Titre du projet (Project title in French)

Utilisation des cellules souche pluripotentes pour etudier les maladies renal hereditaires : mecanismes genetique et development de nouveaux treatement specifiques.

Project title (eng)

Modelling Hereditary Kidney Disease using Induced Pluripotent Stem Cells: Determining Genetic Mechanisms and Developing Novel Targeted Therapies

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OBJECTIVES AND AIMS

Alport syndrome is a genetic kidney diseases that affects one in 10,000 individuals and occurs as a result of collagen IVα5 (*COL4A5*) mutations that affect kidney podocytes. Podocyte depletion is a hallmark of kidney injury, which ultimately leads to end-stage renal failure (ESRF), and for which there is currently no cure (1). Currently, there are no predictive disease models for Alport syndrome and other inherited kidney disorders, which imposes significant challenges for clinicians to provide effective and targeted therapies for these vulnerable patients. However, the recent discovery of adult cell reprogramming to generate induced pluripotent stem (iPS) cells (2) provides an unprecedented opportunity to elucidate disease mechanisms *in vitro*, to provide tools for understanding how mutations cause disease, to develop disease-modifying bioassays, and to advance cell replacement therapy. We will use iPS cell technology to develop an *in vitro*, long-term and self-renewing cellular model of X-linked Alport syndrome. This model will facilitate the development of effective treatments to correct diseased podocytes, and provide a robust platform for the development of new therapeutic interventions for inherited kidney disorders.

We have brought together an internationally renown team of researchers who have an interest in Alport syndrome. We will combine our expertise in cell reprogramming, podocyte biology, morphometric analysis and genomic expertise to generate multiple iPS cell lines from patients with Alport syndrome and to examine how missense and nonsense *COL4A5* mutations produce renal disease. The Chief Investigators (CI) Ricardo and Laslett have generated genetically tailored iPS cell-derived podocytes (hereafter designated as iPS podocytes) using enhanced green fluorescent protein (EGFP)+ tagged to the podocyte-specific gene podocin. The *podocin*^{eGFP}-iPS cells will enable the selective isolation of iPS podocytes. CI Prunotto in collaboration with Dr Aravind Subramanian from the Broad Institute, Boston (see letter of support), will use a chemical genomics connectivity map (cMap), a new tool for biomedical research that is a catalog of gene-expression data collected from human cells treated with chemical compounds and genetic reagents. This small molecule library screen has bought recent paradigm shits in the field and will be used to unravel the therapeutic effects of altered biological processes in *podocin*^{eGFP}-iPS podocytes derived from cells of Alport patients. These iPS cell-derived podocytes will provide a platform for screening therapeutic agents and identify if small molecules can reverse the disease phenotype specific to Alport syndrome mutations. Furthermore, as a proof of concept *in vivo* study, *podocin*^{eGFP}-iPS podocytes will be administered to mice with either podocyte depletion (CI Bertram) or Alport syndrome (CI Perin), and assessed for cell incorporation into damaged glomeruli and improved renal function.

Our **hypothesis** is that Alport mutations induce changes in cellular pathways and protein folding in podocytes that lead to predictable and detectable changes in endoplasmic reticulum (ER) stress, apoptosis, and autophagy. We predict that iPS podocytes generated from Alport syndrome patients will maintain the genetic phenotype of diseased cells, which will enable us to examine patient-specific disease pathogenesis and mechanisms that can be targeted for therapeutic interventions.

The main objectives are:

1. To generate iPS cells from patients with X-linked Alport syndrome and control subjects, and use human iPS cell reporter lines for the selection of iPS podocytes.
2. To investigate altered biological processes in *podocin*^{eGFP}-iPS podocytes from Alport patients using chemical genomics connectivity map (cMap) high-throughput screen.

The Secondary Objectives are:

3. To determine the viability and integration of differentiated mouse podocin-EGFP iPS podocytes following transplantation into podocin-Cre-induced diphtheria toxin receptor (iDTR) mice that have selective podocyte depletion and glomerular pathology.
4. To determine whether delivery of *podocin*^{eGFP}-iPS podocytes into Col4a5$^{-/-}$ Alport mice, with and without administration of an anti-hypertensive agent, slows the progression of renal disease.

Primary End Point (linked with the main objective)

Predicted Outcomes of Objective 1: This study will generate the first podocin-reporter iPS cell lines in Alport and non-Alport patients that will enable the selective isolation of iPS podocytes for use in pharmaceutical screening and therapy. Genome-editing technology using iPS cells is continually advancing, and we anticipate rapid improvements in CRISPR efficiency together with reduction of off-target effects. The collaboration of CI Ricardo and Laslett working with the Monash Gene Targeting Facility will allow for new advances to be rapidly incorporated into the project.

Predicted Outcomes of Objective 2: We predict that Alport iPS podocytes with *COL4A5* mutations will capture the essential aspects of Alport syndrome *in vitro*. We also predict that Alport iPS podocytes will show consistent phenotypic changes, compared control cells, and that in collaboration with CI Prunotto, will for the first time, enable small molecule screening using cMap to identify novel agents that can reverse the Alport phenotype. The outcomes of this study will provide both a basis for understanding how the extracellular matrix contributes to podocyte survival, how Alport mutations affect podocyte viability, as well as the development of an innovative disease model platform for new therapeutic drug discovery.

