Renal Carcinoma/Kidney Progenitor Cell Chimera Organoid as a Novel Tumourigenesis Gene Discovery Model

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Summary statement

Chimeras between embryonic kidney cells and renal carcinoma cells serve as a novel model to assay the roles of co-regulated genes in kidney development and renal carcinogenesis.
Abstract

Three-dimensional organoids provide a new way to model various diseases, including cancer. We made use of recently developed kidney organ primordia tissue engineering technologies to create novel renal organoids for cancer gene discovery. We then tested whether our novel assays can be used to examine kidney cancer development. First we identified the transcriptome profiles of quiescent embryonic metanephric mesenchymes (MM) and of MM in which the nephrogenesis program had been induced ex vivo. The transcriptome profiles were then compared to the profiles of RCC patients and controls. Certain signature genes were identified that correlated in the developmentally induced MM and RCC, including components of the caveolar-mediated endocytosis signalling pathway. An efficient siRNA mediated knock down (KD) of Bnip3, Gsn, Lgals3, Pax8, Cav1, Egfr and Itgb2 gene expression was achieved in renal carcinoma (Renca) cells. The live cell imaging analysis revealed inhibition of cell migration and cell viability in the gene KD RCC cells in comparison to the controls. Upon siRNA treatment, the transwell invasion capacity of the RCC cells was also inhibited. Finally, we mixed the nephron progenitors with the Yellow Fluorescent Protein (YFP) RCC model cells to establish chimera organoids. Strikingly, we found that the siBnip3, siCav1 and siGSN KD RCC-YFP+ cells as a chimera with the MM in 3D organoid rescued, in part, the RCC-mediated inhibition of the nephrogenesis program during epithelial tubules formation. Altogether our research indicates that comparing renal ontogenesis control genes to the genes involved in kidney cancer may provide new growth associated gene screens and that 3D RCC-MM chimera organoids can serve as a novel model with which to investigate the behavioural roles of cancer cells within the context of emergent complex tissue structures.
Introduction

The ability to model diseases such as cancer by targeting candidate genes in embryonic stem cells (ES) and by making in vivo mouse models from multipotent cells revolutionized the pathogenesis studies (Lim et al., 2016). Recently, it has also become possible to reprogram normal and dysfunctional adult cells into stem cells and to develop organoids that form specific cell lineages. These complex organ-like cell aggregates provide a way to model tumourigenesis ex vivo (Lovitt et al., 2016).

Cancer organoid models should offer the possibility to identify the initial steps of tumourigenesis. We propose that the genes responsible for this process can be found among normal developmental regulators. Indeed, processes such as cell proliferation, cell differentiation, cell migration and apoptosis are all involved during normal organogenesis but are associated with malignancy as well. An accumulation of mutational load in the normal developmental signalling pathways may eventually dysregulate and/or reactivate the pathways in adults (Ma et al., 2010; Aiello and Stanger, 2016). Such changes are considered to occur in the kidney (Potter et al., 2010; Sültmann et al., 2005; Yang et al., 2014), where the Wnt, Notch, and Sonic Hedgehog (SHH) growth factor (GF) pathways (Katoh, 2007; Polakis, 2000; Sjölund et al., 2011; Sun et al., 2009) regulate cell division and cell differentiation in a controlled manner but when ectopically activated in the adult, they promote malignant growth (Dormoy et al., 2012; Ohnishi et al., 2014).

The fact that ontogenesis and oncogenesis involve related genetic programs is also reflected at the cellular level in processes such as epithelial-mesenchymal (EMT) and mesenchymal-epithelial transition (MET) (Thiery et al., 2009). Both are necessary for normal renal development. In the context of malignancy EMT activation converts benign cells into more invasive ones (Kalluri and Weinberg, 2009; Pang et al., 2011; Rhim et al., 2012), while MET is linked to the acquired capacity of the cells to colonize ectopic lesions in metastasis (Yao et al., 2011). These multistep processes represent yet another similarity between developmental control and tumourigenesis. In both cases, GF-promoted angiogenesis is essential to ensure the blood supply.
Renal Cell Carcinoma (RCC) accounts for around 90% of all kidney cancers (Ljungberg et al., 2011). Smoking, obesity, certain chemicals, and genetic factors are implicated in RCC promotion (Chow et al., 2010). Chemotherapy for RCC is still very limited. Angiogenesis inhibitors are initially effective, but lose their efficacy because resistance develops (van der Mijn et al., 2014). The small interfering RNAs (siRNAs) are considered promising anti-cancer compounds (Burnett and Rossi, 2012; Castanotto and Rossi, 2009; Sakurai et al., 2013). They are also useful tools to screen candidate oncogenes and their targets in cell transformation.

In light of the similarities between kidney development and carcinogenesis, we assayed whether some developmental genes may be relevant in kidney malignancy. We began by comparing gene expression between human RCC and experimentally induced nephrogenesis, and identified the genes expression of which was changed in both models. To narrow down our research, we identified the pathways of the genes that showed a markedly changed expression both during kidney development and carcinogenesis. Based on our pathway analysis and published research data (Sohn et al., 2016), we selected the caveolin-related genes for further investigation. We found that siRNA mediated silencing of BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3), gelsolin (Gsn), and caveolin-1 (Cav1) diminished the growth and motility of the RCC cells. Next, we investigated whether these cell activities could be modeled in the ex vivo chimeras between the RCC and the kidney progenitor organoids as well. We developed a 3D co-culture method that makes it possible to study the cross-interactions between embryonic and transformed cells under conditions in which expression of certain genes is inhibited by siRNA treatment. In this model the siBnip3, siCav1 and siGSN KD RCC-YFP+ cells partially rescued the Renca-mediated inhibition of the nephrogenesis program. Together, the comparative analysis of the ontogenesis and oncogenesis control genes and their functional analysis in novel chimera organoid between kidney RCC tumour cells and the kidney progenitors illustrate the power of the 3D setups for functional tumourigenesis gene discovery.
Results

Identification of putative growth and differentiation control genes by comparing the transcriptomes between human renal cancer and primary nephrogenesis cells

Given the similarities between cancer and developmental processes, comparative gene expression profiling may serve to identify relevant candidate factors behind dysregulated cell division and cell differentiation in cancer. To test these ideas, we took advantage of the classic metanephric kidney tubule induction model and identified the transcriptome of the uninduced and induced tissue (E11.5; 0h and 96 h). The gene expression profiles were then compared with the human ccRCC generated transcriptome data.

