Single-cell transcriptome of antler stem cells from antlerogenic periosteum

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Abstract

Background: Antler regeneration, a stem cell-based epimorphic process, has potential for applications as a valuable model for regenerative medicine. A pool of antler stem cells (ASCs) for antler generation and regeneration is located in the antlerogenic periosteum (AP). However, this antler stem cell pool has not been fully characterized.

Finding: We produced a comprehensive transcriptome dataset at the single-cell level for antler stem cells based on the 10x Genomics platform. We generated ~252 million sequence reads representing a large RNA-Seq dataset for 4,731 cells from an individual AP tissue sample. Further screening identified 16 key stem cell markers, of which four mesenchymal (CD29, CD90, vimentin, nucleophosmin) and one embryonic (CD9) stem cell markers showed high expression levels. This suggests ASCs are intermediate type between embryonic and mesenchymal stem cells and will help to identify and purify specific ASC types or subtypes.

Conclusion: Our results provide the first comprehensive transcriptome dataset at the single-cell level for ASCs, which may hold the key to unveil the secrets about why antlers are the only mammalian organ to fully regenerate.

Background Information

The 'Holy Grail' of modern regenerative medicine is to grow back lost organs/appendages, which is known as epimorphic regeneration[1,2]. Our current knowledge of epimorphic regeneration is largely gained from the studies on lower vertebrates[3]. Notably, these animals have the ability to reprogram phenotypically committed cells at the amputation plane toward an embryonic-like cell phenotype (dedifferentiation) and to form a cone-shaped tissue mass, known as a blastema[4]. Deer antlers are the only mammalian appendages capable of full renewal and therefore offer a unique opportunity to explore how nature has solved the problem of epimorphic regeneration in mammals[5-8]. Recent studies concluded that antler regeneration is a stem cell-based epimorphic process[9-12], which have potential for application as a valuable model for biomedical research and regenerative medicine. Revealing the mechanism underlying this stem cell-based epimorphic regeneration would undoubtedly place us in a better position to promote tissue/organ regeneration in humans.

Antlers regenerate from permanent cranial bony protuberances, known as pedicles. Growth of a pedicle itself is initiated during puberty from a piece of periosteum, called the antlerogenic periosteum (AP), which covers a crest in the deer skull[13]. Removal of the AP prior to pedicle initiation stops pedicle and antler growth and transplantation of the AP autologously induces ectopic pedicle and antler formation[14-16]. The initial discovery of AP[17] has been hailed as a "hallmark" event in antler research history[1]. The AP tissue, ~2.5 cm in diameter and 2.5-3 mm in thickness, contains around five million cells, which sustain the seasonal renewal of the entire antler throughout the deer's life[7]. The potency of AP cells has been investigated by several laboratories[12,18,19]. The AP cells express some key embryonic stem cell markers, and can be induced *in vitro* to differentiate into chondrocytes, osteoblasts, adipocytes, myoblasts and neural-like cells. Therefore, AP cells have been termed antler stem cells[7] (ASC) and are essential for full regeneration of this unique mammalian organ. It is known that histologically AP consists of two layers: the upper

fibrous layer and the lower cellular layer[13], and the cells released from these two layers reacted to IGF1 and testosterone differently in vitro[20]. In addition, only around 50% AP cells expressed Stro-1 marker (unpublished). All these results imply that the AP cells must not be homogenous. However, it is not known how many subtypes of AP cells exist in the AP tissue.

Differences in cell type in any tissue are an essential feature for their biological state and function. More and more studies on cell biology have now adopted single-cell RNA sequencing by employing new protocols of single cell isolation to characterize functionally heterogeneous cells[21]. Thus far, single cell sequencing technology has not been applied to investigate the ASCs. Here, we have taken this powerful approach to carry out single cell transcriptome sequencing for this unique ASC population using the 10x genomics platform[22], a droplet-based system that enables 3' messenger RNA (mRNA) digital counting of thousands of single cells (Fig. 1).

