Acute drug effects on the human placental tissue: the development of a placental murine xenograft model.

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Abstract

Objective: A pilot study was conducted to establish a human placental xenograft, which could serve as a model to evaluate the effect of toxic exposures during pregnancy. Study Design: The protocol consisted of engraftment of third trimester human placental tissue in immunocompromised mice, after induction of a pseudo-pregnancy state by ovariectomy and progesterone supplementation. To validate the model, the placental tissue before and after engraftment was examined by immunohistochemistry, Fluorescence-activated cell sorting (FACS), single nucleotide polymorphism (SNP) genotyping and whole transcriptome sequencing. The human Chorion Gonadotropin (hCG) production in serum and urine was examined by ELISA. Results: Microscopic evaluation of the placental tissue before and after engraftment revealed a stable morphology and preserved histological structure of the human tissue. Viable trophoblast was present after engraftment and remained stable over time. Vascularization and hormonal secretion (hCG) were present till 3 weeks after engraftment. Thirty-one SNPs were equally present and there was a stable expression level for 56451 genes evaluated by whole transcriptome sequencing. Conclusion: Although this human placental xenograft model cannot copy the unique uterine environment in which the placenta develops and interacts between mother and fetus, it could be a suitable tool to evaluate the acute impact and adaptive processes of the placental tissue to environmental changes.

KEY WORDS: Placenta, Mice, human xenograft model, Drug effects

Introduction

The placenta exerts a diversity of functions to support the normal growth and development of the fetus. An important role for the placenta has been shown in gestational complications like growth restriction, preeclampsia, macrosomia and congenital anomalies after e.g. maternal drug intake. Preeclampsia is related to deficient remodeling of the spiral arteries and increased oxidative stress.[1,2] Also the placenta is equipped with a variety of enzymes and transporters that ensure the detoxification and efflux of xenobiotics, protecting the fetus against exposure to potentially toxic agents.[3] The investigation of drug effects on the human placenta is difficult because of many interfering factors in the clinical setting, like medical condition (for which the drug is taken), maternal habits, gestational age at delivery, etc. Many research is performed in rodents.[4] The problem is that those results cannot be extrapolated to the human setting because of morphological and functional variations in placentation.[5] Comparative studies between species help to understand the physiology and genetic developmental processes, but uncertainty of significance to the human placental function remains.[6]

In vitro models have been established to evaluate and measure biomarkers and the effects of possible treatment options in preeclampsia and intra-uterine growth restriction (IUGR).[7,8] Although feasible, this model doesn't recapitulate the in vivo environment since two of the most important processes

underlying IUGR, neovascularization and villous formation, cannot be evaluated by cell culture.[9-11] In addition isolated cultured trophoblast cannot be used to investigate the interaction between the different cell populations present in placental tissue.

In oncology, patient-derived tumor xenograft (PDX) models have emerged as an important source in multiple applications (eg. preclinical drug evaluation, biomarker identification, translational research) to enable a personalized approach to patient care.[12] PDXs reflect histopathology, tumor behavior, and the metastatic properties of the original tumor, in contrast to in vitro cell lines in which heterogeneity and gene expression levels are lost through continual passage and clonal selection.[13] In addition, the transcriptomic and genomic profile as well as tumour heterogeneity are maintained over the different passages and key features in cancer biology. Even more important, the therapeutic response to treatment in patients can be predicted.[13]

In 1984, Boltenberg et al. published a first report on the successful engraftment of placental tissue in 5 mice with maintenance of the histological structure and the production of placental proteins and hormones.[14] To the best of our knowledge, no other study has evaluated this model further. In line with this previously reported study, and the increased knowledge of PDXs in tumor research, we optimised this model to improve graft viability for in vivo studies on human placental function. The principal aim of this study was to establish a human placental xenograft model, which could serve as a model to evaluate the effect of xenobiotics on placental tissue.

Material and methods

Establishment of a murine placental xenograft model.

All procedures were approved by the Medical Ethical Committee (ML8713) and the Clinical Trial Center (S54185) of the University Hospitals of Leuven, Belgium (for third trimester placental tissue), in accordance with the most recent Helsinki Declaration. All patients provided signed informed consent for participation. Fresh full term (38-40 weeks of gestation, median 39 weeks and 1 day) placental tissue was collected.

