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## Xenotransplantation Transgenesis. Are we there yet?

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### Introduction

The xenotransplantation of pig organs and tissues aimed at overcoming the world-wide shortage that exists for these in humans will require multiple genetic modifications to be incorporated to overcome rejection and associated problems. We have recently produced  $\alpha 1,3$  galactosyltransferase knockout (GTKO) pigs expressing human (h) CD55&59, fucosyltransferase (HTF), hCD39 and hTBM This was done by breeding GTKO pigs to animals expressing human hCD55 and hCD59 plus HTF and then transfecting adult fibroblasts obtained from their progeny with a hCD39 plus hTBM construct. Transfected cells were then selected based on hCD39 expression and used for animal cloning to produce these (Figure 1). The generation of these animals represents almost two decades of research focussed on developing the necessary molecular, cellular and reproductive technologies. Having produced GT KO pigs expressing five transgenes, it is an appropriate juncture to reflect on how this was achieved and what progress still needs to be made in terms of transgenesis technology to advance xenotransplantation to the clinic.

### In the Beginning

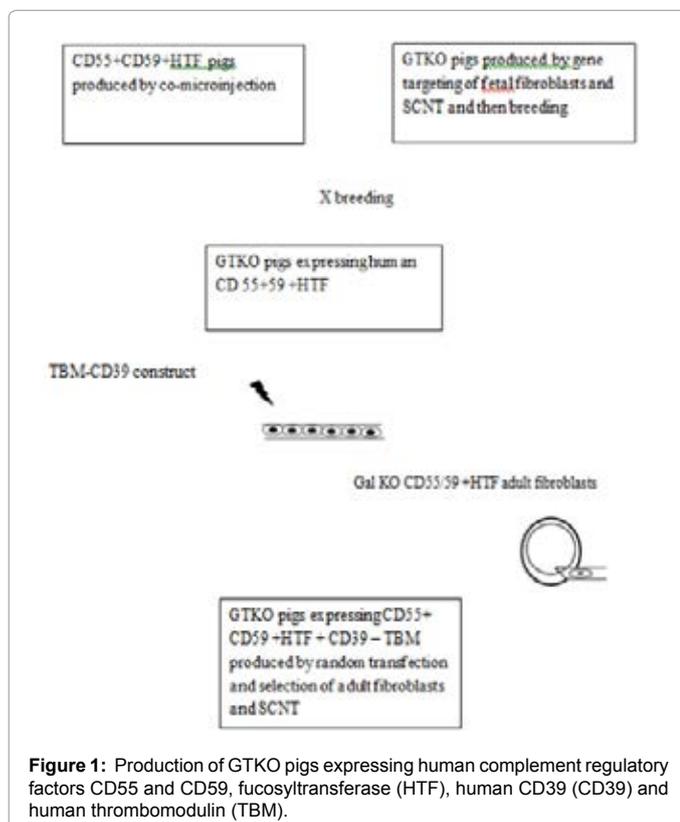
The production of transgenic pigs was originally restricted to using pronuclear microinjection which was beyond the reach of many groups

because of the relatively high cost of doing this. Having developed a large scale technology platform for producing growth hormone transgenic pigs in the early 1990's we used this to produce transgenic pigs for xenotransplantation research [1]. Initial studies focussed on the expression of multiple human complement regulatory factors to overcome hyper acute rejection. However, we realised that we needed a new approach to produce pigs expressing multiple transgenes, because cross-breeding animals containing a single modification took too long. As a result we demonstrated that it was possible to produce animals containing CD55, CD59 and H-transferase by co-injecting these three constructs at the same time [2].