Data Description

AP tissue sampling

The AP tissue was obtained from three 6-month -old male sika deer immediately after slaughtering, according to the previous protocol[23]. To collect the AP tissue, a crescent-shaped incision was made on the scalp skin 2 cm medial to the frontal crest; skin was separated from the frontal bone to expose the AP. The AP was then peeled off from the underlying bone following the incisions cut on periosteum and then placed into cold DMEM medium plus 500 U/ml penicillin and 500 g/ml streptomycin (Invitrogen, USA).

Cell culture

Primary culture for the AP cells was carried out according to our previous methodology[24]. The AP tissue was cut into small pieces and digested in the DMEM culture medium containing collagenase (150 units/ml) at 37℃ for 1-1.5 hour to release cells, and then the digest was washed twice with DMEM only before pelleting.

The AP cell pellet was suspended in 10 ml completed medium (DMEM +10% FBS +100 U/ml penicillin +100 mg/ml streptomycin) and transferred into a 75 ml culture flask (Nunc, Denmark) containing 15 ml completed medium. The AP cells were trypsinized upon reaching about 80% confluence and reseeded in T75 culture flasks at the density of 2×10^5 cells/ml. The cells were detached again when reaching around 85%-90% confluence, transferred to the freezing medium (FBS +10% DMSO) at 1×10^5 cells/ml, and then stored in liquid nitrogen for later use.

Sequencing library construction using ChromiumTM Platform

The AP single-cell library was constructed using the Chromium™ Controller and Chromium™ Single Cell 3' Reagent Version 1 Kit (10x Genomics, Pleasanton, CA) to generate single cell GEMs (Gel Bead-in-Emulsions) as previously described[22]. Briefly, to thaw the ASCs, 1X PBS containing 0.04% BSA was added to wash and dilute the cells. About 1×10^6/ml (1000/ul) suspended cells was obtained and placed on the ice. In total, a 17 μl cellular suspension that contained ~4500 cells was added to the Master Mix (20 μl nuclease-free water, 50.0 μl RT Reagent Mix, 4 μl RT Primer, 1.5 μl RNase Inhibitor, 2.5 μl Additive A and 4.6 μl RT Enzyme) in the tube strip well. The 100 μl Master Mix containing cells was transferred to each well in the Chromium™ Single Cell 3' chip row labeled 1, and 40 μl Single Cell 3' Gel Beads was loaded in the chip row labeled 2, and 135 μl oil-surfactant solution was loaded in the chip labeled 3 for GEM generation and barcoding. Subsequently, GEM-RT was performed using a Thermocycler (BioRad; 55 °C for 2 hrs, 85 °C for 5 mins, hold at 4 °C). Post GEM-RT cleanup and cDNA amplification was performed to isolate and amplify cDNA for library construction. Quality of the cDNA library was assessed using a 2100 Bioanalyzer (Agilent Technologies) to confirm the overall shape of the sample electropherogram shown as the peak at around 2000 bp. The samples were sequenced in two lanes on the HiSeq2500 in rapid run mode using a paired end flow cell. Read1: 98 cycles, Index1: 14 cycles, Index2: 8 cycles, and Read2: 10 cycles

Statistical analysis and quality assessments of single-cell sequence

data

Cell Ranger Software Suite version 1.3.1 (http://support.10xgenomics.com/) by 10x Genomics was used to perform sample de-multiplexing, barcode processing and single-cell 3′ gene counting, as performed previously[22]. Ten bp UMI tags were extracted from Read2. Cellranger mkfastq used bcl2fastq v2.19 (https://support.illumina.com/) to demultiplex raw base call files from Hiseq 2500 sequencer into sample-specific FASTQ files. Cellranger mkref was run to construct a cellranger-compatible reference based on both the Ovir.te1.0 reference sequences and the transcriptome GTF file. These FASTQ files were aligned to the Ovir.te1.0 reference transcriptome with cellranger count that used an aligner called STAR[25], and aligned reads were filtered for valid cell barcodes and UMI to generate filtered gene-barcode matrices. The sequencing and mapping data were summarized in Table 1 and Table 2.

