Liver regeneration during the associating liver partition and portal vein ligation for staged hepatectomy procedure in Sus scrofa is positively modulated by stem cells

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Abstract. This present study investigated the impact of the application of stem cells to liver regeneration following the first stage of associating liver partition and portal vein ligation for staged hepatectomy (ALPPS). The experiment was conducted on a pig model (n=6, 3 that did not receive application of stem cells, 3 that received application stem cells). Collected samples of liver (day 0 and 9 following surgery) were subjected to complete transcriptome sequencing. In total, 39 differentially expressed genes were found in the group without the application of the stem cells (genes of unwanted processes such as fibrosis and inflammation). In the group that did receive application of stem cells, no significantly differentially expressed genes were found, indicating a properly regenerated liver remnant. The present study therefore demonstrated, to the best of our knowledge for the first time, the positive effect of stem cells application in the liver regeneration process during ALPPS procedure in the pig model.

Introduction

The process of liver regeneration is, on the molecular level, an extremely complicated process that requires a perfect interplay of cell-cell signaling and gene expression continuity (1). Liver regeneration has traditionally been divided into three phases: Initiation, proliferation and termination (2). The duration of these phases depends on the organism under examination, including human, pig or rat, and the type of surgical intervention, including partial hepatectomy, intoxication by drugs, or hereditary predispositions (3). The organisms most frequently used to investigate liver regeneration are rats and mice, which are relatively well-investigated model organisms. However, pigs (Sus scrofa) are anatomically and physiologically closer to humans than rodents, and therefore are attractive subjects for biomedical research, despite the higher cost of maintenance (4). Budai et al (5) outlines a detailed comparison of existing associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) animal models and their advantages.

Currently, it is known that application of adipose-derived stem cells may positively modulate tissue regeneration processes (6-8). There are a number of clinical studies that are aimed at verifying the safety and effectiveness of this form of treatment; however, the molecular mechanisms of action remain largely unclear (9,10). It is likely to be the primarily paracrine mechanism of action that produces growth factors and cytokines, which positively modulate regenerative processes, such as improved angiogenesis, and limit inflammatory processes (9,10).

The present study analyzed the effect of the application of stromal vascular fat tissue stem cells on liver regeneration during the first stage of ALPPS procedure. ALPPS is a relatively recent modification of the two-staged hepatectomy, first described in 2010 (11). ALPPS approach allows for surgery on severe liver tumor burden in two associated steps. In the first step, tumor loci are removed.
from less affected liver lobe, the two liver lobes are split by parenchyma transection and the more metastatic region of the liver is deportalized. Deportalization of one liver lobe stimulates the second liver lobe to undergo hypertrophic regeneration (the future liver remnant). The patient is then permitted 1 or 2 weeks to recover. The second step removes the deportalized region of the liver, while the hypertrophic future liver remnant is fully functional (12). This approach significantly increases possibility of curative treatment of severe liver tumor diseases (13).

It is assumed that the application of stem cells obtained from stromal vascular fat tissue accelerates the regenerative process by allowing for improved angiogenesis and modulation of inflammation, as has been previously observed in animal-model studies (14,15); however, to the best of our knowledge, this has not been demonstrated in direct connection with ALPPS approach and Sus scrofa model organism. The aim of the present study was to identify candidate genes that may be used as screening markers for monitoring the process of liver regeneration following the first stage of ALPPS.

