A strange type of zebra to combat diabetes

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Whilst the diabetes epidemic is constantly growing scientists are looking for new treatment paths. Amongst them the grafting of beta cells, the cells which produce insulin within the pancreas, is a promising area. Bernard Peers, Marianne Voz and their colleagues are trying to shed light on the differentiation mechanisms of these cells in order to be able to reproduce them more easily from stem cells.

Situated behind the stomach, in front of and above the kidneys, the pancreas is a digestive gland linked to the duodenum (the initial section of small intestine). Both exocrinic and endocrinic, this gland synthesises secretion products, certain of which are freed into the digestive tract whilst others enter the blood stream. Amongst the substances which spring from the endocrinic pancreas is numbered notably insulin, known to be lacking in patients affected by type 1 diabetes. ‘Type 1 diabetes is an autoimmune disease,’ explains Bernard Peers, who heads the research group on the development of the pancreas within the GIGA’s research unit, Development, stem cells and regenerative medicine. ‘In diabetic people, the immune system attacks the beta cells which produce insulin within the pancreas and destroys them,’ he continues. If the current treatment of type 1 diabetes - which consists of injecting insulin into the patient before meals - allows the patient to survive, this therapy is not optimal. In effect, ‘insulin injections do not allow for a very exact control of glycaemia and the patients frequently go through phases of hyperglycemia and hypoglycaemia. Over the long term, that could lead to complications,’ points out the FNRS research associate. Practically every part of the body could be subject to the side-effects of a badly controlled diabetes: the heart, the blood vessels, the kidneys, the eyes, the nervous system, etc. Finding new remedies against this disease which has been developing in an epidemic manner for a few decades now - the World Health Organisation estimates that the number of diabetics on the planet could reach 300 million by the year 2025 - thus represents a real public health issue.
One of the pathways followed by scientists to respond to this issue is the grafting of beta cells. The transplanting into diabetic patients of pancreatic cells taken from donors who have died is already practiced but the patient has to take powerful immunosuppressants in order to prevent the immune system from attacking these new beta cells, grafted into the pancreas. 'This grafting allows for a temporary remission,' indicates Marianne Voz, a FNRS research associate at the GIGA's' Biology and Molecular Genetics research unit. 'But a major problem of this therapy is the largely insufficient availability of pancreatic endocrine cells from donors. Nevertheless it is now possible to generate stem cells on the basis of the patients' 'mature' differentiated cells. And from these totipotent cells, called iPS, we can thus try to recreate beta cells in order to graft them onto a diabetic patient,' continues Marianne Voz. In order to be able to generate pancreatic cells from stem cells it is important to carry out both research into the regulatory factors and the genetic determinants which control the differentiation of pancreatic cells, but also basic research into the development of the pancreas in a more general manner. In effect, only deeper knowledge of the molecular mechanisms involved in the development of this organ could bring about an optimisation of current treatments and nose out new therapeutic pathways. And that is what Bernard Peers, Marianne Voz and their colleagues in the study group on pancreatic development are currently working on.