We obtained 252,818,309 sequence reads representing 4,731 single cell transcriptomes, which constituted 14,993 genes. The median gene number and transcripts/Unique Molecular Identifier (UMI) counts were 2,568 and 10,309 respectively. On average, 53,438 reads were detected per cell. According to the previous studies[26,27,] ~50,000 mapped reads were sufficient for an accurate analysis by Single Cell 3′ Solution. A steep drop-off was indicative of good separation between the cell-associated barcodes and the barcodes associated with empty partitions (Fig. 2A). Number of genes, UMI counts and percentage of mitochondrial genes per cell were calculated to see if there was a subset of cells at an outlier level as potential multiplets (Fig. 2B, C and D). We observed that a small subset of cells had slightly increased percentage of mitochondrial genes (Fig. 2B and D), i.e., only 85 cells included more than 5% mitochondrial gene referring to the previous threshold criterion[27].

Identification of antler stem cell markers

We utilized t-SNE (t-Stochastic Neighbor Embedding) as a powerful tool in Cell Ranger R kit (http://support.10xgenomics.com/) to screen for known stem cell marker

in 2-d space and selected 16 markers based on the criteria that a marker must be expressed by more than 3% of ASCs and with at least one UMI count. The signatures of these gene markers were visualized in 2-D space (Fig. 3A), including ten mesenchymal stem cell markers (i.e., CD29, CD73, CD105, CD90, Fibronectin1, Vimentin, Nucleophosmin, PDGFRA CD44, CD49), four embryonic stem cell markers (i.e., CD9, SMAD2, MYC, TBX3), three neural stem cell markers (i.e., ID2, NES, Vimentin) and five cancer stem cell markers (i.e., Nestin, CD44. MYC, CD90, CD105) (Fig. 3B). Based on the abundance of their expression (i.e., nUMI>1, 3 and 5) (Fig. 3C), four mesenchymal stem markers (i.e., CD90, CD29, Vimentin and Nucleophosmin) and one embryonic stem cell marker (i.e., CD9) were standout. This suggests that ASCs might display features associated with an intermediate type of cell between mesenchymal stem cells and embryonic stem cells.

Conclusion

In summary, we first report a comprehensive transcriptome dataset at the single-cell level for antler stem cells based on the 10x Genomics platform, which will provide a useful resource for the study of antler biology. The data from the surface marker screening will be helpful in purifying specific cell subtypes of the ASCs for further characterization, which would greatly facilitate dissection of the mechanism underlying annual renewal of deer antlers, the only stem cell-based mammalian organ regeneration. It is also possible to use the raw reads for executing a new experiment to distinguish different cell subtypes with multiple biological samples, such as the pedicle periosteum (PP) cells (another type of ASCs, which are further differentiated derivatives of AP cells) and FP cells (deer facial periosteum, used as a control)[24]

Table 1. Summary of single cell sequencing quality

Sequencing quality metrics	Value
Number of Reads	252,818,309
Valid Barcodes	94.7%
Reads Mapped Confidently to Transcriptome	61.3%
Reads Mapped Confidently to Exonic Regions	63.5%
Reads Mapped Confidently to Intronic Regions	5.4%
Reads Mapped Confidently to Intergenic Regions	8.8%
Sequencing Saturation	58.5%
Q30 Bases in Barcode	72.9%
Q30 Bases in RNA Read	93.6%
Q30 Bases in Sample Index	96.8%
Q30 Bases in UMI	97.0%

Table 2. Summary of single cell sequencing cells

Sequencing cells metrics	Value
Estimated Number of Cells	4,731
Fraction Reads in Cells	82.9%
Mean Reads per Cell	53,438
Median Genes per Cell	2,568
Total Genes Detected	14,993
Median UMI Counts per Cell	10,309

Availability of supporting data

The raw single-cell RNA-seq data in fastq format can be found at SRA under BioProject PRJNA416396.

