

Divergent transcriptional responses to pancreatic transcription factors are dictated by the cellular context

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Objective. The outcome of directed differentiation for cell-based therapies is profoundly influenced by the starting cell's intrinsic state, or cellular context. How pluripotent versus lineage-primed cells interpret the same developmental cues remains unclear. To address this, we performed a comparative transcriptomic analysis using two mouse cell lines engineered for episomal expression: a pluripotent embryonic stem cell line (PLT-ES) and a multipotent pancreatic stem cell line (PLT-PPPD).

Methods. We introduced a core set of pancreatic transcription factors – PMN: Pdx1 (pancreatic and duodenal homeobox 1), MafA (MAF BZIP transcription factor A), and NeuroD1 (neurogenic differentiation 1) – into both cell types and analyzed global gene expression and insulin promoter activity.

Results. The two cell types exhibited strikingly divergent fates. In lineage-primed PLT-PPPD cells, PMN expression induced a mixed pancreatic/hepatic phenotype. In stark contrast, PMN expression in pluripotent PLT-ES cells failed to initiate an endodermal program, instead robustly driving differentiation towards mesodermal lineages. Intriguingly, reporter assays revealed that the PMN factors could activate the insulin promoter in both PLT-ES and PLT-PPPD cells, regardless of the overall differentiation outcome.

Conclusions. Our findings demonstrate that cellular context is a paramount determinant of cell fate. The activation of a single promoter is insufficient to orchestrate a complete differentiation program. While pancreatic stem cells exhibit plasticity within the endodermal lineage, pluripotent cells are misdirected towards entirely different germ layers if not appropriately primed. This study underscores the critical importance of using lineage-committed cells for predictable therapeutic outcomes.

Keywords: cell differentiation, embryonic stem cells, pancreatic stem cells, regenerative medicine

The promise of regenerative medicine for diabetes hinges on our ability to instruct stem cells to become functional, glucose-responsive beta-cells (Kieffer 2016). Embryonic stem (ES) cells, with their boundless pluripotent potential, have long been a focal point of this research (Thomson et al. 1998). While existing protocols have shown that ES cells can be coaxed towards a beta-cell fate, the process is often inefficient and yields cells that lack the full functional maturity

of their endogenous counterparts (Pagliuca et al. 2014; Rezania et al. 2014). This challenge underscores a fundamental question in developmental biology: how does a cell's intrinsic identity shape its response to extrinsic developmental cues (Hochedlinger and Plath 2009)?

The differentiation of a beta-cell is orchestrated by a core set of master regulatory transcription factors, including Pdx1 (pancreatic and duodenal homeobox

1), MafA (MAF BZIP transcription factor A), and NeuroD1 (neurogenic differentiation 1) (PMN) (Odom et al. 2004; Artner et al. 2007). The forced expression of this trio has become a powerful tool to drive cellular reprogramming towards the pancreatic endocrine lineage (Zhou et al. 2008). However, these factors do not operate in a vacuum. Their ability to bind to target genes and initiate a new transcriptional program is profoundly influenced by the epigenetic “soil” of the host cell – the landscape of open and closed chromatin that defines its current state and future potential (Gifford et al. 2013; Atlasi and Stunnenberg 2017).

We hypothesized that a cell already committed to the pancreatic lineage would interpret the command of these master regulators far differently than a naive ES cell. To explore this, we utilized a pancreatic stem cell line (PLT-PPPD) previously established by our group. These cells, isolated from the adult mouse pancreas, display characteristics of ductal cells and possess the proven multipotent ability to generate both endocrine and exocrine lineages upon gene induction (Yamamoto et al. 2006; Yamato et al. 2012). This epigenetically primed identity could provide a more direct and efficient route to beta-cell identity, bypassing the aberrant pathways that may arise from a pluripotent ground state.

