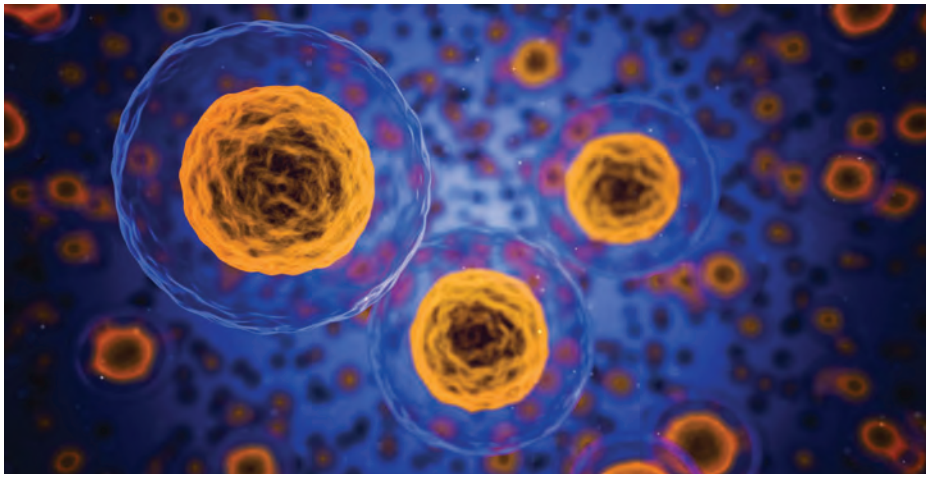
Dr Federico González speaks to *SEQ* about his work using human pluripotent stem cells (hPSCs) to better understand kidney disease

Bioengineering future kidney disease solutions

Advances in tissue and genome engineering in human pluripotent stem cells have unlocked the potential to better understand many diseases, including kidney disease, while the ability to create organoids and, moreover, the development of gene editing techniques such as the CRISPR-Cas9 system, are promising even more breakthroughs in the future.

Here, *SciTech Europa Quarterly* speaks to genome engineer Dr Federico González about his work using human pluripotent stem cells (hPSCs) to better understand kidney disease.



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Can you outline the work that has taken place in using pluripotent stem cells to study kidney disease in recent years?

Human kidney development has been traditionally studied using model organisms such as the laboratory mouse. These studies have been key to defining the basic molecular mechanisms governing this process but they have also provided an opportunity to investigate the mechanisms underlying human kidney pathologies.

For instance, acute and chronic kidney disease can be induced in the mouse through different types of experimental injuries. Moreover, because their genome is easily amenable to manipulation, genetically engineered mice have been particularly useful for studying heritable kidney conditions. For instance, genetic ablation of the gene encoding the α3 chain of collagen type IV in mice mimics human hereditary nephritis.

However, it is important to note that due to the physiological differences existing between mice and humans, mutations can have species-specific effects, which may not be recapitulated in the animal. A classic example is autosomal dominant polycystic kidney disease (ADPKD), caused by the inactivation of only a single copy of the genes PKD1 or PKD2. In mice, when a single copy of these genes is inactivated, only a very mild cystic disease is observed; hence, the necessity of reliable human-based kidney disease models.

Due to their functional properties, human pluripotent stem cells (hPSCs) represent a central asset to achieve this goal: they can proliferate indefinitely *n vitro,* while maintaining the potential of giving rise to virtually any cell type of the human body. They provide an inextinguishable source of cells that can be differentiated on demand to any cell linage relevant for studying kidney disease.

The first hPSC lines were derived from human pre-implantation embryos in 1998. At this time, genetic engineering in hPSCs was extremely laborious and infective, limiting the use of embryo-derived hPSCs for modelling hereditary diseases. Eight years later, the development of a novel technology allowed for the first time the generation of human induced pluripotent stem cells (hiPSCs) by reprogramming adult human skin cells to a pluripotent state.

This approach enables the generation hPSC lines from virtually any individual, therefore allowing creating hPSCs carrying any type of genetic alteration found in the population. This includes monogenic, polygenic diseases and other diseases, for which the precise genetic contribution has yet to be determined.

Over the last decade, hiPSCs from a variety of kidney diseases have been generated, including PKD, renal cysts and diabetes syndrome, Alport syndrome, Wolfram syndrome, Wilms tumour, focal segmental glomerulosclerosis or systemic lupus erythematosus. They provide a promising alternative to animal models for studying kidney disease.

In order to properly achieve this task, the development of efficient and reproducible protocols supporting the generation of kidney cells

from hPSCs has been a major roadblock. The experimental process of guiding the differentiation of hPSCs towards a specific cell type is called 'directed differentiation'.