Abbreviations

AP: antlerogenic periosteum; ASCs: antler stem cells; PBS: phosphate buffer salin; DMEM: dulbecco's modified eagle medium; DMSO: dimethylsulfoxide; GEM: Gel Bead-in-Emulsions; UMI: unique molecular identifier; RT: reverse transcription; bp: base pair; GTF: gene transfer format; PCA: principal component analysis; KNN: K nearest neighbors; t-SNE: t-Stochastic Neighbor Embedding; PP: pedicle periosteum; FP: facial periosteum.

Ethics approval and consent to participate

This study was approved by Animal Ethics Committee of Institute of Special Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences (CAAS2017015).

Competing interests

The authors declare no competing financial interests.

Author Contributions

H.B., D.W. and C.L. conceived the experiment. D.W. collected samples, D.W. and H.S. cultured the cell lines. W.W. extracted RNA samples and prepared them for Single Cell 3' library construction and sequencing. H.B. performed QC and data analysis. H.B., W.W. and C.L. wrote the manuscript. All authors read and approved of the final manuscript.

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Figure Legends

Figure 1. Overview of the design and work flow. The AP was collected from a slaughtered deer head, antler stem cells (ASCs) dissociated from the AP were primarily cultured and then individual cells were selected for the single-cell library construction using Chromium^{TM} Single Cell 3' Reagent Kit and sequencing on Illimina Hiseq 2500 platform. The single-cell data quality control and bioinformatics analysis using the R packages were performed.

Figure 2. Quality metrics of the ASC single-cell transcriptomes using 10x Genomics. A) Barcode rank plot. In the plot, a steep drop-off is indicative of good separation between the cell-associated barcodes and the barcodes associated with empty partitions. B) Distribution of number of genes, Unique Molecular Identifier (UMI) counts and percentage of mitochondria UMIs per cell. C) Plot between number of genes and UMIs counts per cell. D) Plot between percentage of mitochondria UMIs and UMIs counts per cell. A small subset of cells (85 cells) had more than 5% mitochondrial genes.

Figure 3. ASC screening results using currently available stem cell markers. A) *t*-SNE projection of single cells. These cells were labeled by 16 stem cell markers respectively, and the label threshold was set to meet a criterion that a marker must be expressed by more than 3% ASCs and with at least one Unique Molecular Identifier (UMI) count. B) Venn diagram of the 16 stem cell markers across the four types of stem cell markers, including ten mesenchymal stem cell markers, four embryonic stem cell markers, three neural stem cell markers and five cancer stem cell markers. C) Expressed abundance of the 16 stem cell markers based on nUMI>1, 3 and 5.

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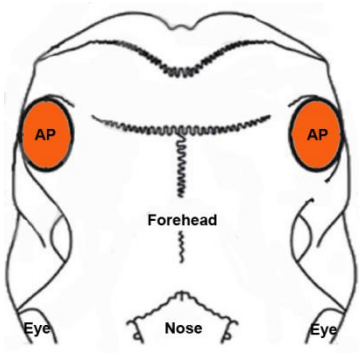
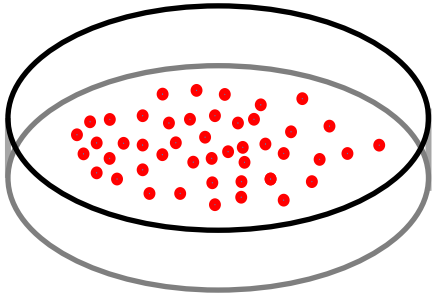
AP tissue collection



