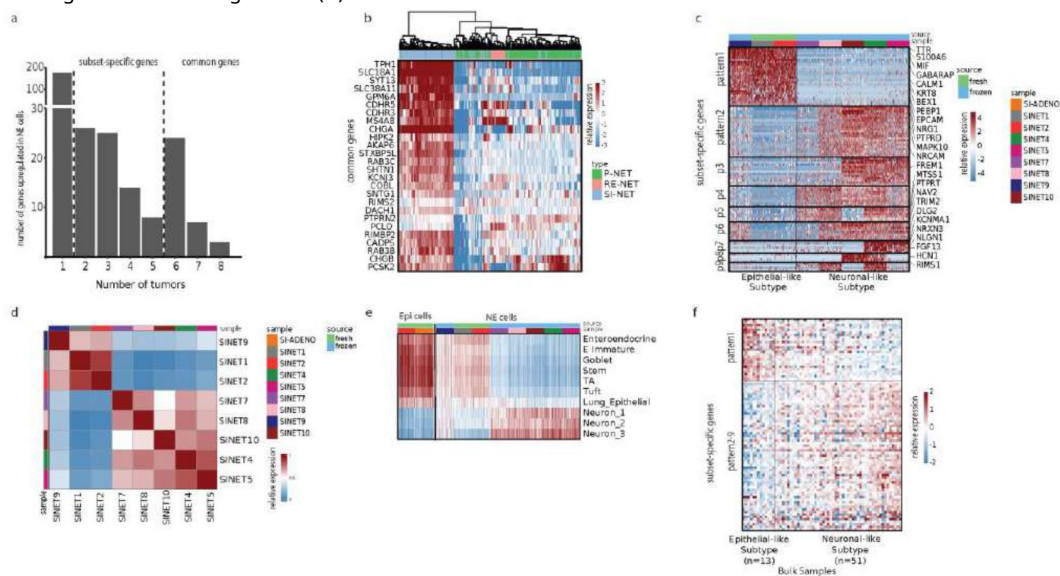


**Figure 2.**

### SiNETs broadly classify into two major subtypes

(A) Bar plot showing the number of upregulated genes against a common threshold, number of genes (y-axis) vs number of tumors (x-axis). (B) Heat map showing a list of 25 representative genes that define SiNET signature of our scRNA seq cohort used to cluster NET samples from a bulk-seq dataset [16]. Type of NET is color coded on the top panel, with P-NET and RE-NET referring to pancreatic and rectal NETs. (C) Heatmap representing clustering of SiNET samples in our cohort based on genes that were differentially expressed and shared between 2-5 samples, showing two major variable gene programs (D) Correlation heatmap between the NET samples. (E) Heatmap showing average expression of epithelial and neuronal gene-sets (rows) in the neuroendocrine and epithelial cells from our SiNET samples (columns). Epithelial gene-sets include signatures of multiple cell types from the small intestine [18], and neuronal gene-sets include three clusters of neurons [36]. (F) Heatmap of the SiNET dominated cluster from the bulk dataset [16] was subjected to differential expression analysis using the same set of genes as (C).



technical confounders. The second subtype (SiNETs 4, 5, 7, 8 and 10) was associated with upregulation of 73 genes, including neuronal-related genes such as *NAV3*, *RYR2*, *CACNA1A/B*, *KCNIP1*. This led us to speculate that even though both subtypes express the neuroendocrine markers described above (i.e. common genes), the first subtype may have higher overall similarity to epithelial cells compared to the second subtype, which might have more neuronal features.

To examine this possibility, we evaluated the expression of other cell type signatures across NE cells from the two SiNET subtypes (**Fig. 2E** [↗](#)). Signatures of multiple epithelial cell types from the small intestine or from the lung, including those of enteroendocrine cells, were expressed more highly in subtype 1 than in subtype 2 SiNETs. Nevertheless, the expression of such epithelial signatures was considerably lower in NE cells from subtype 1 tumors than in bona-fide epithelial cells such as the non-malignant epithelial cells identified in SiNET6 or the malignant cells in the SiAdeno tumor. Thus, epithelial signatures are upregulated in subtype 1 tumors but not at the same level as in bona-fide epithelial cells. Conversely, signatures of neuronal cell types had the opposite expression pattern – highest in subtype 2 SiNETs, intermediate in subtype 1 SiNETs and lowest in bona-fide epithelial cells. Based on these results we renamed subtypes 1 and 2 as the *epithelial-like* and *neuronal-like* SiNET subtypes.

To validate the existence of these two SiNET subtypes, we turned to analyze an external bulk RNA-seq dataset of SiNETs [[16](#) [↗](#)]. The signal of NE cells is diluted in bulk RNA-seq due to the profiling of entire tumor samples, and especially given the wide diversity of cell type compositions that we observe in single cell analysis (see **Fig. 1E** [↗](#)). However, we wondered whether we would still be able to detect the two subtypes when directly analyzing the subset-specific genes defined above. Indeed, the 81 SiNET bulk profiles could be separated into the *epithelial-like* and *neuronal-like* subtypes based on expression of pattern #1 genes vs. the genes of the other patterns (**Fig. 2F** [↗](#)). Moreover, the proportion of the two subtypes (20% vs. 80%) were comparable to those seen in our single cell cohort (30% vs. 70%). This analysis supports the generality of the two SiNET subtypes and their independence from the potential confounding effects of fresh vs. frozen samples in our data, that do not affect the bulk RNA-seq dataset.

## Heterogeneity in the SiNET tumor microenvironment

Next, we examined the diversity of cellular states within each non-NE cell type of the tumor microenvironment (TME). For each cell type, we analyzed the diversity within each tumor, searching for distinguishable subpopulations of cells, their occurrence across tumors and their potential functional implications (see **Fig. 3** [↗](#)). Below we briefly describe the three most notable cases of diversity within cell-type that we observed.

Among fibroblasts, we found the highest diversity in SiNET8, with three distinguishable subpopulations (**Fig. 3A** [↗](#)). To understand their differences, we both conducted a differential expression analysis (**Fig. 3B** [↗](#)) and compared their profiles to recently described fibroblast signatures [[15](#) [↗](#)] (**Fig. 3C** [↗](#)). This analysis indicated that cluster 1 resembles other cancer-associated fibroblasts (CAFs), while cluster 2 and 3 are distinguished by specific expression profiles. Cluster 3 cells were primarily distinguished by expression of MHC-II genes and are thus consistent with previous observations of antigen-presenting CAFs [[19](#) [↗](#)]. Cluster 2 cells upregulate several programs of pericytes and myofibroblasts, but are further distinguished by upregulation of four ABC-transporters (ABCA6, ABCA8, ABCA9 and ABCA10). To our knowledge, such consistent upregulation of multiple ABC transporters was not observed in previous analysis of CAF heterogeneity [[20](#) [↗](#), [21](#) [↗](#)]. Yet, in our data, upregulation of ABC transporters was also detected in a subset of CAFs from SiNET3 and SiNET5 (**Fig. S3A-D** [↗](#)). These results suggest a unique feature of CAFs in a subset of SiNETs, possibly due to the unique microenvironment of SiNETs.

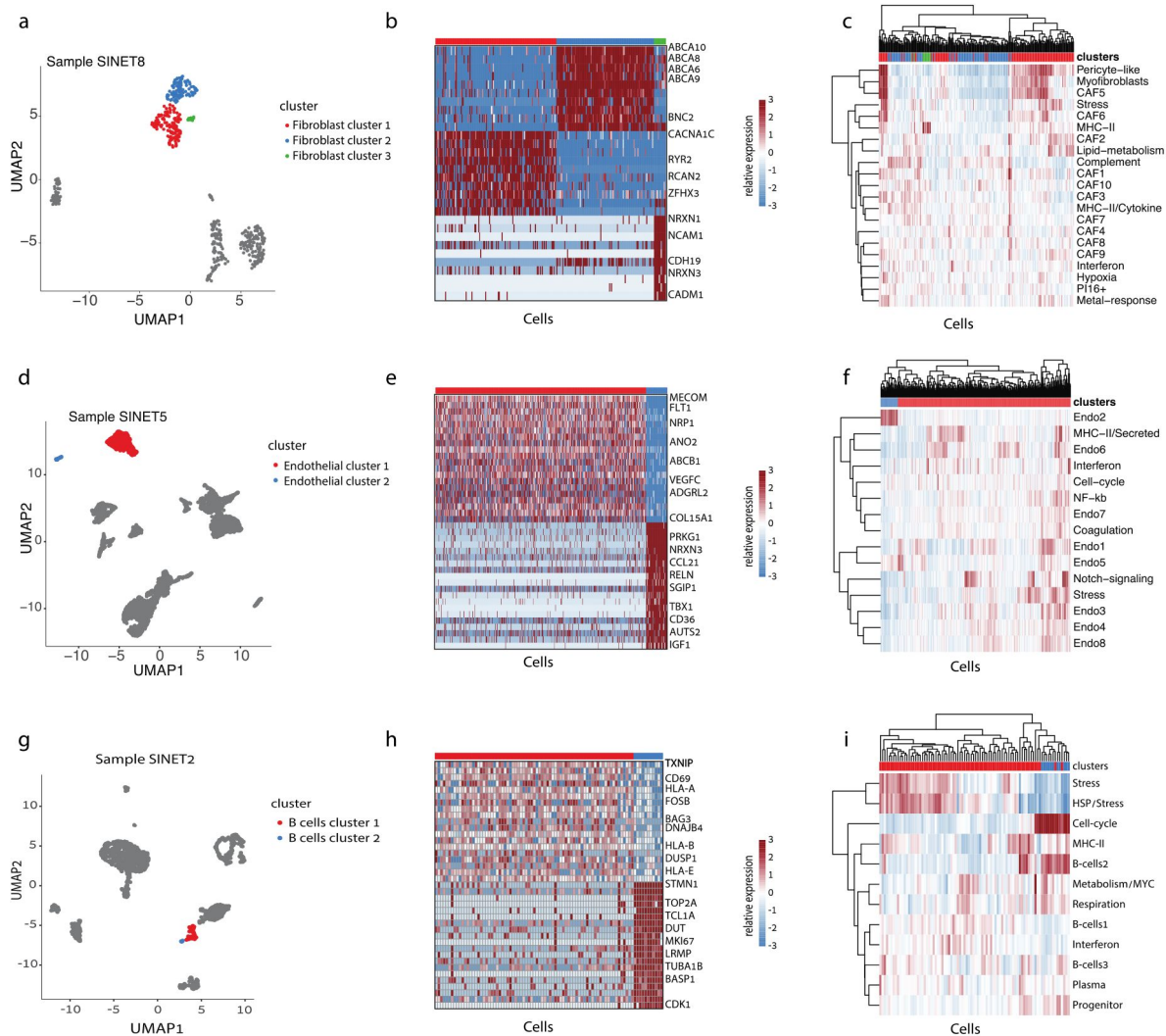


**Figure 3.**

### Heterogeneity in the SiNET tumor microenvironment.

For each of three non-malignant cell types, the diversity of that cell type is shown in one exemplary tumor: fibroblast heterogeneity in SiNET8 is shown in (A-C), endothelial cell heterogeneity in SiNET5 is shown in (D-F), and B-cell heterogeneity in SiNET2 is shown in (G-I). For each cell type, three panels depict three types of analyses. The first panel (A, D, G) is a UMAP plot of the respective tumor, where only the respective cell type is colored, and distinct colors highlight the clusters of that cell type. The second panel (B, E, H) shows differential expression analysis between the first two clusters using heatmaps, with labeling of selected genes. The third panel (C, F, I) shows clustering of cells from that cell type (columns) based on their relative expression of previously defined [15] signatures of diversity in that cell type (rows); the top panel shows assignment of cells to clusters.