To test this hypothesis, we employed an episomal expression system based on the polyoma virus large T antigen (PLT) to engineer both mouse ES cells (PLT-ES) and our PLT-PPPD cells. This method was chosen to maintain robust transgene expression without integration into the host genome, thereby eliminating the unpredictable influences of insertional mutagenesis and chromosomal position effects (Gassmann et al. 1995). We introduced vectors expressing the PMN factors, or a control vector expressing enhanced green fluorescent protein (EGFP), into each cell type. Global gene expression profiles were then compared via Affymetrix microarray analysis, and promoter-specific activity was assessed using a reporter assay. This comparative analysis allowed us to dissect the distinct cellular logic of each starting population, illuminating how cellular context is paramount in dictating developmental outcomes.

Materials and Methods

Cell culture and maintenance. PLT-ES cells, mouse ES cells derived from the E14tg2a line (Hooper et al. 1987) and engineered to express the PLT, were used. These PLT-ES cells were maintained in an undifferentiated state on gelatin-coated dishes without feeder

cells. The culture medium was Glasgow Minimum Essential Medium (GMEM) supplemented with 10% Fetal Calf Serum (FCS; Biowhittaker, Walkersville, MD), 1% non-essential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, and 1,000 U/mL leukemia inhibitory factor (LIF; Chemicon, Temecula, CA).

PLT-PPPD cells. The pancreatic progenitor cell line (PPPD), established and characterized as previously described (Yamamoto et al. 2006), was engineered to stably express the PLT by transfection with the pMGD20neo plasmid and subsequent selection with 200 µg/mL G418 (Sigma-Aldrich, St. Louis, MO). The resulting cell line (PLT-PPPD) was maintained in DMEM/F-12 medium supplemented with 5% KnockOut™ Serum Replacement (KSR; Invitrogen, Carlsbad, CA), 100 ng/mL cholera toxin, 5 µg/mL insulin–transferrin–selenium (ITS), 0.2% bovine serum albumin (BSA), and 25 ng/mL keratinocyte growth factor (KGF). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and the medium, containing 200 µg/mL G418, was changed every two to three days.

Episomal expression vector construction. The pPyCAG expression vector was used for all transgene expression experiments. This vector was constructed from pCAGGS (Niwa et al. 1991) by inserting a puromycin-resistance cassette and a polyoma virus origin of replication (ori) fragment, which allows for stable episomal maintenance in PLT-expressing cells (Yamato et al. 2012). The cDNAs for mouse Pdx1, Mafa, and Neurod1 were amplified by proof-reading PCR, subcloned into pCR4 vectors (Invitrogen), and their sequences were verified. The verified cDNAs were then individually cloned into the pPyCAG vector. A control vector expressing EGFP was constructed in a similar manner.

Transfection and selection. PLT-ES and PLT-PPPD cells were transfected with the episomal expression vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For the experimental group, cells were co-transfected with an equimolar mixture of pPyCAG-Pdx1, pPyCAG-Mafa, and pPyCAG-Neurod1 (collectively termed PMN). For the control group, cells were transfected with pPyCAG-EGFP. Forty-eight hours post-transfection, the culture medium was replaced with fresh medium containing 1.5 µg/mL puromycin (Sigma-Aldrich) to select for transfected cells. The selection medium was renewed every 2–3 days, and cells were harvested for analysis 7–8 days post-transfection.

Insulin promoter reporter assay. To assess the direct activation of the insulin promoter, cells were

co-transfected with the PMN expression vectors (or EGFP control) and an insulin promoter-driven EGFP reporter plasmid (pIns-EGFP). EGFP expression was observed by fluorescence microscopy 7 days post-transfection.

cDNA microarray analysis. Total RNA was extracted from PMN- and EGFP-expressing cells 4 days post-selection using the Acid Guanidinium Thiocyanate-Phenol-Chloroform (AGPC) method. The quality and integrity of the purified RNA were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Approximately 500 ng of high-quality total RNA was used to synthesize cyanine 3 (Cy3)- or Cy5-labeled cRNA with the Low Input Quick Amp Labeling Kit (Agilent). Labeled cRNA was purified using RNeasy Mini Spin Columns (Qiagen, Hilden, Germany). For each comparison, 0.75 µg each of Cy3-labeled (control, EGFP) and Cy5-labeled (experimental, PMN) cRNA were combined, fragmented, and hybridized to an Agilent Whole Mouse Genome Oligo Microarray (4x44K). After washing, the arrays were scanned, and image data were quantified using Agilent Feature Extraction Software.