The profiling screen identified 1616 differentially expressed genes in the induced MMs when compared to the uninduced ones (Table S1). When these expression profiles were further compared to those of the human ccRCC cohort (Eikrem et al., 2016) 930 genes were found the expression of which was changed simultaneously in both data sets (Table S2). These 930 genes were classified into four groups (Table S2). The majority of the genes showed a similar pattern of expression changes in carcinogenesis and normal development: 534 were up-regulated upon MM tubule induction and were abundant also in the ccRCC while only 69 genes were down-regulated in both the RCC and the MM samples. The rest of the genes demonstrated the opposite behaviour. 273 genes were up-regulated in the induced MMs but down-regulated in the ccRCC samples. In the reverse case, there were 56 genes whose expression was down-regulated in the MMs but up-regulated in the ccRCC cohort samples (Table S2). Based on these findings we conclude that the induction of MM and the formation of ccRCC possibly share gene regulation and biological signalling.

The identified non-redundant genes with expression changes of 2-fold or higher and a p-value of <0.05 were subjected to Ingenuity pathway analysis. The Venn diagram (Figure 1A) shows the number of identified pathways in each of the following groups: pathways significantly involved in the process of clear cells renal carcinoma formation (marked ccRCC), pathways induced in the kidney development (marked MM (96 h vs 0 h)), pathways of genes simultaneously changed in kidney development and in renal
carcinogenesis (marked MM vs ccRCC) (the complete lists of pathways are shown in Table S3).

As can be seen from the diagram, 56 pathways were shared between all analyzed groups. In order to find a way to prioritize pathways for further analysis, we checked each group using a much stricter significance threshold ($-\log(p\text{-value})>4$). Using this threshold, we found only six pathways which were simultaneously regulated in all three analyzed groups: atherosclerosis signaling, caveolar-mediated endocytosis signaling, dendritic cell maturation, Fcy receptor-mediated phagocytosis in macrophages and monocytes, hepatic fibrosis/hepatic stellate cell activation, and leukocyte extravasation signaling. Several of these pathways (like hepatic fibrosis) require regulation of multiple genes that have very broad functions and are involved in multiple different processes, while others were more specifically related to tumour development.

For further analysis we preferred to concentrate on a pathway already known to be involved in carcinogenesis. In particular, caveolar-mediated endocytosis signaling, which is one of the top signaling found in ccRCC and induced MM, is known to be responsible for cancer heterogeneity when deregulated (Martinez-Outschoorn et al., 2015). This pathway also showed the highest ratio of the number of genes in a given pathway that meet the cutoff criteria to the total number of genes which belong to the pathway in our study (Figure 1B).

Among the caveolar-mediated endocytosis signaling genes induced in the MMs that were also abundant in the ccRCC were galectin-3 ($Lgals3$), $Gsn$, $Cav1$, epidermal growth factor receptor ($Egfr$) and integrin beta2 ($Itgb2$) (Figure 2A). For the functional studies we selected the signaling genes mentioned above and $Bnip3$, which is up-regulated in the ccRCC but down-regulated in the induced MM.

It has previously been shown that $Bnip3$ expression increases upon $Cav1$ down-regulation in stromal fibroblasts (Martinez-Outschoorn et al., 2010). The paired box 8 ($Pax8$), a carcinogenesis-related transcription factor necessary for kidney development (Sharma et al., 2015), behaves in the opposite manner to $BNIP3$ in the comparative gene array, being down-regulated in the ccRCC but up-regulated in the MM undergoing differentiation. We also analyzed expression of $Lgals3$, $Gsn$, $Cav1$, $Egfr$, $Itgb2$, $Bnip3$, and $Pax8$ using
publically available data from 144 biopsies (77 ccRCC, 77 normal) in Gene Expression Omnibus database GSE53757. The directions of the expression changes for these genes in a bigger GEO dataset were mostly the same as in our smaller collection of human clear cell RCC samples (Table S4).

The siRNAs as putative “drugs” to compromise RCC behaviour in model cells in vitro

After selecting candidate genes for closer study, we chose siRNAs as the tools to screen for functional knockdown of these genes. The efficiency of siRNA-mediated down-regulation of gene expression was studied in the mouse RCC cells (Renca) after transfection with 50nM siRNA in 96-well plates by qRT-PCR (48h, 72h and 96h) and was found to be in the range of 83-97% inhibition (Figure S1A). We also analyzed protein levels for two genes (caveolin-1 and gelsolin) at the same time points by Western blot (Figure S1B), and the protein data was similar to RNA data. In addition, we showed that lowering expression of CAV1 in Renca cells would slightly but significantly increase the mRNA levels of Bnip3, Gsn, Itgb2, Lgals3, while decreasing Egfr expression (Figure 2B).

In order to depict possible changes in the RCC cells after siRNA transfections we first analyzed Renca cell proliferation by live imaging using IncuCyte during a four days culture time. The results showed that the siRNA mediated silencing of the Bnip3, Gsn, and Cav1 genes notably reduced RCC cell proliferation in comparison to the controls, while the other tested siRNAs, namely the Lgals3, Pax8, Egfr and Itgb2, did not influence cell growth (Figure 3A).

To assay whether siRNA mediated gene silencing would have an impact on cell viability, the cells were stained with Sytox Green nuclear acid dye, and the percentage of dead cells at 24 h after cell plating was illustrated. Silencing of the expression of all assayed genes except Egfr promoted cell death as compared to the scrambled siRNA-transfected controls, though to a different extend.

The siRNAs targeting of the Bnip3 and the Gsn mRNA species turned out to be the most effective with respect to cytotoxicity. In these cases, cell death was induced by around 2.5-fold and 2.4-fold, respectively (Figure 3B). Thus, the siRNAs mediated knock down of a
panel of the genes regulated upon nephrogenesis activation in the MM and being highly expressed also in the RCC suggested that the strategy may be useful to identify novel growth control associated genes in RCC.

