Vitamin D3 influences the differentiation of podocytes in situ and in vitro

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Chronic kidney disease (CKD) is a worldwide health concern that affects millions of individuals. It may present as nephrosclerosis, cystic disease or glomerulopathy. The most prevalent subtype of glomerular disorders is podocytopathies. There is currently no causal therapy for CKD, thus highlighting the importance of timely identifying risk factors and high-risk groups to employ preventive measures. Since podocyte dedifferentiation displays a key mechanism in the pathogenesis of podocytopathies, a drug screening tool was recently established based on a transgenic mouse model - the glomASSAY. This model uses mice that express a cyan fluorescent protein (CFP) in podocytes under the control of the nephrin promoter. Glomeruli are isolated from such animals and transferred to an *in vitro* culture to investigate the effects of a certain pharmacological treatment. Freshly isolated glomeruli display strong CFP expression in podocyte cell bodies and foot processes, as well as the typical podocyte markers proteins like nephrin, podocin and WT1. The Glom*Assay* is based on the spontaneous dedifferentiation of podocytes *in vitro*, which is accompanied by a reduction of nephrin expression. As nephrin is one of the first proteins to be downregulated during the differentiation process, this reduction results in a subsequent downregulation of the related CFP expression in a matter of days, which can be quantified using laser scanning microscopy.

Effects of vitamin D3 on podocytes homeostasis

CKD patients are commonly vitamin D3 deficient. This deficiency is associated with albuminuria, disease progression and a higher mortality rate. Vitamin D3 plays a significant role in cell differentiation, proliferation, apoptosis control, and podocyte integrity, although its exact role is still a subject of controversy. One promising result of pre-screening was the positive effect of vitamin D3 on CFP expression, suggesting its role in podocyte differentiation. In order to elaborate on this issue, investigators upgraded the Glom*Assay* to a semi-automated high-throughput screening platform. In this way, they were able to drastically increase the number of samples while decreasing the number of animals required, making this approach more compatible with high-throughput screening.

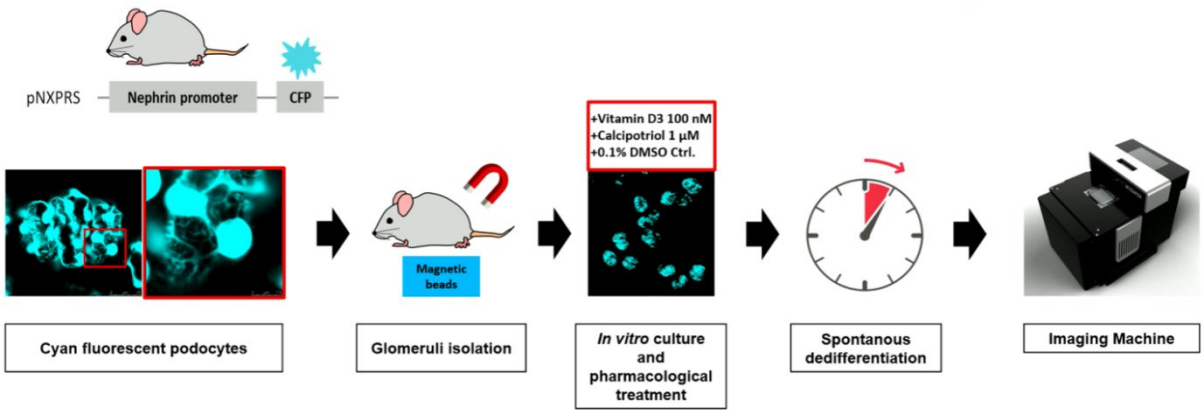

Figure 1. The Glom*Assay* upgrade to a semi-automated high-throughput screening platform

Glomeruli were isolated and treated with 100 nM vitamin D, 1uM calcipotriol (a synthetic vitamin D analogue with a high affinity for the vitamin D receptor and less calcification activity than vitamin D) and 0.1% DMSO as control. All treatments were performed on the glomeruli of the same mouse. Images were acquired on days 3, 6, and 9, and data analysis was performed using a custom-written script. Image analysis showed a spontaneous decrease in CFP fluorescence intensity over time in the control samples. In contrast, vitamin D treatment led to an increase in CFP intensity compared to the control, as did the treatment with calcipotriol. These results were confirmed by statistical analysis, which found a significant increase in CFP fluorescence after vitamin D and calcipotriol treatment compared to the control.

