Human Pancreatic β-Cell G1/S Molecule Cell Cycle Atlas

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Expansion of pancreatic β-cells is a key goal of diabetes research, yet induction of adult human β-cell replication has proven frustratingly difficult. In part, this reflects a lack of understanding of cell cycle control in the human β-cell. Here, we provide a comprehensive immunocytochemical “atlas” of G1/S control molecules in the human β-cell. This atlas reveals that the majority of these molecules, previously known to be present in islets, are actually present in the β-cell. More importantly, and in contrast to anticipated results, the human β-cell G1/S atlas reveals that almost all of the critical G1/S cell cycle control molecules are located in the cytoplasm of the quiescent human β-cell. Indeed, the only nuclear G1/S molecules are the cell cycle inhibitors, pRb, p57, and variably, p21; none of the cyclins or cdks necessary to drive human β-cell proliferation are present in the nuclear compartment. This observation may provide an explanation for the refractoriness of human β-cells to proliferation. Thus, in addition to known obstacles to human β-cell proliferation, restriction of G1/S molecules to the cytoplasm of the human β-cell represents an unanticipated obstacle to therapeutic human β-cell expansion.

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Both type 1 and type 2 diabetes ultimately result from β-cell deficiency. Although β-cell replacement in humans can reverse diabetes, the paucity of β-cells available from adult or juvenile human cadaveric islets, or from hESC cell or iPSC cell sources, makes this approach untenable for β-cell replacement therapy on a public health scale. Accordingly, a major goal of diabetes research is to develop means to induce human β-cell proliferation and expansion, targeting either endogenous human β-cells or β-cells grown ex vivo. This desire to expand human β-cells is complicated by the fact that while there are many models of β-cell replication in juvenile rodents, adult cadaveric human β-cells—the major source of β-cells available for research and therapeutic manipulation—are notoriously refractory to induction of replication: indeed, no growth factors, mitogens, or (patho)physiologic maneuvers (such as pregnancy, partial pancreatectomy, or obesity) are known that are able to induce high rates of adult human β-cell proliferation (1–12). Equally perplexingly, we and others previously delineated the repertoire of G1/S regulatory proteins present in the adult human islet and have used this information to develop a working model of the “human islet G1/S proteome” (12–29), hoping that it might be useful in developing therapeutic approaches to manipulating human β-cell proliferation. Since many, and perhaps most, G1/S molecules are regulated at the level of protein stability, rather than or in addition to transcription (24,26,29), we have focused in this G1/S model on immunoblots of whole human islets rather than exploring mRNA expression of these molecules. The G1/S model has proven useful in predicting approaches to driving human β-cell proliferation in vitro and in vivo systems. For example, the model accurately predicted that it should be possible to induce pRb phosphorylation (and thus its inactivation) and thereby markedly activate adult human β-cell replication (10–15% as assessed using BrdU incorporation or Ki67 immunohistochemistry) by overexpression of combinations of G1/S cyclins and cdks such as the d-cyclins, cyclin E, or cdks 2, 4, or 6 both in cultured adult human β-cells and in transplanted adult human β-cells in vivo (21–23,26). Further, it is also possible to use cyclin/cdk combinations to induce human β-cell proliferation not only constitutively or continuously but also using doxycycline-inducible delivery systems to transiently induce human β-cell proliferation in a regulated, reversible fashion that mimics the transitory replication that occurs in embryonic and neonatal life (28).

However, the human islet G1/S proteome model is not perfect. One major limitation is that it was derived from immunoblots of whole human islets. This is problematic because it is well-known that human islets are composed of many cells types in addition to β-cells. Indeed, β-cells frequently comprise <50% of all islet cells (30–32). Thus, while it may serve as a human islet G1/S proteome, it is not necessarily an accurate human β-cell G1/S proteome. Accordingly, we next wanted to rigorously define which of the 25 or more G1/S molecules are actually present in the human β-cell. More specifically, we wanted to use immunohistochemical and subcellular fractionation approaches.
to develop a “human adult β-cell G1/S proteome atlas” that accurately defines and displays which G1/S molecules are present in the human β-cell, that might suggest hypotheses as to which molecules maintain human β-cell quiescence or drive proliferation, and that might effectively reveal targets for therapeutic induction of human β-cell proliferation.