Directed differentiation consists of a series of steps mimicking the multi-stage process of embryonic development. In the embryo, the differentiation steps experienced by a single cell are guided by the succession of signalling cues provided by the evolving contacts with the surrounding cells, and the molecules that they secrete. Developmental genetic studies performed in animal models have allowed the identifying of which set of molecules mediate the different steps leading an undifferentiated cell to become a specific differentiated cell type. Thank to this knowledge, cell biologists are now able to mimic the temporal series of signals that usually occur *in vivo* and instruct undifferentiated hPSCs to become a particular kidney cell type in vitro by submitting hPSCs to culture medias containing defined growth factors and small-molecule compounds.

The last three years have witnessed the development of a cohort of protocols allowing the efficient differentiation of hPSCs towards defined kidney progenitor populations using two-dimensional (2D) culture systems. Besides their importance for generating relevant therapeutic cell types, these protocols are particularly attractive for studying disease phenotypes manifested at the cellular level.

Instead, the complex interactions occurring between the different cell types constituting the specialised structures of a functional kidney (glomeruli, collecting ducts, and so on) are not recapitulated using these approaches. This limitation has been recently overcome by further directing hPSC differentiation toward kidney organoids.

Kidney organoids represent miniaturised and simplified versions of the human kidney constituted by multiple cell types self-organising three dimensionally into a structure recapitulating many aspects of their *in vivo* counterpart. Between 2013 and 2015, several laboratories have reported the successful generation of kidney organoids from hPSCs. They represent an unprecedented opportunity for studying the complex cellular interactions occurring in the human kidney in both physiological and pathological conditions.

How has the use of organoids obtained from hPSCs opened up new opportunities?

While 2D culture systems allowed us to address how specific cell types function and respond to stimuli. Organoids bring us closer to the *I condition*. In conventional 2D systems, most cell-to-cell and cell-to-matrix interactions important for many cellular functions *in vivo* are not recapitulated. Instead, the 3D cellular organisation of kidney organoids recapitulates more faithfully the actual organisation of a functional kidney.

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While the successful generation of kidney organoids has relied on previous knowledge of the mechanisms underlying kidney development, kidney organoids provide now researchers with an *in vitro* platform for studying the signals governing organogenesis, lineage specification and tissue homeostasis in regions that are usually inaccessible or difficult to study *in vivo* in animal models.

With the possibility of generating hiPSC lines from patients affected by virtually any heritable kidney disease, kidney organoids represent an exceptional platform to investigate disease mechanisms. Studying disease mechanisms in kidney organoids will help identifying novel druggable targets to treat or alleviate kidney disease in patients. Moreover, kidney organoids are useful for precisely identifying the whole range of phenotypes (phenotyping) caused by a specific genetic alteration or kidney injury. This knowledge is fundamental for designing large-scale genetic or drug screens aimed at identifying genes or molecules respectively, capable of improving or correcting the phenotype(s) tested in the screen.

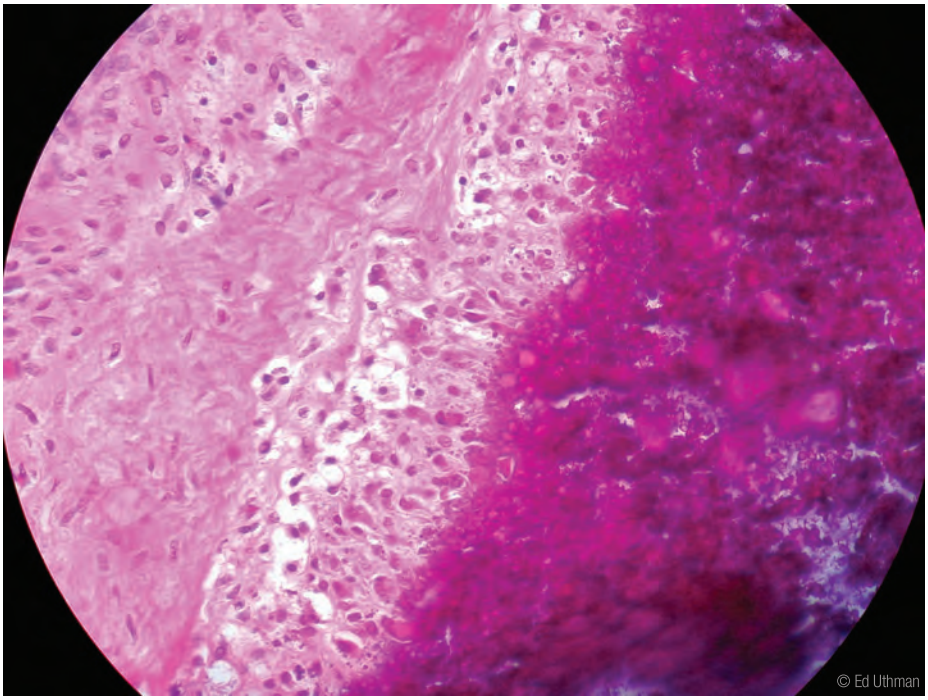
Prior to the development of organoid models, such screens have been extensively performed in animal models and were extremely time consuming, arduous and expensive to realise due to the enormous amount of animals involved. Comparatively, due to the intrinsic properties of hPSCs, the initial material can be easily scaled up and organoids can be easily produce at a scale compatible for large scale screening.