Secondary End Points (linked with the secondary objectives)

Expected Outcomes of Objectives 3 and 4: A significant obstacle for the delivery of iPS cells to treat renal disorders is the lack of data on iPS cell viability and integration after cell delivery *in vivo*. By combining the strengths of this international team we now have the opportunity to assess the viability, integration and therapeutic effects of iPS podocytes identified by GFP+ expression into injured glomeruli using a technique developed by Dr Perin (see Fig 5, preliminary data). In the mouse model of podocyte depletion, the degree of incorporation will vary dependent on the stage of kidney injury, being highest in the early stages following podocyte loss, and less at later stages of glomerular repair.

Leveraging on an established collaboration with Prof Bertram, the use of reporter iPS podocytes and a selective podocyte depletion model, coupled with state-of-the-art confocal morphometric analysis, provides a unique opportunity to complete these studies. In the Alport mouse we anticipate that transplantation of iPS podocytes, combined with an anti-hypertensive agent to aid in cell viability and survival, will reduce kidney pathology and improve kidney function. This finding will provide the first evidence that iPS cell-derived therapy can reduce protect from Alport kidney injury and will represent a major advance in the field.

Originality and innovative aspects

The major strengths of this project include the generation of iPS cells from a national registry of Alport patients with known genetic mutations, coupled with state-of-the-art resources for the CRISPR genomic modification of iPS cells for use in podocyte isolation. Patient-derived iPS podocytes are a potentially valuable tool for future modelling of human Alport syndrome, which overcomes inherent limitations in existing disease model systems. We will generate Alport syndrome iPS podocytes that preserve crucial genotype-phenotype correlations. To do this we will develop *podocin*^{eGFP}-iPS cell lines carrying mapped missense and nonsense mutations, which can be used to isolate podocytes for toxicity assays to test new candidate therapeutics. The delivery of EGFP-tagged iPS podocytes into mouse models with selective podocyte depletion and Alport syndrome will provide proof-of-concept testing and evidence for *podocin*^{eGFP}-iPS podocyte integration into injured glomeruli *in vivo*. Our long-term anticipated goal includes autologous transplantation of patient-derived iPS podocytes with engineered correction(s) of the inherent genetic defect(s), which will establish an innovative and state-of-the-art approach for *in vivo* cellular replacement of diseased kidney tissue.

iPS cell-based modelling of Alport syndrome will fundamentally advance techniques for pharmacological testing before clinical trials by developing a pipeline for high-throughput screening of drugs, natural compounds and toxins. Importantly, these patient-derived podocytes constitute an invaluable resource for understanding how mutations cause kidney disease, for screening new drugs, for developing disease-modifying assays, and for testing autologous cell-replacement therapies. The generation of patient-specific iPS podocytes from patients with inherited renal disease will be embedded in our clinical genetic screening platforms, which offers an innovative approach that will bridge science discovery with population health.

BACKGROUND

The epidemic of chronic kidney disease (CKD) and end-stage renal failure (ESRF) is a crisis for global healthcare. There is urgent need for new therapeutic options considering the high morbidity of dialysis, extensive healthcare costs, and donor-kidney shortages. Alport syndrome results from *COL4A5* genetic mutations that affect kidney podocytes and leads to proteinuria. Therefore, *in vitro* kidney disease modelling provides a powerful tool for the investigation of podocyte defects underlying Alport syndrome and a platform for rapid screening of novel therapeutic compounds. Inherited kidney failure due to Alport syndrome affects one in 10,000 people; currently, there is no known cure. Alport syndrome is the most significant inherited disease affecting glomeruli (the kidney filtration units). Affected males develop progressive haematuria at approximately age 6, and eventually develop proteinuria and ESRF in their teens. Hearing loss is common, and eye abnormalities occur in more than half of these individuals and approximately 15% of affected females with X-linked Alport syndrome develop ESRF by age 60. Most Alport patients (85%) have X-linked disease that causes missense or nonsense mutations in the *COL4A5* gene. How these mutations cause renal disease is not completely known. The majority of remaining patients have autosomal-recessive disease with homozygous or compound heterozygous mutations in the *COL4A3* or *COL4A4* genes.

How *COL4A5* mutations cause disease. More than 600 unique genetic variants have been described in X-linked Alport syndrome (3), and the majority of these are pathogenic. Most inherited kidney diseases are due to multiple mutations. The specific point mutations in Alport disease produce proteins that fail to achieve their correctly folded state and fail to traffic normally from the ER through the Golgi to the lysosome (4). Pathological endpoints of ER stress include apoptosis and autophagy. In Alport syndrome, the accumulation of misfolded proteins in the podocyte ER induces the unfolded-protein response (UPR) for missense mutations and nonsense-mediated decay (NMD) for nonsense mutations. ER stress arising from defective collagen IV chain localisation in human podocytes contributes to Alport syndrome pathogenesis (4), and results in podocyte injury and proteinuria. UPR and ER stress triggers apoptosis by up-regulating pro-apoptotic factors and down-regulating anti-apoptotic factors. The extent of these downstream responses will be examined in Specific Aim 2 using iPS podocytes generated from Alport patients. The results will address an important knowledge gap, allow us to determine genotype-phenotype correlations for the extent of injury, and enable correlation mapping to the patient cohort.

