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Finding NECA: zebrafish screen identifies key signalling pathway in β-cell regeneration

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Summary of and comment on a recent *Cell Metabolism* paper entitled 'Adenosine signaling promotes regeneration of pancreatic β cells in vivo' (Andersson et al., 2012).

The genesis of pancreatic β-cells in humans has been documented in a number of physiological processes, such as pregnancy (Butler et al., 2010), injury (Berrocal et al., 2005) and in response to increased metabolic demand (Saisho et al., 2012). The ability to exploit the intrinsic capacity of the adult pancreas to produce new β-cells would provide exciting new treatment modalities for individuals suffering from diseases involving β-cell depletion.

The development of the mammalian pancreas has been extensively studied and is relatively well understood. In contrast, the mechanisms of β-cell regeneration remain unclear, although model organisms have provided some useful insight into the process. For example, the use of transgenic reporter mouse strains has allowed researchers to perform lineage tracing studies, and to demonstrate that new β-cells in mice arise through proliferation (i.e. the multiplication of existing β-cells) rather than neogenesis (i.e. differentiation from a progenitor population) (Dor et al., 2004; Nir et al., 2007). Nevertheless, few signalling pathways have been identified that drive β-cell proliferation and/or neogenesis. The limited success in this field might be due to the fact that studies of factors affecting β-cell regeneration in vivo have largely been based on a candidate gene approach. The utility of an unbiased in vivo screen to reveal new biology in this area was realised by Andersson et al. in their study involving a chemical screen in zebrafish to identify enhancers of β-cell regeneration (Andersson et al., 2012).

Several factors make zebrafish particularly well suited to this type of experimental screen. Zebrafish are amenable to pharmacological and genetic manipulation. In addition, they are transparent and have a very basic pancreatic structure, allowing for simple and rapid imaging analysis. Zebrafish are also able to regenerate β-cells very efficiently. Andersson et al. used a genetically modified zebrafish line in which the insulin promoter drives the expression of both fluorescent molecules (enabling imaging) and the enzyme nitroreductase in β-cells. Nitroreductase produces a toxic product on exposure to the chemical metronidazole (MTZ); its expression in β-cells enables temporally controlled β-cell ablation on addition of MTZ (Pisharath et al., 2007; Andersson et al., 2012). Pancreatic β-cells were conditionally targeted for ablation in zebrafish embryos at day 3-4 post-fertilisation. Larvae were then exposed to MTZ for 48 hours to ablate β-cells, which began to regenerate between day 4 and 6; compounds tested for their capacity to enhance β-cell regeneration were added during this period. The pancreas was imaged on day 6, when three to seven β-cells had regenerated in control (i.e. MTZ-exposed only) embryos.

In a screen of 7186 compounds, Andersson et al. identified five 'hits' that doubled the number of β-cells that regenerated. This occurred through enhancing β-cell regeneration, rather than survival. Astonishingly, four out of these five compounds were involved in the adenosine signalling pathway. In secondary assays that investigated the underlying mechanisms of the hit compounds, the authors concentrated on the adenosine analogue NECA (5′-N-ethylcarboxamidoadenosine), the most effective compound. The authors measured EdU (5-ethynyl-2′-deoxyuridine) incorporation into the cells to ascertain the rates of proliferation, and found that the proportion of β-cells that incorporated EdU was increased more than threefold in the NECA-treated larvae compared with controls. Thus, NECA increases regeneration by accelerating the multiplication of new β-cells, rather than inducing β-cell neogenesis. Additional experiments indicated that NECA increases proliferation substantially during β-cell regeneration, but only modestly during normal development. The authors propose, therefore, that adenosine signalling restores an optimal β-cell number, rather than increases β-cell proliferation per se.

To test the functionality of the regenerated β-cells, the authors also performed a functional assay by testing glucose levels in MTZ-exposed zebrafish. Although both vehicle-treated and NECA-treated embryos restored normoglycaemia to some extent, the restoration occurred more quickly and effectively in NECA-treated embryos.