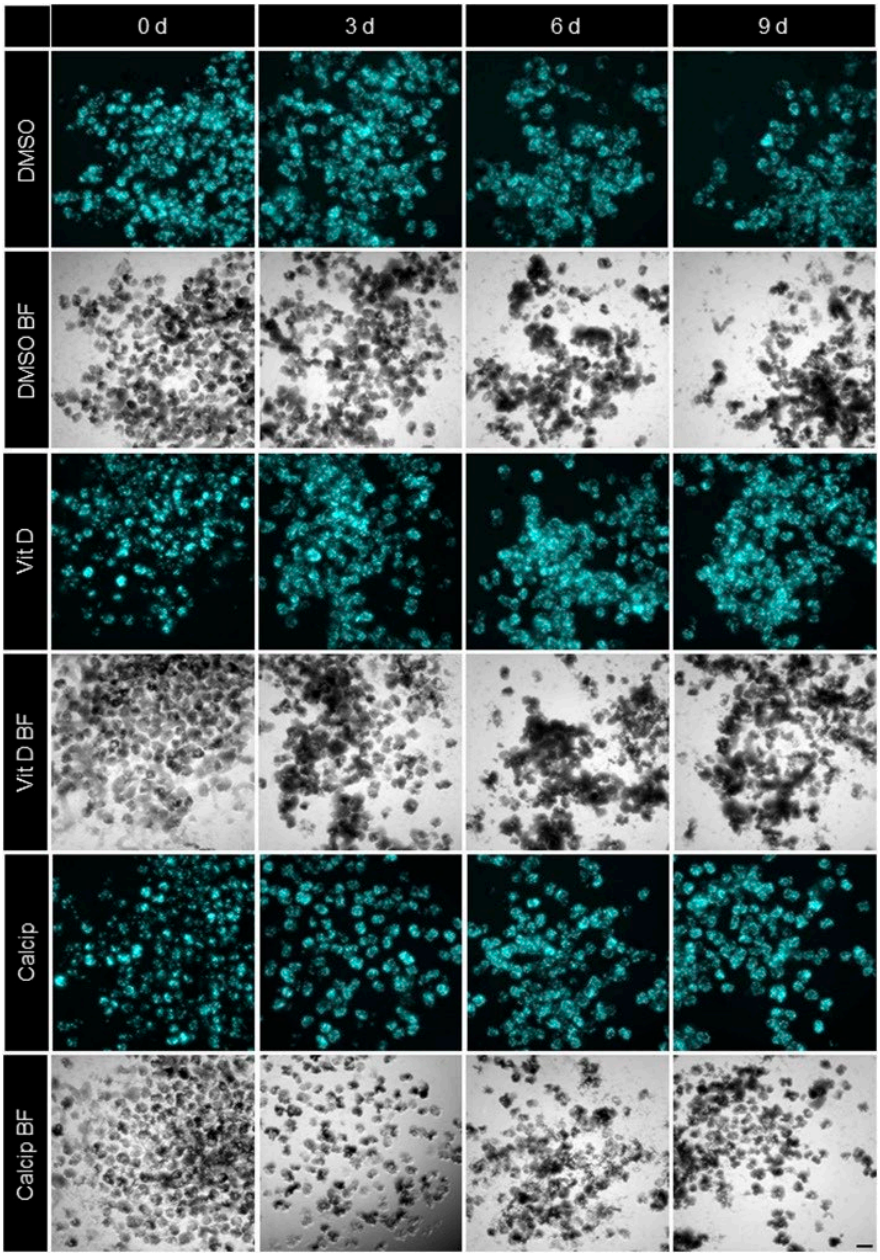

Figure 2. CFP fluorescence intensity in DMSO, vitamin D and calcipotriol-treated glomeruli

To validate these findings, researchers isolated RNA and proteins from the samples and found that the downregulation of CFP and nephrin was partially restored with the treatment by vitamin D and calcipotriol, which supports the imaging data. Additionally, the expression of the vitamin D receptor and retinoic acid receptor was down-regulated after nine days in the control samples, both of which function as nuclear receptors in vitamin D signalling. However, neither vitamin D nor calcipotriol had a significant effect on the mRNA expression. At the protein level, a similar trend was observed with strong down-regulation of nephrin, CFP, vitamin D receptor and retinoic acid receptor after nine days in the control samples. Vitamin D and calcipotriol treatment significantly upregulated the expression levels of CFP, nephrin, and vitamin D receptor. However, the retinoic acid receptor protein levels did not differ significantly.