Pluripotent stem cells targeting the kidney. Similar to other stem cells, iPS cells can proliferate indefinitely *in vitro*, creating a theoretically unlimited source of cells. Additionally, iPS cells are a therapeutically relevant system for modelling diseases, and potentially could be developed as a technology for cellular replacement as we have reviewed (5). Recent reports suggest that iPS cells can provide a powerful tool for modelling kidney diseases: iPS cells have been produced from mesangial cells (6) using four reprogramming factors (*Sox2*, *Oct4*, *Klf4*, and *c-Myc*); certain kidney diseases, including genetically inherited diseases, do not hinder the reprogramming process (7); pluripotent cells were successfully differentiated to an early renal lineage (8); we reported reprogramming iPS cells to *WT-1+/Pax2+/synaptopodin+* podocytes by the addition of bone morphogenetic protein (BMP7), retinoic acid, and activin A (9).

How will iPS podocytes model Alport syndrome? Podocyte research has been hampered by the lack of suitable models for the direct study of factors regulating podocyte survival and function. Primary cultures of human podocytes only replicate for a short time, and they cannot be maintained over long periods. In contrast, podocytes derived from iPS cells maintain characteristic podocyte features (9) and robustly proliferate over long periods. There are clear benefits of using iPS cells compared to immortalized cells. Whilst iPS cells can self-renew indefinitely in an undifferentiated state, immortalized cells do not normally divide indefinitely but have normally gained this ability due to mutation. Moreover, iPS cells have a differentiation capacity that is not observed in immortalized lines and therefore provide opportunities for cell replacement therapy.

Alport syndrome causes GBM and podocyte abnormalities; however, essentially nothing is known about how the X-linked mutations cause disease at the cellular level. We will generate iPS podocytes from X-linked Alport syndrome patients, which will maintain their genetic phenotype and functional characteristics. We will use *podocin*^{eGFP}-iPS cells to isolate podocytes in order to clarify the signalling mechanisms that regulate disease pathogenesis. The established Alport *podocin*^{eGFP}-iPS podocytes will allow us to determine how missense and nonsense *COL4A5* mutations cause renal disease. We will investigate the functions and roles of signalling pathways that are known to function in other progressive collagen IV nephropathies, including NMD, UPR, ER stress, autophagy, and apoptosis (4). Constructing this model system is the first step towards the development of remedial therapies for genetic mutations identified form the small molecular screen.

PRELIMINARY DATA

(i) iPS podocytes generated from Alport syndrome patients

Our team has proven expertise in the distinct skill sets that will be needed to achieve the project goals, including iPS cell technologies and generation targeted at Alport syndrome, experimental nephrology, podocyte biology, and morphometric analysis. We were the first group in the world to derive iPS cells from human kidneys (10), have previously reported the differentiation of embryonic stem (ES) cells to intermediate mesoderm, and also provided extensive transcriptional profiling of these renal progenitors (11). Further, we developed a differentiation protocol for iPS cell-derived podocytes (9) (**Fig. 1**), and have now developed tools using CRISPRs and a targeting construct to make a knock-in

into the podocin gene. Using established Sendai virus cell reprogramming, we have derived iPS cells from patients with Alport syndrome using defined media without mouse embryonic feeder (MEF) cells (**Fig. 1**). To date, we have characterised iPS cells from two X-linked Alport patients and three normal individuals, and mapped the genetic aberrations to nonsense or missense patient mutations.

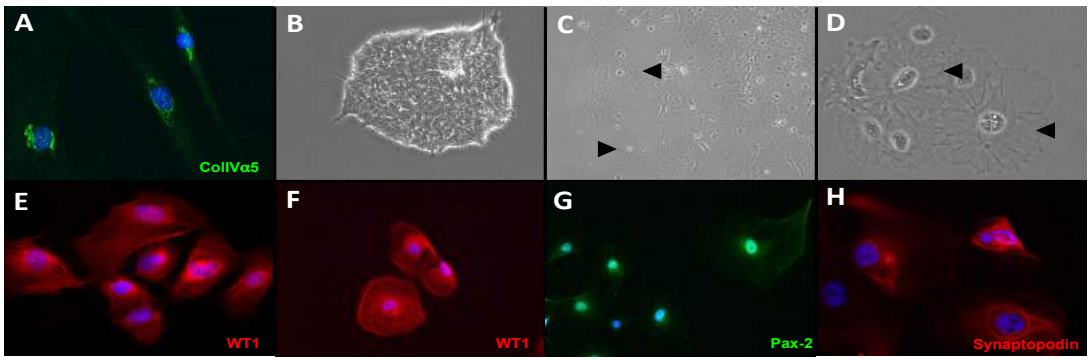


Fig 1. iPS podocytes derived from Alport patients. (**A**) Alport skin fibroblasts immunostained for collagen IVα5 (green). (**B**) Phase contrast image of iPS cells from an Alport patient maintained in feeder-free culture. Cell outgrowths (*arrows*) were propagated in differentiation media (shown at day 3) (**C**), and differentiated into iPS podocytes at day 10 (**D**, *arrows*). iPS podocytes expressed WT-1 (red) at 20 days (**E**), which was comparable to primary human podocytes (**F**). Differentiated iPS podocytes at day 20 expressed Pax2 (**G**, green) and synaptopodin (**H**, red). Nuclei were stained with DAPI (blue in panels A and E–H). Magnification: A and D–G, ×200; B, ×4; C, ×10; H, ×400.

(ii) Alport COL4A5 mutations result in alterations in iPS cell phenotype

There is great therapeutic interest in the application of exogenous chaperone molecules such as 4-phenylbutyrate (PBA) that assist in corrective folding of mutant collagen trimers in Alport syndrome. Using electron microscopy we have, for the first time, shown a reduction in rough ER (RER) size in Alport iPS podocytes following PBA treatment (0.1 mg/ml cultured for 24 hours; **Fig. 2**) with no effect observed following the addition of PBA on control cells.

