

**ESMO Translational Research Fellowship**  
(September 2016 – May 2017)

**Sarah Kammerer**

**FINAL REPORT**

Host Institute: **Max Delbrück Center of Molecular Medicine in the Helmholtz Association, Berlin, Germany**

Mentor: **Prof. Walter Birchmeier**

Project title: **Clear Cell Renal Cell Carcinoma: development of novel treatment strategies targeting cancer stem cells and identification of new predictive biomarkers**

Home Institute: **Medical University of Graz, Austria**

***Introduction***

Kidney cancer accounts for approximately 3% of all malignancies in adults. Clear cell renal cell carcinoma (ccRCC) is the major form of kidney cancer (70-80%), and represents a devastating disease with a high metastatic index and survival rates of 10-20% for metastasized RCC.[1] This rate has been improved by the introduction of targeted therapies using small molecule inhibitors for receptor tyrosine kinases (RTK), and for the mammalian target of rapamycin (mTOR). However, patients present with primary or develop acquired resistance, which leads to progression of the disease and hinders curative therapies.[2] To date, imaging techniques such as CT or MRI generally remain the only option to measure tumor recurrence or progression after surgical removal of the tumor or during therapy surveillance. Only few molecular markers have been proposed for the purpose of disease monitoring and none of them has entered clinical practice.[3,4]

Emerging evidence has shown that tumors contain so-called cancer stem cells (CSCs) with the potential to self-renew and to reconstitute tumor heterogeneity after serial transplantation. CSCs have been identified in various tumors,[5-7] but it remains to be established which cancers follow the stem cell model[8,9] and CSCs have not been extensively studied in kidney cancer.[10,11] There exists great interest in the elucidation of mechanisms that operate in CSCs. Important findings showed that canonical Wnt and other developmental signaling systems are critical in CSCs.[6,7,12] Moreover, Wnt signaling can co-operate with other pathways such as Ras, SHH, HGF-Met or BMP in colon, breast, skin or head and neck cancer.[6,13,14] CSCs are often linked to therapy resistance[15,16] and might therefore represent a prime target for novel treatment strategies.[17]

***Rationale and Aim***

The goal of this project is to develop novel therapy strategies that specifically target cancer stem cells in clear cell renal cell carcinoma and to identify molecular markers that can predict therapeutic responses. To achieve this, a kidney organoid culture system should be established for the study of treatment options in ccRCC. The establishment of such kidney will offer a valuable and close-to-reality model system to study different aspects of kidney cancer.

The results of this project will lead to the development and clinical implementation of new treatment options for patients with ccRCC. The molecular profiling of organoids during treatment and the

identification of relevant biomarkers might give new insights into the mechanisms of disease development, progression and especially into response to therapies and resistance mechanisms.

Taken together, the efforts undertaken within this project will lead to better patient outcomes and higher survival rates in ccRCC.

### **Experimental design**

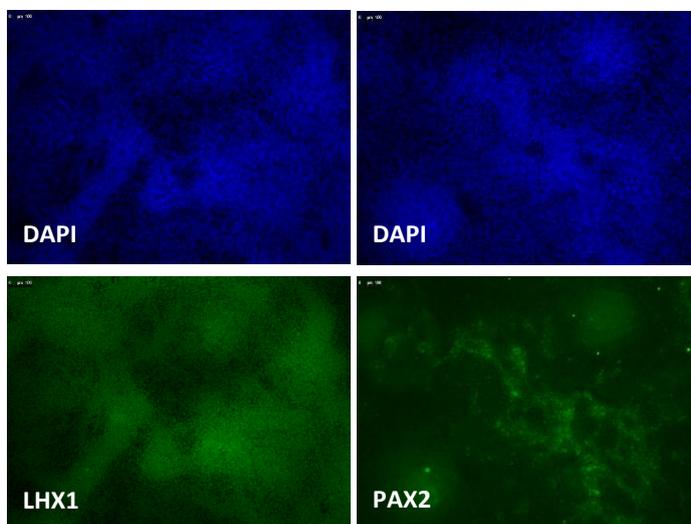
As first important step, kidney organoids will be established from human induced pluripotent stem cells.[18] These will be modified using the CRISPR/Cas9 system to knock out genes that are known to be lost in ccRCC which will lead to the generation of a novel kidney cancer organoid model that closely resembles human tumours.