**siRNA-mediated silencing of certain developmental and RCC-associated genes inhibit cell migration, invasion and colony-forming ability in vitro**

Considering that the Renca cells also serve to address mechanisms of metastases to the lung and liver when implanted in mice (Salup et al., 1985), we investigated whether down-regulation of the expression of the targeted selected genes would influence the migratory or invasive properties of these cells. For this, we applied siRNA cell transfection with the identified efficient siRNA in vitro protocol in the 96-well plates. Putative changes in cell migration were monitored by InCucyte live cell imaging system for 24 h after wounding the confluent cell monolayer. The results revealed that down-regulation of the selected model genes notably inhibited cell migration in the wound assay (Figure 4A and 4B).

Next, we compared the capacity of the siRNA transfected cells for invasion through a porous filter in a Transwell assay. It turned out that transfection of the selected siRNAs, excluding the Pax8 and the Itgb2, significantly inhibited cell invasion across the filter compared to the controls (Figure 4C and 4D). Taken together, the data indicates that the siRNA mediated silencing of the Bnip3, Cav1, Gsn, Egfr, and Lgals3 functions reduces the migratory and invasion capacity of the RCC cells, whereas silencing of the Pax8 and the Itgb2 gene functions only deregulate cell migration.

Given the illustrated capacity of the Bnip3, Cav1, Gsn, Egfr, and Lgals3 genes to influence activities of the model RCC cells, we continued our siRNA analysis by performing a colony-forming assay. As shown in Figure 5A and 5B, the siRNA transfected cells had a poor capacity to form cell colonies when compared to the controls. The Bnip3-specific siRNA turned out to be the most effective, and reduced the amount of colonies by about 70% compared to the controls.
Silencing of the functional RCC/kidney developmental genes influences the EMT/MET markers and phosphorylation of the signal transduction component Akt in the RCC model

Because the embryonic kidney tubulogenesis model serves to study the MET and because the reverse process, EMT, is typical for cancer, we examined whether the identified functional genes have an impact on the EMT/MET. Vimentin served as a marker of the mesenchymal cells, while N-cadherin and E-cadherin were used as epithelial markers for the qRT-PCR.

After siRNA silencing, the EMT markers were detected in Renca cells at 48h. The results showed that introduction of siGsn to the RCC cells notably down-regulated E-cadherin expression (P<0.05), but concurrently up-regulated expression of the vimentin gene (Figure S2 A). Silencing of the siBnip3 function up-regulated E-cadherin expression, but the mesenchymal marker analysis did not reveal any changes. This was also the case for Cav1 and mock siRNAs (Figure S2 A).

Because there is evidence that PI3K/Akt pathway signaling changes can be associated with the RCC phenotype (Cancer Genome Atlas Research Network, 2013), we investigated whether the identified functional siRNAs also have an impact on the Akt signaling in cultured Renca cells. We used the degree of Akt phosphorylation as criterion.

However, among the siRNAs used, only Pax8 siRNA leads to enhancement of Akt phosphorylation, while the Bnip3, Cav1, and Gsn siRNAs did not have a significant influence (Figure S2 B). This points towards a different mechanism of action for Pax8 when compared to all the other genes investigated in our study. To conclude, it appears that the functional consequences of the gene expression inhibition in RCC are unlikely to take place via deregulation of the MET/EMT process or via different degrees of PI3K/Akt activation.
The siRNA gene silenced RCC cells in co-culture with the embryonic kidney cells as a novel functional *ex vivo* organoid tumourigenesis model

Traditionally, cancer associated gene functions have been studied either in monolayer cell cultures *in vitro* or more recently by making *in vivo* models via gene editing in the mouse. To be able to conduct more relevant high throughput functional screens for cancer gene discovery *in vitro*, 3D tumour models that better mimic the natural cellular setting need to be developed.

To develop relevant *ex vivo* diagnostic renal cancer models, we made use of the already described embryonic kidney tubule induction system in which emergence of a complex kidney tubular network can be achieved with a Wnt-pathway-mediated trigger (Halt and Vainio, 2012). Here a 24h induction pulse is enough to trigger tubulogenesis via a transient cell proliferation period *ex vivo* culture setting prior to epithelial differentiation (Lauri Saxen, 1987). We recently developed a number of powerful renal tissue engineering capabilities which allow us to generate chimeric organoids and culture them as 3D explant cultures (Junttila et al., 2015; Halt et al., 2016).

The MM cells were dissociated by collagenase treatment, pelleted by centrifugation, and the nephrogenesis program was induced with a Wnt signaling source (Junttila et al., 2015; Halt et al., 2016). The reaggregated MM cells were placed on a nuclepore filter and cultured for 4 days. The developmental activation led, as expected, to the appearance of epithelial tubular structures formed by the Pax2-positive cells, depicting successful tubule induction (Bouchard et al., 2002) (Figure 6A-D).

In order to test how the addition of immortalized cells would influence the normal nephrogenesis process in these 3D cultures, we mixed mesenchyme cells at a 50:1 ratio with renal carcinoma-derived Renca-Yellow Fluorescent Protein (YFP) cells, cervical cancer-derived HeLa-YFP cells and mK4-YPF cells, derived from induced metanephric mesenchyme undergoing epithelial conversion (Valerius *et al.*, 2002).

Interestingly, we found that various stable cell lines behaved markedly differently in the chimera co-culture with the mesenchymal cells. The addition of mouse Renca cells disturbs the differentiation of the 3D kidney culture (Figure 6E-H) much more strongly than...
the chimerism with the human HeLa cells (Figure 6I-L) or mouse embryonic kidney derived immortalized mK4 cells (Figure 6M-P). The Renca cells distributed rather evenly within the reconstituted embryonic kidney tissue, while HeLa and mK4 grew as small clusters inside the differentiated MM. Therefore, we concluded that renal carcinoma-derived cells must be suitable for use in 3D co-cultures to study whether the down-regulation of certain genes in RCC could lead to differentiation improvements in the kidney organoids.

Our next goal was to investigate whether inhibition of the genes that showed the most consistent results in our in vitro assays (Bnip3, Cav1 and Gsn) would also have an effect in 3D co-cultures. Similarly to the untreated RCC cells, when the mock siRNA transfected RCC cells constitutively expressing YFP were cultured as a chimera with normal MM cells (1:50), the RCC cells notably inhibited tubulogenesis (Figure 7 A-D vs. E-H and Figure 8 A-D vs. E-H).