Kidney organoids are also promising for regenerative medicine. Chronic kidney disease (CKD) affects 3-17% of the adult population in countries of the European Union. Treatment of CKD patients progressing towards end-stage renal disease is currently limited to dialysis and renal transplantation. Since kidney organoids can now be obtained from hiPSC, which can be generated from any patient, in the future they could potentially provide an alternative to heterologous kidney transplantation.

Human liver organoids have already been engrafted into the mouse liver and kidney organoids transplanted under the kidney capsule in mice have become vascularised. It remains to be determined whether kidney organoids will be able to execute all the functions of the adult kidney when engrafted in patients.

In your own research, you have been able to utilise the CRISPR system to study kidney disease with hPSCs. What were the biggest challenges here? What were your most significant discoveries?

Reprogramming has opened the possibility to reproduce any genetic condition in hPSCs. This approach, however, has some limitations. In order to identify disease phenotypes, researchers always need a healthy control to use for a comparison.



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The human population is composed of individuals whose genomes diverge in many aspects. Although their gene composition is the same, they carry slightly different versions of each gene called alleles, which may have slightly different functions. Moreover, the expression of each gene is controlled by regulatory sequences, which activity is modulated by genetic and epigenetic factors (the factors that affect the function of the DNA without affecting its sequence) fluctuating between individuals. This partly explains why even when two patients carry the same mutation, the symptoms and the onset of their disease can be extremely different. Therefore, studying monogenic and polygenic diseases in hiPSCs derived from patients with different genetic backgrounds and histories can become a complicated task.

Moreover, the process of reprogramming itself results in the generation of not only one, but thousands of hiPSC lines from the same patient. Due to the inherent variability of this process, these lines are not 100% equivalent and may vary significantly in their differentiation capacity or in their phenotypic features.

An alternative approach to hiPSC generation for disease modelling is therefore to introduce defined genetic alterations in hPSCs through gene editing. This approach has been widely used to generate engineered mouse models through gene targeting in mouse embryonic stem cells. Applied to hPSCs, this would create a set of genetically matched (isogenic) mutant lines that can be reliably compared with the healthy parental line, which would only differ by the disease causing mutation. Alternatively, disease causing mutations could be corrected in patient-derived hiPSC lines, to obtain isogenic healthy controls to which the mutant lines could now be compared.

To achieve this goal, one needs a powerful genome editing technology. Recently, the type II CRISPR system of the bacteria *Streptococcus pyogenes* has been adapted for effective genome engineering in hPSCs. CRISPR/Cas9 is composed of a protein (Cas9) that can cut the DNA and a small piece of RNA (sgRNA) that binds to Cas9 and direct it to a desired genomic sequence.

The binding specificity of Cas9 is simply determined by 20 nucleotides of the sgRNA recognising and binding a complementary 20 nucleotides sequence found in the genome. The ability to cut the DNA at desired genomic sites provides an entry point allowing the introduction of any type of modifications in the genome through a variety of genome engineering techniques.

Former work I performed at Memorial Sloan-Kettering Cancer Center (New York, USA), in the laboratory of Danwei Huangfu, has been to develop an iCRISPR platform for rapid and Inducible genome editing in hPSCs. This platform considerably facilitates the introduction any genetic modification in hPSCs and represents a powerful tool for disease modelling. It not only simplifies the introduction or the correction of mutations, but also allows very easily introducing genetic elements (reporter genes) that can help monitor any type of cellular behaviour.