A schematic overview of the protocol is shown in figure 1. On D0 immunocompromised nude mice (min. 6 weeks old) were anaesthetized by a mixture of ketamine (Anesketin®, 100 mg/ml, dose 75 mg/kg ip) and medetomidine (Narcostart®, 1 mg/ml, dose 100 mg/kg ip) and body temperature was maintained using a heating plate. 'Pseudo-pregnancy' was induced by ovariectomy via the dorsal route and progesterone (P) (≥99%, Sigma-Alldrich, Bornem, Belgium) supplementation through subcutaneous transplantation of silastic capsules (Dow Corning, Seneffe, Belgium) as previously described by Ström et al. (25mg of progesterone is released at a constant rate for 60 days).[15,16] On D3,

fresh human third trimester placental tissue was derived from sterile placentas obtained through caesarean section. All placentas were acquired from the delivery room at the University Hospitals of Leuven. Exclusion criteria were: maternal medical disorders (Crohn's Disease, colitis, congenital heart disorders, auto-immune disease), maternal obstetrical disorders (preeclampsia, HELLP, cholestasis) and presence of Doppler abnormalities indicating underlying placental developmental problems. Tissue was minced into pieces of $2mm^3$ and within 2 hours after sampling subcutaneously engrafted bilateral in the flanks of the mice. Biweekly, body weight and graft size were monitored. Urine of the mice was collected by individually housing the mice for 4 hours in metabolic cages. In total 30 mice were used. Unless otherwise specified, we show the results of the latest 2 validation projects in which a total of 24 mice were used. All research procedures were executed in accordance with the applicable legal guidelines and under approval of the medical ethical committee for laboratory animals of the KU Leuven (P147/2012 and P038/2015). Postoperative analgesia consisted of buprenorphine (Vetergesic®, 0.3 mg/ml, dose 0.05 mg/kg, subcutaneous (sc) injection). Reversal of anesthesia was performed with Atipamezole (Antisedan®, 5 mg/ml, dose 1 mg/kg, sc). The animals were sacrificed after 1, 2 or 3 weeks. Blood collection was performed by intracardiac puncture. General necropsy was performed and grafts were carefully removed. Tissue was divided and either snap frozen and stored at -80°C, either stored in RNAlater (RNA stabilization reagent, Qiagen, Hilden, Germany) for max 4 weeks at 4°C, or stored in 4% buffered formaldehyde at room temperature upon analysis. Urine and serum samples were stored at -20°C for Elisa (enzyme-linked immunosorbent assay).

Immunohistochemistry (IHC)

Morphology of the tissue before and after engraftment was evaluated with different stainings: hematoxylin and eosin (HE), hCG, and anti-murine CD31 (also known as platelet-endothelial cell adhesion molecule-1 (PECAM-1)). Placental grafts were stored for 24h in 4% buffered formaldehyde, washed with phosphate buffered saline (PBS), transferred to ethanol 70% and embedded in paraffin. Four µm sections were deparaffinated and rehydrated for HE and immunohistochemical expression of hCG and murine CD31. hCG antigen retrieval was performed In Tris-EDTA buffer at pH9, at 80°C for 45 minutes. Sections were rinsed in PBS and incubated at room temperature respectively for 2h and overnight with following primary antibodies hCG (3,55µg/ml, polyclonal, Agilent Technologies, California, USA) and CD31 (15,6µg/ml, monoclonal, BD biosciences, New Jersey, USA). Binding of species-specific biotinylated secondary antibodies was visualized with Dako DAB + Chromogen (K3467, Agilent Technologies, California, USA). Counterstaining was performed with Mayer's hematoxylin before mounting. Negative controls were performed by omitting the primary antibody. Positive staining was identified by the presence of a cytoplasmic trophoblastic or membranous endothelial

brown (DAB) reaction product. Images were taken using the Axioskop microscope (MRc5, Zeiss, Jena, Germany) and the ZEN 2.0 software.