Among endothelial cells, we found two highly distinct subpopulations in SiNET5 (**Fig. 3D-E**). Comparison to pan-cancer endothelial signatures mapped the smaller subpopulation to the Endo2 poorly described signature that was detected primarily in lung and skin tumors [15] (**Fig. 3F**). Similar subpopulations of endothelial cells were also detected in SiNET2, highlighting the Endo2 signature as a recurrent pattern of subsets of SiNET endothelial cells (**Fig. S3E-F**).

Among B cells, two distinct populations were found in SiNET2 (**Fig. 3G**). Differential expression showed that the small B cell subpopulation is distinguished by upregulation of many cell cycle genes including canonical markers (MKI67, TOP2A, CDK1), reflecting proliferating B cells (**Fig. 3H-I**). Moreover, we noticed that, among all SiNET2 cells, the canonical cell cycle markers are expressed in B cells more than in all other cell types, including the malignant NE cells (**Fig. 1C**). This observation prompted us to turn our attention to cell cycle patterns across all cell types within the SiNETs.

## Proliferation of NE and immune cells in SiNETs

Most SiNETs are low-grade tumors with a low mitotic index, but the exact identity of proliferating cells in SiNET is unknown. We used previously defined signatures of the cell cycle to identify all cycling cells. Notably, as we and others demonstrated previously [15, 22, 23], cell cycle involves the consistent upregulation of dozens of canonical genes and therefore the cycling cells can be robustly detected by scRNA-seq along with their phase along the cell cycle (**Fig. S4**).

Surprisingly, extremely few cycling cells were observed among the malignant NE cells (0.246% on average) (**Fig. 4A**). This fraction is considerably lower than what we detect in other cancers types [22], and as an extreme example from the same dataset, in the SiAdeno sample we find ~13% of cycling epithelial cells using the same method (**Fig. S4**). Notably, the fraction of SiNET cycling cells was lower for NE cells than for all other cell types identified in the SiNET ecosystem (**Fig. 4A**; see also **Fig. 1C-D**). Relatively high fractions of cycling cells (>10%) were found only in epithelial or in B/plasma cells. Epithelial cells were only detected in two tumors, and of those only in one tumor they had a high fraction of cycling cells. In contrast, B/plasma cells were detected more commonly – in eight tumors – and in three of those they had a high fraction of cycling cells. The other cell types all had low-to-intermediate fractions of cycling cells across all SiNETs, but even those fractions were consistently higher than for the NE cells.

Thus, at least in our SiNET cohort, proliferation is associated with the tumor microenvironment rather than with the NE cells. Notably, both the low proliferation of NE cells and the higher proliferation of immune cells (especially B/plasma cells, but also T cells and macrophages) were consistently observed in both fresh samples analyzed by single cell RNA-seq (SiNET1 and 2) and frozen samples analyzed by single nuclei RNA-seq (SiNET1 and 2) and therefore are independent of platform and the potential biases in capture of specific cell types. This result raises intriguing questions regarding the manner by which SiNETs grow, and the meaning of their mitotic index (see Discussion).

Next, to ensure that this result is not unique to our cohort we reanalyzed bulk RNA-seq data of 81 SiNET samples [16]. We reasoned that if NE cells are a major source of proliferation, then cell cycle signatures should correlate with the expression of NE marker genes across bulk SiNET samples. Similarly, if other cell types, such as B cells or T cells, are more proliferative than the NE cells, then their markers should correlate with the cell cycle signature. As expected from the single cell analysis, we found that cell cycle signature significantly correlates positively with B cell markers, and to a more limited degree with T cell markers, but not with NE markers (**Fig. 4B**), supporting the broader relevance of our results.

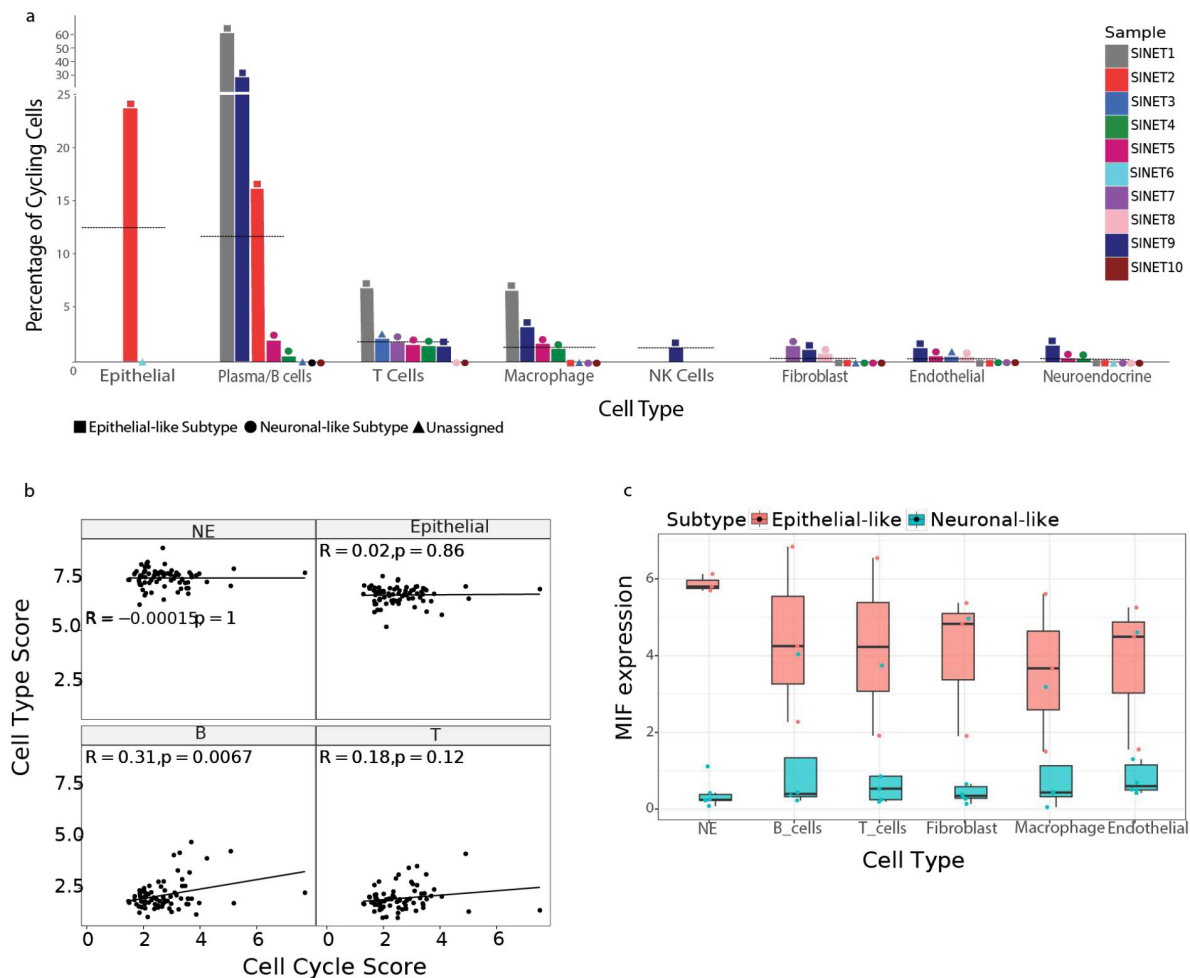


**Figure 4.**

### Cell cycle analysis reveals proliferating B cells in SiNETs.

(A) Bars show the percentage of cycling cells (y-axis) per cell type and per tumor (x-axis). Tumors are color-coded, the two subtypes, Epithelial-like and Neuronal-like, are differentiated by distinct shapes, represented as square and circle, respectively. Information regarding the presence of cell types with zero percentage of cycling cells is provided along the x-axis. Horizontal lines indicate average percentages of cycling cells per cell type. (B) Correlation between cell type and cell-cycle program as computed from an SiNET bulk RNA-seq dataset [16]. Score for each cell type is represented on the top of individual panels. (C) Boxplot depicting the expression of MIF in each SiNET cell type, for each of the two SiNET subtypes.

## B cell proliferation and MIF upregulation in the epithelial-like SiNET subtype

One potential explanation for the high proliferation of B cells is that our SiNET samples may have included germinal centers (GC), in which B cells are expected to proliferate. To assess this possibility, we scored the B cells for a previously defined GC signature [24]. While two tumors with high B cell proliferation also had high GC scores, this was not the case for the third tumor (Fig. S5). Thus, inclusion of GCs may partially explain the unusual proliferation of B cells.

An alternative possibility is that B cells proliferate in response to particular signals in the SiNET microenvironment, for example, due to factors secreted by other cells. The proliferation of B/plasma cells was high only in three of the SiNETs and was absent or low in five other SiNETs (Fig. 4A). Intriguingly, all of the epithelial-like SiNETs had high B/plasma cell proliferation and all of the neuronal-like SiNETs (in which B/plasma cells were detected) had low proliferation of those cells. This perfect agreement with SiNET subtypes suggests that the unique features of the epithelial-like subtype may drive the proliferation of B/plasma cells. To identify such features, we defined the differential expression between the two subtypes for each cell type (Table S4).

This analysis highlighted Macrophage Migration Inhibitory Factor (MIF) as a prominent marker of the epithelial-like subtype that could potentially drive the proliferation of B/plasma cells. First, MIF was one of the top markers of the epithelial-like subtype in our analysis of NE cells (Fig. 2C), and a closer inspection reveals an extreme degree of differential expression, with almost no detected reads in the neuronal-like subtype and a consistently high expression in the epithelial-like subtype (Fig. 4C). Second, upregulation of MIF in the epithelial-like subtype was also detected all other cell types, although the effect was strongest in NE cells (Fig. 4C). Such consistent upregulation across six different cell types is not seen for any other genes, highlighting the unique upregulation of MIF in the ecosystem of the epithelial SiNETs, and supporting its potential causal effect in driving other phenotypes such as B/plasma cell proliferation. Third, MIF is an important cytokine that binds CD74 and was previously shown to regulate B cell proliferation by multiple studies [25, 26].

## Putative progenitors in mixed tumors

Apart from our focus on SiNET, we were intrigued by the phenomena of MiNEN and aimed to profile such tumors by single cell RNA-seq. Due to their rarity and the difficulty of recognizing mixed tumors prior to their surgery, we were so far able to profile only one such tumor. This tumor was a Large Cell Neuroendocrine Carcinoma of the lung (LCNEC), mixed with a squamous cell carcinoma. While this tumor is highly different from SiNETs, we believe that this N-of-1 case study is of interest and raises important questions that might also be of relevance for SiNET and other NENs and hence we describe its analysis below.

We profiled the fresh LCNEC tumor sample by scRNA-seq using the 10x Chromium. Cells were first classified as malignant or non-malignant, based on inference of copy-number aberrations, as described previously [27] (Fig. 5A). Clustering of the non-malignant cells and annotation of the clusters based on standard markers separated them into fibroblasts, endothelial cells, macrophages, T cells and B cells (Fig. 5B).