Materials and methods

Animals. A total of six juvenile domestic swine (Polish white pigs; 6 months; seven females and one castrated male; weight 30-50 kg; Instytut Zootechniki, Grodzic ć Ślaski, Poland) were included in the present study. The pigs were housed in separated boxes at room temperature (15-20°C), air humidity of 50-60%, normal atmosphere, 12 h light/dark cycles and access to food and water ad libitum. Procedures were performed in the Center for Cardiovascular Research and Development, American Heart of Poland S.A. (Ustroń, Poland) between September and October 2014. Approval from the Bioethical Committee from the Center for Cardiovascular Research and Development, American Heart of Poland S.A. (Ustroń, Poland) was obtained. Animals were assigned to two groups: n=3 without stem cell application (pig nos. 1-3) and n=3 with stem cell application (pig nos. 4-6), based on their identification numbers. All animals received an acclimation period of 3 days prior to any procedures, during which and no premedication was administered. Animals were anesthetized following an overnight fast (water was not withheld) based on their body weight using ketamine (20 mg/kg), xylazine (2 mg/kg) and atropine (1 mg/pig). Propofol was also administered as a bolus (1 mg/kg) prior to intubation to induce muscle relaxation. General anesthesia was maintained during procedures with a constant infusion drip of propofol. Fentanyl (100 μg/pig) was administered at the beginning of each procedure to potentiate anesthesia and as an analgesic, and all animals received mechanical ventilation support throughout the procedures. At pre-determined time-points the animals were euthanized with pentobarbital solution (140 mg/kg), and livers were harvested for histological and whole transcriptome analysis. Pigs were necropsied and examined for abnormal findings, and were labeled with the animal identification number, protocol number and date of collection.

ALPPS first phase. Pigs were anaesthetized as aforementioned. Laparotomy and investigation of the abdominal organs was performed, and revision of the liver was conducted, with the preparation of the liver hilus, identification of the portal vein and its branching, identification of the bile duct and hepatic arteries. Confirmation of the injection site was performed by venography using contrast medium (iopromidum) and C-arm fluoroscopy. The entry of hepatic veins into vena cava inferior was identified. The flow of portal blood into four lobes of the liver was interrupted; only the inflow of portal blood into the one selected hepatic lobe (future liver remnant) was preserved. This procedure was followed by splitting of liver between the lobe with preserved perfusion through the portal vein and other lobes, to which the inflow of portal blood was closed. Samples of liver tissue were harvested from the future liver remnant lobes and were stored snap-frozen using liquid nitrogen (-196°C) in a tissue bank. Furthermore, 15 ml of the human adipose stem cells-stromal vascular fraction concentrate (Cytori Therapeutics, Inc., San Diego, CA, USA) was administered intra-arterially to the group of animals with planned administration of stem cells via the hepatic artery during the surgery procedure. For more information about characteristics of this concentrate see a previous study by Lin et al (16). The animals in the group that did not undergo stem cell application were administered 15 ml of saline via an identical route of administration. Hydrocortisone was applied intravenously prior to the administration of stem cells to prevent an autoimmune reaction (rejection). The animals were monitored postoperatively by measuring body temperature (per rectum) and weight daily.

ALPPS second phase. Surgery was performed 9 days after the first stage. Re-laparotomy and investigation of abdominal organs were performed, together with liver revision and identification of pre-marked structures in the hilus and entry of hepatic veins into the vena cava inferior. In total, four liver lobes were removed with the perfused lobe remaining in place.

Tissue sampling, RNA isolation and whole transcriptome sequencing. All samples of liver tissue were collected into separate 5 ml polypropylene tubes prefilled with equivalent volume of RNA later solution and stored at -20°C. Isolation of total RNA was performed using the QuickGene Mini 80 semi-automatic device and appropriate RNA tissue kit SII (both from Kurabo Industries Ltd., Osaka, Japan). RNA concentration and integrity were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). RNA-sequencing libraries preparation and cDNA sequencing was performed by Macrogen, Inc. (Seoul, Republic of Korea), resulting in a set of 101 nucleotide paired-end-read data files.

Transcriptome data analysis. The quality of the raw sequencing data was assessed using FastQC (v0.11.5) (17) and aligned to a reference genome of Sus scrofa (Ensembl v82; Sus scrofa 10.2) using the STAR aligner (v2.4.1b) (18). Up to five mapping reads were used for subsequent analyses. Raw gene counts were obtained by calculating reads mapping to exons and summarized by genes using reference gene annotation (Ensembl v.82, Sus scrofa assembly, GTF) by featureCounts (v1.4.6-p5) (19). Differential gene expression was
calculated using edgeR (v3.10.5) (20). Two states (day 0 and 9) within each experimental group of animals were compared. False discovery rate (FDR) correction was used to correct the P-values for multiple assessments. Genes were determined as differentially expressed when the FDR adjusted P‑value ≤0.1 and log_{2} fold-change (log_{2} FC) ≥0.5. Pathway analyses were performed in STRING (v10.5) (21,22), Panther (23) with the aid of Kyoto Encyclopedia of Genes and Genomes (KEGG) (24,25).