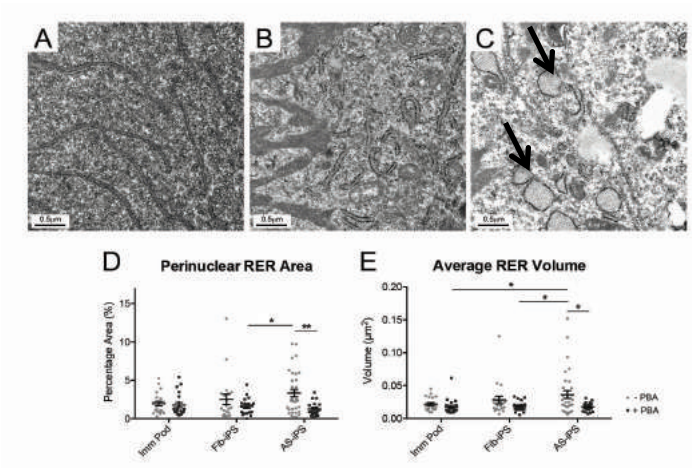
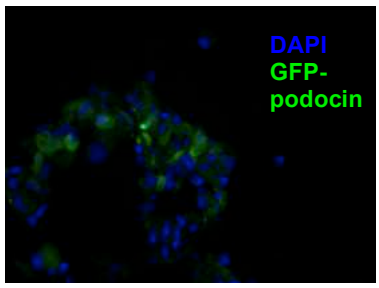


Fig 2 (left). Alport iPS podocytes show differences in RER volume, normalised by PBA treatment. Quantification of perinuclear RER percentage are and volume revealed differences in means of cell lines (n=20) in A) immortalised human podocytes (ImmPod) B) non-Alport fibroblast-derived iPS podocytes (Fib-iPS) or C) Alport iPS podocytes (AS-iPS) at day 10 of differentiation. Alport iPS podocytes showed a significant increase in RER area (D) and RER volume (E) that correlated with distended cytoplasmic RER organelles (C; *arrows*) compared to the uniform RER luminal widths of control human podocytes (A) and control iPS podocytes (B). PBA treatment reduced the total perinuclear RER percentage area of Alport iPS cells ($P<0.05$) and average RER volume ($P<0.05$). 40,000x magnification. Analysis: All data compared by Two-way ANOVA with Tukey's *post hoc* test. Values are mean ± SEM.



(iii) CRISPR-tagged *podocin*^{eGFP}-iPS cells were differentiated into GFP+podocytes and injected intrarenally into day 2 postntal kidneys (**Fig 3,** see right). The cells were found to integrate into postnatal day 3 kidneys following intrarenal injection. GFP expression was used to verify the expression of podocin+ cells. The same strategy

will be used to generate podocin^{eGFP}-modified iPS cells to isolate GFP+ iPS podocytes from Alport patients and inject into podocyte depleted and Col4α5$^{-/-}$ Alport mice.

(iv) *Pod-Cre-iDTR* transgenic mice, generated by Prof Bertram by crossing *Pod-Cre*$^{+/-}$ mice with *iDTR*$^{+/+}$ mice (*Journal of the American Society of Nephrology*, In Press), will be used as an experimental model of kidney disease for the injection of *podocin*^{eGFP}-iPS podocytes. In these mice, only podocytes express the human diphtheria receptor (DTR), and treatment with diphtheria toxin (DT) results in dose-dependent podocyte death, proteinuria, and glomerular pathology (**Fig. 4**).

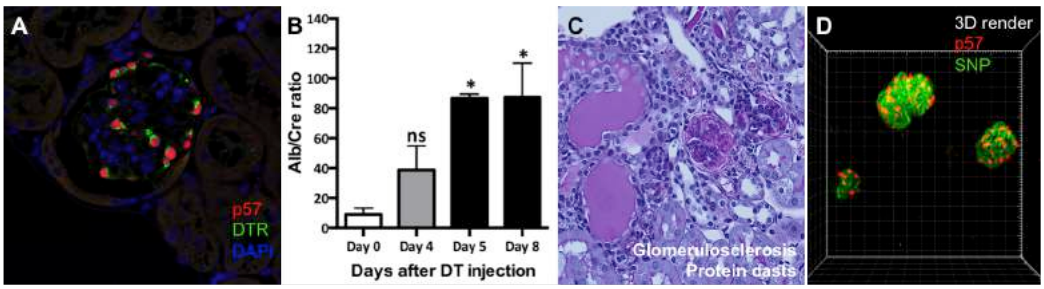
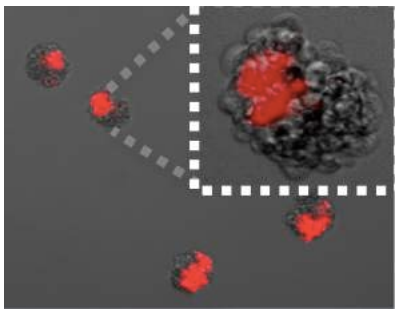


Fig. 4. (**A**) Confocal microscopy shows that in *Pod-Cre-iDTR* mice, the DT receptor (green) is expressed only by podocytes; p57 (red) marks podocyte nuclei. Treating *Pod-Cre-iDTR* mice with DT (0.03 μg/kg) produces (**B**) transient proteinuria, which peaks at day 8 (p<0.05 *versus* day 0) and is resolved by day 25; (**C**) glomerulosclerosis (day 35); and (**D**) the death of ~50% of p57+ podocytes, shown co-localised with the podocyte marker synaptopodin (SNP).