Gene enrichment and bioinformatic analysis. Differentially expressed genes were identified based on a fold-change threshold of >2.0. To understand the

biological significance of these changes, functional enrichment analysis of the gene lists was performed using Metascape (<https://metascape.org/>) (Zhou et al. 2019). This tool performs Gene Ontology (GO) enrichment analysis to identify overrepresented biological processes, molecular functions, and cellular components. Further ontology analysis was conducted using PaGenBase (<http://pagenbase.org/>), a repository of tissue- and time-specific gene expression patterns, to identify the cell types and tissues whose gene expression signatures most closely matched the profiles of our differentially expressed genes.

Results

Forced expression of PMN in pancreatic stem cells (PLT-PPPD) induces a mixed hepatic and pancreatic endocrine fate. Following PMN transduction into PLT-PPPD cells, we observed a significant induction of albumin, a canonical marker for hepatocytes. To explore the global transcriptional changes, we performed Gene Ontology (GO) analysis on the cohort of upregulated genes. This analysis revealed a significant enrichment of terms associated with liver function, including cytochrome P450 pathways and drug metabolism (Figure 1). Furthermore, an ontology search using

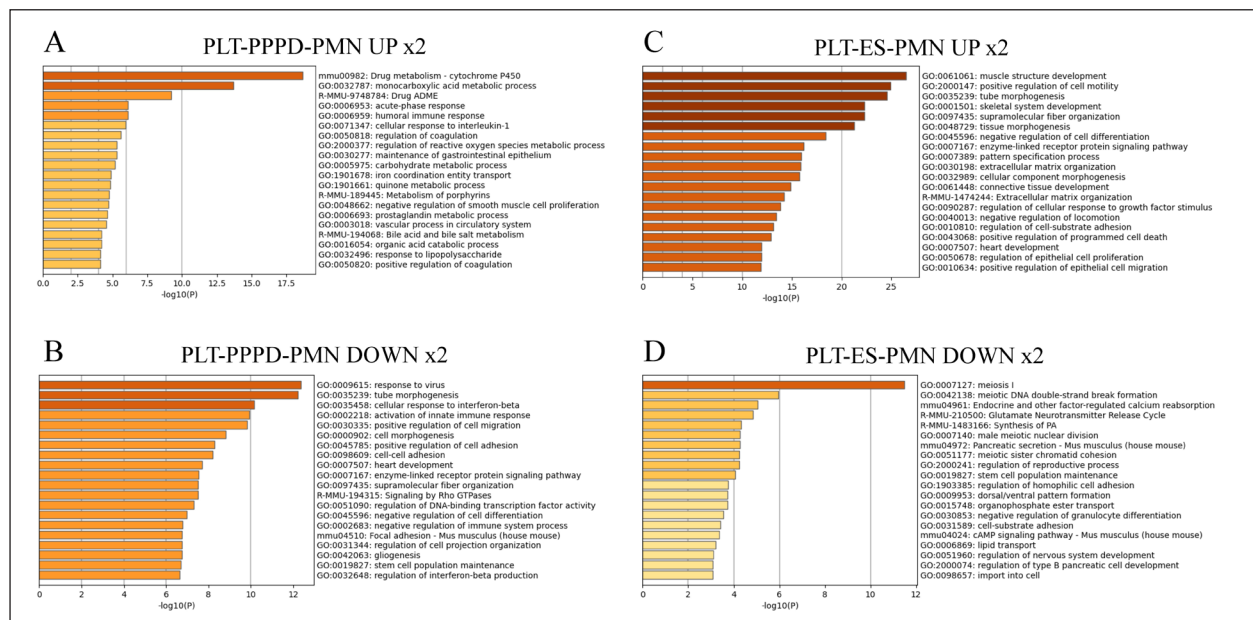


Figure 1. Gene Ontology (GO) enrichment analysis of differentially expressed genes. Metascape analysis was performed on genes differentially expressed between the PMN (Pdx1, Mafa, and NeuroD1) and EGFP (enhanced green fluorescent protein) control groups. The top enriched GO terms are shown for (A) upregulated genes in multipotent pancreatic stem cell line (PLT-PPPD) (1373 genes), (B) downregulated genes in PLT-PPPD cells (1822 genes), (C) upregulated genes in pluripotent embryonic stem cell line (PLT-ES) (1406 genes), and (D) downregulated genes in PLT-ES cells (860 genes).

the PaGenBase repository showed a striking accumulation of both liver-specific gene signatures and gene sets characteristic of the MIN6 pancreatic beta-cell line (Figure 2). Concurrently, GO analysis also indicated an upregulation of genes involved in the maintenance of the gastrointestinal epithelium, suggesting a broad endodermal response. In contrast, analysis of downregulated genes in PMN-transduced PLT-PPPD cells showed a significant suppression of gene sets related to the cardiovascular system.