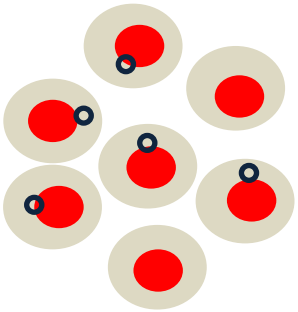
- Age: six month
- Sex: male
- Area: 4-5 cm^2

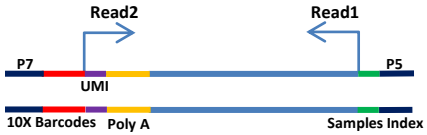
AP cell culture



- Tissue digest
- Primary cell culture

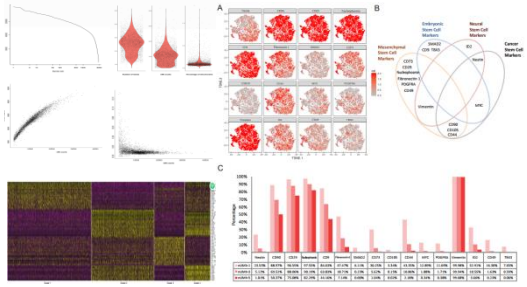
Library construction and sequencing



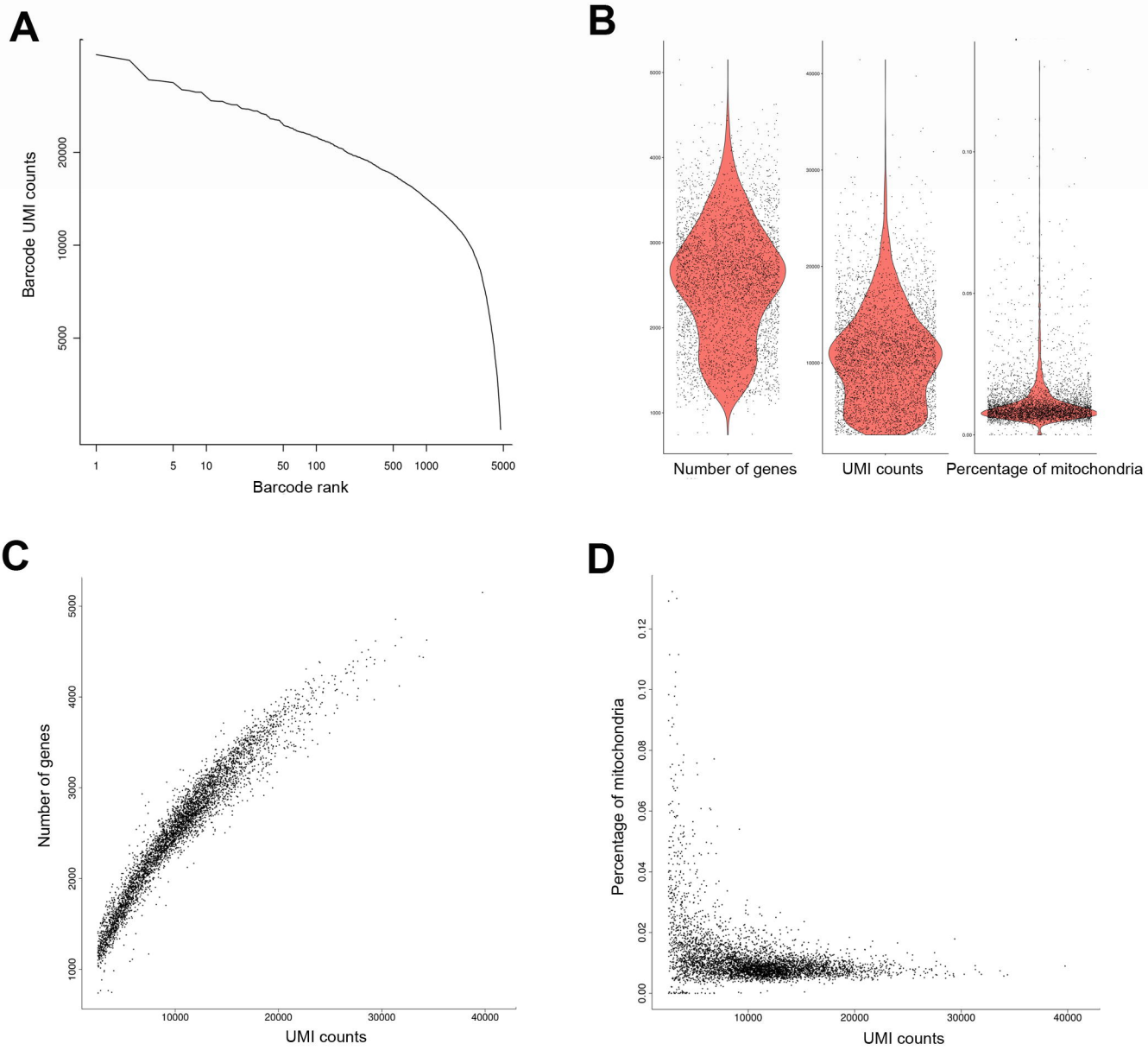


- 10x Genomics Chromium™ Platform
- Illumina Hiseq 2500

Single-cell data analysis



- Cell Ranger Software Suite
- Seurat R package
- Cell Ranger R package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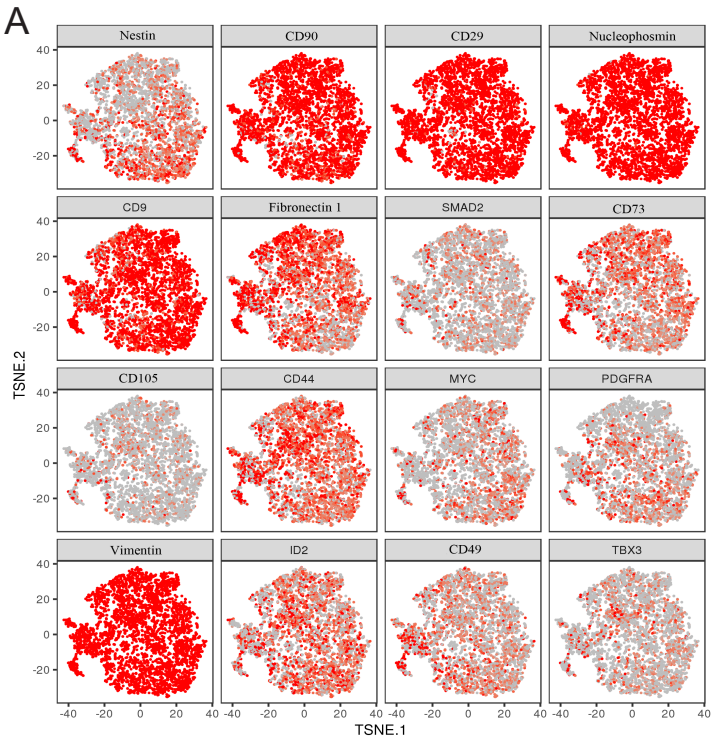