The kidney organoids will then be treated with different combination therapies that are expected to be particularly effective in controlling cancers and preventing resistance.[19-22] Molecular profiles of the samples will be characterized before and during treatment, and the results will be linked to treatment responses, permitting to define molecular subgroups of patients who should benefit from specific treatment regimens.

### **Results, Conclusions and Future Perspectives**

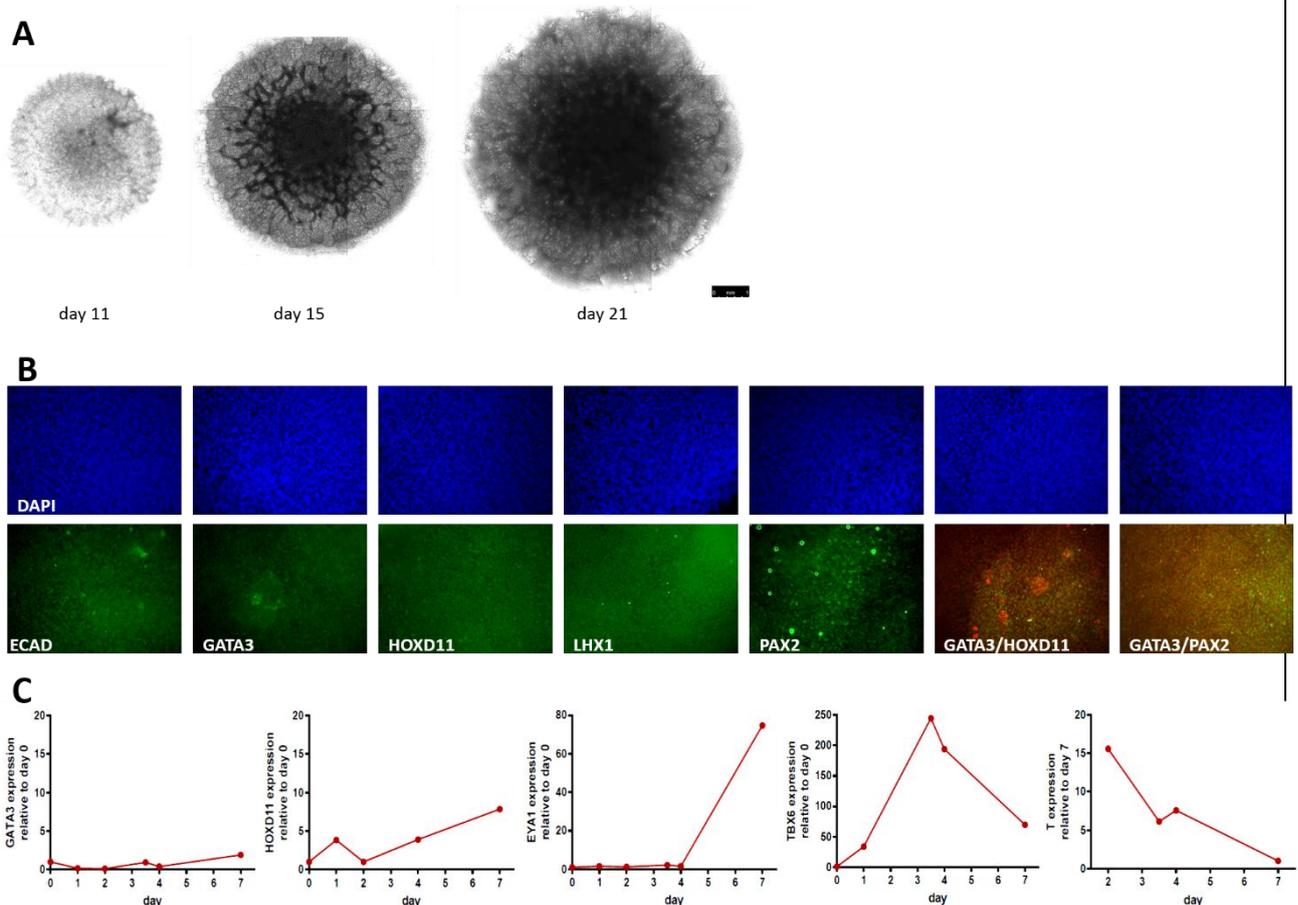
For differentiation of human induced pluripotent stem cells (hiPSCs) into kidney organoids, the LMNA cell line (kindly provided by the Stem Cell Core Facility of the MDC, patient fibroblast cells reprogrammed by mRNA) were used and cultured as described previously.