Preparation of single-cell suspensions from engrafted placental tissue

The placental villous tissue was cut into 1mm^3 pieces on a petri dish with cold PBS. The tissue pieces were then further dissociated using a combination of mechanical dissociation and enzymatic digestion using the Human Tumor Dissociation Kit (Miltenyi Biotec, Leiden, The Netherlands) and the GentleMACS dissociator (Miltenyi Biotec). More specifically, the protocol for soft tissues was followed, according to manufacturer's instructions. The cell suspensions were purified on a 40 µm cell strainer and centrifuged for 5 min at 300 g at 4°C. These single-cell suspensions were then cryopreserved for subsequent flow cytometric analysis.

Fluorescence-activated cell sorting (FACS)

Single-cell suspensions were thawed and centrifuged for 5 min at 300 g at 4 °C. After aspiration of the supernatant, cells were first washed with cold PBS, centrifuged again and Fc receptor blocking was performed by adding a 10% normal goat serum (Sigma-Aldrich, Bornem, Belgium) solution in FACS buffer (PBS supplemented with 0.5% BSA). The cells were extracellularly stained with mouse anti-human CD163-Brilliant Violet 421 (5 µl/test, BD Biosciences, New Jersey, USA) for 30 min at 4 °C, after which red blood cells were lysed by adding 1x Pharm Lyse (BD Biosciences, New Jersey, USA) for 15 min at RT. Next, cells were first stained with a fixable viability dye (0.5 µl/test, Fixable Viability Dye eFluor 780, eBioscience, Vienna, Austria) for discrimination of live and dead cells. Finally, the samples were fixed and permeabilized using a Fixation/Permeabilization Buffer Kit (R&D Systems, Minneapolis, USA), after which the cells were stained intracellularly with mouse anti-human cytokeratin-FITC (2.5 µg/ml, Clone CAM5.2, BD Biosciences, New Jersey, USA) and vimentin-PerCP-Cy5.5 (1.25 µl/test Clone 280618, R&D Systems, Minneapolis, USA) for 30 min on 4 °C. Acquisition was performed with a FACSCanto II flow cytometer using BD FACSDIVA software v6.1.3 (BD Biosciences, New Jersey, USA). A minimum of 30x10^{4}cells was acquired per sample. Data analysis was done using FlowJo v10.3 (FlowJo LLC, USA). Cells were gated as follows: first, dead cells were gated out using the viability dye and subsequently, fibroblasts and macrophages were gated out using vimentin and CD163 respectively. Finally, trophoblasts were gated as cytokeratin-positive cells.

Single Nucleotide Polymorphism (SNP) Genotyping

The 31 SNPs used for sample tracking have been selected from Haplotype Map (HapMap) and were selected based on their exonic position and availability of population frequencies in the HapMap position. The power of the SNP panel was estimated by assessing the probability of match for a real

match pair. Using the 20 least informative SNPs from our panel, there is a one in 187,000 chance of finding two different individuals with the same genotypes in the least polymorphic population (44 unrelated Japanese individuals from Tokyo, Japan (JPT)) and one in 1,000,000 chance in the CEU population (30 trios of Utah residents of northern and western European ancestry). When all SNPs are used, the chance is above 2.3×10^9 in the CEU population.

Total DNA was extracted from 20 milligrams of fresh frozen tissue using magnetic bead technology with the Chemagic DNA tissue 10 kit for automated DNA isolation with the Chemagic MSM I instrument (Perkin Elmer, Zaventem, Belgium). Briefly, sample tissues were incubated with protease and lysis buffer, and magnetic beads were added to the mixture. After incubation, nucleic acids bound to the beads were separated with a magnetic separator. After two washing steps, the nucleic acids were eluted in a volume of 200µl of elution buffer and the magnetic beads were separated from the solution. The final concentration of total DNA is 50ng/µl. A multiplex PCR reaction was performed to amplify regions targeting 31 SNPs used for sample tracking (GeneAmp PCR System 9700; denaturation at 94°C for 2 min followed by 45 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min followed by 72°C for 1 min). PCR products were then purified with magnetic beads (HighPrep PCR beads; MB AC-60050; Magbio) and the DNA length was assessed on the Fragment analyser (Advanced Analytical). P5 and P7 tails were incorporated in a second PCR reaction (GeneAmp PCR System 9700; 30 sec of denaturation at 94°C followed by 15 cycles of 94°C for 10 sec, 50°C for 30 sec, 72°C for 30 sec followed by 72°C for 1 min). The resulting product was sequenced on a HiSeq2500 (Illumina, Paired-end 125 bp).