However, when Renca-YFP cells that have been transfected with Bnip3-specific siRNA or Cav1-specific siRNA were cultured as chimera organoids with wild type MM cells, multiple Pax2-positive tubular structures and proximal tubes (Aq1+) were observed (Figure 7I-P and Figure 8I-P). The siGsn treated Renca cells in the reaggregated pellet disrupted the tubule structure formation, but to a lesser extent than the siRNA control (Figure 7 Q-T vs. A-D and Figure 8 Q-T vs. A-D).

We next aimed to investigate whether increased cell death upon Renca treatment with different siRNAs was the only reason for better formation of Pax2-positive tubular structures in chimeric organoids. Since the siBnip3 application to the Renca cells induced the cell death most (by about 250%), we mixed siBnip3 treated Renca cells with MM at a higher ratio (1:20). The results showed that many renal tubules were still formed even at this ratio, showing that in addition to apoptosis some other changes in Renca cells upon siRNA treatment contribute to their growth in co-culture with MM (Figure S3).

In general, our data indicate that down-regulation of certain genes’ expression in RCC co-cultured with MM could rescue tubule formation, which was disrupted by wild type Renca cells, showing parallels with the in vitro results described earlier (Figure 3-5). In summary, the results reveal that embryonic kidney nephron progenitor cells which are first
dissociated and then later used to reconstitute the nephrogenesis potential provides a novel and complex cancer gene discovery model.
Discussion

Despite the fact that some of the genetic changes underlying the ccRCC have been described before (Cancer Genome Atlas Research Network, 2013), there are still no clinically applicable prognostic markers for this disease (Parkinson et al., 2014). Better understanding of the molecular pathogenesis of RCC elucidates that carcinogenesis and nephrogenesis share certain properties (Aiello and Stanger, 2016). This consideration is based on the fact that some of the gene expression signatures observed in kidney development are shared with cancer cells (Kim and Orkin, 2011).

Given the depicted facts, we first aimed to find out whether a comparison of the expression profiles during nephrogenesis and kidney cancer development may help identify novel renal cancer-inducing genes. Next, we planned to address the putative roles of the identified genes as functional players in cell growth control using not only classical in vitro assays but also a novel ex vivo assay system based on our recent success in dissociating and reconstituting the embryonic kidney from founder cells (Junttila et al., 2015; Halt et al., 2016). More specifically, we aimed to establish novel and relevant organoids representing a chimera between embryonic kidney cells that normally assemble the nephron and renal carcinoma cells. The data indicates that our development of a novel complex assay system was successful. The generated setup allows co-culture of the RCC cancer cells with the host embryonic kidney cells to mimic RCC tumourigenesis in an ex vivo setting suitable for RCC functional studies.

Given the similarities between developmental processes and the processes involved in cancer, including for example extensive cell proliferation and changes in cell differentiation, we used the classic mammalian kidney tubule induction model in the hope of identifying candidate developmental control genes. The candidate genes were then compared to genes highly expressed in human RCC cohort.

The microarray-mediated analysis led to the identification of multiple genes with similar expression patterns in developing embryonic kidney cells and in renal carcinogenesis. Ingenuity pathway analysis found that products of these genes are involved in multiple intracellular processes. Interestingly, pathways found by only analyzing genes that are changed in both processes simultaneously gave different results compared to analyzing
the cancer dataset and developmental dataset separately (Figure 1A and Table S3). Taking into account the p-values and percentages of involved genes from Ingenuity analysis software as well as literature data outlined below, we selected caveolin-1 and other members of caveolar-related pathways to be studied in further detail.

Caveolae represent around 50-100 nm invaginations of the plasma membrane connected to endocytosis, lipid regulation, and signal transduction (Parton and del Pozo, 2013). Loss of caveolae, as a result of mutations or gene expression changes in caveolins or cavin, results in the development of various diseases, including cancer (Martinez-Outschoorn et al., 2015). Caveolae interact with membrane lipid rafts and the galectin lattice controlling cell signaling both under normal physiological conditions and in cancer (Dumic et al., 2006; Lajoie et al., 2009; Shankar et al., 2015). Caveolins, a major constituent of the caveolae, have been shown to be important for the regulation of many signaling cascades involved in cancer development.

In the past 20 years, dysregulated Cav1 expression has consistently been detected in various cancers (Martinez-Outschoorn et al., 2015). Still, there is no universal pattern linking Cav1 expression in cancer cells and patient outcomes: high Cav1 levels are associated with poor prognosis and aggressive disease in melanoma, breast, prostate, and lung cancer, while at the same time high Cav1 expression is correlated with good clinical outcomes in head and neck cancer and extrahepatic biliary carcinoma cells (Martinez-Outschoorn et al., 2015). In renal cancer, Cav1 has been shown to be a tumour progression factor, but in general its involvement in kidney tumourigenesis has been investigated relatively little (Campbell et al., 2013; Steffens et al., 2011; Waalkes et al., 2011).

Caveolins, including caveolin-1, are expressed in mouse embryo in a tissue-specific manner starting from E6.5 (Sohn et al., 2016). Caveolin-1 is also involved in the regulation of signaling pathways critical for nephrogenesis, including the Wnt pathways (Parton and Simons, 2007), and is known to be connected to Wnt/β-catenin signaling in podocytes (Jing et al., 2015). Still, no studies that explain the function of caveolins in kidney organogenesis, or even in embryogenesis in general, have been reported to date (Sohn et al., 2016).
Due to the presence of a scaffolding domain, caveolins interact directly with signaling molecules, including cytokine receptors, and suppress the activation of EGFR upon EGF stimulation (Agelaki et al., 2009; Martinez-Outschoorn et al., 2015; Lajoie et al., 2009; Park et al., 2001). On the other hand, galectin-glycoprotein lattices can sequester EGFRs, protecting them from interacting with Cav1 scaffolds, and hence promoting growth factor signaling and tumour growth (Lajoie et al., 2007).