A first experiment was set up (*end of November to mid of December*): the iPS cells were used to differentiate them into human kidney organoids as described by Takasato et al. (Nature, 2015). For this purpose, several conditions were tested to determine the optimal culture conditions for kidney differentiation. It could be shown that cells grew and differentiated best with a mouse embryonic fibroblast conditioned medium with 10 ng/ml bFGF when compared to essential 8 or mTeSR1 medium. In the second step, APEL2 medium with 5% protein free hybridoma medium (PFHM) proved to be superior to APEL2 medium alone. After seven days of differentiation as monolayer culture, no organoids were made, but the cells were fixed and immunofluorescence staining for the markers LHX1 and PAX2 was performed to test whether this first step towards kidney differentiation was successful. Positive stainings are indicative for successful differentiation into intermediate mesoderm. As depicted in figure 1, the cells expressed both markers.



**Figure 1:** Kidney differentiation to day 7 with MEF conditioned medium and APEL2 medium supplemented with 5% PFHM. Targets are stained in green, corresponding DAPI staining is shown in blue.

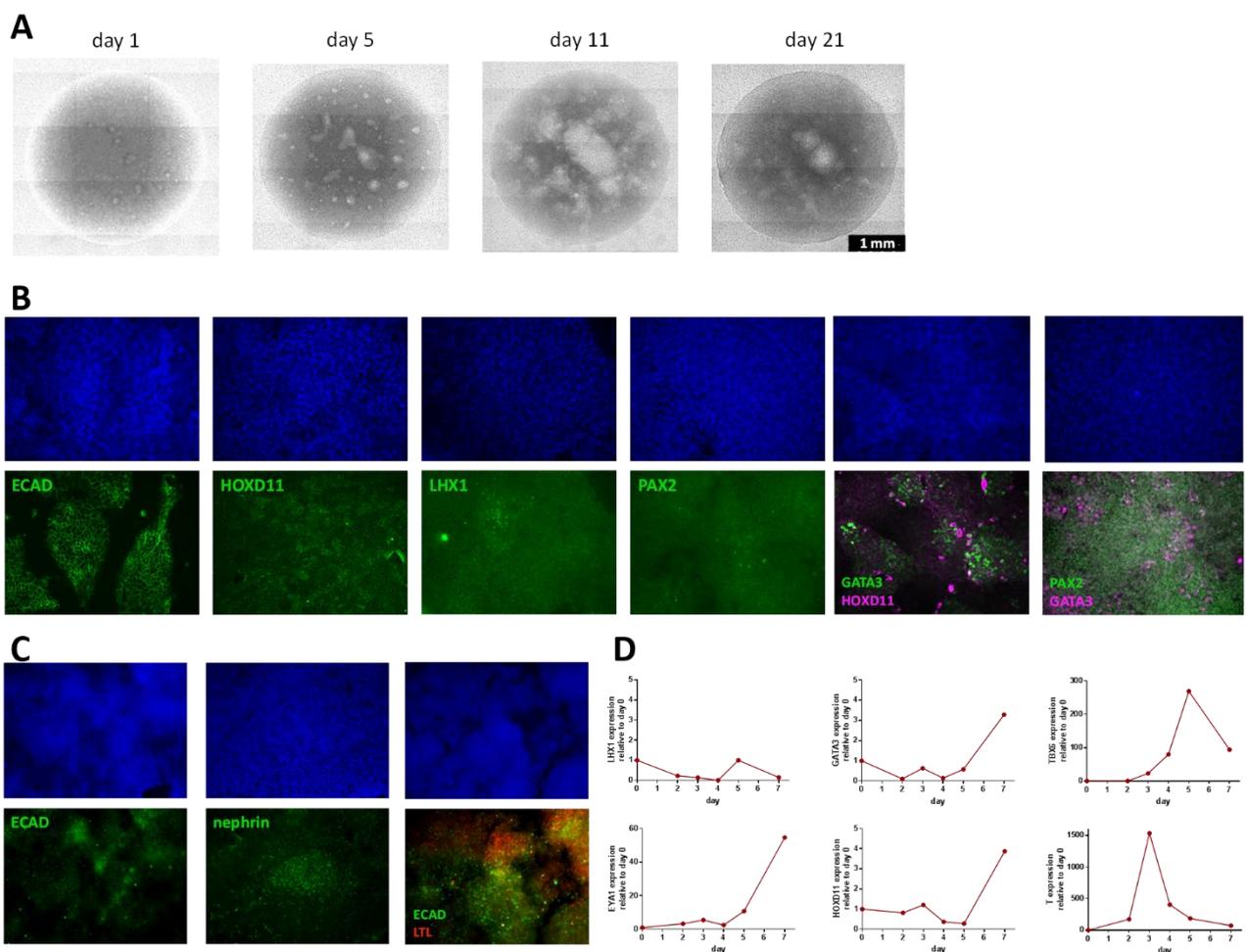
After this first exploratory experiment, kidney organoids were produced according to the Takasato protocol with the culture media tested before and characterized (*beginning of January to mid of March*). Figure 2A shows a representative bright field image of a kidney organoid. Signs of self-organization within the organoid as well as reasonable growth over time could be observed. At day 7 of differentiation, monolayer cells were fixed for immunofluorescence stainings. Antibodies against LHX1, PAX2, ECAD, GATA3 and HOXD11, all of them indicative for successful differentiation towards kidney lineages, were used. As shown in figure 2B, all markers were expressed as expected. During differentiation, cell pellets were harvested during the first 7 days of monolayer culture for qRT-PCR. The markers GATA3, HOXD11 and EYA1 showed an increase during differentiation, while the markers TBX6 and T decreased over time (figure 2C). The findings were expected and are comparable to previously published results (Takasato et al.).



