Stem Cells as a Possible Cell-based Therapy for the Treatment of Type I Diabetes

Since the last reporting period, we have completed the experiments aimed at demonstrating the functional activation of Cx43 Gap Junction channel. In these experiments, we have been able to demonstrate that treatment of our stem cell cultures with the Cx43-activatin peptide AAP10, promotes a dramatic increase in Gap Junction communication, as demonstrated by the increased exchange of a fluorescent dye between cells in contact in a dye-transfer assay.

As shown in the Figure below, cells grown in the presence of the AAP10 peptide (Figure, lower panels)

, under conditions that promotes their differentiation toward Definitive Endoderm, and later into Pancreatic Progenitors,

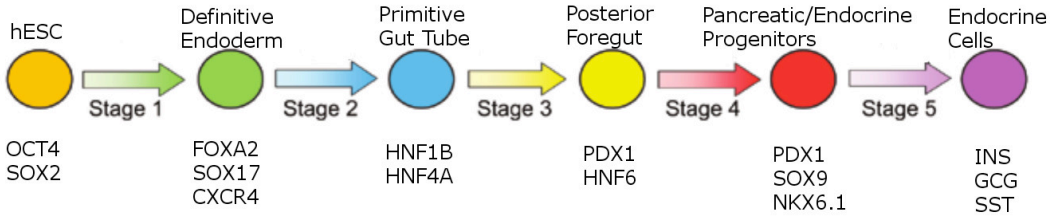

Figure 0 *Stage specific markers of differentiation.*
Each stage of differentiation can be identified by the presence of specific cell markers. Some representative markers are shown in the figure. The stages are as follows: embryonic stem cell (hESC), definitive endoderm (DE), primitive gut tube (PG), posterior foregut (PF), pancreatic progenitors (PP), endocrine progenitors (EP), and β-cells (EC).

Modulation of cell-to-cell communication as a possible strategy to improve the specification of stem cells toward the islet cell lineage.

Our lab has a long-standing interest in the study of mechanisms that regulated cell-to-cell communication mechanisms and its function in hastening development of differentiating stem cells. In this regard, a specialized form of intercellular communication is the one mediated by Gap Junction (GJ) channels. GJ channels are unique in that they allow for the rapid exchange of biochemical signals between cells in contact bypassing the extracellular space.

Building on these notions, a major tenet of my thesis work is that Connexins (Cxs), the building blocks of GJ channels, contribute to stem cell differentiation toward select cell lineages. Specifically, my work tested the hypothesis that the targeted manipulation of select Cxs at specific stages of stem cell differentiation may be exploited to enhance the *ex vivo* derivation of the pancreatic islet cell types.

SUMMARY OF PROGRESS

Over the past six months, I performed studies that focused on the activation of Cx43 function using a novel peptide (AAP10) that once added to cell cultures induce the phosphorylation of this connexins, and opening of Gap Junction made of Cx43, which in turn results in a greater cell-to-cell communication between adjacent cells (21,22).

Specifically, we conducted a series of experiments in which stem cells were differentiated in the presence or absence of the AAP10 peptide, and monitored for their ability to produce Definitive Endoderm, and, subsequently, pancreatic cell lineages.

The results of these studies show that addition of AAP10 to the culture media during the directed differentiation of stem cells toward pancreatic cell lineages leads to a significant increase in the number of Definitive Endoderm (DE) cells. Interestingly, when we continued the differentiation of these AAP10-treated cultures in the presence of this Gap Junction-activating peptide, we also observed a significant increase in the number $Pdx1^+$ and $Nkx6.1^+$ cells, as well as $Pdx1^+/Sox9^+$ co-expressing cells. Subsequently, we also discovered that, to achieve this enhanced differentiation of stem cells toward pancreatic progenitors, the treatment with AAP10 is only required during the early stages of differentiation (i.e. up to DE stage), when Cx43 expression levels are most prominent. These results suggest that AAP10 (or increased utilization of Cx43-Gap Junctions) promote the development of a Definitive Endoderm that has a high propensity to commit toward the pancreatic cell lineage. These are important discoveries since recent evidence indicate that without an effective early induction of Definitive Endoderm the commitment toward pancreatic endoderm and islet progenitors remain relatively low.