One potential role for galectins and caveolins might be to regulate crosstalk between integrin and various signaling molecules (del Pozo et al., 2005; Lajoie et al., 2009). Electron microscopy has revealed complex interactions between caveolae and cytoskeleton components such as actin filaments (Parton and del Pozo, 2013). Gelsolin, an actin-binding protein with a well-established role in actin organization (McGough et al., 2003), has been recently described for the first time as a caveolar raft-associated molecule (Chilla et al., 2013). Among the downstream targets of caveolin-1 are proteins involved in autophagy induction, including Bnip3 (Martinez-Outschoorn et al., 2015). We found here that developmentally regulated genes Cav1, Egfr, Bnip3, Gsn, Itgb2, and Lgals3 are all activated in human ccRCC.

When comparing the effects of gene down-regulation by siRNA on cell viability, we observed that only Cav1, Gsn and Bnip3 siRNA-treated cells exhibited lower cell proliferation than the controls. The strongest reduction in colony number in the colony-forming assay was observed after transfection of cells with Bnip3-specific siRNA. Moreover, all the siRNAs tested, except for Egfr siRNA, induced cell death, though they did so to a different extend. Again, the most prominent effects were seen for Bnip3 and Gsn. In general terms, these results suggest that higher cell death rather than a decrease in cell proliferation explains the reduction in Renca cell numbers after transfection of the Bnip3- and Gsn-specific siRNAs.

We also found here that down-regulation of all the selected genes reduced the motility of Renca cells in the wound healing assay. In addition, the Transwell assay showed that Cav1, Gsn, Bnip3, Lgals3 and Egfr, but not Pax8 or Itgb2, have an effect on Renca cell invasion. Given this fact, we may conclude that a number of developmental genes related to caveolar signaling promote kidney tumourigenesis in a coordinated manner. Indeed, our data demonstrated that down-regulation of each of the selected genes had an effect on at
least some of the cell behavioural features commonly associated with carcinogenesis. Only three genes, *Bnip3*, *Cav1*, and *Gsn*, however, were found to be effective in all the assays tested.

Our results are generally in line with previously reported data. *Cav1* gene expression has been observed to coordinate with *Lgals3* in several cancers, and these proteins together have been seen to regulate the downstream pathway signaling (Goetz et al., 2008; Lajoie et al., 2007; Shankar et al., 2012). These observations had not been reported for renal cancer before.

*In vitro* down-regulation of *Gsn* in breast cancer MDA-MB 231 and in prostate cancer PC-3 cell lines reduced the cells’ invasive and motile properties, as well as cell aggregation (Van et al., 2007). Indeed, we noted here that down-regulation of *Gsn* influenced RCC cell behaviour, probably by targeting the process of EMT, which is critical for normal nephrogenesis and when deregulated also for tumourigenesis. Depending on the cellular context, expression of *Bnip3* either induces or delays cell death, and overexpression of *Bnip3* has been reported in several types of cancer (Burton et al., 2006). Our data indicate that in renal carcinoma cells *Bnip3* acts as an anti-apoptotic factor.

Components of the PI3K/Akt pathway belong alongside HIF/VHL to the group of the most frequently altered genes in ccRCC (Guo et al., 2015). The PI3K pathway has recently also been shown to balance self-renewal and differentiation of nephron progenitor cells during kidney development (Lindström et al., 2015). Moreover, there is a link between caveolin and Akt signaling. The activation of caveolin-1-PI3K/Akt/GSK3β pathway has been found to mediate the cardioprotective effect of epigallocatechin-3-gallate in cardiac cells (Hsieh et al., 2013), but it is also responsible for ammonium-related toxicity in astrocytes (Wang et al., 2017).

We studied whether down-regulation of caveolin-related genes influences Akt phosphorylation in Renca and found that inhibition of these genes did not lead to significant changes in Akt activation. However, down-regulation of *Pax8* resulted in a strong induction of Akt phosphorylation. This may indicate that Pax8 has a different action mechanism from the other genes used in our study.
Next, we developed 3D co-cultures that make it possible to study the cross-interactions between embryonic and transformed cells under conditions in which the expression of certain genes that are relevant both for normal development and carcinogenesis, is inhibited. We found that the addition of immortalized cells of different origin reduces kidney tubule formation in the 3D chimera assay. The level of this reduction varies from quite slight for the mK4 embryonal-derived cell line to very severe for the Renca renal carcinoma-derived cells.

The fact that the RCC cells inhibit normal tubulogenesis is consistent with our earlier finding. We have shown that for example the Wilm’s tumor inhibits Wnt4 function and promotes kidney tumourigenesis. Thus likely occurs by mutant WT1 mediated locking the cells to the progenitor state. This also prevents the MET (Murugan et al., 2012).

Importantly, in the present study, *Bnip3*, *GSN* and *Cav1* down-regulation in Renca with the siRNA compounds enabled tubule formation as judged from the induction of Pax2 and Aq1 expression in the induced kidney mesenchymal cells. Therefore, the kidney tubules are able to develop when the expression of certain genes was silenced with siRNAs prior to establishment of the organoid chimera.

To conclude, it appears that the down-regulation of several embryonic developmental genes by siRNA treatment reduced the malignant behaviour of renal cancer cells. In addition to providing a way to identify putatively novel oncogenes, the current work also offers a novel platform to study the capacity of kidney cancer cells to influence normal cell proliferation, differentiation and morphogenesis in a close to *in vivo* setting. Interestingly, this model also provides a way to monitor how normal kidney cells influence cancer growth upon silencing of carcinogenesis-related genes.

The *ex vivo* setup may also be extended to an *in vivo* situation by grafting the reaggregated organoid under the kidney capsule. The proposed techniques make it possible to monitor cancer cells and correlate their behaviour to the process of organogenesis. The siRNA-mediated inhibition of nephrogenesis genes in kidney cancer may not only help to identify new putative oncogenes but may also provide new therapeutic opportunities.
Materials and Methods

Patients and RCC samples
A bio bank of human clear cell RCC (ccRCC) biopsies was generated from donors (n=16) treated in the Haukeland University Hospital (Bergen, Norway) during 11.2013-08.2014. All the experiments were approved by the regional ethics committee of Western Norway (REC West no. 78/05) and by a written consent of the participants.

Animals
Embryos from wild type CD1 mice were used to prepare the embryonic kidneys at E11.5 for the ex vivo chimera organoid assays. Maintenance of the colonies and collection of the embryos were performed in accordance with the Finnish national legislation, European Convention ETS 123, and EU Directive 86/609/EEC and were approved by the local ethics committee.

