# Identification of Pancreatic Endocrine Regulators in Real-Time at Single-Cell level


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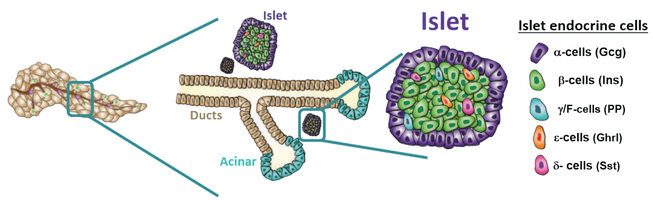
## 1 Introduction



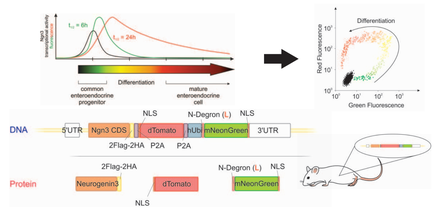

Although they constitute only 1-2% of the total pancreatic mass, dysfunction of pancreatic endocrine cells, caused by mutations, deregulated differentiation or hormone secretion, can result in diseases such as pancreatic neuroendocrine tumours (PNETs) and diabetes. Identification of factors driving differentiation of the various types of pancreatic endocrine cells will, in the long-term, aid the development of treatment strategies for diseases involving these cells.

Differentiation of pancreatic endocrine cells is initiated by a brief expression of the master transcription factor Neurogenin3 (Neurog3). This leads to both transient and constitutive expression of downstream factors (such as transcription factors, inhibitors and ubiquitin ligases), ultimately resulting in maturation of the various types of endocrine cells. This dynamic differentiation process requires distinct gene sets to be expressed at specific stages. Tight regulation of this process is mainly achieved through transient expression of factors regulating the expression of these gene sets. However, compared to constitutively expressed factors, which have been relatively easy to identify, transiently expressed factors have remained more elusive.

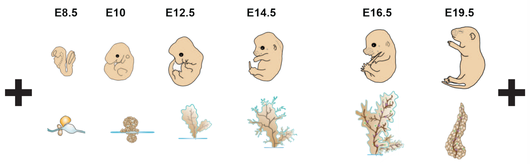
## 2 Aim and Approach

### Identification of Pancreatic Endocrine Regulatory Factors

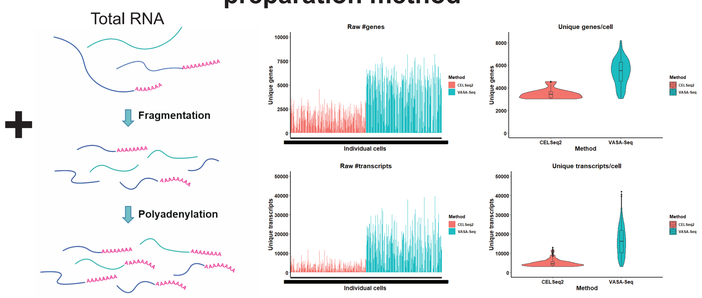
**Neurog3Chrono, a bi-fluorescent knock-in reporter**




**Embryonic Pancreas Development**



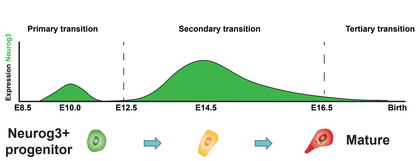

**VASA-Seq, a novel full transcriptome single-cell library preparation method**




To identify transiently expressed factors along the endocrine cell differentiation trajectory we utilized the novel Neurog3Chrono reporter mouse. In this model a bi-fluorescent reporter (mNeonGreen and dTomato) is endogenously knocked-in in the Neurog3 allele. The mNeonGreen protein folds and can be detected faster compared to dTomato. However, since mNeonGreen is destabilized it also degrades faster. Following trnasient expressin of Neurog3 endocrine progenitors will express a ratio between the two proteins.
Reporter positive cells from the pancreata of Neurog3Chrono reporter mice at different embryonic days of development were isolated by flow cytometry and single cell transcriptional libraries were prepared using a library preparation method called VASA-seq, where the total RNA is first fragmented and later polyadenylated. This new method provides increased sensitivity, full-length RNA sequences and detection of non-coding transcripts, which is not possible with 3'-biasedsed methods such as CELSeq2 (only captures coding transcripts).