(v) Assessing stem cell integration into kidney glomeruli from Alport mice.



CI Perin has developed methodology for assessing the integration of injected labelled stem cells into damaged kidneys that will be used for the current study. Following injection of fluorescent-labelled cells via intracardiac injection of Col4a5$^{-/-}$ mice, the kidneys will be excised at 1 week, 1.5 and 3 months and glomeruli isolated using mechanical sieving and the cells visualised using fluorescence microscopy as shown in Fig 5.

Fig 5 (left). Fluorescent-labelled stem cells in glomeruli isolated from Alport mice at 5 days after injection into Alport mice.

RESEARCH DESIGN AND METHODS

Objective 1. To generate iPS cells from patients with X-linked Alport syndrome and control subjects, and use human iPS cell reporter lines for the selection of iPS podocytes.

Phase 1. Identification of patients with X-linked Alport syndrome and establishment of cell lines. We will initially target three missense mutations and three nonsense *COL4A5* mutations (n=*6* skin biopsies total), and compare these with our previously generated and mapped Alport fibroblasts and Alport iPS cell lines. We have access to X-linked missense mutations in *COL4A5* in >40 families with Alport syndrome through a national registry, and developed mutation-detection assays based on DNA or mRNA. Consent will be obtained from patients (18–60 years of age) with genetic diagnosis of X-linked Alport syndrome to provide a skin biopsy. Control specimens from patients who do not have Alport syndrome (n=3) will also be obtained. Skin fibroblasts will be reprogrammed to iPS cells, tested for pluripotency, and characterised as described previously by CI Ricardo (6).

Phase 2. Generation of transgenic human and mouse iPS podocyte reporter cells using RNA-guided cell engineering (CRISPR via Cas9). Gene editing is becoming an important component of patient-specific iPS cell modelling and therapeutic strategies [for review, (16)]. A powerful new tool that enables targeted manipulation of the human genome is the CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 system (29), which has high targeting efficiency and low toxicity. We have used CRISPRs and a targeting construct (IRES-EGFPFRTneomycinFRT cassette) to make a knock-in into the podocin gene in normal skin fibroblast-derived iPS cells **(Fig 3)**. We will use the same approach to generate a podocin^{eGFP}-Alport iPS cell line using the Monash Gene Targeting Facility, initially in an already established Sendai viral reprogrammed Alport iPS cell line (**Fig. 1**).

Moreover, the human iPS cell line that expresses eGFP in the *NPHS1* locus, which encodes the podocyte-specific marker nephrin (12) is currently in-house in the laboratory of CI Ricardo. These nephrin-positive, *NPHS1*^{eGFP}-expressing podocytes showed phenotypic podocyte characteristics, which was confirmed by immunolabelling and morphological analysis (13). Bioinformatics analysis of sorted eGFP-expressing cells confirmed the homology of podocyte genes, and when these cells were transplanted into recipient animals, they formed glomerular structures (12). We will use our pioneered differentiation method for the cultivation and differentiation of the *podocin*^{eGFP}-iPS cells (9) and use *NPHS1*^{eGFP}-iPS cells (12) as a comparison of our differentiation protocol to generate GFP+podocytes that are isolated by flow cytometry for disease modelling and cell transplantation.

Objective 2. To investigate altered biological processes in *podocin*^{eGFP}-iPS podocytes from Alport patients using chemical genomics connectivity map (cMap) high-throughput screen.

Podocin^{eGFP}-iPS podocytes are a valuable tool for investigating how Alport mutations modulate protein misfolding and collagen IV abnormalities, which contribute to the pathogenesis of a number of nephropathies (4). Three cell lines will be studied: (1) Alport *podocin*^{eGFP}-iPS podocytes; (2) non-Alport control *podocin*^{eGFP}-iPS podocytes (already generated); and (3) primary cultured podocytes (in house, purchased from Celprogen Inc). At least three biological replicates (from each clone) and three technical replicates will be analysed for the culture experiments.

Phase 1: Generation of Alport *podocin* GFP-iPS podocytes and cell phenotype characterisation. Alport *podocin*^{eGFP}-iPS podocytes will be transfected with wild type COL4A5 shRNA or empty vector. The two generated cell populations will be then characterised A) by mRNA sequencing evaluating their similarity to primary Alport or non-Alport iPS podocytes, B) functionally tested with a very simple battery of well characterised stimuli (e.g., TNF-α, TGF-β, etc.).

Phase 2: Chemical Genomic screen. The two populations will be then expanded and be submitted to incubation with a 1000 compound library. The compound library will be designed by the Broad Institute (laboratory of Dr. Aravind Subramanian) in order to cover all possible cellular pathways. Treated cells will be submitted to CMap analysis, a high-throughput transcriptomic platform allowing quantification of 1000 transcript in one single screen. Compounds showing differential effects in the two cell populations will then be used to assess biological processes altered in the Alport syndrome phenotype. Hit expansion using the Broad Institute cMap historical database will be then performed and these compounds will be used to validate the approach on additional 5 Alport *podocin*^{eGFP}-iPS podocytes to validate results of the screen.

Phase 3: Bioinformatics. The compounds from the screening and the validation set will then be used to interfere with the altered biological processes in Alport iPS podocytes and based on this information, and the historical records of CMap screened 'conditional probability distributions', a compound will be selected and its ability to revert the Alport phenotype will be assessed *in vivo* in Col4α5$^{-/-}$ Alport mice.