Following development by transparency

The animal model the researchers have turned to for their research is a little untypical. In effect, it is not the small fruit fly, regularly used as a model organism for research into genetics, but which does not possess a pancreas; nor is it the mouse, a common model for scientific experiments which aim to study the differentiation of cells over the course of embryonic development. The creature which allows Bernard Peers and his colleagues to see clearer into pancreatic development is…the zebrafish. Also called danio rerio, this little animal has advantages which the mouse does not, and vice versa, from which springs the complementarity of these two models for scientific research. The use of zebrafish in laboratories has moreover been booming over the last ten years.
Amongst the advantages the zebrafish has for scientists is notably the fact that embryonic development takes place in the water and not in the females' stomachs. ‘The female lays the eggs and fertilisation takes place in the outside. The whole of this animal's development then occurs in the water, which means we can follow it through binocular instruments or a microscope,’ points out Marianne Voz. The second of the zebrafish's plus points: the embryos are translucent. ‘We can see all the cells through the skin of the embryo and we can thus observe the formation of pancreatic cells in the living embryo, without interfering in their development,’ picks up Bernard Peers. Finally 'it is very easy to study the function of a gene in zebrafish as they generate a large quantity of embryos which develop very rapidly. Three days after the fertilisation of the eggs we have already obtained small fish, totally autonomous larvae which will take three months to reach maturity,’ he stresses. Scientists can easily block the expression of a gene in zebrafish. 'A gene is a segment of DNA, which, to be expressed, must be transcribed into messenger RNA to be transformed into protein,’ explains Bernard Peers. ‘In injecting what are called morpholinos, in other words small oligonucleotides, into the embryo, we can block the translation of messenger RNA or interfere with RNA gene splicing,’ continues the researcher. Splicing is
the process by which RNA transcribed from the basis of DNA can be subject to disconnections leading to the suppression of certain regions in the final messenger RNA. The segments preserved bear the name 'exons' whilst the suppressed fragments are called 'introns'. The parts of the genes which code for proteins are contained within the exons. 'Certain morpholinos specifically target the extremities of the same exon and thus provoke the deletion of this fragment of RNA. The result: the corresponding protein is not produced,' points out Bernard Peers. The goal of this type of manipulation is to characterise new regulatory genes involved in the development of the pancreas.

From the zebrafish to the diabetic patient

In the light of these explanations we can easily understand the 'practical' aspects of the zebrafish in the study of the development of the pancreas. But what weight can these discoveries carried out thanks to this animal have in the search for therapeutic pathways against diabetes in humans? 'The majority of transcription factors which regulate gene expression, identified as being very important for the differentiation of pancreatic cells in mice, are also present over the course of the development of the pancreas in zebrafish, and conversely,' specifies Marianne Voz. 'The gene sequences which code for proteins have been very well preserved over the course of evolution and the discoveries made through zebrafish are generally applicable to mice and humans,' she continues. At the beginning of this year, 2010, the zebrafish allowed Bernard Peers and Marianne Voz’s team to make two new discoveries concerning the development mechanisms of the pancreas. The first, published in the journal Development (1) in January, carried out in collaboration with a team of French researchers from the University of Salzburg within the framework of the European 'Betacelltherapy' project, concerns the transcription factor called RFX6.

'The Salzburg researchers had identified the RFX6 gene and had observed that it was expressed within pancreatic cells in mice but they had no idea as to the gene’s function,' explains Marianne Voz. On hearing this news, Lydie Flasse, a doctoral student in the Liège team, set about trying to check if there existed an ortholog gene in the zebrafish and whether it was also expressed within the pancreas. 'We carried out function tests which consisted of injecting morpholinos in order to block the expression of the RFX6 gene and we noted that this gene is required for the differentiation of pancreatic cells,' reveals the Liège student. 'If this gene is not expressed the cells of the pancreas remain in their progenitor state,' she specifies. One month after the publication by the Liège researchers an American team published a study which confirmed that the RFX6 gene played the same role in mice. Finally, another study has recently showed that patients whose RFX6 gene is mutated have defects in hormonal secretions in the pancreas. According to Bernard Peers these recent studies confirm that controlling the differentiation of pancreatic cells has been very well preserved in vertebrates and the bringing to light of the transcription factor RFX6 could in the long term allow new remedies against diabetes to be found.
Comparison of the phenotype of wild zebrafish larvae (wild-type) and mutated in the gene pax6b (sa0086). The upper part of the figure illustrates the location of the mutation sa0086 introducing a ‘stop’ codon in the paired domain (PD) of pax6b. Photos A and B present the morphology of wild and mutant larvae showing a reduction of the size of crystalline in the mutants. The cells expressing insulin are detected in wild larvae (photo C) and not in the mutant larvae sa0086 (photo D).

When two studies confront each other...