We found extensive diversity among the malignant cells, which highlighted several subpopulations of cells (Fig. 5B-C). These include the two malignant components expected based on the classification as a mixed tumor – neuroendocrine cells (e.g. expressing CHGB and SSTR2) and epithelial squamous cells (e.g. expressing KRT5 and KRT8). The epithelial cells further varied in their expression of a program that we previously termed “epithelial senescence” [28], which was indeed associated with lack of proliferation (Fig. 5D). Interestingly, we also found additional subpopulations of malignant cells with intermediate expression levels of both the

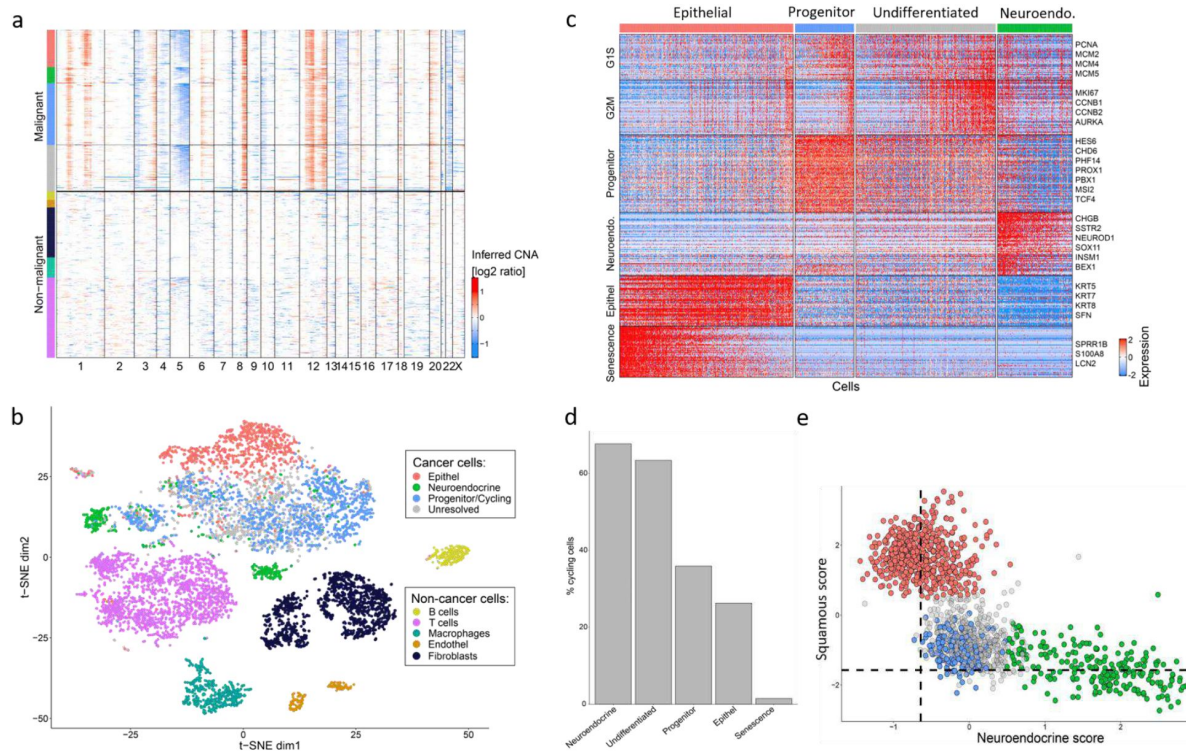


**Figure 5.**

### A putative progenitor population in mixed LCNEC.

(A) Copy number variation (CNV) profiles inferred from scRNA-seq data for all cells from the LCNEC sample. Malignant and non-malignant cells are annotated based on their CNV profiles, using the same color codes as in next panels. (B) tSNE plot showing the diversity of single cells from the mixed lung tumor, colored by their clustering. (C) Heatmap shows relative expression of differentially expressed genes (rows), separated by horizontal lines into programs that distinguish between the four populations of cells detected in the LCNEC sample. Also included are two cell cycle programs (G1/S and G2/M). Columns correspond to malignant cells, separated into the four populations by vertical lines and as indicated by color at the top. Selected genes are labeled for each program. (D) Bars show the percentage of cycling cells in each malignant cluster. (E) Malignant cells scored against an epithelial vs. neuroendocrine programs (gene set), colored by their assignment into four populations.

neuroendocrine genes and the squamous genes (Fig. 5E). The “intermediate” cells included two clusters that we termed *undifferentiated* and *progenitors*, since they were distinguished by expression of developmental and stemness-related regulators (Fig. 5C). These include the homeobox transcription factors PBX1 and PROX1 [29, 30], The RNA-binding protein MSI2 [31], the WNT-related TCF4 [32, 33], and the NOTCH-related transcription factor HES6 [34] (Table S5). All four malignant populations were highly proliferative, except for the subset of squamous cells that express the epithelial senescence program (Fig. 5D). The progenitor cluster, with intermediate expression of the two lineage programs and upregulation of stemness factors, is consistent with the possibility that stem/progenitor cells may give rise to the two differentiated components that constitute the mixed phenotype. Additional MiNENs would need to be profiled at single cell resolution to explore the generality of this observation.

## Discussion

scRNA-seq is a promising approach to improve our understanding of SiNET biology, and in particular their composition and diversity. A recent study described scRNA-seq analysis of NETs but only had samples from two SiNET patients and had enrichment of immune cells with a limited amount of NE cells [35]. Here, we profiled at single cell resolution ten SiNET samples from eight patients and focused our analysis primarily on the NE cells. While low-grade SiNETs are known to have a low mitotic index, any cancer is driven by cell proliferation such that malignant cells are expected to have higher cell cycle activity than non-malignant cells. Surprisingly, this does not seem to be the case in our analysis of SiNETs, where malignant cells appear to be less proliferative than multiple types of non-malignant cells. In such cases, it is conceivable that pathological evaluation of tumor proliferation (Ki67 counts) may be confounded by non-malignant cycling cells and therefore inflated. But a more fundamental question is how could those tumors initiate and progress when the NE cells appear to be largely non-proliferative?

We can envision multiple potential answers although further studies are needed to resolve these possibilities. First, since growth is determined by the balance between cellular proliferation, death and migration, SiNETs may still grow despite their very low proliferation through abnormally low death or abnormal patterns of migration. We are not aware of any evidence in support of this possibility but cannot exclude it. Second, proliferation of NE cells may be inhibited by prior treatments with Somatostatin analogues. Third, proliferation may be spatially or temporally restricted. Accordingly, higher proliferation may occur only in particular niches or at a particular time of tumor progression. This possibility could be examined in future work using spatial omics.

A related possibility is that proliferation may be restricted to a hidden stem/progenitor population that we have not captured, either because it is rare or because it may be sensitive to our experimental workflows. Such proliferative stem/progenitor cells were described in other cancer types and are consistent with the cancer stem cell hypothesis. For example, we previously described the cellular hierarchy of oligodendroglioma, in which proliferation is restricted to cells resembling neural progenitor, while the tumor is dominated by more differentiated cells resembling oligodendrocytes and astrocytes [27]. A second, and more relevant example, is presented here with the N-of-1 analysis of a mixed LCNEC. In this case, high proliferation is seen across all malignant components, but the mere presence of a proliferative stem/progenitor population suggests that bipotent progenitors may explain the diversity within mixed LCNECs and possibly even the wider phenomena of MiNENs. The existence and the properties of such bipotent progenitors in MiNENs would require further validation by future studies, yet it is tempting to speculate that such progenitors may also exist in other neuroendocrine tumors (e.g. SiNETs).

SiNETs deviate from classical oncogenesis not only by their low malignant cell proliferation, but also by their paucity of mutations and apparent driver events. Accordingly, previous work proposed that SiNET may be driven by epigenetic aberrations in NE cells or by effects of the TME.

Our observation of minimal proliferation in NE cells supports the latter possibility of TME oncogenic effects. In particular, SiNETs of the epithelial-like subtype are associated with extremely high levels of MIF, an important and pleiotropic cytokine that may influence the TME in multiple important ways and possibly create a unique inflammatory TME. One potential effect that we observe is high proliferation of B or plasma cells. Notably, the three SiNETs of the epithelial-like subtype are not associated with higher numbers of B/plasma cells compared to other SiNETs, but rather only with increased cell cycle of B/plasma cells, suggesting that their proliferation is balanced by high death. The implications of high B/plasma cell turnover, and of other downstream effects of high MIF expression, are unclear, but raise the possibility that MIF-CD74 interaction may constitute a relevant target for the epithelial-like SiNET subtype. Notably, MIF-CD74 inhibitors have already been developed and initially tested for the treatment of solid tumors [36–38].

In summary, single cell profiling of SiNETs revealed two distinct subtypes that differ in NE profiles and in the proliferation of B-cells, possibly justifying different therapeutic strategies (e.g. MIF inhibition). The two subtypes share a minimal proliferation of NE cells, which raises questions about the initiation and growth of SiNETs and the potential role of TME cells, in line with the limited mutations observed in SiNETs. Our analysis of a mixed tumor further suggests the potential presence of bipotent progenitors that may account for mixed phenotypes. Taken together, these results improve our understanding of neuroendocrine neoplasms while highlighting their complex biology.

## Methods

### Human samples

The study was approved by the Institutional Helsinki Committee at Sheba Medical Center (SMC-18-5674). For tissue preparation for Immunohistochemistry, flash frozen SiNETs were stored at  $-80^{\circ}\text{C}$  until cutting. Blocks were prepared in OCT followed by freezing and sectioning. Frozen tissue was sectioned using a  $-20^{\circ}\text{C}$  temperature on a cryostat (Leica CM3050 S) onto microscopic slides (Thermo, Superfrost Plus).

### Tumor dissociation and library preparation

Fresh tumors were collected directly from the operating room at the time of surgery. Tumors were washed in ice-cold HBSS, minced using a pair of Noyes spring scissors (Fine Science Tools) and then enzymatically dissociated using a tumor dissociation kit (Miltenyi Biotec) in a GentleMACS Octo-dissociator on low speed settings (Filbin et al., 2018; Patel et al., 2014; Tirosh et al., 2016b). Single cell suspension was obtained by passing dissociated slurry through a 60-100 micron cell strainer (Miltenyi) and the filtrate was subjected to RBC removal using a RBC lysis buffer (Roche). Dead cell removal (Miltenyi) was performed on cell suspension following manufacturer's protocol and cells were assessed for viability and density. 5  $\mu\text{l}$  of Trypan blue (Thermo Fisher Scientific) was mixed with 5  $\mu\text{l}$  of the sample and loaded onto chip disposable automated hemocytometer (Countess II). Cell concentration was adjusted such that a total of 8000-10,000 cells were loaded onto each channel of the 10x Genomics Single-Cell Chromium Controller.

For frozen tumors, a similar dissociation procedure was performed with the following modifications. Nuclei isolation was achieved using either ST-based buffers as done before (Slyper et al) or by EZ-lysis method (Sigma). Briefly, tissue samples were cut into pieces  $<0.5$  cm using spring noyes scissors and then homogenized in ice-cold buffer (ST based or EZ lysis) using a glass Dounce tissue grinder (Sigma). Nuclei were centrifuged at 500g, with low acceleration/deceleration for 5 min at  $4^{\circ}\text{C}$ , washed with 5 ml ice-cold ST/EZ lysis buffer and incubated on ice for 5 min. A suspension buffer containing PBS, BSA and RNase inhibitor was used to wash and resuspend the

nuclei pellet. Nuclei suspension was filtered through a 40  $\mu$ m cell strainer (Miltenyi) and counted. A final concentration of 1,000 nuclei per  $\mu$ l was used for loading on a 10x channel with a target of 10,000 nuclei per channel in the 10x controller.