Among the genes upregulated by at least two-fold upon PMN gene transfer in PLT-PPPD cells and PLT-ES cells, 204 genes were common between the two cell lines. Conversely, among the genes downregulated to half or less, 107 genes were shared.

PMN overexpression drives mesodermal differentiation in embryonic stem cells (PLT-ES). In sharp contrast to the endodermal fate induced in PLT-PPPD cells, GO analysis of genes induced by PMN in PLT-ES cells revealed a strong enrichment for mesodermal lineages. Specifically, we observed a significant upregulation of gene sets characteristic of muscle cells, vascular cells, and kidney tubular cells (Figure 1). This finding was further corroborated by PaGenBase ontology analysis, which demonstrated an accumulation of gene signatures specific to kidney collecting duct cells, fibroblasts, and myocytes

(Figure 2). Conversely, genes suppressed by PMN in PLT-ES cells were strongly associated with pluripotency and self-renewal. Notably, PaGenBase identified various ES cell lines as the cell types most representative of the downregulated gene expression signature. GO analysis also showed a significant suppression of meiosis-related genes.

PMN factors activate the insulin promoter in both pluripotent and progenitor contexts. To determine if the PMN factor combination could directly engage the insulin gene promoter regardless of cellular context, we co-transfected an insulin promoter-EGFP reporter plasmid. Interestingly, after 7 days, EGFP-positive cells were observed not only in the PLT-PPPD cell population, as expected, but also within the PLT-ES cell culture (Figure 3). Although the overall differentiation trajectory of PLT-ES cells was mesodermal, this result indicates that the PMN factors could still access and activate the specific insulin promoter locus even in an inappropriate pluripotent context.

PMN differentially regulates key stem and progenitor cell markers. To further dissect the divergent fates, we examined the expression of specific stem and progenitor cell markers. Nestin, a marker for neural stem cells but also expressed in pancreatic progenitors (Humphrey et al. 2010), was highly