Next, RNA sequencing was performed on glomeruli after six days of vitamin D and control treatment to further interpret the pathways influenced by vitamin D treatment. Results showed several significantly differentially expressed genes. Podocyte markers nephrin, tcf21 and kirrel2 were most strikingly upregulated. Significant up-regulation of genes involved in epithelial cell differentiation (Hdac1), extracellular matrix organization (Mmp8) and actin cytoskeleton (Pdlim2) was also observed. The largest effects were noticed on day 9 of *in vitro* culturing, leading to further detailed analysis of the transcriptome and proteomic analysis.

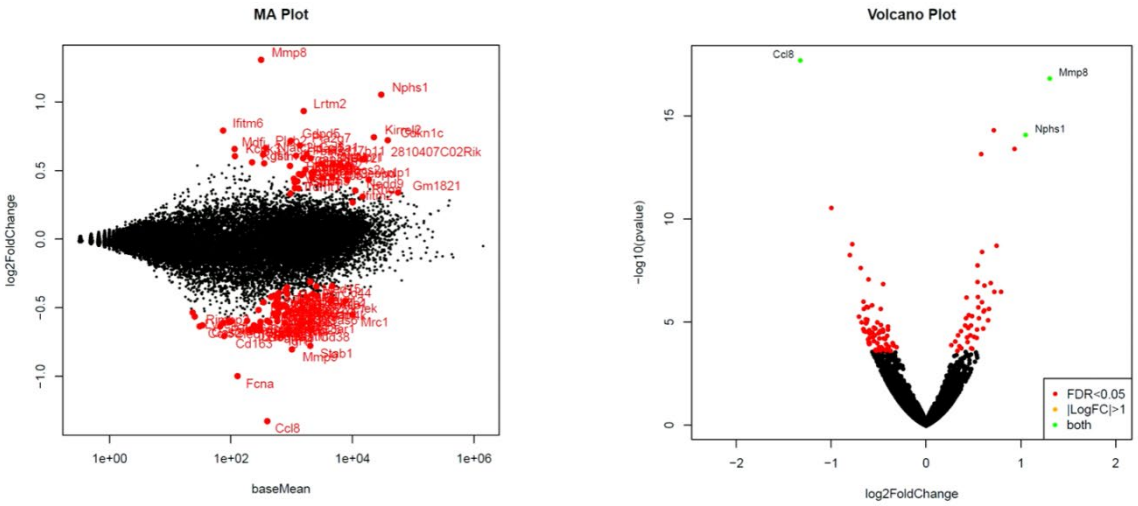

Figure 3. Transcriptome on Day 6

At the transcriptome level, similar patterns of data distribution were found among vitamin D and calcipotriol-treated samples compared to controls, as revealed by the principle component analysis. Nearly 28,000 genes were found, including several significantly deregulated genes after vitamin D and calcipotriol treatment. Interestingly, there was no differential expression of genes between vitamin D and calcipotriol-treated glomeruli. Raw GTP signalling, extracellular matrix organization, and cell-cell communication were identified as the most affected pathways. Interestingly, vitamin D and calcipotriol treatment regulated mostly the same pathways. The proteomic data showed more than 4,700 proteins, including several differentially expressed proteins after vitamin D and calcipotriol treatment. Similar to the transcriptomics data, only one significantly deregulated protein between vitamin D and calcipotriol treatment was found. Looking at affected pathways, extracellular matrix organization and collagen formation and laminin interactions were again found to be most affected.

The study further combined both data sets and analyzed single differentially expressed genes corresponding to different signalling pathways, such as actin cytoskeleton, cell differentiation, EGF signalling, FGF signalling, focal adhesions, podocyte development, slit diaphragm and *Wnt* signalling. The upregulation of Pdlim 2 and Ablim 1 on both the transcriptome and proteome levels was among the highest in the cytoskeleton-related genes.