**Volumetric measurements.** All magnetic resonance imaging (MRI) experiments were performed using a 1.5 T scanner (GE Healthcare, Chicago, IL, USA), and an eight-channel phased array head coil was used. MRI measurements were performed at baseline (day 0) and on day 9, prior to second ALPPS stage.

**Statistical analysis.** The non-parametric paired Wilcoxon test was used for statistical comparison of changes in liver volume between day 0 and 9. According to the experimental design, this comparison was performed separately for the group that did not receive the application of stem cells and for the group that did. Software R was used for statistical analysis (version 3.4.1; The R Foundation for Statistical Computing, Wien, Austria). P<0.05 was considered to indicate a statistically significant difference. Data in the barplots are presented as the mean ± standard deviation.

**Results**

Although each step of the ALPPS procedure was performed successfully, no significant changes in total liver volume were observed following the first ALPPS stage (Fig. 1) (P=0.5 without application of stem cells; P=0.75 with application of stem cells). This may be due to the fact that only future liver remnants are expected to increase in size over a longer period of time.

Comprehensive transcriptome analysis of samples from future liver remnant was performed to examine for changes in the gene expression between groups with and without application of stem cells. We hypothesized that the application of stem cells would accelerate liver regeneration by inhibition of undesirable processes, such as fibrosis and inflammation.

A total of 39 significantly differentially expressed genes were identified in the group without application of stem cells between day 0 and 9 (Table I), there of 37 genes were upregulated and two downregulated. In the group with stem cell treatment there were no differentially expressed genes between day 0 and 9. The highest significantly different gene expression was observed for collagen type IV α1 chain (COL4A1). COL4A1, COL4A2, laminin subunit γ1 and nidogen 2 (all of which were upregulated; Fig. 2) form major components of the basement membrane (with COL4A1 and COL4A2 constituting a functional heterotrimer with 2:1 stoichiometry) (26,27). The greatest positive change (upregulation) in the gene expression was for latent transforming growth factor-β binding protein 2 (LTBP2) and the greatest negative change (downregulation) was for heme binding protein 2. LTBP2 together with thrombospondin 1, transglutaminase 2 and fibrillin 1, all of which were upregulated (detailed changes in gene expression are depicted in Fig. 3) and serve an important role in the transforming growth factor-β pathway in the extracellular matrix remodeling process (28).

Functional classification revealed that the majority of differentially expressed genes from the group of pigs that received the application of stem cells are associated with their functional interactions and localization (primarily in the extracellular matrix and cytoplasmic membrane); Fig. 4 contains a detailed interactome, with mainly collagens making up a strong interaction network. Analysis of molecular functions revealed 19 significantly enrichment categories, as ‘growth factor binding’, ‘extracellular matrix structural constituent’ or ‘semaphorin receptor activity’ (Table II). This is in congruence with a previous study by Rychtrmoc et al (29), where they observed changes in expression in a number of genes
involved in extracellular matrix remodeling pathways in liver regeneration termination using microarray and reverse transcription-quantitative polymerase chain reaction analysis in a rat model (29). At the level of biological processes the most relevant significantly enriched categories were 'anatomical structure morphogenesis', 'circulatory system development' and 'axon development' (Table III). The most enriched KEGG pathways were 'PI3K-Akt signaling pathway',