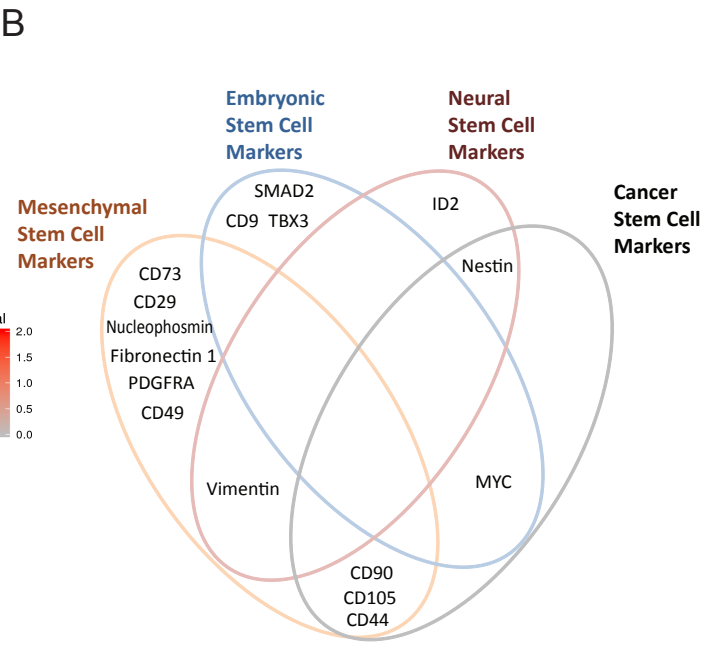

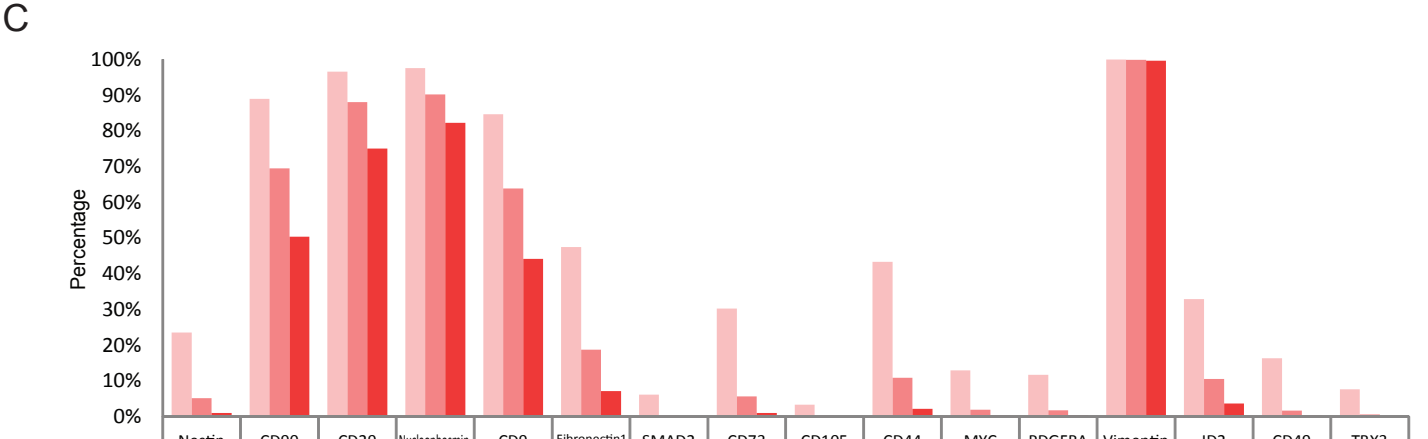

	Nestin	CD90	CD29	Nucleophosmin	CD9	Fibronectin1	SMAD2	CD73	CD105	CD44	MYC	PDGFRA	Vimentin	ID2	CD49	TBX3
nUMI>1	23.53%	88.97%	96.55%	97.55%	84.63%	47.47%	6.11%	30.25%	3.34%	43.35%	12.89%	11.69%	99.98%	32.91%	16.30%	7.65%
nUMI>3	5.12%	69.52%	88.06%	90.19%	63.83%	18.71%	0.23%	5.62%	0.15%	10.86%	1.88%	1.71%	99.94%	10.55%	1.63%	0.59%
nUMI>5	1.01%	50.37%	75.08%	82.29%	44.16%	7.14%	0.00%	1.04%	0.02%	2.18%	0.34%	0.38%	99.68%	3.66%	0.23%	0.06%