Renal Cell Carcinoma cell culture
The mouse Renal Carcinoma (Renca) cells (ATCC® CRL-2947™) and HeLa cells were obtained from the American Type of Culture Collection and had been verified via short tandem repeat profiling. mK4 cell line was described before (Valerius et al., 2002). The cells were passaged in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin in 5% CO₂ at 37°C. Yellow Fluorescent Protein (YFP) Renca, HeLa and mK4 cells were generated by transfecting the RCC cells with the pcDNA3.1+ YFP cDNA construct. Cells in which the construct had integrated into the genome were enriched via puromycin mediated selection and FACS (BD FACSArtaTM IIIu). The YFP expressing cells were used to make the chimeric organoids with embryonic kidney nephron progenitor cells. A panel of siRNAs (Sigma-Aldrich) was used to knock down their respective target mRNAs. The MISSION siRNA (Sigma-Aldrich) was used as a control.

Transfilter kidney tubule induction assay
Embryonic kidneys were dissected from CD1 embryos at E11.5 and incubated in 1.125% pacreatin- 2.25% trypsin for 30-40 seconds. The ureteric bud (UB) was separated from the nephron progenitor/stem cell containing metanephric mesenchyme (MM) in media supplemented with 10% FBS and antibiotics (Lauri Saxen, 1987). The MM was treated
with 40µl of 2mg/ml collagenase III in 280µl of physiological buffer at 37°C for 10min to obtain single cell suspension. The MM cells were washed twice with cell culture medium, placed on Nuclepore polycarbonate membrane (Whatman, pore size 1.0µm), and a piece of embryonic dorsal spinal cord (E11.5) was glued onto the other filter side as a robust tubulogenesis inducer. The conjugate was cultured in 37°C, at 5% CO\textsubscript{2} for 96h, which is sufficient to induce and advance nephrogenesis (Lauri Saxen, 1987), snap frozen in liquid nitrogen, and stored at -80°C until used for RNA purification. Collectively 40 freshly prepared control and induced MM were processed for the oligonucleotide gene chip analysis. The analyses were conducted in triplicates.

**Microarrays and statistical analysis**

Total RNA (200 ng) that had been extracted from control and induced MM was subjected to the Affymetrix Mouse Genome 430 2.0 array analysis. The Affymetrix CELL-derived data files were processed with the R Bioconductor LIMMA package (Ritchie et al., 2015). The Robust Multichip Average (RMA) (Irizarry et al., 2003) tool served to adjust the signal-to-noise ratio and was also normalized with Quantile by median polished probe set summarization with the perfect data match. The data values with log2>6.64 scores in at least three of the six analyzed gene chip samples were considered a relevant change. The expression was counted as significant when its fold change was >2.0 with a P-value of <0.05 adjusted with the Benjamin-Hochberg multiple testing correction tools.

For the patient derived RCC samples, two biopsies from each patient were taken. The first one was processed for histolopathological inspection and ccRCC grading. The other sample was taken from the same tissue but from a region that appeared histologically normal and stored until subjected to RNA purification for the generation of a gene library. The genes that depicted a fold change of >2.0 with \(p<0.05\) were considered to be differentially expressed (Eikrem et al., 2016).

The Ensemble IDs of the genes that turned out to be differentially expressed in the induced MM cultured for 96h and the human ccRCC samples were analyzed with the Ingenuity Knowledge Base canonical pathway collection tool (www.ingenuity.com, version 23814503). The significance of the genes in the illustrated signal transduction pathways was evaluated first. The data was then presented as the p-values corrected with multiple testing via the Benjamin-Hochberg algorithm. Only the data that depicted an adjusted p-
value of <0.05 are reported. Venn diagram was generated using the online tool http://www.pangloss.com/seidel/Protocols/venn.cgi.

Groupwise comparison with linear model for microarray data from GSE53757 (Smyth, 2005) was performed in Geo2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/). P-value adjustment is reported as Benjamini-Hochberg False Discovery Rate.

**Analysis of cell proliferation and cell death**
The degree of proliferation of the RCC cells was estimated with the IncuCyte ZOOM (Essen BioScience Inc.) live cell imaging system. The siRNAs (50nM) were transfected to the Renca cells by Lipofectamin. Transfected cells were plated and cultured for two days, after which the cells were detached by trypsinization, counted, and plated into the 96-well plates at a density of 3000 cells/well. The degree of cell confluence in the cultures was monitored every two hours for four days starting two hours after setting the cultures. For the cell death measurements, 1µM Sytox® Green Nuclear Acid Stain (Life Technologies) was added to the cells after seeding into 96-well plates. The putative siRNA induced cytotoxicity was assayed by comparing the confluence metrics of the total cells and the dead cells using the IncuCyte integrated software.

**Wound healing induced cell migration**
The putative changes in cell migration caused by siRNA were analyzed by *in vitro* wound-healing assay using the IncuCyte ZOOM system. The RCC cells were transfected with 50nM siRNAs for two days in culture. After trypsinization and washing with PBS the cells were plated into the Essen ImageLock 96-well plates (40,000 cells per well) and cultured for 20h to reach 90-100% confluence. Then 0.4µg/ml mitomycin C (Sigma) was added and incubated for 4h to inhibit cell proliferation. The cells were washed and the wounds were generated with the IncuCyte Wound Maker (Essen BioScience Inc.). After a 24h migration time, the wounds were photographed and analyzed with the software tool included in the IncuCyte platform. Wound confluence (in %) was taken as a measure of cell motility.

**Estimation of cell invasion potential**
The invasion capacity of the RCC cells was evaluated by using the Transwell filter assay (6.5mm diameter, 8.0µm pores; Corning Costar) as described (Zhou et al., 2013). The Renca cells were transfected with 50nM siRNAs for two days in culture. After trypsinization
20,000 cells were plated into upper Transwell chamber. After a 24h culture period, the culture medium was aspirated. The wells were washed twice with PBS and the cells on the plated filter side were removed. Cells that had already migrated across the filter were fixed with 3.7% formaldehyde, stained with 0.4% crystal violet, and counted.