| Identifier | Symbol | Gene name | log₂ FC | FDR |
|------------|--------|-----------|---------|-----|
| ENSSSCG00000009544 | COL4A1 | Collagen, type IV, α1 | 0.93 | 0.00 |
| ENSSSCG00000007000 | FAT1 | FAT tumor suppressor homolog 1 (Drosophila) | 0.78 | 0.01 |
| ENSSSCG0000000712 | VWF | Von Willebrand factor | 1.15 | 0.01 |
| ENSSSCG00000023522 | TGM2 | Transglutaminase 2 | 0.75 | 0.01 |
| ENSSSCG0000004658 | FBN1 | Fibrillin 1 | 0.94 | 0.01 |
| ENSSSCG00000011859 | HEG1 | HEG homolog 1 (zebrafish) | 0.95 | 0.01 |
| ENSSSCG0000001725 | GPR116 | G protein-coupled receptor 116 | 0.69 | 0.01 |
| ENSSSCG0000009545 | COL4A2 | Collagen, type IV, α2 | 0.84 | 0.01 |
| ENSSSCG0000014442 | PDGFRB | Platelet-derived growth factor receptor, β-polypeptide | 0.82 | 0.01 |
| ENSSSCG0000028022 | COL6A2 | Collagen, type VI, α2 | 0.74 | 0.02 |
| ENSSSCG0000002368 | LTB2 | Latent transforming growth factor-β binding protein 2 | 1.64 | 0.02 |
| ENSSSCG0000004150 | HEBP2 | Heme binding protein 2 | -1.09 | 0.02 |
| ENSSSCG0000008749 | SLIT2 | Slit homolog 2 (Drosophila) | 1.28 | 0.02 |
| ENSSSCG0000011443 | STAB1 | Stabilin 1 | 0.84 | 0.02 |
| ENSSSCG0000005751 | COL5A1 | Collagen, type V, α1 | 0.76 | 0.03 |
| ENSSSCG0000009045 | HHIP | Hedgehog interacting protein | 0.71 | 0.03 |
| ENSSSCG0000004387 | FOXD3 | Forkhead box O3 | 0.65 | 0.04 |
| ENSSSCG000001834 | MAGE8 | Milk fat globule-EGF factor 8 protein | 0.74 | 0.04 |
| ENSSSCG0000027969 | AHNAK | AHNAK nucleoprotein | 0.91 | 0.04 |
| ENSSSCG0000009320 | FLT1 | Fms-related tyrosine kinase 1 | 0.85 | 0.04 |
| ENSSSCG000004091 | AKAP12 | A kinase (PRKA) anchor protein 12 | 0.81 | 0.04 |
| ENSSSCG0000028239 | FBXL7 | F-box and leucine-rich repeat protein 7 | 1.10 | 0.04 |
| ENSSSCG0000011075 | KIAA1217 | KIAA1217 | 0.61 | 0.04 |
| ENSSSCG0000022000 | COL1A2 | Collagen, type I, α2 | 0.80 | 0.05 |
| ENSSSCG0000029189 | DCHS1 | Dachshous 1 (Drosophila) | 0.91 | 0.05 |
| ENSSSCG0000017548 | NGFR | Nerve growth factor receptor | 0.79 | 0.05 |
| ENSSSCG0000009111 | SYNPO2 | Synaptopodin 2 | 0.91 | 0.06 |
| ENSSSCG0000015068 | APOA4 | Apolipoprotein A-IV | -0.62 | 0.06 |
| ENSSSCG0000015555 | LAMC1 | Laminin, γ1 | 0.74 | 0.07 |
| ENSSSCG0000005030 | NID2 | Nidogen 2 (osteonidogen) | 0.68 | 0.07 |
| ENSSSCG0000011102 | NRP1 | Neuropilin 1 | 0.55 | 0.08 |
| ENSSSCG0000026383 | NRP2 | Neuropilin 2 | 0.78 | 0.08 |
| ENSSSCG0000015326 | COL1A2 | Collagen, type I, α2 | 0.78 | 0.09 |
| ENSSSCG0000027331 | COL6A3 | Collagen, type VI, α3 | 0.71 | 0.09 |
| ENSSSCG0000011743 | MECOM | MDS1 and EVI1 complex locus | 1.28 | 0.09 |
| ENSSSCG0000005494 | TNC | Tenascin C | 1.40 | 0.10 |
| ENSSSCG0000015426 | RELN | Reelin | 0.61 | 0.10 |
| ENSSSCG0000016035 | COL5A2 | Collagen, type V, α2 | 0.67 | 0.10 |
| ENSSSCG0000004789 | THBS1 | Thrombospondin 1 | 1.10 | 0.10 |

Log₂FC >|0.5| and FDR <0.1 were chosen as a threshold. log₂FC, log₂ fold change; FDR, false discovery rate.
Table II. Molecular function enrichment in the group without application of stem cells between day 0 and 9, sorted by FDR value.