Objective 3. To determine the viability and integration of differentiated mouse podocin-EGFP iPS podocytes following transplantation into podocin-Cre-induced diphtheria toxin receptor (iDTR) mice that have selective podocyte depletion and glomerular pathology.

We will test if mouse *podocin*^{eGFP}-iPS podocytes can incorporate into injured glomeruli and replace lost podocytes *in vivo* using the Pod-Cre-iDTR transgenic mice (Fig. 4, *Journal of the American Society of Nephrology*, CI Bertram). *Podocin*^{eGFP}-iPS podocytes will be delivered to two groups of *Pod-Cre-iDTR* mice (n=10 mice/group): (1) *Pod-Cre-iDTR* mice with injection of DT (0.03 mg/kg) to induce reversible kidney damage, and (2) *Pod-Cre-iDTR* with vehicle saline injection. *Podocin*^{eGFP}-iPS podocytes (0.5×10^6 cells) will administered via the renal artery [routinely used by CI Ricardo (14, 15)] into male mice in both groups at three different time points after DT administration [day 0, day 10 (during the course of injury), and day 25 (established glomerular pathology)]. The mice will be euthanized 3 days after cell transplantation. The selected time points will allow comparisons of podocyte replacement after 50% podocyte depletion (day 10) or during established disease (day 25). We will use our combined expertise of podocyte biology (9), podocyte loss (16, 17), and *in vivo* cell tracing using confocal microscopy to assess GFP+ podocytes. This will be confirmed by immunofluorescence analyses using an anti-GFP antibody, DT receptor staining, and the podocyte

nuclear marker P57. Kidney histopathology will be assessed and renal function will be determined using serum BUN/Cr and albuminuria as reported by CI Ricardo (18, 19). We predict that the *podocin*^{eGFP}-iPS podocytes will incorporate into injured glomeruli of the recipient animals. The degree of incorporation will vary depending on the stage of glomerular injury; it will be highest during the early stages following podocyte loss, and then decline during later stages of glomerular disease. The use of *podocin*^{eGFP}-iPS podocytes and a selective podocyte depletion model, coupled with state-of-the-art confocal morphometric analysis, provides a unique strategy to achieve the project goals.

Objective 4. To determine whether delivery of *podocin*^{eGFP}-iPS podocytes into Col4a5$^{-/-}$ Alport mice, with and without administration of an anti-hypertensive agent, slows the progression of renal disease.

We will deliver normal *podocin*^{eGFP}-iPS podocytes to Col4α5$^{-/-}$ Alport mice (B6.Cg-*Col4a5*^{tm1Yseg}/J; perfomrd by CI Perin at USC), to determine if iPS podocyte therapy ameliorates progression of disease. After 7 weeks, hemizygous (X-linked) mutant male Col4α5$^{-/-}$ mice exhibit proteinuria resulting from collagen IV podocyte mutations, and eventually develop renal failure (20). *Podocin*^{eGFP}-iPS podocytes (8×10^5 cells) will be delivered, via the renal artery or intracardic injection, into four groups of male mice (6–8 weeks of age): (1) Col4α5$^{-/-}$ Alport mice; (2) wildtype Col4α5$^{+/+}$ mice; (3) Col4α5$^{-/-}$ Alport mice receiving the ACEi ramipril (10 mg/kg/day in drinking water) (21); and (4) wildtype Col4α5$^{-/-}$ mice receiving ramipril (n=20 mice/group). An additional cohort of Col4α5$^{-/-}$ Alport mice will receive 100µl PBS control (diluent volume for iPS podocytes).

The injected *podocin*^{eGFP}-iPS podocytes will be visualised in glomeruli isolated from recipient mice using the methodology developed by Dr Perin (**Fig 5**). Cells will be visualised using phase and confocal microscopy of EGFP expression, and confirmed with an anti-GFP antibody. We anticipate increased viability and survival of differentiated iPS podocytes when co-administered with ramipril, which is the major therapeutic treatment for Alport patients. This represents a clinically relevant therapeutic approach. Kidney function, renal histopathology, and survival rates will be determined in Alport mice with and without cell delivery or therapy. Mice will be euthanized during early (1 wee and 1.5 months old; before structural damage) and late disease (3 months old), as reported by Dr Perin (22), and EGFP+ podocyte integration will be assessed at both time points.

Statistical analysis will be performed using GraphPad Prism (GraphPad Software Inc., USA). Our previous *in vivo* studies in experimental renal disease models (14) determined that a group size of >8 provides sufficient statistical power to test the therapeutic effects of cell therapy. For cell culture comparisons between treatments, an unpaired *t*-test will be used to analyse the data. For multiple intergroup comparisons, data will be analysed via ANOVA with Tukey's *post hoc* test.

Team Quality and Capability

Strength of the team and how will the team work together? The research team is unique, in that it draws together world-renowned researchers with complimentary capabilities in stem cells, genomics, and experimental nephrology, and who have a focus on Alport syndrome. This is an internationally collaborative project that will build a platform between Monash University, the University of Southern California and the University of Geneva to develop a new way in exchanging the knowledge and technologies and also strengthen the scientific communications between top-research corporations in Europe, USA and Australia. The iPS podocytes and CRISPR lines will be cultured at Monash University (CIs Ricardo and Laslett) and delivered into podocyte-depleted mice housed at Monash (CI Bertram). We currently successfully ship iPS cells internationally using a 'dry shipper' unit via FedEx. Therefore, the *podocin*^{eGFP}-iPS podocytes will be shipped to University of Geneva and the Broad Institute, Boston (Prunotto/ Subramanian) for propagation to perform the genomics cMAP small molecule screen. Likewise, the *podocin*^{eGFP}-iPS podocytes will be shipped to CI Perin at USC for injection into Alport mice.