RNA Isolation and Whole Transcriptome Shotgun Sequencing (WTSS)

Tissue for RNA extraction was stored in RNAlater (RNA stabilization reagent, Qiagen, Hilden, Germany) for max 4 weeks at 4°C upon analysis. Total RNA was isolated from the placental grafts using Tripure Isolation Reagent (Roche, Bazel, Switzerland). The quantity and quality of the extracted RNA were respectively photometrically tested (NanoDrop, Isogen Life Science, Temse, Belgium) and evaluated for RNA integrity with the Bioanalyzer 2100 (Agilent Technologies, California, USA) on RNA 6000 Nano Chips (Agilent Technologies, California, USA)**.** 500ng of total RNA was used as input material for sequencing library preparation, which was performed with the QuantSeq 3' mRNA_Seq Library Prep Kit (Lexogen Inc., Greenland, USA) according to the manufacturer's protocol. Denaturation of RNA was performed at 85°C in a thermocycler and cooled down to 42°C. 12 PCR cycles were used for the PCR enrichment step. Samples were indexed to allow for multiplexing. Sequencing libraries were quantified using the Qubit fluorometer (Thermo Fisher Scientific, Massachusetts, USA). Library quality and size range was assessed using the Bioanalyser (Agilent Technologies, California, USA) with the High Sensitivity DNA 1000 kit (Agilent Technologies, California, USA) according to the manufacturer's

recommendations. Each library was diluted to a final concentration of 4nM and sequenced on Illumina HiSeq2500 according to the manufacturer's recommendations, generating 50 bp single-end reads.

Enzyme-linked immunosorbent assay (Elisa)

The concentration of hCG (human chorionic gonadotrophin) in mIU/ml and creatinine in mg/ml was determined using respectively a commercial available Elisa kit based on appropriate and validated sets of monoclonal antibodies and a quantitative colorimetric determination kit (Pathozyme, OD347, OmegaDiagnostics, Alva, GB; Quantichrom DICT-500, BioAssay Systems, California, USA). The assays were performed as instructed by the manufacturer. hCG levels were related to those of the standard reference curve. The concentration of hCG is directly proportional to the colour intensity of the test sample. The concentration of creatinine is directly proportional to the colour intensity of the test sample. All experiments were performed in duplicate and measured at 450 nm (hCG) and 490 nm (creatinine) (Multiskan FC, Thermo Fisher Scientific, Massachusetts, USA). The concentration of hCG in mIU/mg was calculated by dividing the hCG concentration in mIU/ml with the creatinine concentration in mg/ml.

Data analysis

SNP Genotyping

Adapters were trimmed using ea-utils v1.2.2 and overlapping paired end reads from a same fragment were merged using FLASH 1.2.11.[17,18] Reads were mapped against the reference genome (build 19) with bwa-mem 0.7.8.[19] Duplicate reads were kept. All positions were genotyped with GATK HaplotypeCaller 3.6 with the option EMIT_ALL_SITES to get reference genotypes.[20] For each SNP position, calls (reference and variant) were retained if their genotype quality was above 30.

WTSS

Quality control of raw reads was performed with FastQC v0.11.5.[21] Adapters were filtered with ea-utils v1.2.2.[18] Xenome software was used to classify xenograft-derived raw reads into human, mouse, both or neither according to their origin.[22] Splice-aware alignment was performed with TopHat v2.0.13 against the hg19 human reference genome, Ensembl version 75 for human-classified reads, and against the mm10 mouse reference genome, Ensembl version 75 for mouse-classified reads.[23] The number of allowed mismatches was 2. Reads that mapped to more than one site to the reference genome were discarded. Reads mapping to the human genome only were considered for differential expression analysis. The minimal score of alignment quality to be included in count analysis was 10.