The Chromium Next GEM Single Cell 3' GEM, Library & Gel Bead Kit v3.1, Chromium Single Cell 3' Feature Barcode Library Kit, Chromium Next GEM Chip G, and 10x Chromium Controller (10x Genomics) was used for generation of libraries of fresh tumors/single cells and the Chromium Next GEM Single 5' GEM was used for frozen tumors/single-nuclei.

As per the standardized protocol of creating 10x libraries, single cells, reagents and single gel beads containing barcoded oligonucleotides were emulsified and encapsulated into nanoliter-sized droplets followed by reverse transcription (RT). Following manufacturer's recommendations RT samples were subjected to cDNA amplification, fragmentation, adapter and sample index attachment. Libraries from two 10x channels were pooled together and sequenced on one lane of an Illumina NovaSeq-6000, using an sp-100 kit or 4 were pooled together on two lanes with an S1-100 kit, with paired end reads as follows: read 1, 26 nt; read 2, 55 nt; index 1, 8 nt; index 2, 0 nt.

## Sample Normalization, Filtering and annotations

Four samples (SiAdeno, SiNETs 1-3) underwent single nuclei sequencing, while the remaining samples (SiNETs 4-10 and the LCNEC sample) were subjected to scRNA-seq. To facilitate comparative analysis, we converted counts of unique molecular identifier (UMI) counts to transcripts per million (TPM) values, which were divided by 10 since the actual complexity of cells is assumed to be in the realm of  $\sim$ 100,000 transcripts and not 1 million as implied by the TPM measures. The resulting values were added 1 and log<sub>2</sub>-transformed. Finally, to derive relative expression we centered the value of each gene in each tumor, except in analysis where we were interested in absolute expression levels.

To ensure data quality, we excluded low-quality cells based on the number of detected genes. For the nuclei-seq platform, we used a threshold of 1000 detected genes, while for the scRNA-seq platform, we used a threshold of 700 in order to retain lymphocytes that tend to have low numbers of detected genes. Additionally, we performed most analysis with a filtered set of genes, retaining only the genes in which the logged row-means per gene across all cells passed 4.5. After filtering, the number of cells analyzed per sample ranged from 287 to 10,802.

For further downstream analyses we have avoided the use of integration methods as we believe that they tend to distort the data and decrease tumor-specific signals. Instead, we primarily analyzed one tumor at a time and never directly compared cell profiles across distinct tumors but only compared the differences between subpopulations in a given tumor; specifically, as described below, we normalized the expression of NE cells by subtracting the expression of reference non-NE cells from the same tumor as a method to decrease batch effects.

## Neuroendocrine cell signatures and their classification

To identify NE-specific genes, we performed a differential expression analysis between NE cells and a reference group consisting of Macrophages, Fibroblasts, and Endothelial cells. For each sample, we sampled 50 NE cells along with 50 cells from the reference group. Differentially expressed (DE) genes were defined as those with fold change greater than 8 and a p-value lower than 0.05. False discovery rate (FDR) correction was applied to the p-values of individual genes to account for multiple comparisons. In samples that had insufficient reference cells (SiNET7 and SiNET8), we sampled the reference cells from a different sample that exhibited the highest correlation with the given sample. Samples that did not contain NE cells (SiNET3 and SiAdeno) or had too few NE cells (SiNET6), based on a defined threshold of 100 cells, were excluded from this

analysis. Accordingly, SiNET9 was included in the analysis due to its high absolute number of profiled cells (130) which resulted in a sufficient count of NE cells, despite having a relatively small fraction of NE cells.

We classified the DE genes into three groups: Common genes, Subset-specific genes, and Sample-specific genes. Common genes were defined as those differentially expressed in at least 6 samples, Subset-specific genes were those differentially expressed in 2-5 samples, and Sample-specific genes were differentially expressed only in one sample.

## Clustering of Subset-specific NE genes into nine patterns

We divided the subset-specific NE genes based on their expression pattern across the 8 tumors that contained enough NE cells. To this end, we first considered all potential binary patterns, in which a gene is considered as either expressed (1) or not expressed (0) in each tumor. This defined  $2^8 = 256$  theoretical patterns. Next, for each gene, we calculated the correlation between its relative expression across the eight samples, and each of the 256 binary patterns; the gene was then assigned to the pattern with highest correlation. This uncovered 9 patterns that were each assigned to >5 genes (**Fig.2C** [↗](#), Table S3).

## Cell Cycle Analysis

To investigate the cell cycle dynamics within the dataset, we utilized a canonical cell cycle gene-set [27 [↗](#)]. Each cell in every sample was scored based on the expression of these cell cycle genes. To identify cycling cells, we established a threshold based on the distribution of scores within each sample. We chose a relatively lenient threshold (log2 fold change of 1.5), and further verified that our main claims are qualitatively maintained when using a more strict threshold of 2-fold. In particular, NE cells had an extremely low fraction of cycling cells (0.127%), while B and plasma cells had a high fraction of cycling cells in epithelial-like SiNETs (32.25%) but not in Neuronal-like SiNETs (0.178%).

## Within Cell-Type analysis

In each tumor, we examined clusters that were annotated as the same cell type but were not grouped together in the initial clustering analysis. For each such pair of clusters, we defined differential expression (with fold change greater than 3 and p-value lower than 0.05) and analyzed the expression of previously defined meta-programs associated with the corresponding cell type. FDR correction was applied to the p-values of individual genes to account for multiple comparisons.

## Comparing Gene Expression between SiNET Subtypes across Cell Types

We performed a comparative gene expression analysis, examining epithelial-like and neuronal-like subtypes of SiNETs. The significance threshold for gene selection was established at an uncorrected p-value < 0.05. Due to the limited sample size, we did not apply an FDR correction but added a second strict criterion of fold change > 4. Additionally, we extended this analysis to bulk data, where each sample was assigned to the subtype for which the signature score was higher.

## Cell cycle correlations with cell type signatures in bulk SiNET samples

From GSE98894, we used the 81 SiNET samples. Counts were converted to TPM, and log2-transformed with an offset of 1. We then averaged the marker genes of Epithelial, T cells, B cells, NE cells and cell cycle to create scores of their expression. Few outlier samples with very low NE

score were excluded from further analysis. Finally, we calculated Pearson correlation between the cell-type scores and cell cycle score.

## Supplementary figures

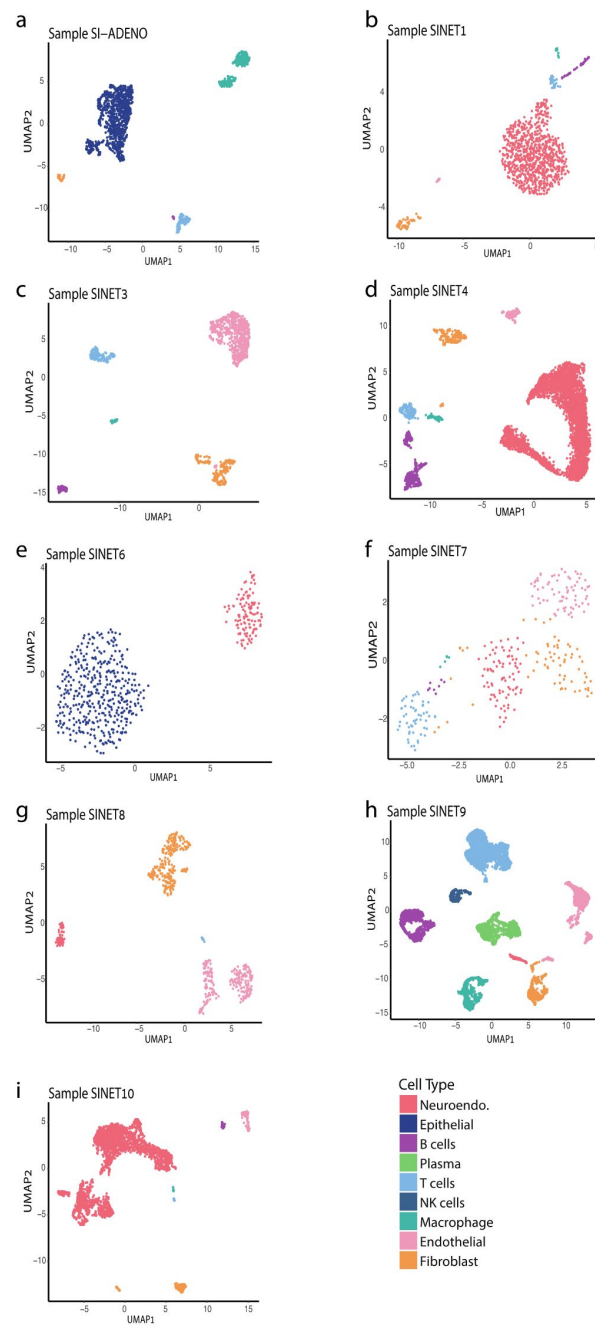


**Figure S1.**

**Cellular composition of SiNETs as determined by single cell and single nuclei sequencing.**

(A-I) UMAP plots showing the diversity of single cells from each sample, colored by their clustering.

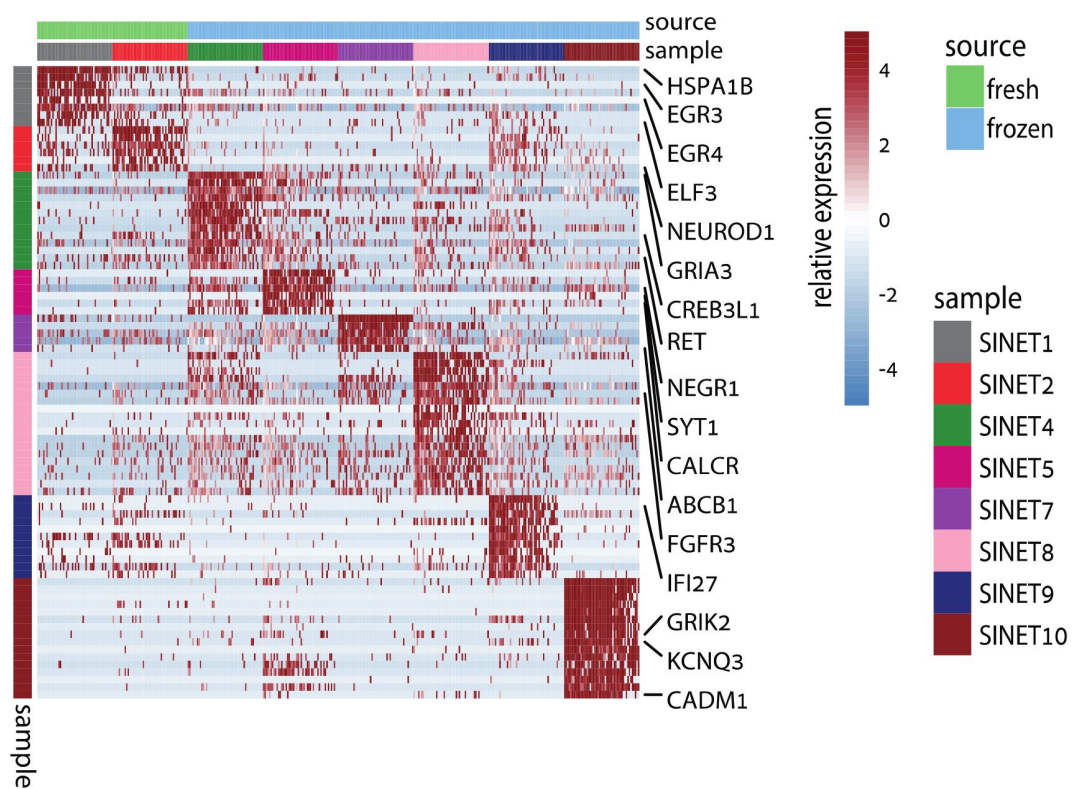


**Figure S2.**

Specific upregulated genes expressed in neuroendocrine cells per siNET sample.