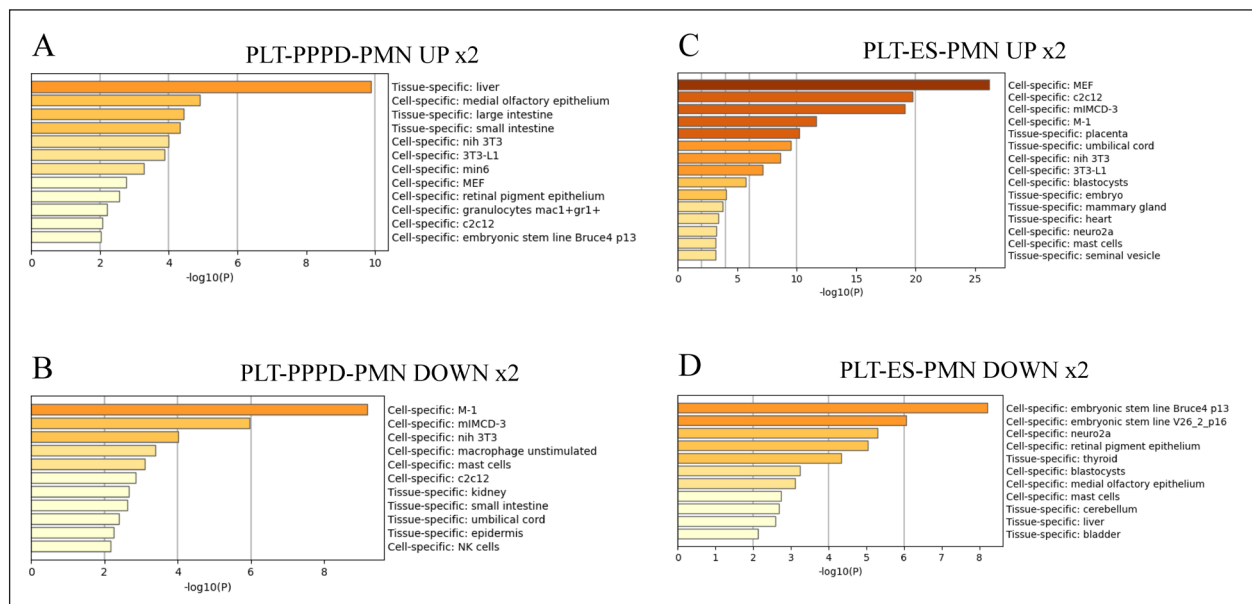


Figure 2. PaGenBase ontology analysis of differentially expressed genes. PaGenBase was used to identify the cell types whose gene expression signatures most closely matched the differentially expressed gene sets. The analysis was performed for (A) upregulated genes in multipotent pancreatic stem cell line (PLT-PPPD) (1373 genes), (B) downregulated genes in PLT-PPPD cells (1822 genes), (C) upregulated genes in pluripotent embryonic stem cell line (PLT-ES) (1406 genes), and (D) downregulated genes in PLT-ES cells (860 genes).