Therefore, we believe that this is an important milestone in stem cell biology, as increasing the yields of pancreatic islet cells from undifferentiated stem-cell preparations will have significant implications for perfecting protocols for the derivation of insulin-producing cells to treat Type 1 Diabetes.

Cx43 Expression Pattern in hESC under spontaneous differentiation

To investigate the expression pattern of Cx43 during the specification of stem cells toward the three main germ cell lineages in an unbiased manner, we used shESC cultures stontaneously differentiating into embryoid bodies (EBs), a model system that is known to produce Ectoderm,

Mesoderm and Endoderm lineages. In these experiments we discovered that Cx43 is prominently expressed in Definitive Endoderm cells (Figure 2), and at lower levels in Mesendoderm cells (see below).

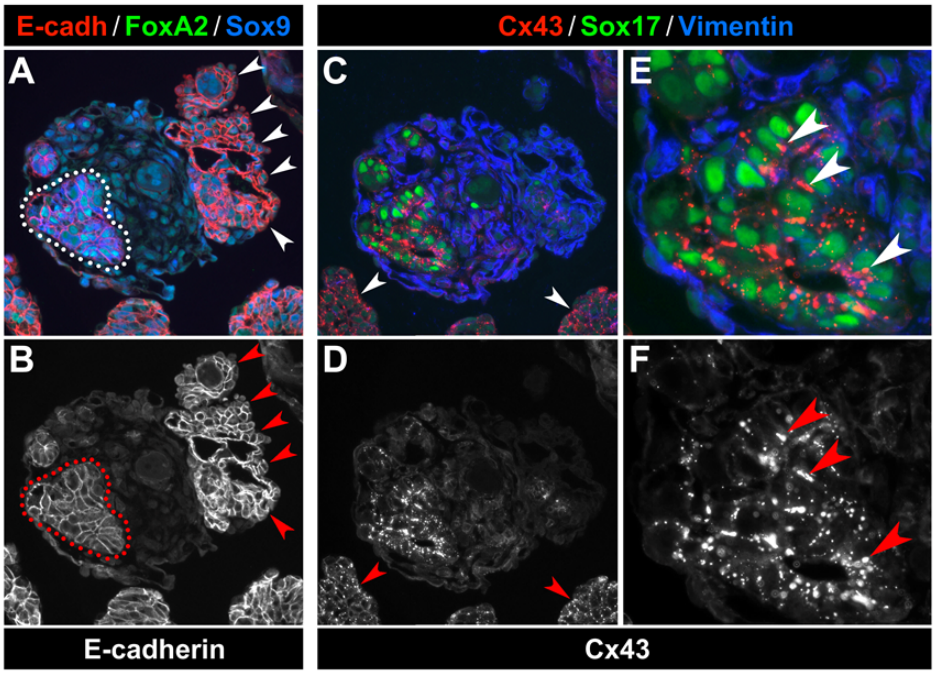

Figure 1: *Cx43 is preferentially expressed in Mesendoderm and Definitive Endoderm developing from hESC in vitro.*

Embryoid Bodies (EBs), used as an in vitro *model of spontaneous hESC differentiation, and immunostained for E-cadherin (red), FoxA2 (green), and Sox9 (blue) identified all three germ layers: Ectoderm (**A**, arrowheads), Endoderm (**A**, dotted line) and Mesoderm (**A**, cells negative for E-cadherin). (**B**) Monochromatic imaging from the microscopic field shown in panel A allows better identification of E-cadherin^{+} cells. (**C**) Serial sections from the same EBs immunostained for Cx43 (red), Sox17 (Green) and Vimentin (blue) revealed that Cx43 expression was enriched in Definitive Endoderm cells, as defined by the co-expression of E-cadherin, FoxA2, and Sox9 (**A**, dotted line), and Sox17 (**C** and **E**, green). Significant Cx43-specific immunoreactivity was also detected in ME cells, identified in a sequential section by the expression of E-cadherin and Sox9, but not Sox17 (**C** and **D**, arrowheads). Panels **E** and **F** show higher magnification of Definitive Endoderm cells identified in **C** and **D**. Note that immunoreactivity specific for Cx43 is not detected at significant levels in Mesoderm cells, identified by Vimentin expression and lack of E-cadherin immunoreactivity. Images are representative of n=12 fields (Cx43-Sox17-Vimentin) and n=10 fields (Ecad-Foxa2-Sox9).*