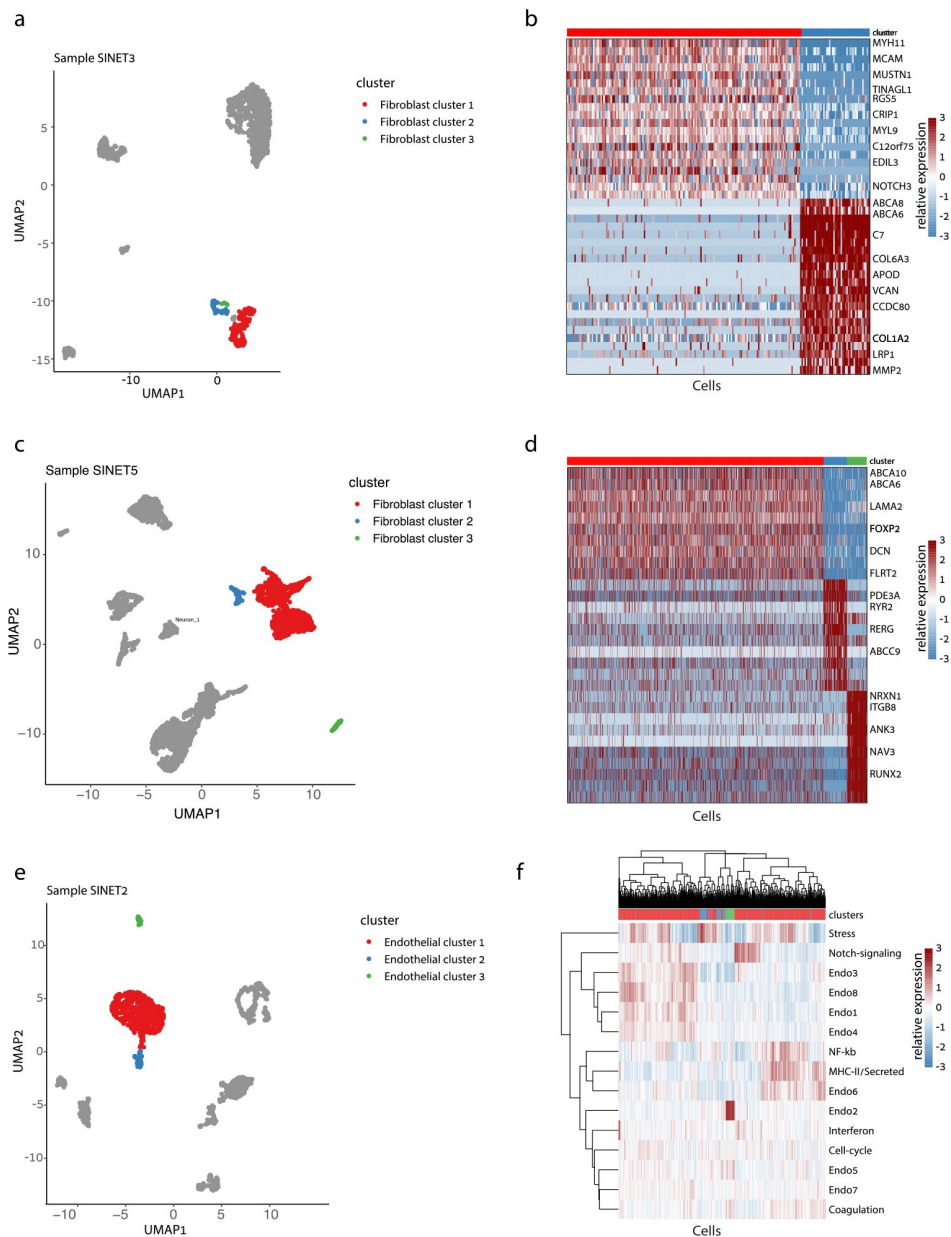


**Figure S3.**

### Heterogeneity in the SiNET tumor microenvironment.

For each of three non-malignant cell types, the diversity of that cell type is shown in one exemplary tumor: fibroblast heterogeneity in SiNET3 is shown in (A, B), fibroblast cell heterogeneity in SiNET5 is shown in (C, D), and endothelial heterogeneity in SiNET2 is shown in (E, F). For each cell type, panels depict types of analyses. The first panel (A, C, E) is a UMAP plot of the respective tumor, where only the respective cell type is colored, and distinct colors highlight the clusters of that cell type. The second panel (B, D) shows differential expression analysis between the first two clusters using a heatmap, with labeling of selected genes. The third panel (F) shows clustering of cells from that cell type (columns) based on their relative expression of previously defined [18] signatures of diversity in that cell type (rows); the top panel shows assignment of cells to clusters.



**Figure S4.**

**Heat map illustrating the expression of G1/S and G2/M genes across various cell types in the Epithelial-like Subtype (A), Neuronal-like Subtype (B) and the siAdeno sample (C).**

The annotated cell types include Epithelial cells, Macrophages, T cells, and Fibroblasts that were sampled for illustrative purposes.

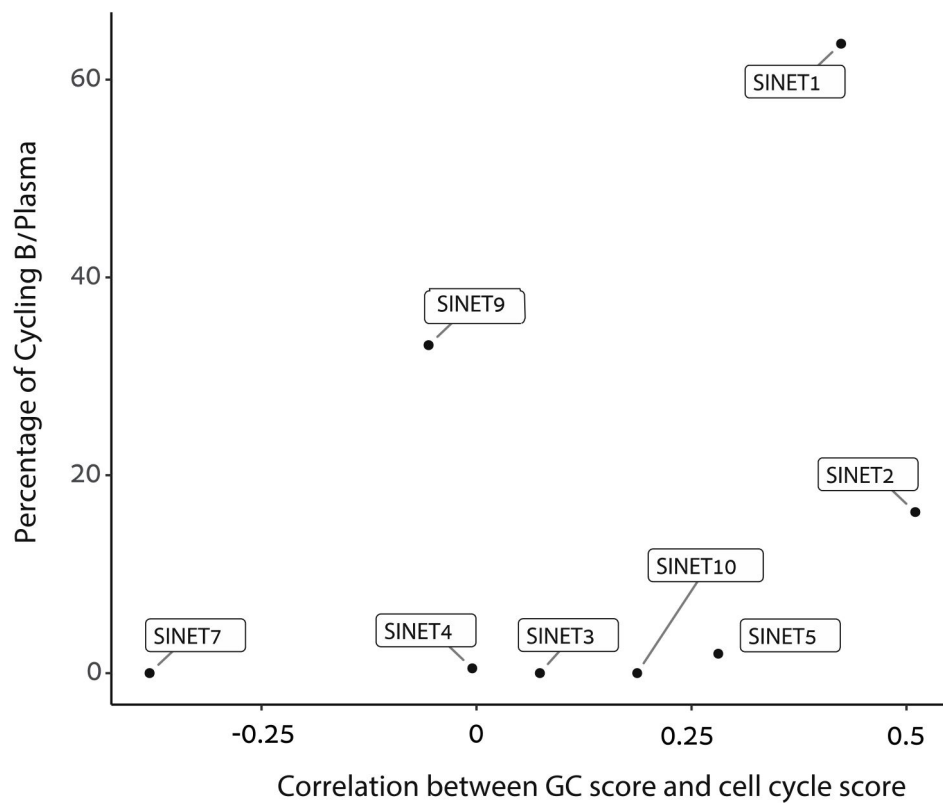


**Figure S5.**

**Scatter plot illustrating the percentage of cycling B/Plasma cells and the correlation between the germinal center signature and cycling B/Plasma cells signature.**

In SiNET1 and SiNET2 we observe high correlation between cell cycle and GC score, but not in SiNET9.

## Data availability

Data is available through GEO with accession number GSE292163.

## Acknowledgements

This work was supported by grants from the Neuroendocrine Tumor Research Foundation (to I.T. and A.T.). I.T. is further supported by the Zuckerman STEM Leadership Program, the Mexican Friends New Generation, the Benozio Endowment Fund and E. Harari. I.T. is the incumbent of the Dr. Celia Zwillenberg-Fridman and Dr. Lutz Zwillenberg Career Development Chair.

## Additional files

**Table S1** [↗](#)

**Table S2** [↗](#)

**Table S3** [↗](#)

**Table S4** [↗](#)

**Table S5** [↗](#)

## References

1. Dasari A., et al. (2017) **Trends in the Incidence, Prevalence, and Survival Outcomes in Patients With Neuroendocrine Tumors in the United States** *JAMA Oncol* **3**:1335–1342 [Google Scholar](#)
2. Clift A.K., et al. (2020) **Neuroendocrine Neoplasms of the Small Bowel and Pancreas** *Neuroendocrinology* **110**:444–476 [Google Scholar](#)
3. Niederle M.B., et al. (2010) **Gastroenteropancreatic neuroendocrine tumours: the current incidence and staging based on the WHO and European Neuroendocrine Tumour Society classification: an analysis based on prospectively collected parameters** *Endocr Relat Cancer* **17**:909–18 [Google Scholar](#)
4. Gangi A., et al. (2018) **Multifocality in Small Bowel Neuroendocrine Tumors** *J Gastrointest Surg* **22**:303–309 [Google Scholar](#)
5. Kalifi M., et al. (2021) **Unifocal versus Multiple Ileal Neuroendocrine Tumors Location: An Embryological Origin** *Neuroendocrinology* **111**:786–793 [Google Scholar](#)
6. Elias E., et al. (2021) **Independent somatic evolution underlies clustered neuroendocrine tumors in the human small intestine** *Nat Commun* **12**:6367 [Google Scholar](#)
7. Makinen N., et al. (2022) **Whole genome sequencing reveals the independent clonal origin of multifocal ileal neuroendocrine tumors** *Genome Med* **14**:82 [Google Scholar](#)
8. Hofving T., et al. (2018) **The neuroendocrine phenotype, genomic profile and therapeutic sensitivity of GEPNET cell lines** *Endocr Relat Cancer* **25**:367–380 [Google Scholar](#)
9. Hofving T., et al. (2021) **SMAD4 haploinsufficiency in small intestinal neuroendocrine tumors** *BMC Cancer* **21**:101 [Google Scholar](#)
10. Scarpa A. (2019) **The landscape of molecular alterations in pancreatic and small intestinal neuroendocrine tumours** *Ann Endocrinol (Paris)* **80**:153–158 [Google Scholar](#)
11. Di Domenico A., et al. (2017) **Genetic and epigenetic drivers of neuroendocrine tumours (NET)** *Endocr Relat Cancer* **24**:R315–R334 [Google Scholar](#)
12. Francis J.M., et al. (2013) **Somatic mutation of CDKN1B in small intestine neuroendocrine tumors** *Nat Genet* **45**:1483–6 [Google Scholar](#)
13. Crona J., et al. (2015) **Somatic Mutations and Genetic Heterogeneity at the CDKN1B Locus in Small Intestinal Neuroendocrine Tumors** *Ann Surg Oncol* **22**:S1428–35 [Google Scholar](#)
14. Frizziero M., et al. (2020) **Mixed Neuroendocrine Non-Neuroendocrine Neoplasms: A Systematic Review of a Controversial and Underestimated Diagnosis** *J Clin Med* **9** [Google Scholar](#)
15. Gavish A., et al. (2023) **Hallmarks of transcriptional intratumour heterogeneity across a thousand tumours** *Nature* **618**:598–606 [Google Scholar](#)