**Figure 2:** Characterization of kidney organoids. **A.** Representative images of a kidney organoid showing growth and inner-organoid organization at several time points. **B.** Immunofluorescence staining of several markers indicative for kidney differentiation at day 7 of differentiation. Targets are stained in green or red, corresponding DAPI staining is shown in blue. **C.** qRT-PCR of several markers at different time points during differentiation (day 0 to day 7).

The procedure was repeated in a third experiment to validate and reproduce the data (*mid of March to end of Mai*). Growth of organoids was less pronounced that in the experiment before, but cell density was again highly increased over time as shown by a representative image of one organoid in figure 3A. Immunofluorescence staining of day 7 monolayer culture was performed again using the markers ECAD, HOXD11, LHX1, PAX2 and GATA3. As shown in figure 3B, ECAD staining was more pronounced and more

clearly localized in the cell membrane than last time; all other results were comparable to previous findings of us and of Takasato et al. Immunofluorescence staining was also performed of organoids of day 11 in culture (figure 3C). Single stainings show that both ECAD and nephrin are expressed in distinct regions. ECAD expression is indicative for formation of tubuli and nephrin expression for the presence of glomeruli structures. A double staining of ECAD and LTL showed that the two markers are expressed in different adjacent regions with only small overlaps. This distinct staining pattern is expected and indicative for the presence of yet immature proximal tubules while overlapping staining as seen in the top right of the picture is indicative for the presence of mature proximal tubules. Figure 3D shows the qRT-PCR results of different marker genes as expressed over time during the first seven days of differentiation. Also these results were fully reproducible compared the former experiment in figure 2C and to the results by Takasato et al.