Communication: We will have monthly teleconferences to discuss the project and will meet collectively at least once a year during the European Alport meeting and the American Society of Nephrology annual scientific meeting. Laboratory exchanges are planned for the Postdoctoral Fellow and PhD students recruited to the project. Results generated from this project will be of wide scientific interest including patient groups. We will publish in the highest-ranking peer-reviewed journals that

have open access options where possible. Results will be presented by oral and poster communication at national and international conferences, both by the CIs, postdoctoral fellows and PhD students.

Track Record of Research Team

Professor Sharon Ricardo has over 22 years of experience in kidney disease and stem cell research. Her laboratory was the only one worldwide with the capabilities to reprogram kidney cells to iPS cells, having made pioneering discoveries in this area (6). Her international standing is evident by being in the top 5 published authors on 'iPS cells and kidney' worldwide over the past 10 years (ISI Web of Science); and being on the Editorial Board of *Kidney International.* The work forming the basis of this grant has won awards (e.g., AMGEN science award); has been presented at the American Society of Nephrology (ASN) meeting, the American Transplant Society meeting, and as the Keynote presentation at the International Society of Nephrology meeting. She will direct the study, co-ordinate meetings and organise regular teleconferences and oversee manuscript publication and PhD and postdoc travel between laboratories.

Dr Laura Perin has more than 15 years of experience, notably she is an expert in the application of stem cells as potential therapeutics to treat disease progression in Alport Syndrome. She has extensive experience with animal models of Alport Syndrome and disease progression. Dr Perin has been an invited speaker at international meetings, such as the ASN meeting and the American Transplant Society meeting. In addition to her scientific accomplishments Dr Perin is highly involved within the American Alport Foundation, specifically, Dr Perin is organizing the first Family Alport Workshop this year in her institution. This workshop will provide Alport Syndrome patients the possibility of interacting with highly experts on the field ranging from genetics, scientists and nephrologists. Dr Perin will perform the iPS podocyte injection into Alport mice and assessment of kidney pathology and function in her laboratory at USC.

Dr Marco Prunottto has an academic appointment at the Institute of Clinical Pathology, University of Geneva. Dr Prunotto will perform the cMap small molecule screen on the iPS podocytes in collaboration with Dr Aravind Subramanian from the Broad Institute of MIT and Harvard (see letter of support). It constitutes a new collaborative effort that will be performed independent of Dr Prunotto's position at Roche. Dr Prunotto and Subramanian have initiated a basic science collaboration on Alport syndrome between Geneva University (Prunotto), the Broad Institute @ the MIT (Subramanian). The use of iPS podocytes from patients with Alport syndrome provides and innovative approach to probe pathological pathways and therapeutic targets using this technology that has not been done previously.

Professor John Bertram has conducted kidney research for 30 years (>250 publications) on topics including renal stem cells, nephron number and fetal origins of adult disease. We is internationally recognised for his work on podocyte biology, developing new imaging and morphometric methods for assessing podocyte number in glomeruli. Profs Bertram and Ricardo are co-located in the Biomedical Discovery Science Precinct at Monash University and have an established research collaboration with 13 co-authored papers, past joint-grant funding and joint supervision of PhD students. Prof Bertram will use his colony of *PodocinCre/iDTR* mice to test the therapeutic effects of iPS podocytes and quantitate integration using imaging techniques that are established in his laboratory.

A/Prof Andrew Laslett is internationally known for his high impact research on pluripotent stem cells and creating novel tools that enhance pluripotent cell research translation. This work has been published in *Nature Methods*, *Cell Stem Cell*, *Nature Biotechnology* and *Stem Cells*. CIs Laslett, Ricardo and Bertram have a long-standing research collaboration with the fist co-authored publication in 2010 reporting a differentiation method for ES cells to renal progenitors. A/Prof Laslett's will contribute intellectually to experimental design and provide technical expertise on the CRISPR-tagged cell lines at Monash University, in addition to data collection and preparation of manuscripts.

Five recent publications for team:

1. Song B, Niclis J, Alikhan M, Sakkal S, Sylvain A, Kerr PG, **Laslett AL**, Bernard CA and **Ricardo SD**. Generation of induced pluripotent stem cells from human mesangial cells. ***J Am Soc Nephrol*** 22(7):1213-1220, 2011. Citations 66. *The first successful reprogramming of induced pluripotent stem cells from human kidneys - featured on the cover of the top-ranked kidney journal and was highlighted in an editorial article.*

2. Puelles VG, …J Li, IS Harper, DJ Nikolic-Paterson and **JF Bertram**. A novel 3D method for counting and sizing podocytes in whole glomeruli: validation in a model of podocyte depletion. ***J Am Soc Nephrol*** (in press, Jan 21 2016). *Describes a new method based on kidney clearing, immunofluorescence and confocal microscopy for imaging, counting and sizing podocytes in whole mouse glomeruli. This technique will be employed in this AIRG project.*