16. Alvarez M.J., et al. (2018) **A precision oncology approach to the pharmacological targeting of mechanistic dependencies in neuroendocrine tumors** *Nat Genet* **50**:979–989 [Google Scholar](#)
17. Andersson E., et al. (2016) **Expression profiling of small intestinal neuroendocrine tumors identifies subgroups with clinical relevance, prognostic markers and therapeutic targets** *Mod Pathol* **29**:616–29 [Google Scholar](#)
18. Haber A.L., et al. (2017) **A single-cell survey of the small intestinal epithelium** *Nature* **551**:333–339 [Google Scholar](#)
19. Elyada E., et al. (2019) **Cross-Species Single-Cell Analysis of Pancreatic Ductal Adenocarcinoma Reveals Antigen-Presenting Cancer-Associated Fibroblasts** *Cancer Discov* **9**:1102–1123 [Google Scholar](#)
20. Lavie D., et al. (2022) **Cancer-associated fibroblasts in the single-cell era** *Nat Cancer* **3**:793–807 [Google Scholar](#)
21. Luo H., et al. (2022) **Pan-cancer single-cell analysis reveals the heterogeneity and plasticity of cancer-associated fibroblasts in the tumor microenvironment** *Nat Commun* **13**:6619 [Google Scholar](#)
22. Puram S.V., et al. (2023) **Cellular states are coupled to genomic and viral heterogeneity in HPV-related oropharyngeal carcinoma** *Nat Genet* **55**:640–650 [Google Scholar](#)
23. Kowalczyk M.S., et al. (2015) **Single-cell RNA-seq reveals changes in cell cycle and differentiation programs upon aging of hematopoietic stem cells** *Genome Res* **25**:1860–72 [Google Scholar](#)
24. Brescia P., et al. (2018) **MEF2B Instructs Germinal Center Development and Acts as an Oncogene in B Cell Lymphomagenesis** *Cancer Cell* **34**:453–465 [Google Scholar](#)
25. Gore Y., et al. (2008) **Macrophage migration inhibitory factor induces B cell survival by activation of a CD74-CD44 receptor complex** *J Biol Chem* **283**:2784–92 [Google Scholar](#)
26. Noe J.T., Mitchell R.A. (2020) **MIF-Dependent Control of Tumor Immunity** *Front Immunol* **11**:609948 [Google Scholar](#)
27. Tirosh I., et al. (2016) **Single-cell RNA-seq supports a developmental hierarchy in human oligodendroglioma** *Nature* **539**:309–313 [Google Scholar](#)
28. Kinker G.S., et al. (2020) **Pan-cancer single-cell RNA-seq identifies recurring programs of cellular heterogeneity** *Nat Genet* **52**:1208–1218 [Google Scholar](#)
29. Chan K.K., et al. (2009) **KLF4 and PBX1 directly regulate NANOG expression in human embryonic stem cells** *Stem Cells* **27**:2114–25 [Google Scholar](#)
30. Muggeo S., et al. (2021) **PBX1-directed stem cell transcriptional program drives tumor progression in myeloproliferative neoplasm** *Stem Cell Reports* **16**:2607–2616 [Google Scholar](#)
31. Hope K.J., Sauvageau G. (2011) **Roles for MSI2 and PROX1 in hematopoietic stem cell activity** *Curr Opin Hematol* **18**:203–7 [Google Scholar](#)

- 32. Lu R., et al. (2012) **Transcription factor TCF4 maintains the properties of human corneal epithelial stem cells** *Stem Cells* **30**:753–61 [Google Scholar](#)
- 33. Sun S., et al. (2020) **TCF4 promotes colorectal cancer drug resistance and stemness via regulating ZEB1/ZEB2 expression** *Protoplasma* **257**:921–930 [Google Scholar](#)
- 34. Krossa I., et al. (2022) **Recent advances in understanding the role of HES6 in cancers** *Theranostics* **12**:4374–4385 [Google Scholar](#)
- 35. Hoffman S.E., et al. (2023) **Intertumoral lineage diversity and immunosuppressive transcriptional programs in well-differentiated gastroenteropancreatic neuroendocrine tumors** *Sci Adv* **9**:eadd9668 [Google Scholar](#)
- 36. Mahalingam D., et al. (2020) **Phase I study of imalumab (BAX69), a fully human recombinant antioxidantized macrophage migration inhibitory factor antibody in advanced solid tumours** *Br J Clin Pharmacol* **86**:1836–1848 [Google Scholar](#)
- 37. Varinelli L., et al. (2015) **4-IPP, a selective MIF inhibitor, causes mitotic catastrophe in thyroid carcinomas** *Endocr Relat Cancer* **22**:759–75 [Google Scholar](#)
- 38. O'Reilly C., et al. (2016) **Targeting MIF in Cancer: Therapeutic Strategies, Current Developments, and Future Opportunities** *Med Res Rev* **36**:440–60 [Google Scholar](#)

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**Reviewer #1 (Public review):**

Summary:

The authors have assembled a cohort of 10 SiNET, 1 SiAdeno, and 1 lung MiNEN samples to explore the biology of neuroendocrine neoplasms. They employ single-cell RNA sequencing to profile 5 samples (siAdeno, SiNETs 1-3, MiNEN) and single-nuclei RNA sequencing to profile seven frozen samples (SiNET 4-10).

They identify two subtypes of siNETs, characterized by either epithelial or neuronal NE cells, through a series of DE analyses. They also report findings of higher proliferation in non-malignant cell types across both subtypes. Additionally, they identify a potential progenitor cell population in a single lung MiNEN samples.

In the revised study, they have addressed my points and I have no further comments.

<https://doi.org/10.7554/eLife.101153.2.sa3>

**Reviewer #2 (Public review):**

## Summary:

The research identifies two main SiNET subtypes (epithelial-like and neuronal-like) and reveals heterogeneity in non-neuroendocrine cells within the tumor microenvironment. The study validates findings using external datasets and explores unexpected proliferation patterns. While it contributes to understanding SiNET oncogenic processes, the limited sample size and depth of analysis present challenges to the robustness of the conclusions.

## Strengths:

The studies effectively identified two subtypes of SiNET based on epithelial and neuronal markers. Key findings include the low proliferation rates of neuroendocrine (NE) cells and the role of the tumor microenvironment (TME), such as the impact of Macrophage Migration Inhibitory Factor (MIF).

## Weaknesses:

However, the analysis faces challenges such as a small sample size and lack of clear biological interpretation in some analyses.

<https://doi.org/10.7554/eLife.101153.2.sa2>

**Reviewer #3 (Public review):**

This study profiles small intestine NETs and one mixed lung NET at single cell resolution and identifies two subtypes of neuroendocrine cells, as well as explores the proliferation patterns in malignant and nonmalignant cell types, identifying MIF as a potential factor that promotes proliferation of B and plasma cells in siNETs. Furthermore, they explore the single-cell landscape of a mixed LCNEC and squamous cell carcinoma, from which they identify a putative stem cell population with expression of features from both lineages.

## Strengths:

This work showcases single-cell profiling of a rare tumor type, which is very informative for the field of NETs. The authors highlight very interesting observations, including the identification of the epithelial and neuronal subtype of siNETs, which they validated with an independent bulk RNA sequencing cohort. Furthermore, the observation of low cycling in malignant cells and high cycling in nonmalignant cells is an interesting one which may be applicable to other NETs.

## Weaknesses:

- The authors do not connect their findings to clinical outcome. For example, is the epithelial or neuronal subtype enriched in tumors with worse or better prognosis or high grade vs. low grade siNETs or in patients who metastasize vs. who don't? As the authors show they can identify epithelial vs. neuronal subtypes in bulk RNA seq, perhaps they can take advantage of these other studies with larger sample sizes to investigate this. Additionally, the authors identify that the phenomenon of higher B/plasma cell proliferation is particular to epithelial siNETs and write that "The implications of high B/plasma cell turnover, and of other downstream effects of high MIF expression, are unclear, but raise the possibility that MIF-CD74 interaction may constitute a relevant target for the epithelial-like SiNET subtype." However, if this interaction contributes to survival in these patients, targeting this interaction may not be beneficial. Thus, it is important for the authors to try to connect their finding to clinical outcomes to enhance the translational relevance of this paper.

- The generalizability of this study would be enhanced if the authors analyzed other available single cell studies of NETs and found a similar phenomenon of high proliferating nonmalignant cell types. Although these studies are also very limited in sample size, seeing concordance in findings across independent cohorts and different experimental techniques would help to strengthen the findings. While the authors rationalize that these other studies are too distinct from their own due to enrichment for immune cells, this limitation should be noted but does not prevent such an analysis from being attempted.
- On page 3, the authors claim that "Technical effects (e.g. single cell analysis of fresh samples vs. single nuclei analysis of frozen samples) could also impact the capture of distinct cell types, although we did not observe a clear pattern of such bias." Can the authors show that cell type frequencies are not significantly different between the samples profiled with these two methods?
- Why did siNET3 and siNET9 have much lower recovery of neuroendocrine cells compared to other samples? It would be interesting to see how similar or different the transcriptional profiles are of the samples that were obtained from the same patient, considering that multifocal siNETs are found to derive from distinct clones, although this analysis is understandably not possible in this case due to the lack of neuroendocrine cells in one of two samples from the same patient.
- It should be more clearly stated in the text that these samples were previously treated with somatostatin analogues, as this impacts the interpretation of the findings.
- The identification of a potential progenitor subtype in the miNEN is very intriguing, albeit a case study and represents a distinct cancer from the lowly proliferating siNETs. While the authors mention this in the text, the case study feels rather tangential to the other parts of the paper.
- How the authors compared the DE genes to known signatures for the fibroblast and endothelial cells should be clarified and discussed in the Methods section.

<https://doi.org/10.7554/eLife.101153.2.sa1>

#### Author response:

The following is the authors' response to the original reviews

##### **Public Reviews:**

##### **Reviewer #1 (Public review):**

##### *Summary:*

*The authors have assembled a cohort of 10 SiNET, 1 SiAdeno, and 1 lung MiNEN samples to explore the biology of neuroendocrine neoplasms. They employ single-cell RNA sequencing to profile 5 samples (SiAdeno, SiNETs 1-3, MiNEN) and single-nuclei RNA sequencing to profile seven frozen samples (SiNET 4-10).*

They identify two subtypes of siNETs, characterized by either epithelial or neuronal NE cells, through a series of DE analyses. They also report findings of higher proliferation in non-malignant cell types across both subtypes. Additionally, they identify a potential progenitor cell population in a single-lung MiNEN sample.