To assess the specificity of NECA on β-cell proliferation, Andersson et al. investigated its effects on other endocrine cell types in the pancreas. They found that NECA did not cause a change in the proliferation of glucagon-producing α-cells or somatostatin-producing δ-cells, indicating that NECA specifically increases β-cell proliferation, rather than non-specifically stimulating the proliferation of pancreatic endocrine cells. In addition, there were no changes in cell proliferation in the gut, liver or neural compartment. These in vivo studies are important because the production of adenosine is increased under conditions of stress, and it is thought to have roles in cytoprotection and tissue damage and repair (Fredholm, 2007).

Andersson et al. then investigated in greater detail which adenosine receptor was involved in the NECA-mediated effects on β-cell regeneration. NECA nonselectively agonises four G-protein coupled receptors (A1, A2a, A2b and A3) that are well conserved among vertebrates (Fredholm et al., 2001). Activation

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of A1 and A3 decreases intracellular levels of cAMP, whereas activation of A2a and A2b increases cAMP. Because two of the hits in the screen were drugs known to increase cAMP, the authors focused on A2a and A2b as putative mediators of the regenerative response. Expression analysis revealed that A2a was highly expressed in cells budding from the extrapancreatic duct, supporting a role for this receptor in β-cell regeneration. There are two genes for the A2a receptor in zebrafish, *a2aa* and *a2ab*; however, only mRNA for *a2aa* was detected in the pancreas. When the authors used an *a2aa* morpholino to knock down the expression of the A2a receptor, NECA no longer enhanced β-cell regeneration in the MTZ exposure assay. The morpholino also blocked the proliferation of β-cells in the vehicle-treated group, suggesting that adenosine is an endogenous signal that promotes β-cell regeneration.

Finally, the authors showed that NECA has a similar effect on β-cell regeneration in mammals. In culture, mouse islets treated with NECA showed an increase in the rate of β-cell proliferation. Furthermore, in adult mice initially treated with streptozotocin (which depletes β-cells) and then treated or not with NECA for 8 days, a 30% decrease in glycaemia was observed in NECA-treated mice compared with controls. Histological analysis showed that the β-cell mass in the NECA-treated mice was eight times greater than in controls. In addition, β-cells in the NECA group were eight times more likely to be proliferating.

In summary, Andersson et al. identified a signalling pathway that was not previously associated with β-cell regeneration that might provide new therapeutic avenues for the treatment of diabetes. The fact that their screen was carried out in vivo allowed for the complex and interrelated pathways that control β-cell differentiation and maintenance to be preserved. It also provided the opportunity to easily monitor the effect of candidate compounds on other tissues and cell types in the body.

The finding that four of the five hit compounds are involved in the adenosine signalling pathway highlights the importance of this pathway in β-cell regeneration. This finding is supported by results from another high-throughput screen in which Annes et al. identified that adenosine kinase inhibitors (ADK-Is) promote the replication of primary β-cells in mouse, rat and pig (Annes et al., 2012). ADK-Is block the conversion of adenosine into adenosine monophosphate (AMP), thereby increasing the pool of available adenosine. Thus, ADK-Is increase the activation of adenosine receptors, similarly to how an adenosine receptor agonist increases receptor activation.

Adenosine is a known stress signal that can be released from dying cells. It therefore might constitute an endogenous signal that increases β-cell proliferation during the regenerative response to β-cell death. This finding generates many questions. What is the mechanism underlying the selectivity of adenosine signalling for β-cells? What signalling pathways mediate the proliferative signal following adenosine receptor activation? It would also be interesting to know whether NECA has a synergistic effect on β-cell proliferation when combined with other compounds that are known to increase β-cell mass, such as glucagon-like peptide-1 (GLP-1) (Stoffers et al., 2000). The next step is to test agonists of the adenosine A2a receptor and/or ADK-Is in human fetal or neonatal cadaveric islets, or, alternatively, in β-cells derived from human induced pluripotent stem cells or embryonic stem cells. If the outcome of such studies is promising, we might be a step closer to a new treatment for diabetes.

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