Here, we describe this human β-cell G1/S proteome atlas that we believe is a valuable resource for the β-cell research community. One major finding is that each of the G1/S proteins, previously reported to be present in human islets, is actually present in the human β-cell, permitting the deployment of an accurate human β-cell G1/S proteome model. A second major and wholly unanticipated finding is that the majority of the G1/S molecules, which we had presumed to be nuclear proteins, are in fact cytoplasmic in location in human β-cells, presumably unavailable to drive cell cycle progression. This may represent an unanticipated obstacle to induction of proliferation of human β-cell.

RESEARCH DESIGN AND METHODS

Human cadaveric islets. Human islets were purchased from the National Institutes of Health– and JDRF-supported Integrated Islet Distribution Program (iidp.coh.org) and also generously provided by Dr. Tatsuya Kin at the Clinical Research Center for Diabetes and Endocrinology, National Institutes of Health. Islets from two sources. One was the Network for Pancreatic Organ Donors developed by the JDRF (www.jdrfnpod.org). These pancreas specimens were obtained from four donors. Intact normal adult pancreas was obtained from two sources. One was the Network for Pancreatic Organ Donors developed by the JDRF (www.jdrfnpod.org). These pancreas specimens were purchased from Vector Biolabs (Philadelphia, PA). Dispersed islets on coverslips with 50 μg/mL trypsin and then incubated for 20 min at 37°C. During the digestion, the cells were then centrifuged for 3 min at 600 × g and washed with PBS and incubated in blocking buffer (1.0% BSA, 0.5% Triton, and 1.5 mmol/L MgCl2; 10 mmol/L EDTA; 10% glycerol; 0.1 mmol/L Na3VO4; 1% phenylmethylsulfonyl fluoride; and 20 μg/mL aprotinin) and then cooled at 25°C. As a positive control, when possible dispersed islets on coverslips with 50 μg/mL trypsin and then incubated for 2 min at 37°C were added to a fresh tube. The nuclear pellet was then resuspended in 0.35 mol/L sucrose buffer (0.35 mol/L sucrose; 10 mmol/L MgCl2; 10 mmol/L HEPES, pH 7.5, and 1% Triton), layered over 0.35 mol/L sucrose buffer (0.35 mol/L sucrose; 10 mmol/L MgCl2; 10 mmol/L HEPES, pH 7.5, and 1% Triton), and centrifuged at 4°C for 5 min at 1,400g. The nuclear pellet was then resuspended in 50 μL nuclear extract buffer (20 mmol/L HEPES, pH 7.5; 0.1 mmol/L EDTA; 5 mmol/L MgCl2; 0.5 mol/L NaCl; 20% glycerol; 1% NP-40; 0.1 mmol/L Na3VO4; 1% phenylmethylsulfonyl fluoride; and 20 μg/mL aprotinin) and then plated onto ice on a rotating shaker for 4 h, sonicated, and centrifuged at 16,000g for 10 min at 4°C. The supernatant (nuclear fraction) was collected and transferred into a fresh tube. The nuclear and cytoplasmic extracts were compared with extracts of whole islets prepared as previously described (21,22). Results shown are representative of three to six separate human islet preparations.

Immunoblotting. Islet extracts were resolved using 10 or 12% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Antibodies used to detect the G1/S molecules are described in detail in Supplementary Table 1. Each experiment shown is representative of three to six human islet preparations.

RESULTS

Localization of the pocket proteins and E2F family of transcription factors in human β-cells. Our initial objective was to comprehensively document which of the G1/S family of proteins are present in the human β-cell, using immunohistochemical colabeling for insulin and cell cycle proteins in dispersed cadaveric adult human islet cells. As can be seen in Fig. 1, the retinoblastoma protein pRb is present in the human β-cell (as well as in non-β-cells) and, as anticipated, is principally in the nuclear compartment, being observed in the nuclei of 65 ± 7% (mean ± SEM) of human β-cells in five different islet preparations. The other two pocket proteins, p107 and p130, are also present in the β-cell but, surprisingly, appear in the cytoplasmic compartment and not the nuclear compartment. The specificity of the labeling for the pocket proteins was confirmed for pRb (using SA02 human osteosarcoma cells, which are null for pRb) and supported for p107 and p130 by the absence of labeling when the primary antisera for p107 and p130 were omitted.