Resulting SAM and BAM alignment files were handled with Samtools v0.1.19.[24] Quantification of reads per gene was performed with HT-Seq count v0.5.3p3.[25] Count-based differential expression analysis was done with R-based (The R Foundation for Statistical Computing, Vienna, Austria) Bioconductor package DESeq.[26] Three replicates per conditions were considered except for F0, where only two samples were available with sufficient usable reads. Reported p-values were adjusted for multiple testing with the Benjamini-Hochberg procedure, which controls false discovery rate (FDR). List of differentially expressed genes were selected at a FDR of 0.1. The resulting list of differentially expressed genes was further analyzed with Qiagen's Ingenuity Pathway Analysis (IPA) software (Qiagen, Hilden, Germany) with input genes, p-values and adjusted p-values reported by DESeq.The expression changes from our mRNA-sequencing were evaluated based on the knowledge from prior experimental reports on causal effects between molecules (endogenous and exogenous) compiled in the IPA Knowledge Base.

Results

Macroscopic evaluation

One week after engraftment the small minced pieces (2mm^3) clustered together to form a solid tumor. Mean volume of the grafts remained stable till 3 weeks after engraftment (Figure 2).

Microscopic evaluation

Histological assessment of the placental tissue before and after engraftment revealed preserved structural features of the trophoblast layers till 1 to 2 weeks after engraftment. A higher percentage of fibrosis (up to 50%) with lower expression of hCG was observed over the different time periods of engraftment (Figure 3). Eighty-five to 95% expression of hCG at the syncytiotrophoblast was detected before engraftment, after one week of engraftment the expression remained high (50 tot 80%), but declined steep to max. 30% respectively 15% after 2 and 3 weeks of engraftment. Positive staining for CD31 revealed the presence of murine vessels in the human placental tissue, increasing over time (Figure 4).

Circulatory hormone concentration

The expression of hCG was still detectable 3 weeks after engraftment in urine as well as in serum. Although the hCG level in urine increased, in serum the level decreased (Figure 5). The hCG levels in urine were correlated to the creatinine level (considering the concentration or dilution of urine), nevertheless the levels between urine and serum remained inconsistent.

Flow cytometric analysis

After engraftment, there was a steap decrease of the viability of all placental cells, from 90,85% to 12,9% (Figure 6A). However, the percentage of viable trophoblasts remained stable at different time points after engraftment, from 4,675% to 4,875% to 6,425%. (Figure 6B). A representative flow cytometry plot showing the gating strategy is shown in Figure 6C.

Genetic and transcriptome evaluation

The analysis of 31 selected SNP's revealed no differences between the samples before engraftment and after engraftment at any time point (Table 1).

188 out of 56639 explored genes were significantly up- or downregulated 1 week after engraftment (FC<-2.5 or >2.5; FDR<0.1). Between respectively week 1 and 2, and week 2 and 3, only 1 and 3 genes were additionally found significantly differential expressed. Table 2 shows the highest significant differentially expressed genes between the different groups. Running the IPA pathway analyzer for the 188 differentially expressed genes between before and after engraftment, the genes were sorted by function and involvement in (patho-) physiological processes. Most differentially expressed human genes after engraftment were involved in the pathway of inflammation (upregulated) or were relevant to the hormonal activity of the placental tissue (downregulated). We investigated the differential expression ratios of important genes relevant to oxidative damage and stress reactions as well as apoptosis, inflammation, angiogenesis and hormonal activity, to reveal the stability of these pathways after engraftment. In figure 7 the heatmap of 30 selected genes related to these pathways shows that the oxidative damage and stress reactions (eg. NOS3, NOX4, TXN), as well as most genes related to apoptotic reactions (eg. Caspase's, MTOR, KRAS, RAF1) remain stable till 3 weeks after engraftment. Inflammatory response genes (IL8, IL1RN) and genes related to angiogenesis (eg. VEGF-A FLT1, PAPPA2) were upregulated after engraftment, placental hormones (CSH1, CSH2) were downregulated.