| Pathway ID     | Pathway description                                           | Observed gene count | FDR          | Matching proteins                                                                 |
|----------------|---------------------------------------------------------------|---------------------|--------------|-----------------------------------------------------------------------------------|
| GO.0019838     | Growth factor binding                                         | 8                   | 5.25x10^-9   | COL1A2, COL4A1, COL5A1, FLT1, NRP1, NRP2, DGFRB, THBS1                             |
| GO.0048407     | Platelet-derived growth factor binding                       | 4                   | 4.28x10^-6   | COL1A2, COL4A1, COL5A1, PDGFRB                                                   |
| GO.0005539     | Glycosaminoglycan binding                                    | 7                   | 3.18x10^-5   | COL5A1, LTBP2, NRP1, NRP2, SLIT2, STAB1, THBS1                                   |
| GO.0005201     | Extracellular matrix structural constituent                   | 5                   | 6.82x10^-5   | COL1A2, COL4A1, COL4A2, COL5A1, FBN1                                            |
| GO.0097493     | Structural molecule activity conferring elasticity            | 3                   | 6.88x10^-5   | AHNAK, COL4A1, FBN1                                                              |
| GO.0005021     | Vascular endothelial growth factor-activated receptor activity| 3                   | 8.59x10^-5   | COL5A1, LTBP2, NRP1, NRP2, SLIT2, THBS1                                          |
| GO.0008201     | Heparin binding                                              | 6                   | 8.59x10^-5   | AHNAK, AKAP12, APOA4, COL1A2, COL4A1, COL5A1, FBN1, FLT1, FOXO3, HHIP, MECOM, NGFR, NID2, NRP1, NRP2, PDGFRB, RELN, SLIT2, SYNPO2, THBS1, TNC |
| GO.0005515     | Protein binding                                              | 21                  | 0.000139     | AHNAK, AKAP12, APOA4, COL1A2, COL4A1, COL5A1, FBN1, FLT1, FOXO3, HHIP, MECOM, NGFR, NID2, NRP1, NRP2, PDGFRB, RELN, SLIT2, SYNPO2, THBS1, TNC |
| GO.0005509     | Calcium ion binding                                          | 9                   | 0.00039      | DCHS1, FAT1, FBN1, HEG1, LTBP2, MECOM, NID2, SLIT2, THBS1                        |
| GO.0043394     | Proteoglycan binding                                         | 3                   | 0.000866     | COL5A1, LTBP2, THBS1                                                            |
| GO.0004714     | Transmembrane receptor protein tyrosine kinase activity      | 4                   | 0.0106       | FLT1, NRP1, NRP2, PDGFRB                                                        |
| GO.0030023     | Extracellular matrix constituent conferring elasticity        | 2                   | 0.0044       | COL4A1, FBN1                                                                    |
| GO.0038085     | Vascular endothelial growth factor binding                   | 2                   | 0.0044       | NRP1, PDGFRB                                                                    |
| GO.0046872     | Metal ion binding                                            | 17                  | 0.0117       | APOA4, COL1A2, COL5A1, DCHS1, FAT1, FBN1, HEG1, HHIP, LTBP2, MECOM, NID2, NRP1, NRP2, RELN, SLIT2, TGMB, THBS1 |
| GO.0017154     | Semaphorin receptor activity                                 | 2                   | 0.0259       | NRP1, NRP2                                                                      |
| GO.0019955     | Cytokine binding                                             | 3                   | 0.0335       | NRP1, NRP2, THBS1                                                               |
| GO.0005178     | Integrin binding                                             | 3                   | 0.0461       | COL5A1, FBN1, THBS1                                                             |
| GO.0005198     | Structural molecule activity                                 | 6                   | 0.0461       | AHNAK, COL1A2, COL4A1, COL4A2, COL5A1, FBN1                                     |
| GO.0030169     | Low-density lipoprotein particle binding                     | 2                   | 0.0476       | STAB1, THBS1                                                                    |

FDR=0.05 was chosen as a threshold. FDR, false discovery rate.
Table III. Biological process enrichment in the group without application of stem cells between day 0 and 9, sorted by lowest FDR value.