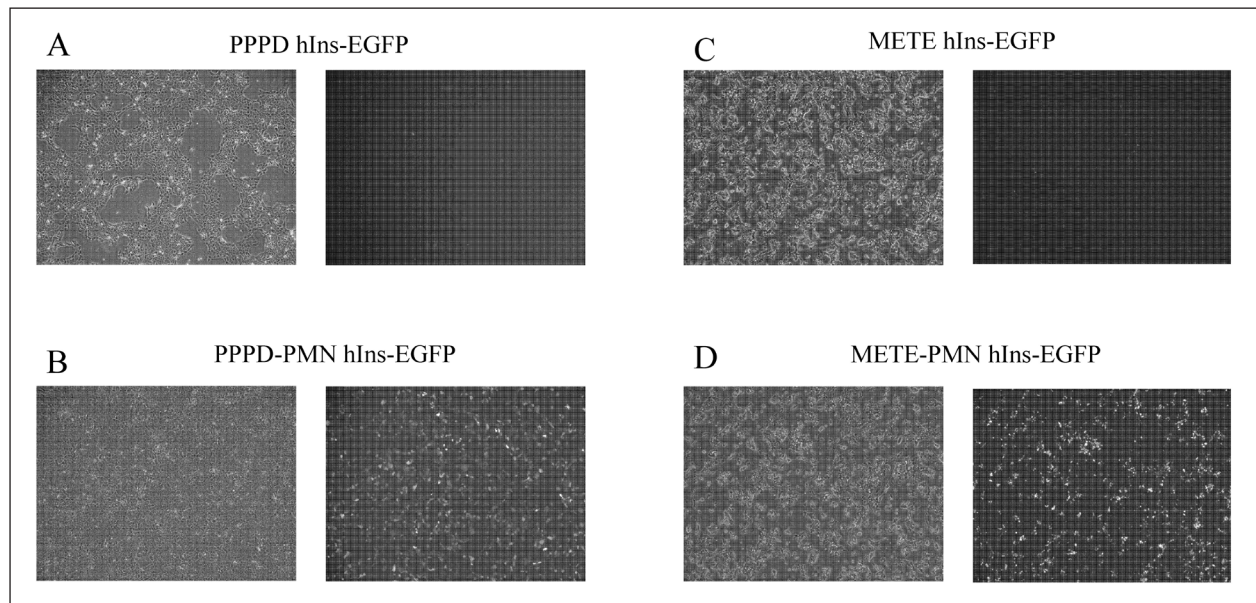


Figure 3. PMN factors (Pdx1, Mafa, and NeuroD1) activate the insulin promoter in both progenitor and pluripotent cells. Representative images of multipotent pancreatic stem cells (PLT-PPPD) (**A, B**) and pluripotent embryonic stem cell line (PLT-ES) (**C, D**) were taken 7 days post transfection. Cells were co-transfected with an insulin promoter-enhanced green fluorescent protein (EGFP) reporter plasmid and either a control vector (**A, C**) or PMN expression vectors (**B, D**). For each panel, the left image shows the phase-contrast view and the right image the corresponding fluorescence view. EGFP-positive cells appear bright white indicating insulin promoter activation.

expressed in our PLT-PPPD cells and was significantly suppressed upon PMN transduction. In PLT-ES cells, the opposite was observed, Nestin expression was low basally and was markedly upregulated by PMN introduction (Table 1). Sox9, a critical transcription factor for pancreatic progenitors (Seymour et al. 2007), was highly expressed in PLT-PPPD cells and, like Nestin, was downregulated following PMN-induced differentiation. In PLT-ES cells, Sox9 was expressed at low levels and remained unchanged by PMN. Finally, the core pluripotency factor Pou5f1 (Oct4) was, as expected, highly expressed in PLT-ES cells and was strongly suppressed by PMN. In PLT-PPPD cells, Pou5f1 expression was negligible and unaffected by the transgenes.

Divergent modulation of immune signaling pathways. Our transcriptomic analysis revealed a significant and divergent regulation of immune-related pathways. In PLT-PPPD cells, PMN transduction led to the activation of signals related to humoral immunity including a notable activation of the interleukin 1 (IL-1) signaling pathway. However, ontology analysis also indicated a suppression of the “response to virus” gene set. This was accompanied by a broad suppression of gene expression related to the interferon (IFN) signaling pathway and innate immunity.

Table 1
Expression levels of selected genes

Gene	PLT-PPPD		PLT-ES	
	EGFP	PMN	EGFP	PMN
Ins1	58.6	666.9	63.4	40.6
Ins2	52.6	869.2	19.0	23.5
Gcg	33.5	31.5	2.2	2.6
Sst	3.8	6.3	2.6	2.4
Alb	240.3	7210.7	2.1	2.4
Nes	236.2	68.0	86.6	216.0
Sox9	401.3	117.8	2.2	4.7
Pou5f1	56.3	89.8	5010.0	3491.6

Data represent relative expression levels obtained from the GeneChip microarray analysis. Abbreviations: EGFP – enhanced green fluorescent protein; PLT-ES – pluripotent embryonic stem cell line; PLT-PPPD – multipotent pancreatic stem cell line; PMN – Pdx1, MafA, NeuroD1.