**Colony-forming assay**
To study the putative siRNA-mediated cell growth inhibition, the Renca cells were transfected with a Lipofectamine siRNA (50nM) cocktail and cultured for two days. The cells were trypsinized, counted, and 200 cells/well were seeded and cultured for seven days. The formed cellular colonies were fixed with 4% paraformaldehyde (PFA) for 15min, stained with 0.4% crystal violet and counted. The number of colonies containing more than 50 cells each was counted. Triplicates containing 20-150 colonies per well were counted for each treatment.

**Western blotting**
Total proteins (30-50μg per sample) were extracted from the siRNA transfected cells. The proteins were separated by gel electrophoresis (10% polyacrylamide gel) and transferred to a nitrocellulose membrane for immunoblotting by using routine procedures. Anti-Akt (Cell Signaling #4691, 1:1000), anti-phospho-Akt (Ser473, Cell Signaling #4060, 1:1000), anti-caveolin-1 (Abcam, ab2910, 1:1000), anti-gelsolin (ThermoFisher, PA5-27350, 1:1000), and anti-alpha-tubulin (Sigma-Aldrich #T6074, 1:5000) primary antibodies were incubated overnight at 4°C with the membranes, and washed several times in PBST buffer. The respective secondary peroxidase-conjugated IgG antibodies (Invitrogen) were then applied to the membranes. The LumiGLO Peroxidase chemiluminescence kit (Cell Signaling) was used to visualize the bound antibodies.

**RNA isolation and quantitative RT-PCR**
The total RNA was extracted from the cells two days after siRNA transfection with the RNeasy mini kit (Qiagen). The cDNA was synthesized from 1μg of total RNA with the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), and 2μl of the cDNA in 1:10 dilution was subjected to the qRT-PCR reaction. The Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies) was used according to the manufacturer’s instructions.
The forward and reverse primers for the qRT-PCR were: 5’-CAGTGAGCTTCCCGTTCAG-3’ and 5’-AGAACATCATCCCTGCATCC-3’ (GAPDH), 5’-GCTTGGGTGAAGACAGGGAG-3’ and 5’-GTGAAGGCTTGAGCACAACA-3’ (E-cadherin), 5’-CCATCCTGACAGACCCCAAC-3’ and 5’-ACTGAGGTGGGTGCTGAATG-3’ (N-Cadherin), 5’-TCCAGAGAGAGCCGAA-3’ and 5’-AAGGTCAGACGTGCCAGAG-3’ (Vimentin). The qRT-PCR reactions were done in the Mx3005P qRT-PCR System machine (Agilent Technologies). The GAPDH probe served as a control to normalize the data. The experiments were repeated at least three times.

Generation of the chimeras
The dissociation and reaggregation assay of the MM has been described (Junttila et al., 2015). The separated mouse embryonic MM (E11.5) was mechanically dissociated into a single cell suspension after incubation with 9400 U/ml collagenase Type IV for 15 min. Chimeric organoids were assembled by mixing the MM cells and either Renca-YFP cells, HeLa-YFP, or mK4-YFP cells at a 50:1 ratio. In the siBnip3 treated condition, we also tried 1:20 ratio mixture with MM. They were then reaggregated in the presence of the nephrogenesis inducer bromoindirubin-3-oxime (BIO) for 20h in DMEM with 4.5g/L glucose and 10% FBS at 37°C.

The Renca-YFP cells were treated with siRNA twice: first 2 days before and a second time 3 hours before co-culture onset. After removal of the BIO 24h after reaggregation start, the chimeric organoids were subcultured for four days, fixed with 4% PFA and stained with anti-Pax2 (Abcam ab79389) and anti-Aquaporin1 (Cell Applications.INC CA0648) antibodies as reported (Itaranta et al., 2009).
Acknowledgements

We thank Paula Haipus, Hannele Härkman and Johanna Kekolahti-Liias for their technical assistance, Virpi Glumoff for the FACS, Hang-mao Lee for the bioinformatics, Kimmo Halt and Antti Railo for testing the siRNAs.

Competing interests

The authors declare no competing or financial interests.

Author contributions

S.J.V, Q.X., A.S. and S.E.Q. conceived and designed the experiments. Q.X., J.S., S.J., K.R.G., O.K. and A.S. performed the experiments. Q.X., A.S., A.Sch. and H.-P.M. analyzed the data. Q.X. wrote the paper with comments and revisions from S.J.V., A.S., J.R. and I.S.

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Data availability

The microarray data of the patient derived RCC samples are available in the repository Gene Expression Omnibus, (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76207) under accession number GSE76207.

Supplementary information

Supplementary information available online at: http://dmm.biologists.org/XXX
References:


Figures

A

MM vs ccRCC

50

36

56

7

13

7

60

ccRCC

ccRCC

B

-Log(p-value)

Hepatic Fibrosis/Heptocellular Stellate Cell Activation
Fcγ Receptor-mediated Phagocytosis in Macrophage and Monocytes
Leukocyte Extravasation Signaling
Atherosclerotic Signaling
Phagosome Formation
Caveolar-mediated Endocytosis Signaling
Endothelin-1 Signaling
Dendritic Cell Maturation
UVA-Induced MAPK Signaling
Virus Entry via Endocytic Pathways
Gap Junction Signaling
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages
Macropinocytosis Signaling
Cellular Effects of Sildenafil (Viagra)
Growth Hormone Signaling
Figure 1. Ingenuity pathway analysis of the genes enriched in both 96h-induced MMs and human ccRCC. (A) Venn diagram shows the number of pathways identified by Ingenuity pathway analysis for three groups of genes: regulated in the kidney development model (MM (96h vs 0h)), regulated in renal carcinoma patients (ccRCC), genes simultaneously regulated in both groups. The full pathway list is shown in Table S3. (B) Ingenuity pathway analysis of the genes with differential expression in both the ccRCC and the 96h-induced MMs showing significantly modulated pathways ($p< 0.05$). 15 top pathways with significant changes are shown. The p-value for each pathway is indicated by the bar and is expressed as -1 times the log of the p-value. The line represents the ratio of the number of genes in a given pathway that meet the cutoff criteria to the total number of genes which belong to the pathway.
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**EGFR**
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**ITGB2**
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**LGALS3**
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**PAX8**
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$P_{adj}=4.50\times10^{-06}$