### Cloning and GTKO Pigs

While we were one of the first groups to knock out Gal the major xeno antigen, in mice using gene targeting in embryonic stem cells (ESCs) [3]. The lack of pig embryonic at the time meant that we and others were unable to do this in pigs. The advent of animal cloning with the birth of Dolly [2] changed this however as ESCs were longer needed to perform a knockout. Subsequently we and several others produced cloned pigs [4,5] and then GTKO pigs [6] within a decade of Dolly. This was a remarkable achievement given the number of technologies that had to be developed, from *in vitro* oocyte maturation [7] to the use of promoter-less constructs to increase targeting efficiencies to workable levels [8] for this to happen. Previously we had reported that GTKO mice developed cataracts and there was concern that GTKO pigs would also. However by outbreeding our original Gal founder females to males of different breeds and then crossing GTKO animals we showed that GTKO pigs were viable and produced normal sized litters and. We also demonstrated that GTKO pigs could be produced by cloning using GTKO fetal fibroblasts [9].

The production of GTKO pigs using gene targeting of somatic cells and cloning heralded a new approach to the production of transgenic pigs. By transfecting somatic cells and then selecting those cells which expressed the transgene two of the major limitations associated with pronuclear injection could be overcome namely, the production of animals which did not contain the transgene (typically 80-90%) as well as those that did but did not express it (normally around 50%) could be overcome. In parallel, advances in *in vitro* embryo production



**Figure 1:** Production of GTKO pigs expressing human complement regulatory factors CD55 and CD59, fucosyltransferase (HTF), human CD39 (CD39) and human thrombomodulin (TBM).

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meant that we could use oocytes from slaughterhouse ovaries rather than collect one cell embryos from tens of live animals for pronuclear microinjection, further reducing costs and improving efficiencies. For example, we were able to produce 15 CD39 pigs for experimental and breeding purposes from eight transfers, whereas using pronuclear microinjection would have required up to three times this number of transfer to account for its inherent inefficiencies.

Despite these advances the major limitation to producing animals expressing multiple transgenes remained, namely the need to breed animals together to produce animals expressing multiple transgenes. While we had used co-injection in the past we had long recognised that the best approach would be one where multiple transgenes could be incorporated into the GTKO targeting construct so that they could be inserted as single copies at the same time as knocking out GT [10]. This approach would also allow all modifications to be inherited as a single unit, eliminating the production of those animals that did not contain all the modifications as a result of segregation during meiosis. To overcome this problem we have used the 2A ribosome sequence to produce transgenic mice expressing three genes on the one construct [11]. We then used this sequence to produce a construct consisting of CD39 and hTBM. This was then transfected onto our existing GTKO CD55+59 plus h transferase background and cells screened for CD39 expression. Suitably expressing cells were then used to produce GTKO pigs expressing all five transgenes as described above

### Stem Cells, Meganucleases and Beyond

In reviewing the literature in 2011 we suggested that for xenotransplantation to advance to the clinic, a cell type which could last longer in culture than fibroblasts was needed to produce pigs containing multiple modifications [10]. As such much of our research in recent years has focussed on isolating embryonic stem cells [10]. These together with other cell types such as induced pluripotent stem cells survive longer in culture allowing multiple modifications to be made and examined before using these for animal cloning. The ability to target specific sites in the genome using zinc finger nucleases has provided an alternative approach to knocking out GT using homologous recombination [12]. The use of Transcription activator-like effector nucleases (TALENs) also allows DNA to be inserted at the same time as knocking out gene function [13] and may provide an alternative approach to producing GTKO pigs expressing multiple transgenes inserted as single copies at this site.

This is a rapidly advancing field and it is now possible to envisage a technology platform in the near future where stem cells or similar are transfected *in vitro* using one of these approaches, screened to confirm integration and expression and then used for cloning to produce animals expressing multiple transgenes inserted at the GT locus. Furthermore the use CRISPR/Cas9 is allowing changes to be made by microinjecting directly into the cytoplasm of zygotes at similar if not higher rates than that achieved with cloning [14] and the production of GTKO pigs has already reported using this method in cells [15]. Whatever approach is ultimately used, we are already witnessing a wave of targeted modifications in pigs as a result of these technologies which has changed the way we make transgenic pigs. However the challenge for xenotransplantation is not only to be able to knockout both GT alleles but at the same time insert multiple transgenes at each allele. Something which is yet to be achieved despite more than two decades of research.

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