The E2F family of transcription factors, E2Fs1–8, was examined next (Fig. 2). E2Fs1–7 were observed in the human β-cell, whereas E2Fs2 was not easily detected. Surprisingly, despite being transcription factors, each was absent from the nuclear compartment but was predominantly present in the cytoplasmic compartment. The only exception to this was E2F4 (Fig. 2C), which was predominantly cytoplasmic but also nuclear, as confirmed with nucleolin immunolabeling (Fig. 3). The
specificity was supported, where possible, by blocking the primary antibody using the cognate antigenic peptide or, where no antigenic peptide was available, by omitting the primary antibody. E2F1–3 immunochemistry was also enhanced by their adenoviral overexpression as shown in Fig. 2B, where it can be seen that labeling for each of these E2Fs increased in intensity, and most remained cytoplasmatic, even when overexpressed—an exception being E2F2, which also became nuclear in some cells.

Collectively, these observations indicate that with the exception of pRB, all of the other pocket proteins and most of the E2Fs are located in the cytoplasm of the human β-cell.

Cyclins A and E and cdk1 and -2 in the human β-cell.

We next examined the “late G1/S cyclins,” cyclins E and A, and their cdk partners, cdk1 and cdk2 (Fig. 4A). Once again, each of these molecules was present in the β-cell, but each was present predominantly in the cytoplasm. Specificity was confirmed where possible with competing peptides (Fig. 4A) or supported by omission of primary antiserum. As in the accompanying manuscript, the ages were 23, 34, 35, 39, 42, 45, and 45 years old, with a mean of 37.8 years. The BMIs of the subjects were 24, 25, 25.6, 26.6, 26.9, 26.9, 29, and 32.5 kg/m², with a mean BMI of 27.1 kg/m². These values were not significantly different from those of the group as a whole, and there was no correlation between age, BMI, and nuclear versus cytoplasmic location of p21. The specificity of the p21 and p27 immunostaining was supported by lack of primary antiserum (Fig. 6B), and adenoviral overexpression enhanced their immunolabeling (Fig. 6C). The nuclear-cytoplasmic distribution of the INK4s and CIP/KIPs is quantified and statistically validated in the accompanying manuscript (34). Interestingly, cdk6, p27, and p16 also display a cytoplasmic localization in α- and Δ-cells, as shown in Supplementary Fig. 1.
Parenthetically, the ability to observe p57, as with pRb, in the nuclear compartment indicates that the immuno-labeling techniques used were able to detect endogenous G1/S molecules in the nuclear compartment if they were present in that compartment.

Subcellular fractionation independently confirms that all G1/S molecules except pRb, p57, and p21 are cytoplasmic and not nuclear in human β-cells. The predominant nuclear localization of G1/S molecules was unanticipated. For independent confirmation of this intracellular distribution, whole human islets were subjected to subcellular fractionation into cytoplasmic and nuclear components. Figure 7 reveals the results for each G1/S molecule in each fraction: nuclear and cytoplasmic compared with whole unfractionated human islets. The cytoplasmic marker, tubulin, confirmed the cytoplasmic fraction of each prep, and a nuclear marker (histone 3 or RNA polymerase) confirmed the nuclear fraction of each prep; there was little contamination between nuclear and cytoplasmic fractions.

Using this independent technique, once again pRb was clearly nuclear, as was p57. p21 was either occasionally partly nuclear or entirely cytoplasmic, depending on the human islet preparation studied. The remainder of the G1/S molecules—even the E2F transcription factors—was principally or entirely cytoplasmic. Thus, there was near-perfect concordance between results obtained using whole islets and dispersed islets and between results obtained using immunohistochemistry or immunoblots of fractionated islet extracts: of the 27 molecules examined, two were principally nuclear, 24 were exclusively cytoplasmic, and one, p21, varied among human islet preparations. The only exceptions

FIG. 2. The E2F transcription factors. A: The proproliferative E2Fs 1–3. B: Adenoviral overexpression of E2Fs enhances E2F immunocytochemistry. C and D: E2Fs 4–8. Each panel is representative of 3–5 experiments on different human islet preparations. Labeling with no primary antibody (Ab) or with primary antibody plus blocking peptide is shown as negative controls. Ins, insulin.

FIG. 3. E2F4 both is cytoplasmic and colocalizes with nucleolin. Coimmunolabeling for E2F4 and nucleolin suggests that E2F4 is a cytoplasmic as well as nucleolar molecule.
to this near-perfect correlation were E2F3 and E2F7, which appeared to be nuclear by immunoblot but cytoplasmic by immunohistochemistry. It is uncertain which of these observations is most accurate. Importantly, the only G1/S molecules in the human β-cell nucleus are the cell cycle inhibitors, pRb, p57, and p21. In contrast, all of the cell cycle activators appear in the cytoplasmic compartment.