Discussion

In this study, we demonstrate that the developmental fate induced by a defined set of transcription factors is fundamentally dictated by the identity of the starting cell population. Our comparative transcriptomic analysis revealed that the pancreatic master

regulators Pdx1, MafA, and NeuroD1 initiate vastly different genetic programs in pluripotent PLT-ES cells versus lineage-primed PLT-PPPD pancreatic stem cells.

The most striking finding in our PLT-PPPD cells was the induction of a mixed pancreatic and hepatic phenotypes. The upregulation of albumin and cytochrome P450-related genes strongly indicates a transdifferentiation towards a hepatocyte-like fate. This is consistent with the shared developmental origin of the liver and pancreas from the embryonic foregut endoderm, which is governed by a partially overlapping set of transcription factors (Gualdi et al. 1996). It suggests that while our PLT-PPPD cells are committed to a pancreatobiliary lineage, their potential is broader than previously appreciated and can be redirected towards a hepatic fate by this specific combination of factors.

In stark contrast, introducing the same factors into PLT-ES cells led to a robust mesodermal differentiation program. This result serves as a critical cautionary tale for directed differentiation strategies. It clearly shows that without prior lineage specification, key developmental regulators can function aberrantly pushing pluripotent cells down unintended pathways. Our findings strongly support the necessity of a stepwise differentiation protocol, where ES cells are first committed to definitive endoderm before being exposed to pancreas-specific factors (Pagliuca et al. 2014). Any residual undifferentiated PLT-ES cells in such a protocol would pose a risk of generating undesirable mesodermal cell contaminants. Notably, our observation aligns with recent reports suggesting a role for MafA in myogenesis highlighting the pleiotropic nature of these factors (Kim et al. 2021).

A particularly intriguing finding of this study was the activation of an insulin promoter-reporter in PLT-ES cells, despite their wholesale commitment to mesodermal lineages. This apparent paradox provides a deeper insight into the mechanism of directed differentiation. It suggests a decoupling between the ability of transcription factors to bind and activate a specific promoter locus and their ability to orchestrate the complex genome-wide program required for stable cell fate determination. While the PMN factors could find and switch on the insulin promoter even in the foreign epigenetic landscape of an ES cell, they failed to execute the complete

endodermal differentiation program. This powerfully reinforces our central conclusion that the appropriate cellular context, as found in our PLT-PPPD cells, is not merely permissive, but instructive providing the necessary framework for transcription factors to function correctly and drive a cell towards a stable functional identity.

The differential regulation of stem cell markers further illuminates these divergent paths. In PLT-PPPD cells, the downregulation of progenitor markers like Nestin and Sox9 is consistent with their differentiation and exit from the progenitor state. Conversely, the upregulation of Nestin in PLT-ES cells suggests that PMN may be inducing an intermediate tissue-specific progenitor-like state, but one that is mesodermally rather than endodermally specified. The suppression of Pou5f1 in PLT-ES cells confirms the loss of pluripotency, which appears to be a prerequisite for any differentiation.

Finally, our data point to a complex and intriguing interplay between differentiation and the immune system. The activation of IL-1 signaling in differentiating PLT-PPPD cells is noteworthy, as IL-1 has been reported to play a role in beta-cell function and maintenance (Mandrup-Poulsen et al. 2010). Simultaneously, we observed a suppression of innate immune pathways including IFN signaling. This is reminiscent of embryonic development, during which IFN-mediated antiviral responses are known to be attenuated to allow for proper morphogenesis (Eggenberger et al. 2019). It is plausible that the PMN factors recapitulate this developmental program suppressing certain innate immune modules to create a permissive environment for differentiation.

Conclusion

Our study provides a high-resolution map of how cellular context dictates the function of master regulatory transcription factors. We show that pancreatic stem cells can be driven towards a mixed pancreatic/hepatic fate, while pluripotent stem cells are misdirected towards mesoderm. These findings underscore the critical importance of selecting a well-defined lineage-primed cell population for predictable and efficient cell-based therapies and reveal an unexpected link between cellular differentiation and the modulation of innate immune pathways.

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