**Figure 3:** Characterization of kidney organoids. **A.** Representative images of a kidney organoid showing growth and cell density at several time points. **B.** Immunofluorescence staining of several markers indicative for kidney differentiation at day 7 of differentiation. Targets are stained in green or pink, corresponding DAPI staining is shown in blue. **C.** Immunofluorescence staining of several markers indicative for kidney differentiation at day 11 of organoid growth. Targets are stained in green or red, corresponding DAPI staining is shown in blue. **D.** qRT-PCR of several markers at different time points during differentiation (day 0 to day 7).

**All these results prove that the organoid model system could be successfully established, that it is stable and reproducible and that it is ready-to-use for further experiments.**

**Outlook:**

In a next step, the CRISPR/Cas9 system will be used to knock out VHL and BAP1 during kidney differentiation. Loss of those two genes represent one of the major subtypes of clear cell renal cell carcinoma. In parallel to the work described above, those genes were knocked out in HK-2 cells, a proximal tubules cell line, to test the efficiency of the system (*since beginning of January*). For both genes, two sgRNAs were designed, one located in exon 1 for both genes and one in exon 3 for VHL and in exon 4 for BAP1. The sgRNAs were cloned, isolated and purified and transfected to the cells. Selection of positive clones was possible by using vectors with different fluorescent markers. Both vectors with sgRNAs for one gene were co-transfected to HK-2 cells, one containing a green fluorescent protein (GFP), the other one containing a red fluorescent protein (mCherry). Transfection efficiency was low as estimated by fluorescent microscopy (about 3-5%), but transfected cells were double positive for the two sgRNAs transfected. Cells were FACS-sorted, which confirmed the transfection efficiency of about 5% and cells were generally double positive. Double positive cells were sorted for single cell cloning. Seven single cell clones could be successfully generated for VHL knockout, and four clones for BAP1 knockout. Due to time limitations, a detailed characterization of the clones were was not possible before termination of the fellowship. **My colleague, Adam Myszczyzyn, PhD student in the lab of Walter Birchmeier, will continue this work.**

As soon as the pilot experiment in HK-2 cells proofed to be successful, the technology will be used on iPSC cells. In order to cover also other molecular subtypes of clear cell renal cell carcinoma, the following combination knockouts will be performed: VHL/PBRM1, VHL/SETD2, and VHL/Notch/ $\beta$ -catenin. Also this part of work could not be finalized within the fellowship period, and **Daniel Bauer, now Master student in the Birchmeier lab, will take over the project** as his PhD project. He will use the organoid model that I could successfully establish and proceed with still unmet project goals.

***List of Publications and Presentations Resulting from the Translational Research Project "Clear Cell Renal Cell Carcinoma: development of novel treatment strategies targeting cancer stem cells and identification of new predictive biomarkers"***

None so far, because further work will be performed before publication of results. As mentioned above, Adam Myszczyzyn and Daniel Bauer will continue the project. Any future publication that includes results of my work will include me as an author and reference the ESMO fellowship according to the guidelines.

***List of Publications and Presentations resulting from other projects during the fellowship period (if applicable)***

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***Selection of Courses and Workshops Attended During the Fellowship***

- weekly lab meetings
- journal club on kidney cancer (once a week)
- journal club on CRISPR/Cas technology (once a month)
- training on handling and maintenance of human pluripotent stem cells (3 days)
- working with Graph Pad Prism (1 day)
- R basics (2 days)
- R advanced (2 days)
- How to shape your future - career planning (2 days)
- Image analysis with Fiji (2 days)

### **Acknowledgements**

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Last, but not least, I would like to thank my family, my partner and my friends who have always supported me despite great distances.

### **Personal Statement (not mandatory)**

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At the farewell dinner in Berlin with my colleagues

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