Comment

In this study we created a human placental xenograft model. This model showed a preserved morphology, detectable hormone production, and similar SNPs and transcriptome expression, up till 3 weeks after engraftment, but especially in the first week after engraftment. Therefore this model could be a suitable model to investigate the acute impact of exposure of xenobiotics to human placental tissue.

This model is the first in vivo model based on healthy third trimester human trophoblasts.[14] Our principal aim was to develop a model to examine the individual effect of chemotherapeutic agents

during pregnancy. Since cancer treatment cannot be performed in first trimester, we used third trimester placental tissue.[27] Second trimester placental tissue would have been the most appropriate to examine, but can only be taken either by invasive procedure (chorionic villous sampling (CVS)) or after termination of pregnancy in cases with serious congenital anomalies. We showed a preserved histological structure of the trophoblastic layers up till 2 weeks after engraftment with sustained expression of hCG in the tissue as well as in the urine and serum. Although we correlated the urine hCG level to the creatinine level, the inconsistency of hCG levels between urine and serum can be related to the decrease of the number of samples between the different time points, but can also still be related to the differences in glomerular filtration. Anti-murine CD31 indicated the presence of murine vascularization, increasing over time. Comparing the morphology before and after engraftment, trophoblastic layers and stromal cells were still identified but decreased in time, replaced by murine fibroblasts and vessels. This was also observed by flow cytometric analysis revealing a decrease of cytokeratin positive cells. The infiltration of murine fibroblasts and vessels might be a result of the processing technique of the spongy placental tissue. Although this limitation in our model, artificial in vitro models exhibit additional limitations, especially when investigating the impact of environmental changes and drug treatments. Most cell lines are derived from commercially available choriocarcinoma cells (eg. BeWo, JEG3), and therefore there's uncertainty about the expression of transporters and enzymes and accordingly the response to drug treatment or environmental changes as compared to the original cells.[28] In contrary, primary trophoblast cultures can be used and have the capacity to differentiate in syncytio- and cytotrophoblast. However, the establishment of a primary trophoblast culture is laborious and the 3-dimensional structure is still lost.[29,30]

An adverse fetal or placental environment has been shown to alter the placental function, and impact the long-term outcome of the offspring.[4] Understanding the maternal-placental interface and the interaction between the uterine endometrium, fetal membranes, and trophoblast is very challenging. Investigating the human placenta directly, in its own environment would be the optimal condition. Since this is ethical not possible, comparative studies between animals can help, and the establishment of this xenograft model has the advantage to be able to directly assess the human placental tissue. Although 188 differentially expressed genes in the pathway of inflammation (upregulated) or hormonal activity (downregulated) were demonstrated, the majority of the expression levels were maintained upon engraftment. These results indicate the stability of the engrafted tissue at a transcription level and confirms a potential application of the established human placental xenograft to investigate drug toxicities and the protective function of the placenta to the acute adverse environmental effects as smoking, alcohol or drug intake. In addition, SNPs were maintained upon engraftment and confirmed genealogy. Latest research on cancer in pregnancy showed a good

cognitive and general outcome of the children in utero exposed to chemotherapy, but also an increased risk for decreased fetal growth was noticed. In these cases the placenta could be considered as protective for the adverse effects of chemotherapeutic agents, however it does not seem to prevent growth restriction in 22.0% to 24.2% of the cases.[31,32] Since up to 90% of fetal growth restriction is caused by placental dysfunction,[9] and because the exact mechanism of the effects of chemotherapeutic agents on the placental metabolism and function are still unknown, this xenograft model can be helpful to examine the direct effect of individual chemotherapeutic agents on the placental structure and function.

Our model has some limitations. A major limitation is that the post-engraftment viability and morphology is dependant on the pre-engraftment state of the placental tissue. The individual differences of the placental development and the level of stress factors present during pregnancy and delivery, will potentially affect the viability and morphology pre- and also post-engraftment. This model can only serve to evaluate acute toxicities and short-term drug effects due to its limited lifespan, because of the increasing percentage of fibrosis seen over time after engraftment, resulting in a decreased placental function (hormonal production). Moreover the terminal villi and fetal endothelial cells could not be identified. Therefore teratogenic impact can only be evaluated on the trophoblastic layers and stromal cells. At last, some pathways are affected by the engraftment itself. Few genes enrolled in the pathway of inflammation and the placental function (hormonal production) showed differential expression ratios. Besides this, using a xenograft model implicates a high cost and time investment in order to obtain good results.