##### *Strengths:*

*Overall, this study adds interesting insights into this set of rare cancers that could be very informative for the cancer research community. The team probes an understudied cancer type and provides thoughtful investigations and observations that may have translational relevance.*

*Weaknesses:*

*The study could be improved by clarifying some of the technical approaches and aspects as currently presented, toward enhancing the support of the conclusions:*

*(1) Methods: As currently presented, it is possible that the separation of samples by program may be impacted by tissue source (fresh vs. frozen) and/or the associated sequencing modality (single cell vs. single nuclei). For instance, two (SiNET1 and SiNET2) of the three fresh tissues are categorized into the same subtype, while the third (SiNET9) has very few neuroendocrine cells. Additionally, samples from patient 1 (SiNET1 and SiNET6) are separated into different subtypes based on fresh and frozen tissue. The current text alludes to investigations (i.e.: "Technical effects (e.g., fresh vs. frozen samples) could also impact the capture of distinct cell types, although we did not observe a clear pattern of such bias."), but the study would be strengthened with more detail.*

We thank the reviewer for the thoughtful and constructive review. Due to the difficulty in obtaining enough SiNET samples, we used two platforms to generate data - single cell analysis of fresh samples, and single nuclei analysis of frozen samples. We opted to combine both sample types in our analysis while being fully aware of the potential for batch effects. We therefore agree that this is a limitation of our work, and that differences between samples should be interpreted with caution.

Nevertheless, we argue that the two SiNET subtypes that we have identified are very unlikely to be due to such batch effect. First, the epithelial SiNET subtype was not only detected in two fresh samples but also in one frozen sample (albeit with relatively few cells, as the reviewer correctly noted). Second, and more importantly, the epithelial SiNET subtype was also identified in analysis of an external and much larger cohort of bulk RNA-seq SiNET samples that does not share the issue of two platforms (as seen in Fig. 2f). Moreover, the proportion of samples assigned to the two subtypes is similar between our data and the external data. We therefore argue that the identification of two SiNET subtypes cannot be explained by the use of two data platforms. However, we agree that the results should be further investigated and validated by future studies.

The reviewer also commented that two samples from the same patient which were profiled by different platforms (SiNET1 and SiNET6) were separated into different subtypes. We would like to clarify that this is not the case, since SiNET6 was not included in the subtype analysis due to too few detected Neuroendocrine cells, and was not assigned to any subtype, as noted in the text and as can be seen by its exclusion from Figure 2 where subtypes are defined. We apologize that our manuscript may have given the wrong impression about SiNET6 classification (it was labeled in Fig. 4a in a misleading manner). In the revised manuscript, we corrected the labeling in Fig. 4a and clarified that SiNET6 is not assigned to any subtype. We also further acknowledge the limitation of the two platforms and the arguments in favor of the existence of two SiNET subtypes.

*(Additional specific recommendations for the authors are provided below)*

*(2) Results:*

*Heterogeneity in the SiNET tumor microenvironment: It is unclear if the current analysis of intratumor heterogeneity distinguishes the subtypes. It may be informative if patterns of tumor microenvironment (TME) heterogeneity were identified between samples of the*

*same subtype. The team could also evaluate this in an extension cohort of published SiNET tumors (i.e. revisiting additional analyses using the SiNET bulk RNAseq from Alvarez et al 2018, a subset of single-cell data from Hoffman et al 2023, or additional bulk RNAseq validation cohorts for this cancer type if they exist [if they do not, then this could be mentioned as a need in Discussion])*

We agree that analysis of an independent cohort will assist in defining the association between TME and the SiNET subtype. However, the sample size required for that is significantly larger than the data available. In the revised manuscript we note that as a direction for future studies.

*(3) Proliferation of NE and immune cells in SiNETs: The observed proliferation of NE and immune cells in SiNETs may also be influenced by technical factors (including those noted above). For instance, prior studies have shown that scRNA-seq tends to capture a higher proportion of immune cells compared to snRNA-seq, which should be considered in the interpretation of these results. Could the team clarify this element?*

We agree that different platforms could affect the observed proportions of immune cells, and more generally the proportions of specific cell types. However, the low proliferation of Neuroendocrine cells and the higher proliferation of immune cells (especially B cells, but also T cells and macrophages) is consistently observed in both platforms, as shown in Fig. 4a, and therefore appears to be reliable despite the limitations of our work. We clarify this consistency in the revised manuscript.

*(4) Putative progenitors in mixed tumors: As written, the identification of putative progenitors in a single lung MiNEN sample feels somewhat disconnected from the rest of the study. These findings are interesting - are similar progenitor cell populations identified in SiNET samples? Recognizing that ideally additional validation is needed to confidently label and characterize these cells beyond gene expression data in this rare tumor, this limitation could be addressed in a revised Discussion.*

We do not find evidence for similar progenitors in the SiNET samples, but they also do not contain two co-existing lineages of cancer cells within the same tumor, so this is harder to define. We agree about the need for additional validation for this specific finding and have noted that in the revised Discussion.

#### **Reviewer #2 (Public review):**

##### *Summary:*

*The research identifies two main SiNET subtypes (epithelial-like and neuronal-like) and reveals heterogeneity in non-neuroendocrine cells within the tumor microenvironment. The study validates findings using external datasets and explores unexpected proliferation patterns. While it contributes to understanding SiNET oncogenic processes, the limited sample size and depth of analysis present challenges to the robustness of the conclusions.*

##### *Strengths:*

*The studies effectively identified two subtypes of SiNET based on epithelial and neuronal markers. Key findings include the low proliferation rates of neuroendocrine (NE) cells and the role of the tumor microenvironment (TME), such as the impact of Macrophage Migration Inhibitory Factor (MIF).*

##### *Weaknesses:*

However, the analysis faces challenges such as a small sample size, lack of clear biological interpretation in some analyses, and concerns about batch effects and statistical significance.

### **Reviewer #3 (Public review):**

#### **Summary:**

*In this study, the authors set out to profile small intestine neuroendocrine tumors (siNETs) using single-cell/nucleus RNA sequencing, an established method to characterize the diversity of cell types and states in a tumor. Leveraging this dataset, they identified distinct malignant subtypes (epithelial-like versus neuronal-like) and characterized the proliferative index of malignant neuroendocrine cells versus non-malignant microenvironment cells. They found that malignant neuroendocrine cells were far less proliferative than some of their non-malignant counterparts (e.g., B cells, plasma cells, epithelial cells) and there was a strong subtype association such that epithelial-like siNETs were linked to high B/plasma cell proliferation, potentially mediated by MIF signaling, whereas neuronal-like siNETs were correlated with low B/plasma cell proliferation. The authors also examined a single case of a mixed lung tumor (neuroendocrine and squamous) and found evidence of intermediate/mixed and stem-like progenitor states that suggest the two differentiated tumor types may arise from the same progenitor.*

#### **Strengths:**

*The strengths of the paper include the unique dataset, which is the largest to date for siNETs, and the potentially clinically relevant hypotheses generated by their analysis of the data.*

#### **Weaknesses:**

*The weaknesses of the paper include the relatively small number of independent patients ( $n = 8$  for siNETs), lack of direct comparison to other published single-cell NET datasets, mixing of two distinct methods (single-cell and single-nucleus RNA-seq), lack of direct cell-cell interaction analyses and spatially-resolved data, and lack of in vitro or in vivo functional validation of their findings.*

*The analytical methods applied in this study appear to be appropriate, but the methods used are fairly standard to the field of single-cell omics without significant methodological innovation. As the authors bring forth in the Discussion, the results of the study do raise several compelling questions related to the possibility of distinct biology underlying the epithelial-like and neuronal-like subtypes, the origin of mixed tumors, drivers of proliferation, and microenvironmental heterogeneity. However, this study was not able to further explore these questions through spatially-resolved data or functional experiments.*

### **Recommendations for the authors:**

#### **Reviewer #1 (Recommendations for the authors):**

##### **(1) Methods:**

*a) Could the team clarify the discrepancy in subtype assignment between two samples from the same patient? i.e. are these samples from the same tumor? If so, what does the team think is the explanation for the difference in subtype assignment?*

As noted above in response to the public review of reviewer #1, SiNET6 was in fact not assigned to any subtype (due to insufficient NE cells) and hence there was no discrepancy. We apologize for the misleading labeling of SiNET6 in the previous version and have corrected this in the revised version of Figure 4.

*b) What is the rationale for scoring tumor-derived programs on samples with no tumor cells? For instance, SiNET3 does not contain NE cells, and SiNET9 has a very low fraction of NE cells. Please clarify how the scoring was performed on these samples, as the program assignments may be driven by other cell types in samples with little to no NE cells.*

Scoring for tumor-derived programs was done only for the NE cells. Accordingly, SiNET3 was not scored or assigned to any of the programs. SiNET9 was included in this analysis - although it had a relatively small fraction of NE cells, the absolute number of profiled cells was particularly high in this sample and therefore the number of NE cells was 130, higher than our cutoff of 100 cells.

*c) Given the heterogeneity of cell types within each sample, would there be a way to provide a refined sense of confidence for certain cell type annotations? This would be helpful given the heterogeneity in marker gene expression and the absence of gold-standard markers for fibroblasts and endothelial cells in this cancer type. Additionally, there seems to be an unusually large proportion of NK and T cells - was there selection for this (given that these tumors are largely not immune infiltrated)?*

Author Response: Except for the Neuroendocrine cells, there are six TME cell types that we consistently find in multiple SiNET samples: macrophages, T cells, B/plasma cells, fibroblasts, endothelial and epithelial cells. Each of these cell types are identified as discrete clusters in analysis of the respective tumors (as shown in Fig. 1a,b and Fig. S1), and these are exactly the six most common non-malignant cell types that we and others found in single cell analysis across various other tumor types (e.g. see Gavish et al. 2023, ref. #15). The signatures used to annotate these cell types are shown in Table S2, and they primarily consist of classical markers that are traditionally used to define those cell types. We therefore believe that the annotation of these typical tumor-associated cell types is robust and does not include major uncertainties. In addition to these five common cell types, there are three cell types that we find only in 1-2 of the samples – epithelial cells, plasma cells and NK cells. Again, we believe that their annotation is robust, and these cell types are primarily not used for further analysis.

There was no selection for any specific cell types in this study. Nevertheless, single cell (or single nuclei) analysis may lead to biases towards specific cell types, that we cannot evaluate directly from the data. NK cells were detected only in one tumor. T cells were detected in eight of the ten samples; but in four of those samples the frequency of T cells was lower than 5% and only in one sample the frequency was above 20%. Therefore, while we cannot exclude a technical bias towards high frequency of T/NK cells, we do not consider these frequencies as high enough to suggest this specific type of bias. In the revised manuscript, we clarify that the commonly observed cell types in SiNETs are the same as those commonly observed in other tumors and we acknowledge the possibility of a technical bias in cell type capture.

*d) Evaluating the expression of one gene at a time may not effectively demonstrate subtype-specific patterns, particularly when comparing NE cells from one tumor to non-NE cells from another, which may not be an appropriate approach for identifying differentially expressed genes. DE analysis coupled with concordance analysis, for example, could strengthen the results.*

We apologize, but we do not fully understand this comment. We note that the initial normalization by non-NE cells was done in order to decrease batch effects when combining the data of the two platforms. We also note that the two subtypes were identified by two distinct approaches, as shown in Fig. 2c and in Fig. 2f.