Examination of intact human pancreas confirms the subcellular location of G1/S molecules. It is conceivable that the enzymatic and mechanical rigors of human islet isolation from cadaver pancreases, culturing human islets in 10% FBS, or islet cell dispersal of these into single cells on coverslips might lead to nonphysiological or artifactual localization or shifting of G1/S molecules into the cytoplasm. For determination of whether the results described herein were also observed in intact normal human β-cells, pancreas sections were obtained from the Network for Pancreas Organ Donors (nPOD), and immunohistochemistry was performed on these intact human pancreatic sections. These nPOD sections were fixed in 10%
FIG. 5. The early G1/S cyclins/Cdk4. A: The D-cyclins. Note that cyclins D1 and D3 are principally cytoplasmic and that cyclin D2 is not apparent. B: Cdk5 and 6. Again, these two molecules are preferentially cytoplasmic in the control insulin islet. C: Adenoviral overexpression of D-cyclins and cdk4 and 6 enhances their abundance and, for the D-cyclins, their nuclear presence. Each panel is representative of 3–5 experiments on different human islet preparations. Labeling with no primary antibody (Ab) or with primary antibody plus blocking peptide is shown as negative controls. Ins, insulin.
formalin, rendering them difficult to immunolabel. Therefore, only two antisera, those for pRb and p18, were able to lead to clear visualization of G1/S molecules. As can be seen in Fig. 8, for these two antisera the results in intact pancreas faithfully reproduced the results observed in β-cells from isolated islets: pRb was principally nuclear, and p18 was principally cytoplasmic. In an effort to extend these observations to additional G1/S molecules, additional fresh human pancreas samples were generously provided by Dr. R. Bottino at the University of Pittsburgh and fixed overnight in 4% paraformaldehyde, as had been used for the isolated dispersed β-cells in the preceding figures. As shown in Fig. 8, p107 appeared in the cytoplasm and not the nucleus of β-cells, as in dispersed β-cells. Conversely, p57 was nuclear and not cytoplasmic in β-cells—exactly as had been observed in dispersed β-cells and by immunoblot and as described by Kassem et al. (35). In addition, although p16 was less well visualized, it appeared to be cytoplasmic in most β-cells (Fig. 8), although occasional β-cells contained p16 in the nucleus, as shown in the inset. The remaining G1/S molecules were not able to be visualized using these longer-term formalin- or paraformaldehyde-fixed intact pancreas sections. Thus, all five results available from intact pancreas corresponded to those in isolated islets (subcellular fractionation) as well as in dispersed human β-cells on coverslips.

DISCUSSION
Although all agree that induction of adult human β-cell proliferation is important, achieving this goal in a therapeutically meaningful manner has been frustratingly difficult. In part, this is the result of an incomplete understanding of the mechanistic details of cell cycle progression in the human β-cell, i.e., the lack of a “wiring diagram” or accurate model of the relevant regulatory molecules. Prior models of the control of the G1/S transition were based on extracts of whole islets (12,18–24,26), which are comprised of multiple different cell types (30–32). Here, we endeavored to develop a comprehensive β-cell–specific G1/S model of cell cycle control. The two broad results of this endeavor, based on 164 separate human cadaver islet preparations, are that we now have an atlas of G1/S molecules that are present in the human β-cell. Equally importantly, we now have a complete and very substantially revised working model of G1/S control in the human β-cell. More specifically, the atlas, the model, and the data presented herein 1) reveal that most G1/S molecules are located in the cytoplasm in human β-cells and are not nuclear as anticipated; 2) reveal that the only nuclear G1/S molecules—pRb, p57, and sometimes p21—are all cell cycle inhibitors; 3) may provide unanticipated insight into why human β-cells are quiescent and resistant to proliferation; and 4) may have broad applicability beyond β-cells, extending to quiescent mammalian cells in general.
The prior model of the human β-cell G1/S control was in part correct in assuming that most of the G1/S molecules are present in the β-cell. It was incorrect, however, in assuming that they are nuclear. Thus, the new “atlas” presented in Figs. 1–8 provides a more accurate cell biological description of the quiescent human β-cell, showing that all of the G1/S molecules, except cyclin D2, as reported previously (12,21,22,24), are found in β-cells but are found in the cytoplasm, with three notable exceptions: pRb, p57, and in some human islet preparations, p21. Thus, we now have a reasonably complete and concrete model for understanding human β-cell G1/S control and for hypothesis generation as to approaches to therapeutic induction of proliferation.