Next, to determine the expression profile of Cx43 in stem cell development toward pancreatic progenitors, we profiled Cx43 transcript and protein in stem cells collected at specific stages of differentiation (Figure 3A). We found that gene expression of Cx43 steadily rose throughout the first three stages of differentiation: Mesendoderm (ME), Definitive Endoderm (DE), and Primitive Gut Tube (PGT). After these stages, e.g. Posterior Foregut (PF), Cx43 gene expression sharply declined (Figure 3B). Western blot for Cx43 showed a large increase in expression during the first three stages of differentiation followed by a drop off at later stages. Cx36 protein was upregulated at DE and slowly dropped off from there (Figure 3C).

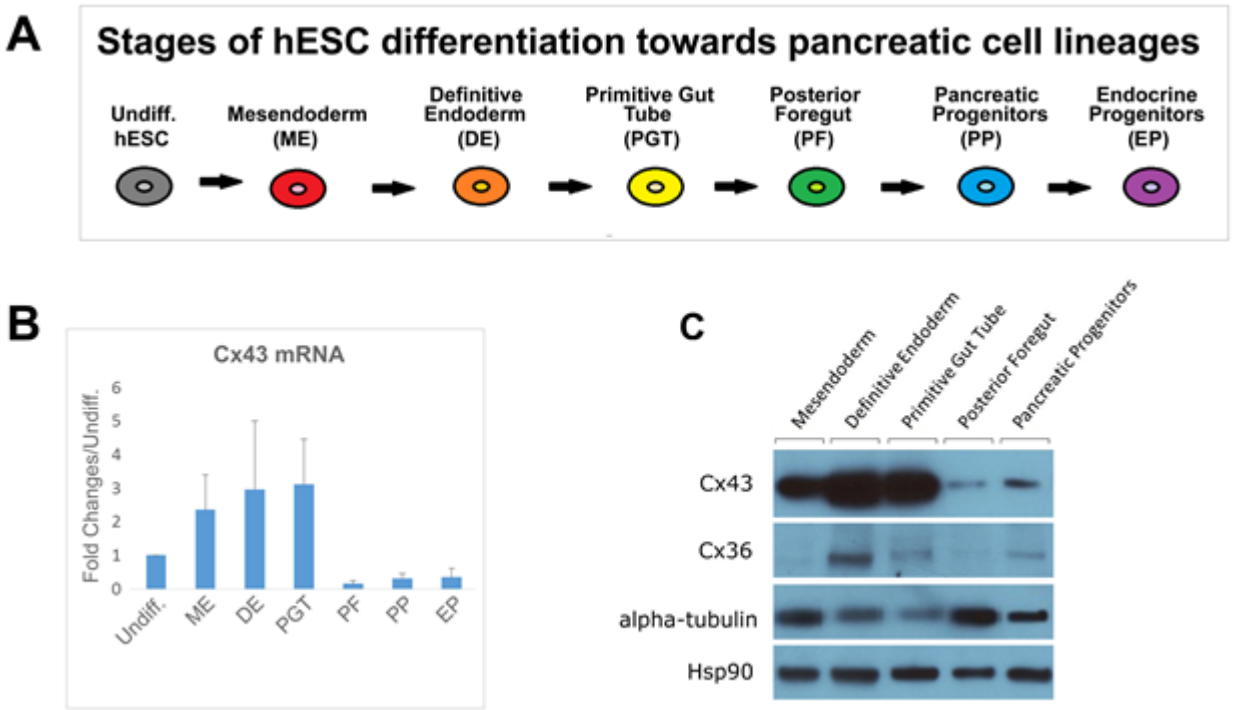

Figure 0: *Expression profile of Cx43 in hESC directed to differentiate towards pancreatic cell lineages. (**A**) Schematic of step-wise protocol for the directed differentiation of hESC into pancreatic cell lineages. (**B**) Gene expression of Cx43 throughout all stages of hESC differentiation (n=4). Expression of Cx43, normalized to levels present in undifferentiated hESC, progressively increases during early stages of differentiation into ME, DE, and PGT, followed by a sudden decline following the specification of downstream cell lineages (i.e., PF). (**C**) Gene expression data for Cx43 was validated at the protein level by Western blotting using α-tubulin and Hsp90 as a loading control.*