3. Laurent LC, Ulitsky I, Slavin I, Tran H, Schork A, Morey R, Lynch C, Harness JV, Lee S, Barrero MJ, Ku S, Martynova M, Semechkin R, Galat V, Gottesfeld J, Izpisua Belmonte JC, Murry C, Keirstead HS, Park HS, Schmidt U, **Laslett AL**, Muller FJ, Nievergelt CM, Shamir R, Loring JF. *Cell Stem Cell* 7;8(1):106-18, 2011 (citations 502). *This high impact* paper *provides critical information regarding genomic stability for the use of human pluripotent stem cells (hPSC).*

4. Polanco JC, Ho MS, Wang B, Zhou Q, Wolvetang E, Mason E, Wells CA, Kolle G, Grimmond SM, Bertoncello I, O'Brien C, **Laslett AL**. *Stem Cells* 31(8):1498-510, 2013 (citations 10). *Manuscript describes novel methodology to identify unsafe human IPS cell lines with CI Ricardo as senior author.*

5. Sedrakyan S, Da Sacco S, Milanesi A, Shiri L, Petrosyan A, Varimezova R, Warburton D, Lemley KV, De Filippo RE, **Perin L**. Inejction of amniotic fluid stem cell delays progression of renal fibrosis. ***J Am Soc Nephrol*** 23(4):661-73, 2012 (Citations 47). *Stem cell injection into colIVa5 -/- mice shown to ameliorate kidney fibrosis that will be used for this study.*

BUDGET

To complete the aims a total of €150.000 is requested over two years. The funds will be distributed to for research to be conducted at Monash University, University of Southern California (Alport mice) and the University of Geneva (cMap/bioinformatics).

Personnel: €80,000. An experienced postdoctoral Fellow is requested and will be based at University of Geneva and Monash University. A postdoctoral researcher is requested as the derivation of iPS cells from primary cultures (Monash) and in vivo testing (Monash/USC) requires specialised expertise. The requested postdoc will work with the Monash platform to generate the EGFP-podocin iPS cell lines. FACS isolation of *podocin*^{eGFP}-iPS podocytes is required for all Aims. The requested postdoc will generate the iPS podocytes for delivery into the in vivo models of podocyte depletion and Alport Syndrome. They will also perform the qPCR, ELISAs, and immunofluorescence microscopy.

Cell culture €12,500. iPS differentiation and cell reprogramming requires a large amount of media for the extensive experiments planned. These costs include growth factor supplements and reagents i.e.: cell reprogramming vectors @ €750.00 each and supplements including Activin A 3@875.00 = €2625.00, BMP7 3@2088.00 = €6264.00, Retinoic acid €179.00.

Animal costs €8,000. Pod-Cre-iDTR Mice @ 6-8 week old = €29/mouse x 2 groups x 10 per group x 2 = € 1150.00. Agistment @€ 4.5/box/week = €140.00. Diphteria toxin 1@680.00 = €680.00. Col4a5 -/- Alport Mice @ 6-8 week old = € 29/mouse x 2 groups x 20 per group x2 = €2272.00. Col4a5 +/+ Wildtype Mice @ 6-8 week old = € 29/mouse x 2 groups x 20 per group x2 = € 2272.00. Col4a5 -/- Alport mice Agistment € 4.5@€7/box/week : 5 mice/box : 2 boxes x 6 weeks x2 = €105.00 and €212 .

cMap and Genomics analysis €15,000. Following the amplification and sequencing of Alport patient-derived iPS cells genome by unique single cell sequencing technology, a detailed CMap analysis of the Alport cells will be conducted. The costs for screening and analysis and bioinformatics are estimated at 15,000. Technical support will also include data analysis in differential analysis, correlation analysis and comparison analysis.

qPCR, flow cytometry and immunofluorescence microscopy and kidney pathology €12,000. Regent costs for qPCR including PicoPure RNA isolation kits $1854.29 each and SuperStrand III first-strand synthesis 2@747.00 = €1494.00, TaqMan Universal PCR Master Mix 6@602.61 = €3615.66. Costs also include primary and secondary antibodies and ELISAs.

CRISPR cell lines €9800. Mouse and human Podocin- IRESEGFP- polyAFRTneomycin- FRT targetingconstruct €3030 each Mouse and human Podocin Southern Screening Package €1280 each Cas9 nickase / sgRNA plasmids for Podocin. €950 each Sequencing and BAC clones €4000

Travel €6,000. Travel expenses, including flights and accommodation, are requested for exchange programme for the postdoctoral research and PhD students working on the project.

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2 April 2016

Professor Dominique Chauveau

Head, Scientific Council of AIRG France.

Re: Letter of Support AIRG grant 2016

Dear Professor Chauveau,

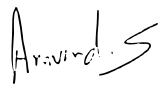
I am delighted to write a letter of support for the grant titled 'Modelling Hereditary Kidney Disease using Induced Pluripotent Stem Cells: Determining Genetic Mechanisms and Developing Novel Targeted Therapies' submitted to the AIRG France.

In my role as Director of the Connectivity Map (CMap) program at the Broad Institute, Boston, USA, we will design a compound library to screen the Alport *podocin*^{eGFP}-iPS podocytes. The cells will be submitted to CMap analysis, a state-of-the-art high-throughput transcriptomic platform that has generated over 2M gene expression profiles from ~4,500 drugs.

This is an exciting project that will unravel novel biological processes altered in the Alport syndrome phenotype with the intent to develop new therapeutic targets.

Funding from the AIRG will allow for a future international research program with Dr Marco Prunotto and Prof Sharon Ricardo focussed on Alport syndrome.

Yours sincerely,



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