When I moved from New York to the Institute for Bioengineering of Catalonia (IBEC), I joined the Laboratory of Nuria Montserrat, who was awarded by a European Research Council (ERC) grant aiming at studying kidney development and disease using hiPSC-derived kidney organoids. A major effort of her lab has focused in establishing efficient, reproducible and scalable protocols for generating kidney organoids from hiPSCs and the team responsible for this work has made significant progress recently, producing organoids displaying a high level of organisation.

As a genome engineer, I have focused my effort in applying the iCRISPR technology to generate a panel of hPSC lines enabling the study of different aspects of kidney biology. Briefly, we were able to inactivate a large set of developmental genes for dissecting their function in kidney organoids. We also edited an extensive array of patient-specific mutations to study a range of kidney diseases, including renal cancers.

Cancer modelling in kidney organoids is particularly attractive because one can mimic and observe the effect of the series of genetic events occurring during renal cancer initiation, something that is impossible to observe in a patient. Our medium term goal is to establish a multipurpose iCRISPR platform in hPSCs allowing studying any aspect of kidney biology in kidney organoids with superior speed and precision.

What other genome editing techniques will you be looking to in the future? Do you have any concerns over how ethical and safety issues are currently being addressed with regard to such technologies?

The fantastic aspect of CRISPR/Cas9 is that the system is not only limited to targeting modifications in the DNA. Different labs have engineered Cas9 variants that have lost their capacity to cut DNA but can exert novel functions such as activate or repress gene expression on demand; modify the epigenetic marks that a are found in a specific genomic location; mark desired areas of the genome with fluorescent proteins that can then be visualised under a microscope, etc. The integration of all these variants in our current iCRISPR platform will provide a fantastic toolbox for addressing gene function and efficiently investigate disease mechanisms.

Another important area of research is to improve the specificity of editing techniques. As previously mentioned, Cas9 is driven to its genomic target sequence by only 20 nucleotides of homology between the sgRNA and the genomic sequence. Therefore, the likelihood of finding a sequence of 20

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nucleotides identical or very similar across the whole extent of the human genome is in fact quite high.

The presence of such sequences (called 'off-targets') allows Cas9 to cut both on target and, to different extents, off targets. This is obviously a major concern if Cas9 is used, for instance, to correct mutations in a patient *in vivo:* there is a reasonable possibility that off-target mutations may alter in unpredictable ways the behaviour of the edited cells, which could have negative impact on the patient. This concern is justified and in many ways similar to what has been argued during many years against the safety of viral-based gene therapy protocols.

Recent work suggests, however, that Cas9 specificity can be improved through genetic manipulation and many other genome editing system have been described in the last few years, which could represent potentially safer alternatives to Cas9. Whether Cas9 or any other genome editing system will ever be used to correct mutations in embryos, as a recent study suggests the feasibility, will be a major collective decision in which scientists need to play a critical role for explaining objectively the whole range of outcomes that such an approach may have for human beings in the long term.

Moving forwards, where will your own research interests lie?

Regarding tissue engineering, the main challenges in the organoid field are the scaling up of the production of organoids, making the whole differentiation process faster, more reproducible, and generating organoids with a level of maturation closer to the adult organ. These aspects are crucial if we want to use these organ surrogates for large scale genetic and drug screens.

Improvements will come on one side from the automation of the differentiation when- and wherever possible and on the other side from the development and usage of 100% chemically defined components for directing the fate of hPSCs. For instance, Matrigel, which is used in many 3D differentiation protocols, is a poorly characterised protein mixture purified from a mouse sarcoma cell line. Replacement of Matrigel with defined synthetic hydrogels has helped to define the signalling and mechanical properties of the matrix that are important for differentiation and organoid formation. A precise formulation of the chemical and structural requirements for organoid culture will necessarily reduce the variability in their production and potentially improve maturation. The use of 3D bioprinters to design and generate defined scaffolds supporting organoid growth is also something we are investigating, though many technical problems still need to be solved.

On the genomic engineering side, the technology is actually quite mature, and there are few things that can't be done in terms of gene editing. Modelling kidney cancer using either a step up approach, in which we sequentially introduce cancer-initiating mutations in hPSC-derived kidney organoids, is something we have already started and will contribute to a better understanding of the early steps of this disease.

This approach also provides an ideal platform for identifying biomarkers for early cancer detection. Renal carcinomas are currently detected at advanced stages, when the disease has already spread to other organs. Being able to detect early tumoural markers either in the blood or urine would dramatically improve the lifespan of kidney cancer patients.

Alternatively, by generating hiPSC lines from metastatic kidney tumours, we will be able to generate kidney canceroids useful for personalised medicine. Drug-response could be efficiently tested in these avatars, helping oncologists to tailor the treatment to the patient.

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