Cx43 Gain of Function using AAP10

The AAP10 peptide was originally reported as an antiarrhythmic peptide due to its ability to constitutively open Cx43 channels and synchronize beating heart cells (21,22). We utilized this property as a gain-of-function strategy in our experiments of hESCs differentiation toward pancreatic cell lineages. Hence, we conducted gene expression analysis of DE markers FoxA2 and Sox17 (**Figure 2**A). Gene expression of FoxA2 and Sox17 was significantly increased at the DE and PGT stages in cultures treated with 400ng/mL AAP10 compared to untreated cells ($p<0.01$). FoxA2 and Sox17 steadily declined through the later stages of differentiation. This result was recapitulated by western blotting where there was an induction of Sox17 in DE nuclear lysates of cells treated with peptide (**Figure 2**D). This indicated a greater induction of DE cells as a result of Cx43 activation by AAP10.

To quantitatively validate these biochemical data, we performed a FACS analysis on both, control (no peptide) and in AAP10-treated cultures following immunostaining for E-cadherin and CXCR4. As shown in Figure 4B, we observed that addition of AAP10 to stem cell cultures increased the percentage of DE cells from an average of 36% to 51.5% (i.e. a 52% increase in DE cells). The induction of DE with AAP10 was further quantified by immunostaining cells fixed at the DE stage. Cells were stained using intracellular markers for DE such as FoxA2 and Sox17 in combination with Cx43 and Nanog. In these experiments, we observed that cells treated with AAP10 displayed a marked increase in FoxA2 nuclear immunoreactivity demonstrating an induction of DE type cells. In addition, while AAP10 did not increase total protein content of Cx43, peptide treated cells were found to exhibit much larger Cx43 plaques (**Figure 2**C).