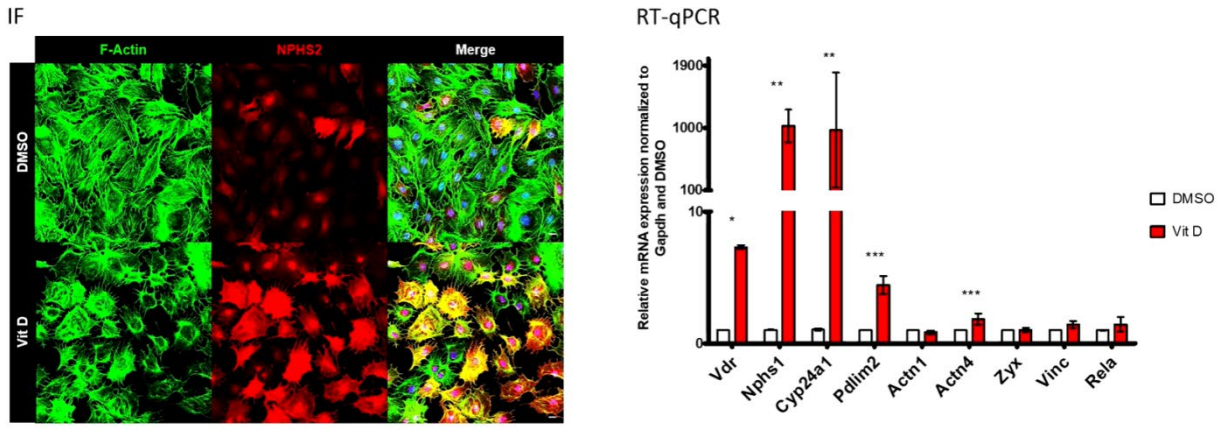

Figure 4. Vitamin D *in vitro* treatment

The influence of vitamin D on podocytes was further analysed *in vitro*, where an immortalized murine podocyte cell line was treated with 100 nM vitamin D3 and 0.1% DMSO for two days. The results showed a reorganization of the actin cytoskeleton, with the development of cellular protrusions and a rearrangement of the cortical actin fibres, as well as an upregulation of podocin and vitamin D receptor. A significant upregulation of the vitamin D receptor, nephrin, and the vitamin D inactivator, Cyp24a1, was also observed. Furthermore, the actin cytoskeleton-associated genes were also upregulated as yet another beneficial effect of vitamin D treatment. Immunofluorescence staining confirmed strong upregulation of the vitamin D receptor and showed that morphological changes after vitamin D treatment were indeed dependent on the vitamin D receptor. Calcipotriol treatment showed comparable morphological effects to those of vitamin D treatment, as well as a similar upregulation of podocin on the protein level and vitamin D receptor, nephrin and Cyp24a1 at the mRNA levels.

Key points

1. There is an ongoing search for potential therapeutic options for podocyte injury in glomerular diseases.
2. A recent study presented the implementation of the already established mouse Glom*Assay* as a semi-automated high-throughput screening method—shGlom*Assay*—allowing the analysis of several hundreds of compounds in combination with downstream pathway analysis like transcriptomic and proteomic analyses from the same samples, using a small number of animals.
3. The beneficial effects of vitamin D3 on podocytes in a de-differentiation model were confirmed by imaging data and molecular biological methods and omics data, as well as *in vivo* experiments and injury models.
4. Calcipotriol had a comparable effect on podocytes and should be considered as a potential alternative to vitamin D3 due to its lower calcific side effects.

Further reading

(1) Kindt F, Hammer E, Kemnitz S, et al. A novel assay to assess the effect of pharmaceutical compounds on the differentiation of podocytes. Br J Pharmacol. 2017;174(2):163-176. doi: 10.1111/bph.13667.
(2) Ristov MC, Lange T, Artelt N, et al. The ShGlomAssay Combines High-Throughput Drug Screening With Downstream Analyses and Reveals the Protective Role of Vitamin D3 and Calcipotriol on Podocytes. Front Cell Dev Biol. 2022;10:838086. doi: 10.3389/fcell.2022.838086.