(2) Results:

*See the above public review.*

(3) Minor Comments:

a) Results: Single cell and single nuclei RNA-seq profiling of SiNETs

*The results say ten primary tumor samples from eight patients. Later in the paragraph it says, "After initial quality controls, we retained 29,198 cells from the ten patients." Please clarify to either ten samples or eight patients.*

Indeed these are ten samples rather than ten patients. We corrected that in the revised version and thank the reviewer for noticing our error.

b) Methods:

*- Please specify which computational tools were used to perform quality control, signature scoring, etc.*

The approaches for quality control, scoring etc. are described in the methods. We implemented these approaches with R code and did not use other computational tools.

*- Minor point but be consistent with naming convention (ie, siAdeno vs SiAdeno) throughout the paper. For example, under "Sample Normalization, Filtering and annotations" change "siAdeno" to "SiAdeno."*

Thank you for noting this, we corrected that.

*- Add processing and analysis of MiNEN sample to the methods section. It is not mentioned in the methods at all.*

As noted in the revised manuscript, the MiNEN sample was analyzed in the same way as the SiNET fresh samples.

c) Supplementary Figures:

*Figure S1: Change (A-H) to (A-I) to account for all panels in the figure.*

*Figure S4: Add (C) after "the siAdeno sample" in the legend.*

Thank you for noting this, we corrected that.

(4) Font size is quite small in the main figures.

We enlarged the font in selected figure panels.

**Reviewer #2 (Recommendations for the authors):**

*(1) The small number of samples used in some analyses affects the robustness of the findings. Increasing the sample size or including more validation data could improve the*

*statistical reliability and make the results more convincing. The authors should consider expanding the cohort size or integrating additional external datasets to increase statistical power.*

We agree with the reviewer that adding more samples would improve the reliability of the results. However, the external data that we found was not comparable enough to enable integration with our data, and we are unable to profile additional SiNET samples in our lab. We hope that future studies would support our results and extend them further.

*(2) The biological significance of differentially expressed genes needs more depth, limiting the insights into SiNET biology. The authors should perform a comprehensive pathway enrichment analysis and integrate findings with existing literature. Tools like Gene Set Enrichment Analysis (GSEA) or Overrepresentation Analysis (ORA) could provide a more holistic view of altered biological processes.*

We thank the reviewer for this suggestion. We did examine the functional enrichment of differentially expressed genes and did not find additional enrichments that we felt were important to highlight beyond what we described. We report the genes in supplementary tables, enabling other researchers to examine these lists further.

*(3) The unexpected finding of higher proliferation in non-malignant cells requires further investigation and plausible biological explanation. The authors should perform additional analyses to explore potential mechanisms, such as investigating cell cycle regulators or performing in vitro validation experiments. The authors should consider single-cell trajectory analysis to explore these highly proliferative non-malignant cells' potential differentiation or activation states.*

We agree that our results are descriptive and that we do not fully explain the mechanism for the high level of non-malignant cell proliferation. We did attempt to perform follow up computational analysis. These analyses raised the hypothesis that high levels of MIF are causing the proliferation of immune cells. Additional analyses that we performed were not sufficient to conclusively identify a mechanism, and we felt that they were not informative enough to be included in the manuscript. Further *in vitro* (or *in vivo*) studies are beyond the scope of the current work.

*(3) More details are required on methods used for p-value adjustment, and criteria for statistical significance should be clearly defined. Additionally, integrating scRNA-seq and snRNA-seq data needs a more thorough explanation, including batch effect mitigation and more explicit cell clustering representation. The authors should clearly describe p-value adjustments (e.g., FDR) and batch correction methods (e.g., Harmony, FastMNN integration) and include additional figures showing corrected UMAP plots or heatmaps post-batch correction to enhance the confidence in results.*

We now clarify in the Methods our use of FDR for p-value adjustments. As for batch correction, we have avoided the use of integration methods as we believe that they tend to distort the data and decrease tumor-specific signals. Instead, we primarily analyzed one tumor at a time and never directly compared cell profiles across distinct tumors but only compared the differences between subpopulations; specifically, we normalized the expression of NE cells by subtracting the expression of reference non-NE cells from the same tumor as a method to decrease batch effects. We now clarify this point in the Methods section.

*(4) The lack of analysis of interactions between different cell types limits understanding of tumor microenvironment dynamics. The authors should employ cell-cell interaction*

*analysis tools (e.g., CellPhoneDB, NicheNet) to explore potential communication networks within the tumor microenvironment. This could provide valuable insights into how different cell types influence tumor progression and maintenance.*

We thank the reviewer for this suggestion. We have tried to use such methods but found the results difficult to interpret since these approaches generated very long lists of potential cell-cell interactions that are largely not unique to the SiNET context and their relevance remains unclear without follow up experiments, which are beyond the scope of this work. We therefore focused only on ligand/receptors that came up robustly through specific analyses such as the differences between SiNET subtypes. In particular, MIF is highly expressed in the epithelial subtype, and remarkably, MIF upregulation is shared across multiple cell types. Thus, the cell-cell interactions that are suggested by the SiNET data as somewhat unique to this context are those involving MIF and its receptor (CD74 on immune cell types), while other interactions detected by the proposed methods primarily reflect the generic ligand/receptors expressed by corresponding TME cell types.

**Reviewer #3 (Recommendations for the authors):**

*(1) For a relatively small dataset, the mixing of single-cell versus single-nucleus RNA-seq should be discussed more. It would be nice to have 1-2 tumors that are analyzed by both methods to compare and increase our understanding of how these different approaches may affect the results. This could be accomplished by splitting a fresh tumor into two parts, processing it fresh for single-cell RNA-seq, and freezing the other part for single-nucleus RNA-seq.*

We agree with the reviewer that the different techniques may bias our results and we refer to this limitation in the Results and Discussion sections. However, it is important to note that we do not directly integrate the primary data across these modalities, but rather analyze each tumor separately and only combine the results across tumors. For example, we first compare the NE cells from each tumor to control non-NE cells from the same tumor and then only compare the sets of NE-specific genes across tumors. Moreover, the subtypes that we detect cannot be explained by these modalities, as the first subtype contains samples from both methods and these subtypes are further demonstrated in external bulk data. Similarly, the results regarding low proliferation of NE cells and high proliferation of B/plasma cells are observed across both modalities. We therefore argue that while the combination of methods is a limitation of this work it does not account for the main results.

*(2) The authors state that they defined the siNET transcriptomic signature by comparing their siNET single-cell/nucleus data to other NETs profiled by bulk RNA-seq. Some of the genes in the signature, such as CHGA, are widely used as markers for NETs (and not specific for siNET). The authors should address this in more detail.*

To define the SiNET transcriptomic signature we first analyzed each tumor separately and compared the expression of Neuroendocrine (NE) cells to that of non-NE cells to detect NE-specific genes. Next, we compared the lists of NE-specific genes across the 8 SiNET patients and found a subset of 26 genes which were shared across most of the analyzed SiNET samples (Fig. 2a). Thus, the signature was defined only from analysis of SiNETs and not based on comparison to other types of NETs and hence it is expected that the signature could contain both SiNET-specific genes and more generic NET genes such as CHGA.

*Only after defining this signature, we went on to compare it between SiNETs and other types of NETs (pancreatic and rectal) based on external bulk RNA-seq data. In this comparison, we observed that the signature was clearly higher in SiNETs than in the other NETs (Fig. 2b). This result supports the accuracy of the signature and further*

*suggests that it contains a fraction of SiNET-specific genes and not only generic NET genes such as CHGA. Thus, we would expect this signature to perform well also for distinguishing between SiNET and types of NETs, but it does contain a subset of genes that would be high in the other NETs. Finally, we note that even though CHGA is a generic NET marker, the bulk RNA-seq data would suggest that, at least at the mRNA level, this gene is still higher expressed in SiNETs than in other NETs. To avoid confusion regarding the definition and specificity of the SiNET transcriptomic signature we have extended the description of this section in the revised manuscript.*

*(3) The authors only compare their data to bulk transcriptomic data on NETs. While in some instances this makes sense given the bulk dataset has >80 tumors, they should at least cite and do some comparison to other published single-cell RNA-seq datasets of NETs (e.g., PMID: 37756410, 34671197). The former study listed has 3 siNETs, 4 pNETs, and 1 gNET. Do the epithelial-like and neuronal-like signatures show up in this dataset too?*

We examined these studies but concluded that their data was inadequate to identify the two SiNET subtypes. The latter study was of pNETs, while the former study had 3 SiNET samples but only from 2 patients, and furthermore it was enriching for immune cells with only very low amounts of NE cells. Therefore, we now cite this work in the discussion but cannot use it to extend the results from our work.

*(4) How did the authors statistically handle patients with more than one tumor sample (true for  $n = 2$ )? These tumor samples would not be truly independent.*

In both cases where we had two distinct samples of the same patient, only one sample had sufficient NE cells to be included in NE-related analysis and therefore the other samples (SiNET3 and SiNET6) were excluded from all analysis of NE differential expression and subtypes. These samples were only included in the initial analysis (Fig. 1) and in TME-related analysis (Fig. 3-4) in which there was no statistical analysis of differences between patients and hence no problem with the inclusion of 2 samples for the same patient. We clarified this issue in the revised version.

*(5) The association between siNET subtype and B/plasma cell proliferation is very interesting, as is the hypothesis regarding MIF signaling. It would be illuminating for the authors to perform cell-cell interaction analyses with methods such as CellChat in this context rather than just relying on DE. Spatial mapping would be helpful too and while this may be outside the scope of this study, it should at least be expounded upon in the Discussion section.*

Indeed, spatial transcriptomic analysis would add interesting insight to our data and to SiNET biology. Unfortunately, this is not within the scope of the current project but we note this interesting possibility in the Discussion. Regarding additional methods for cell-cell interactions, we have performed such analysis but found it not informative as it highlighted a large number of interactions that are not unique SiNETs and are difficult to interpret, and therefore we do not include this in the revised version.

*(6) The authors note that in the mixed lung tumor, the NE component was more proliferative than that observed with siNETs. How does the proliferation compare to pNETs, gNETs, in other published studies? How about assessing the clonality of the SCC and LNET malignant cells with various genomic or combined genomic/transcriptomic methods?*

The percentage of proliferating NE cells in the mixed lung tumor was higher than 60%. This is extremely high, approximately four-fold higher than the average that we found in a pan-cancer analysis and higher than the average of any of the >20 cancer types that we analyzed (Gavish et al. 2023, ref. #15). This remarkably high proliferation serves as a control for the low proliferation that we found in SiNET NE cells.

(7) In the Discussion on page 13, the authors write "Second, proliferation of NE cells may be inhibited by prior treatments with somatostatin analogues." How many patients were treated in this manner? This information should be made more explicit in the manuscript.

Details on pretreatment with somatostatin analogues are provided in Table S1. All patients were pre-pretreated with somatostatin analogues, with the possible exception of one patient (P8, SiNET10) for which we could not confidently obtain this information.

(8) On page 5, "bone-fide" is misspelled.

(9) On page 8, "exact identify" is misspelled.

We thank the reviewer and have corrected the typos.

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