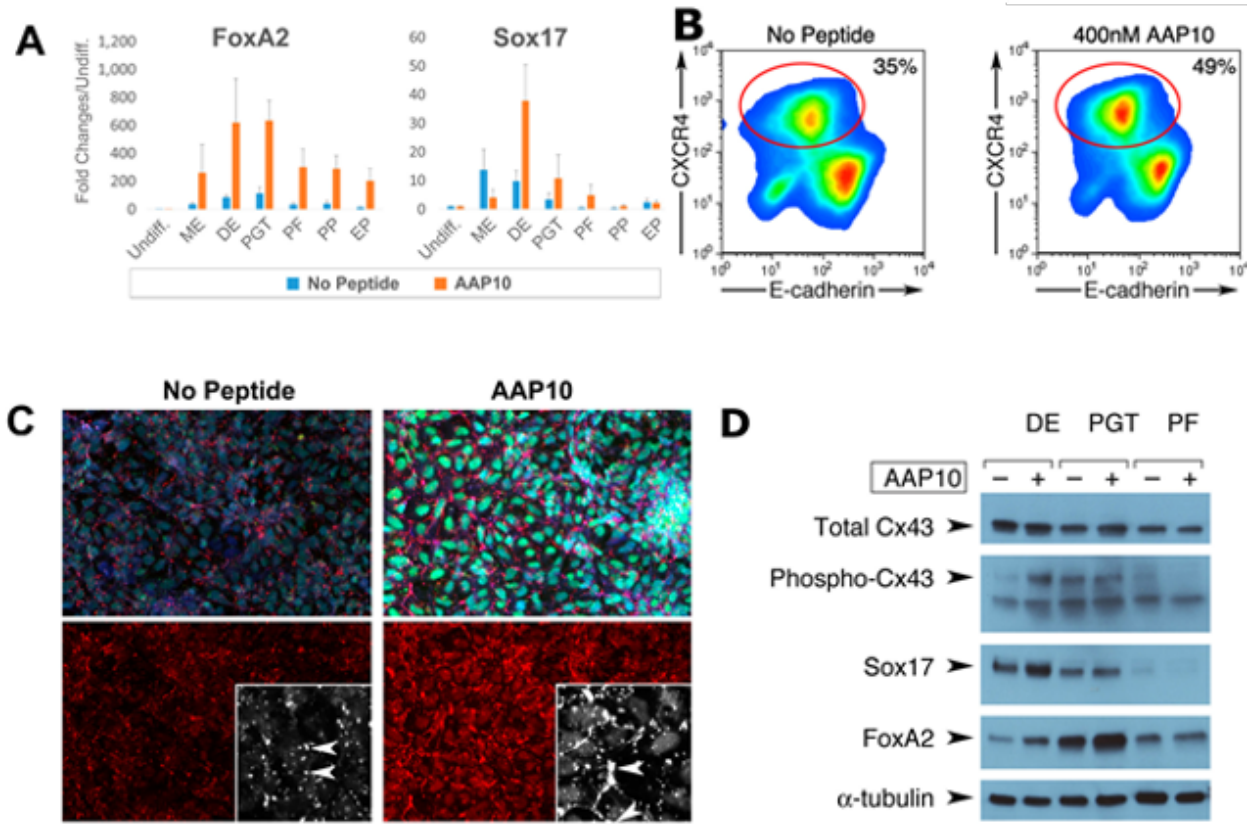

Figure 2: *Induction of Definitive Endoderm is enhanced by the Cx43 agonist AAP10 peptide. (**A**) Gene expression of FoxA2 and Sox17 throughout all stages of differentiation. Gene expression is significantly increased at DE and PGT stages in cultures treated with 400ng/ml AAP10 ($p < 0.01$), followed by a decline during subsequent stages. This indicates a greater induction of DE cells as a result of Cx43 activation by the AAP10 peptide. (No peptide n=4, Peptide Thru n=3) (**B**) FACS analysis of DE cells. Cells were differentiated to the DE stage and stained for E-cadherin and CXCR4. Addition of AAP10 resulted in a marked increase of double positive cells from $\bar{x} = 36\%$ to $\bar{x} = 51.5\%$ double positive cells (i.e., 52% increase). (FACS is representative of n=4) (**C**) Corresponding immunostaining of H1 cells at DE with and without peptide treatment. Cells treated with AAP10 show a marked increase in frequency and levels of expression of FoxA2 demonstrating an induction of DE cells. In addition, while AAP10 did not increase total protein content of Cx43, peptide treated cells were shown to have much larger Cx43 plaques (insets, arrowheads). (Staining is representative of n=3 fields). (**D**) Western blot in differentiated H1 cells showed a greater induction of Sox17 with addition of peptide compared to untreated cells at the DE stage.*

AAP10 is only necessary during early stages of differentiation

To determine the effects of AAP10 on the developmental potential of these DE cells, later stages of differentiation were analyzed for the expression of endocrine progenitor-specific transcription factors Pdx1, Sox9, and Nkx6.1. Cells grown on coverslips were fixed at the end of differentiation and then immunostained by two-color immunofluorescence for Pdx1/Nkx6.1 and Sox9/Pdx1. In these studies we observed that AAP10-treated cells were significantly enriched for pancreatic progenitors as defined by co-expression of Pdx1 and Sox9. Interestingly, while treatment with AAP10 throughout the entire duration of the differentiation resulted in a significant increase in the number of $Pdx1^+$ pancreatic progenitors, compared to control cultures (i.e., no peptide), when AAP10 treatment was applied only up to the DE stage (referred to as Pepstop) we observed a higher number of cells co-expressing Pdx1 and Sox9 (One way ANOVA p = 0.0664, post test for linear trend p =0.0069), with a concomitant increase of the total number of $Sox9^+$/$Ecadherin^+$ cells (One way ANOVA p = 0.0048, post test for linear trend p= 0.0007) (**Figure 3**A). Collectively, there was a significant increase in $Pdx1^+$ cells in later stages of differentiation in both peptide-treated conditions compared to no peptide (One way ANOVA p = 0.0246, post test for linear trend p = 0.0017) (**Figure 3**B). These results indicate that activation of Cx43 gap junction channels by AAP10 is an effective treatment for the derivation of a significantly larger number of $Pdx1^+$ progenitors compared to conventional protocols of differentiation. Furthermore, based on the observation that AAP10 treatment was effective in producing significantly higher numbers of $Pdx1^+$/$Sox9^+$ progenitors when applied up to the DE stage of differentiation (Pepstop), we speculate that these results may be explained by the requirement of Cx43-mediated cell-to-cell communication, or signaling, during early induction of DE cells that holds a higher propensity to further differentiate toward pancreatic progenitors.