B

- si Control
- si Cav1

Gene Relative Expression

- Cav1
- Bnip3
- Edg
- Gsn
- Itgb2
- Lgals3
Figure 2. Gene expression profile in kidney tumours. (A) The genes that are differentially expressed in both 96h-induced MMs and human ccRCC were selected after the pathway analysis. The gene expression microarray values for ccRCC samples and normal tissue from the same patient (n=16) are shown. (B) Gene expression profiles after 48h siCav1 or control siRNA treatment in Renca cells. The results are presented as means ± SD, and data from three independent experiments are shown. * p<0.05 and *** p<0.001 compared by t-test with control siRNA-transfected Renca cells.
Figure 3. Effect of siRNAs transfection on the viability of Renca cells. (A) Proliferation assay; (B) Cell death. The Renca cells were pre-transfected with various siRNA oligonucleotides (50 nM each) for 2 days, seeded on 96-well plates at a density of 3000 cells/well and imaged by Incucyte. Cell proliferation was presented as a percentage of the confluent area. To study cell death, Sytox Green was added, both phase contrast and the green channel were measured and the percentage of the green area was calculated. The results are presented as means ± SD. Data from three independent experiments are shown. * \( p<0.05 \) and ** \( p<0.01 \) compared by t-test with control siRNA-transfected Renca cells.
Figure 4. Effect of siRNAs transfection on the migration and invasion of Renca cells. (A and B) Cell migration analyzed by wound healing assay, as compared with siRNA controls. (C and D) Invaded Renca cells analyzed by Transwell assay, as compared with siRNA controls. The results are presented as means ± SD, and data from three independent experiments are shown. *p<0.05; **p<0.01 and ***p<0.001 compared by t-test with control siRNA-transfected Renca cells.
Figure 5. Effect of siRNA transfection on colony formation by Renca cells. (A) The number of colonies after siRNAs transfection; (B) Representative photomicrographs. The colony-formation assay was performed by seeding 200 cells/well on a 24-well plate. The number of colonies was counted after one week of incubation at 37°C, 5% CO₂. Data are presented as means ± SD. Results from three independent experiments are shown. *p<0.05; **p<0.01 and *** p<0.001 compared by t-test with control siRNA-transfected Renca cells.
Figure 6. Disruption of the tubule formation by immortalized cells during nephrogenesis in vitro. After dissociation and reaggregation of embryonic kidney, MM cells were mixed at a ratio of 50:1 with either Renca-YFP, HeLa-YFP or mK4-YFP cells. (A-D) The Pax2+ tubular epithelial structures (in red) and glomeruli (arrowheads) were formed from dissociated and reaggregated embryonic kidney mesenchymal progenitor cells. Bringing of Renca-YFP cell to MM (E-H) strongly impaired tubular structure formation, while addition of Hela-YFP (I-L) and mK4-YFP (M-P) cells had a much milder effect on the nephrogenesis in vitro. 3D cultures were maintained for 4 days. Blue - nuclear stain (Hoechst); green – YFP; red – Pax2 immunostaining. Bar: 20µm.
Figure 7. Nephrogenesis *in vitro* can be rescued in 3D co-cultures by siRNA treatment of Renca cells. (A-D) The Pax2+ tubular epithelial structures (in red) and glomeruli (arrowheads) were produced by dissociated and reaggregated embryonic kidney mesenchymal progenitor cells. (E-H) Few Pax2+ tubular epithelial structures were formed by MM after mixing with the *siControl* treated Renca cells. The Pax2+ tubular epithelial structures and glomeruli formation was again well visible after addition of the *siBnip3* (I-L) and *siCav1* (M-P) treated Renca cells. These structures also partially formed if the Renca cells were treated by *siGsn* (Q-T). 3D cultures were maintained for 4 days. Blue - nuclear stain (Hoechst); green – YFP; red – Pax2 immunostaining. Bar: 20µm.
Figure 8. Formation of proximal tubes can be rescued in 3D co-cultures by siRNA treatment of Renca cells. (A-D) The proximal tubes (Aquaporin1 staining in red) were well formed in dissociated and reaggregated MM pellet in vitro. Few Aq1+ structures were formed after mixing with the siControl treated Renca cells (E-H). The proximal tubes were well visible after addition of the siBnip3 (I-L) and siCav1 (M-P) treated Renca cells. siGsn treatment also partially rescued tubes formation (Q-T). 3D cultures were maintained for 4 days. Blue - nuclear stain (Hoechst); green – YFP; red – Aquaporin 1 immunostaining. Bar: 20µm.
Supplementary Figure 1 Gene expression and protein profiles in siRNA-transfected Renca cells. (A) The mRNA expression level was determined by qRT-PCR. The results are expressed as relative mRNA levels in cells treated with siRNAs for 48h, 72h and 96h. The results obtained with the control siRNA were given the value 1. (B) Western blot analysis of Gsn and Cav1 levels in siRNA treated Renca cells.
Supplementary Figure 2 Analysis of EMT and Akt pathways in siRNA silenced Renca cells. (A) EMT marker gene expression measured by qRT-PCR. Data are presented as means ± SD, and results from three independent experiments are shown. *p<0.05 compared by t-test with control siRNA-transfected Renca cells. (B) Western blot analysis of phosphorylated Akt, total Akt and alpha-tubulin levels in Renca cells treated with siRNAs.
Supplementary Figure 3. Different ratios of *siBnip3* treated Renca to MM cells in the coculture organoids. (A-D) Formation of Pax2+ tubular epithelial structures was disrupted by the siControl treated Renca cells. (M-P) The Pax2+ tubular epithelial structures were well formed in the organoids with 1:20 ratio (I-L) as well as with the 1:50 ratio of cancer to normal cells (E-H). 3D cultures were maintained for 4 days. Blue - nuclear stain (Hoechst); green – YFP; red – Pax2 immunostaining. Bar: 20µm.
Supplementary Table 1 Differentially expressed genes in the induced MM.

Click here to Download Table S1

Supplementary Table 2 Genes differentially expressed in both induced MM and ccRCC.

Click here to Download Table S2

Supplementary Table 3 VENN diagram and pathway lists.

Click here to Download Table S3

Supplementary Table 4 Groupwise comparison with linear model for microarray data from Gene Expression Omnibus database GSE53757.
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