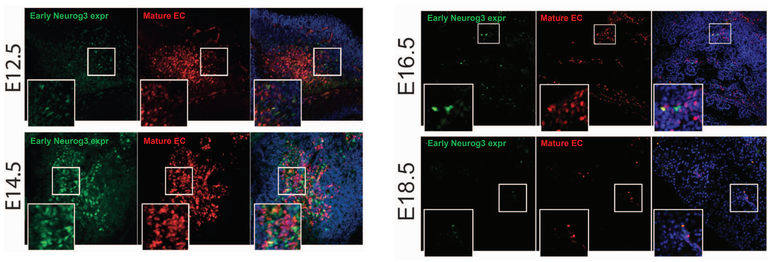
## 3 Neurog3Chrono reveals temporal heterogeneity during endocrine development

A



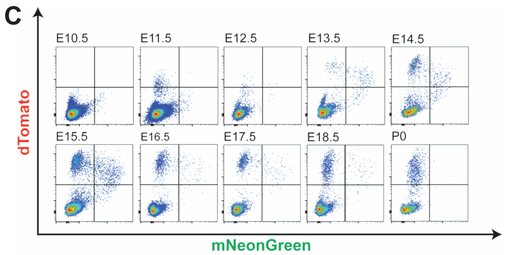

During pancreatic development Neurog3, key transcription factor initiating the endocrine differentiation program, is expressed in two waves. The first wave is shorter and the Neurog3 expression lower compared to the second wave, where the majority of the endocrine cells are born.

B



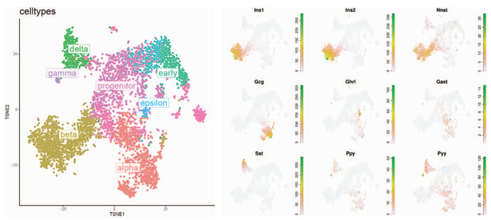

Pancreata isolated from different embryonic time points reveals temporal heterogeneity on a single-cell level among the endocrine progenitors, as single cells expressing different ratios of the two fluorescent proteins can be observed simultaneously (green, yellow and red). Cells recently starting to express Neurog3 only express mNeonGreen (green) are at an early stage of their differentiation while cells being further along the differentiation trajectory express both mNeonGreen and dTomato (yellow/orange) or only dTomato (red).

C




The profiles of FACS isolated Neurog3Chrono+ cells reflect Neurog3 expression during development.
At the beginning of each wave the number of mNeonGreen+ cells are higher, and at the end of each wave most cells are dTomato+.

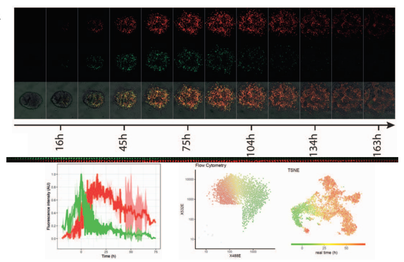
## 4 Reporter positive cells comprise all the different pancreatic endocrine cell types




Following FACS isolation the sorted endocrine cells are prepared using VASA-Seq and single cell sequenced. The resulting transcriptional t-SNE map shows that we can identify the five different pancreatic endocrine lineages alpha-cells expressing glucagon (Gcg), beta-cells expressing insulin (Ins1, Ins2), gamma-cells secreting pancreatic polypeptide (PP), delta-cells releasing somatostatin (Sst) and finally epsilon-cells secreting ghrelin (Ghrl).

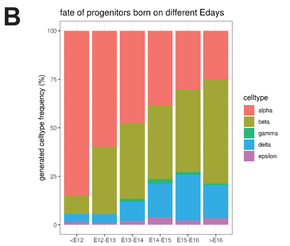
## 5 Fluorescence allows for inference of real-time

A



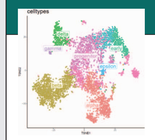

By performing live imaging of pancreatic explant cultures and subsequently track individual cells, real-time information can be inferred from the fluorescence intensity. This enables determination of how long ago (hours) a cell started to express Neurog3. Subsequently it is possible to identify the day of birth of cells sorted at a certain embryonic day. For example, a cell sorted at E15.5 could have been born on E14.5.

B



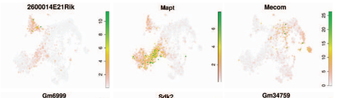

Progenitors born earlier have a higher chance to become alpha-cells whereas progenitors born at a later stage have a higher chance to become beta-cells.

## 6 Identification of transiently expressed genes and lncRNAs

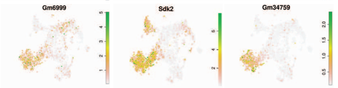



**Early**


**Middle**




**Late**




Our time-resolved, single-cell transcriptional map allows for identification of novel constitutive and transiently expressed regulatory factors. To the right are examples of factors expressed early, middle or late stage of differentiation.

## 7 Summary and Future directions

### Summary

- We generated a time-resolved map of transcriptional changes during pancreatic endocrine development by combining the Neurog3Chrono reporter mouse and VASA-seq
- Reporter positive cells comprise all the different pancreatic endocrine cell lineages
- Fluorescence allows for inference of real-time, which is used to determine the fate (the type of endocrine cell) of progenitors born on different days of pancreatic development
- We identified transiently expressed novel coding as well as non-coding molecular regulators.

### Future directions

- Perform isoform analysis of the transcriptome data to identify isoform switching of e.g. growth factor and hormone receptors durind endocrine cell development
- Integrate various time components to generate a differentiation time-line with minute resolution
- Confirmation knockouts of new transient and mature candidate endocrince regulators

Comparing regulators from our time-resolved transcriptional map of pancreatic endocrine differentiation to regulator found to be enriched in PNETs - to determine whether certain regulator or stages of differentiation are associated with certain types or stages of this disease.