The observation that the majority of G1/S molecules are cytoplasmic was unanticipated. Yet, it is strongly supported by three independent methods: first, immuno-histochemistry with appropriate negative (no primary antibody, human cell lines that lack specific G1/S molecules, and competition with excess antigenic peptide) controls; second, subcellular fractionation of human islets; and, third, confirmation that this is not an artifact of islet isolation and dispersal, since the same pattern is observed, when technically possible, in intact islets in intact pancreas. The observation that pRb is nuclear was expected, since it is widely viewed as a nuclear protein (36–38). Similarly, the observation that p57 is nuclear was also anticipated, since this has been reported previously by Kassem et al. (35). Interestingly, here as well as in the study by Kassem et al. (35), p57 is not only abundant in the nuclei of β-cells (it is present in 30–40% of β-cell nuclei in both reports) but is also apparently restricted to β-cells compared with other endocrine and exocrine cell types. Again, the observation that the only G1/S molecules that are consistently nuclear are these two cell cycle inhibitors may provide insight into why human β-cells are quiescent.

Most cell cycle biologists, as illustrated in current reviews of G1/S control (36–42), have considered the G1/S molecules to be nuclear molecules, perhaps best exemplified by the E2Fs that are transcription factors. But it is also true that most who study G1/S biology are focused...
on replication in rapidly proliferating cells such as cancer cells, embryonic cells, or cells in lower organisms that are replicating rapidly or are prokaryotes. Thus, it was a surprise to observe these molecules in the cytoplasm. On the other hand, scattered reports indicate that many G1/S molecules may be present in the cytoplasm of β-cells or other quiescent cells. For example, we had previously noted that p21 is principally cytoplasmic in the quiescent human β-cell but could be translocated to the nucleus by overexpression of cdk4 and cyclin D1 (32). Others have reported that D-cyclins are present in the cytoplasm of the mouse β-cell and that they can translocate into the nucleus with glucose-induced β-cell proliferation (43,44). E2Fs have also been reported to be present in the cytoplasm of quiescent cells, such as differentiated skeletal muscle cells and keratinocytes (45,46). In other mammalian cell types, p107 and p130 have been observed under some conditions in the cytoplasm of prostate cells (47), and p53 has also been observed in the cytoplasm of many cell types and to undergo cytoplasm-to-nuclear translocation (48). Cdk6 has also been reported to exist in the cytoplasmic compartment of astrocytes (49) and lymphocytes (41), as have cyclins A and E and cdk5 1 and 2 in HeLa cells (50). Thus, there is ample precedent for G1/S molecules being cytoplasmic proteins. Interestingly, the possibility exists that the association between cytoplasmic localization of G1/S molecules and the enforced quiescence of the cell types involved may represent a broad biologic principle among quiescent mammalian cells—not solely restricted to β-cell biology.

Construction of this atlas and the model described in the accompanying article (34) is a work in progress and has limitations, as discussed in the DISCUSSION section of the accompanying article.

In conclusion, these studies provide both a G1/S cell cycle molecule atlas for the human β-cell and a working model for hypothesis generation. The atlas suggests that restraint or tethering of cell cycle molecules in the cytoplasm may be a regulatory step that restrains or prevents replication in adult human β-cells. It further suggests that if one were able to develop mechanisms to direct cyclins and cdk5 to traffic into the nucleus, one might be able to effectively drive human β-cell proliferation. This hypothesis is demonstrated to be true in the accompanying article (34).

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N.M.F.-T. researched data, contributed to the discussion, and wrote the manuscript. J.W.K., F.S., R.T., R.W., M.T., G.C., A.E.C., and K.K.T. researched data. D.K.S. contributed to the discussion. A.F.S. contributed to the discussion and

FIG. 8. Confirmation that subcellular localization observed in isolated and dispersed human β-cells is also observed in intact human islets in situ. Human pancreatic sections obtained from nPOD (pRb and p18, fixed in 10% formalin) or fresh human pancreas blocks fixed in 4% paraformaldehyde (p57, p107, and p16) were labeled as described in RESEARCH DESIGN AND METHODS and confirm the observation in dispersed human islets and subcellular fractionation studies. Each panel is representative of 3–5 experiments on different human pancreas preparations. The inset in the p16 panel shows that it is generally cytoplasmic but occasionally nuclear, particularly in smaller islets. See the accompanying article (34) for quantification. Ins, insulin.
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