Collectively, our results suggest that enhancing the activity of Cx43-Gap Junctions with AAP10 drives the specification of a larger number of DE cells toward pancreatic progenitors. We conclude that cell-to-cell communication through Cx43 Gap Junctions is required to recruit heterogeneous hESC populations into differentiation programs toward Definitive Endoderm and Pancreatic Progenitors.

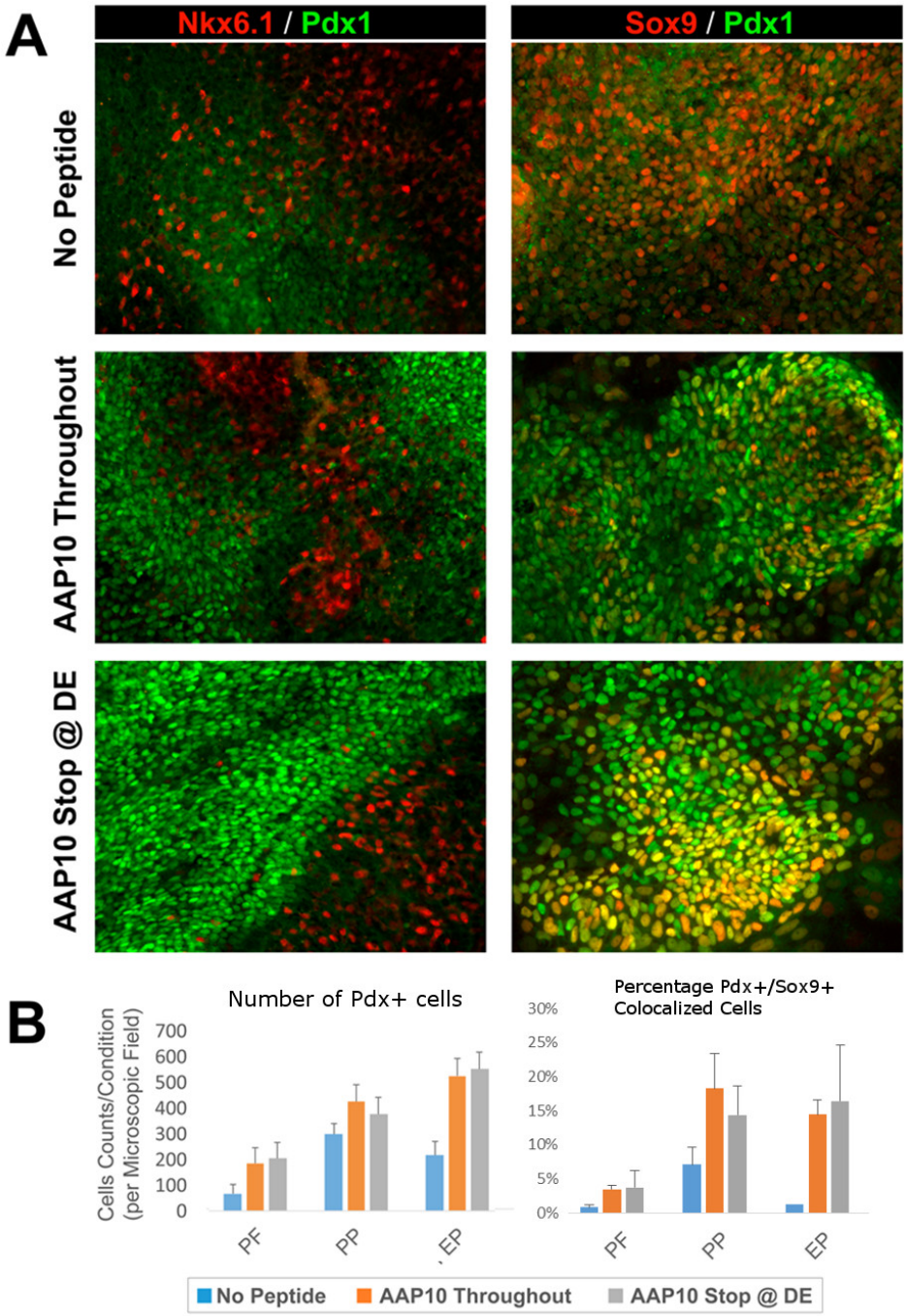

Figure 3: *Addition of AAP10 during early stages results in greater induction of Pdx1^{+} cells.* (***A***) *Immunostaining of end-stage cells (i.e. Endocrine Progenitors; EP) for Pdx1/Nkx6.1 and Sox9/Pdx1. In "no peptide" condition (n=3), there is very little Pdx1 and a greater number of Nkx6.1 and Sox9 indicating a possible pancreatic population but few cells that will further develop into endocrine cells. In samples treated with AAP10 throughout the duration of the differentiation (n=3), there is a greater induction of Pdx1 cells compared to no peptide. Interestingly, when the AAP10 peptide was added to the cultures only up to the DE stage (i.e., Peptide stop @ DE) (n=2), we*

*observed an even higher induction of Pdx1 positive cells, suggesting that the purposeful activation (i.e., phosphorylation) of Cx43 by AAP10 during stages of highest Cx43 expression (see Figures 2 and 3) plays an important role in early commitment towards islet cell lineage, as opposed to a steady activation of Cx43 throughout all stages of differentiation. This possibility is suggested not only by a significant increase in the number of Pdx1 positive cells, (**B**) but also by the higher frequency of Sox9/Pdx1 co-expressing cells, known to represent endocrine-committed progenitors likely to further develop into beta cells. There is also a significant difference in the number of Sox9 cells.*

Mechanistic evidence for increased cell-cell communication in AAP10-treated cells:

During the last three months of the project we conducted a final set of experiments in which we tested the impact of AAP10 treatment on cell-to-cell communication. In these experiments, using a dye transfer assay, we have been able to demonstrate that treatment of our stem cell cultures with the Cx43-activating peptide AAP10 promotes a dramatic increase in Gap Junction communication, as demonstrated by the increased exchange of the fluorescent dye *Fluorescein isothiocyanate* (FITC) between cells in contact (Figure 6). In this assay, cells were let to reach confluence under conditions that promotes their differentiation toward Definitive Endoderm. Subsequently, the green-fluorescent FITC was added to the medium, and a scratch made across the cell monolayer with a micropipette tip. This procedure causes a transient wounding along the edge of cells scraped that allows entry of the FITC dye into cells lining the wound. As it can be observed in Figure 6, by 5 minutes following the wound a significant fluorescent signal can be detected in both, the control (upper panels) and the AAP10-treated cells (lower panels). Interestingly, after 15 minutes, while in control cells the FITC dye remained confined to the cells lining the wound, AAP10-treated cells exhibit a significant increase in the territory of FITC transfer among cells in contact. These results indicate that activation of Cx43-Gap Junction channels by the AAP10 peptide promotes an increased cell-to-cell communication that allows

Figure 6: Dye transfer assay to test Cx43 Gap Junction function. *Under control culture conditions minimal FITC transfer is observed between cells in contact (Upper Panels). Treatment with the Cx43 activating peptide AAP10 promotes a significant time-dependent FITC dye transfer between cells in contact, as measured by the increase territory of green-fluorescent cells (Lower panels).*

the exchange of molecules between contacting cells. Hence, from these studies we conclude that activation of Cx43 by the AAP10 peptide represents a fundamental mechanism by which stem cells can be fostered to adopt a Definitive Endoderm phenotype, and functionally validate our earlier results of increased production of Sox9+Pdx1+ Pancreatic Progenitors in cultures supplemented with the AAP10 peptide. We are now writing a manuscript that we will submit for publication by the end of the summer.

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