The second study recently published by the Liège team in *The Journal of Biological Chemistry* (2) bears on the transcription factor baptised PaX6. The gene coding for PaX6 was discovered twenty years ago and causes, when it is mutated, defects within the eyes and the brain in the fruitfly and the mouse as well as defects in the development of the pancreas in the latter. A few years ago, Bernard Pees and his colleagues had begun a study to determine if PaX6 existed in the zebrafish and if it was also expressed in its brain, eyes and pancreas. ‘At the time we had shown that there are two PaX6 genes in the zebrafish; both are expressed in the brain and the eyes but only one, the PaX6b gene, is expressed in the pancreas and controls the differentiation of pancreatic cells, as is the case in mice,’ points out Bernard Peers. But last year a Scottish study revealed the existence of zebrafish, in which the PaX6b gene had mutated, whose eyes had defects but whose pancreases were intact. The mutants used in the Scottish researchers experiments are called ‘sunrise’ mutants. This new study thus appeared to contradict that which had been carried out earlier by Dr. Peers’ team. To get to the bottom of this the Liège researchers set themselves the challenge of resolving this enigma. Thanks to their investigations they succeeded in identifying another line of zebrafish originating from the Welcome Trust Sanger Institute (GB). In these mutants, contrary to the ‘sunrise’ mutants, the mutation which affects the PaX6b gene brought about exactly the same problems as those observed in the fish Bernard Peers and his colleagues had injected with morpholinos. But how is it that these two mutants do not have the same anomalies in the pancreas?

To answer this the researchers in the development of the pancreas study group established a preliminary hypothesis, according to which the ‘sunrise’ mutant would be hypomorphous, in other words that the mutation caused a reduction of protein activity but not a complete elimination of it. ‘This reduction of activity could affect the forming of the eyes whilst it wouldn't be sufficient to affect the pancreas, which would explain why the sunrise mutants had eye defects but none in the pancreas,’ points out Bernard Peers. To check this hypothesis the Liège team once again made use of the morpholino technique. ‘Normally, the more you inject morpholinos
the more you block gene expression and thus the quantities of proteins produced,' he continues. The result: the ocular and pancreatic defects appeared for the same reduced doses of protein Pax6b. This preliminary hypothesis did not thus hold water.


'An original and surprising explanation'

Far from being stumped for ideas, Bernard Peers' team thus suggested a second hypothesis: out of the two domains of the Pax6b protein which bear the name 'homeodomain' and 'paired domain' and which permit this protein to fix itself to DNA in order to regulate the expression of other genes, a single one would be required to ensure the function of the protein in the pancreas whilst both would be necessary for the protein to act within the eyes. In effect, the sunrise mutation is brought about by the modification of a single amino acid within the Pax6b's homeodomain, whilst the Welcome Trust Sanger Institute's mutants have a large deletion in the Pax6b gene which leads to protein activity being completely blocked.

To verify this second hypothesis the researchers injected into zebrafish embryos two morpholinos which, within the transcribed RNA, targeted the exon which codes for the homeodomain. These fish were consequently lacking this domain within the Pax6b protein. And it was thus that the scientists were able to observe that these specimens, in the same way as the sunrise mutants, showed abnormal development within the eyes but not in the pancreas. 'The explanation for the differences obtained by the Scottish team and our team concerning the development of the pancreas in zebrafish, mutated in the Pax6b gene, thus comes down to the location of this mutation. In the sunrise mutant this mutation results from the modification of a single amino acid in the Pax6b's protein's homeodomain and brings about ocular abnormalities because this domain is indispensable to the smooth functioning of the protein in this region of the body, but not for the correct functioning of the pancreas. The Welcome Trust Sanger Institute's mutants for their part have a larger mutation which blocks completely the production of protein Pax6b, which thus also affects the development of the pancreas,' sums up Bernard Peers. From this arises the conclusion of the study recently published in The Journal of Biological Chemistry: the homeodomain of the Pax6b protein is not indispensable for the differentiation of pancreatic cells in the zebrafish.

If this second discovery, made during the beginning of 2010 by the Liège team, is in the field of basic research and not that of applied research as it cannot be directly linked to any research path to combat diabetes, it is far from being without interest for the overall understanding of the development of the pancreas. In the light of the results obtained in their laboratory these recent months, Bernard Peers and Marianne Voz are moreover determined to continue to explore the involvement of proteins Rfx6 and Pax6b in the development of this organ, with the goal of putting their finger on the mechanisms which allow beta cells to be more easily created from stem cells and to understand why diabetes develops in some people and not in others.