This establishment of a human placental xenograft model offers the opportunity to investigate the acute impact and adaptive processes of the placental tissue to environmental changes (drug effects, maternal habits, deficient oxygen or nutrient supply). Nevertheless, we are aware that our model as the in vitro models cannot copy the unique uterine environment in which the placenta develops and interacts between mother and fetus. The decreased viability in time after engraftment and non-proliferative state of the trophoblast post-engraftment by using term placental tissue can be adapted and improved by injecting proliferative cells derived from primary trophoblast culture. Nevertheless in this case the interaction between different placental cell types cannot be evaluated. Further research to improve this model is warranted.

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Tables and Figures

Figure 1: Schematic overview of the protocol.

Figure 2: Macroscopic view of the placental grafts, respectively 1 (A), 2 (B) and 3 (C) weeks after engraftment.

Figure 3: H&E (A) and hCG (B) stainings of the placental tissue before (0) and 1, 2, and 3 weeks (1,2,3) after engraftment. The arrows indicate the trophoblast layers. After one week, the morphology of the placental tissue remained clearly present. After time progressing further we noticed less clear morphology and the expression of hCG also declined. Images were taken at 12,5 magnification, scalebar 50µm.

Figure 4: Anti-murine CD31 staining after 1 (A), 2 (B) and 3 (C) weeks of engraftment. The arrows indicate murine vessels. Vessel formation increases over time of engraftment. Images were taken at 12,5 magnification, scalebar 50µm.

Figure 5: Mean expression levels of hCG in urine (A) and serum (B). Data are shown as boxplots with the box length showing the interquartile range, and whiskers showing the smallest and largest value. Respectively 24, 16 and 8 samples were analyzed during the first, second and third week. In urine we see an increase of hCG production as time progresses, whereas in the serum samples a decrease was seen.

Figure 6: Flow cytometric analysis performed in 1 experiment of 12 mice shows the percentage of viable placental cells (6A) and viable trophoblast (6B) before and after engraftment. Immediately after engraftment the viability decreased, but during engraftment the viable trophoblast remained stable. Bars indicate the median percentages (Graphpad Prism Version 5). A representative flow cytometry plot showing the gating strategy is shown in Figure 6C.

Figure 7: Heat map of selected genes relevant to oxidative damage, stress reactions, apoptosis, angiogenesis, inflammation and placenta hormonal activity. Colors indicate the expression level (high = red, low = yellow). F0 = before engraftment, F1 = after 3 weeks of engraftment. Genes related to oxidative damage and stress reactions (eg. NOS3, NOX4, TXN), and most genes related to apoptotic reactions (eg. Caspase's, MTOR, KRAS, RAF1) remained stable. Inflammatory response genes (IL8, IL1RN) and genes related to angiogenesis (eg. VEGF-A, FLT1, PAPPA2) were upregulated after engraftment, placental hormones (CSH1, CSH2) were downregulated.

Table 1: Single Nucleotide Polymorphism (SNP) Genotyping results in 1 experiment of 12 mice. Analyses of 31 different SNPs revealed the presence of the same nucleotides before and after engraftment. With the knowledge that when using all SNPs the chance of finding two different individuals with the same genotypes in the CEU population (30 trios of Utah residents of northern and western European ancestry) is above 2.3×10^9 in the CEU population, this indicates that the samples before and after engraftment are related and do not exhibit major mutations.

Table 2: Whole Transcriptome Shotgun Sequencing (WTSS) results. Ten highest statistically significant down- and upregulated genes between study before and after engraftment. *Significantly differentially expressed between 1 and 2 weeks of engraftment. **Significantly differentially expressed between 2 and 3 weeks of engraftment. Genes in bold are relevant to inflammation and vasculogenesis, genes underlined are relevant to the hormonal activity of the placenta. IPA: Ingenuity Pathway Analysis, FC: fold change, FDR: false discovery rate.