| Pathway ID     | Pathway description                                      | Observed gene count | FDR         | Matching proteins                                                                 |
|----------------|----------------------------------------------------------|---------------------|-------------|-----------------------------------------------------------------------------------|
| GO.0009653     | Anatomical structure morphogenesis                        | 21                  | 7.36x10^{-10} | COL1A2, COL4A1, COL4A2, COL6A2, COL6A3, DCHS1, FAT1, FBN1, FLT1, FOXO3, HEG1, HHIP, MECOM, NGFR, NRP1, NRP2, PDGFRB, SLIT2, TGM2, THBS1, TNC |
| GO.0072358     | Cardiovascular system development                         | 14                  | 1.8x10^{-8}  | COL1A2, COL4A1, COL4A2, COL5A1, DCHS1, FBN1, FLT1, HEG1, MECOM, NRP1, NRP2, PDGFRB, SLIT2, THBS1 |
| GO.0072359     | Circulatory system development                            | 14                  | 1.8x10^{-8}  | COL1A2, COL4A1, COL4A2, COL5A1, DCHS1, FBN1, FLT1, HEG1, MECOM, NRP1, NRP2, PDGFRB, SLIT2, THBS1 |
| GO.0044243     | Multicellular organismal catabolic process                | 7                   | 1.38x10^{-7} | COL1A2, COL4A1, COL4A2, COL5A1, COL6A2, COL6A3                                    |
| GO.0001568     | Blood vessel development                                  | 11                  | 1.51x10^{-7} | COL1A2, COL4A1, COL4A2, COL5A1, FLT1, HEG1, NRP1, NRP2, PDGFRB, SLIT2, THBS1      |
| GO.0001944     | Vasculature development                                   | 11                  | 1.58x10^{-7} | COL1A2, COL4A1, COL4A2, COL5A1, FLT1, HEG1, NRP1, NRP2, PDGFRB, SLIT2, THBS1      |
| GO.0006935     | Chemotaxis                                               | 10                  | 1.72x10^{-7} | COL1A2, COL4A1, COL4A2, COL5A1, COL6A2, COL6A3, FBN1, NID2, THBS1, TNC            |
| GO.0030198     | Extracellular matrix organization                         | 10                  | 1.72x10^{-7} | COL1A2, COL4A1, COL4A2, COL5A1, COL6A2, COL6A3, FBN1, NID2, THBS1, TNC            |
| GO.0061564     | Axon development                                          | 11                  | 2.00x10^{-7} | COL1A1, COL4A1, COL4A2, COL5A1, COL6A2, COL6A3, NGFR, NRP1, NRP2, RELN, SLIT2, TNC |
| GO.0007411     | Axon guidance                                             | 10                  | 3.15x10^{-7} | COL1A1, COL4A1, COL4A2, COL5A1, COL6A2, COL6A3, NGFR, NRP1, NRP2, RELN, SLIT2, TNC |
| GO.0022617     | Extracellular matrix disassembly                          | 7                   | 5.34x10^{-7} | COL1A1, COL4A1, COL4A2, COL5A1, COL6A2, COL6A3, NGFR, NRP1, NRP2, RELN, SLIT2, TNC |
| GO.0040011     | Locomotion                                               | 14                  | 6.1x10^{-7}  | COL1A1, COL4A1, COL4A2, COL5A1, COL6A2, COL6A3, FAT1, FLT1, NGFR, NRP1, NRP2, PDGFRB, SLIT2, THBS1 |
| GO.0048666     | Neuron development                                        | 12                  | 1.04x10^{-6} | COL1A1, COL4A1, COL4A2, COL5A1, COL6A2, COL6A3, MECOM, NGFR, NRP1, NRP2, RELN, SLIT2, TNC |
| GO.0035704     | Collagen catabolic process                                | 6                   | 1.23x10^{-6} | COL1A2, COL4A1, COL4A2, COL5A1, COL6A2, COL6A3                                    |
| GO.0071386     | Cellular response to growth factor stimulus               | 11                  | 1.3x10^{-6}  | COL1A2, COL4A2, FBN1, FLT1, FOXO3, LTBP2, MECOM, NGFR, NRP1, NRP2, PDGFRB          |
| GO.0000904     | Cell morphogenesis involved in differentiation            | 11                  | 1.57x10^{-6} | COL1A1, COL4A2, COL5A1, COL6A2, COL6A3, HEG1, NGFR, NRP1, NRP2, RELN, SLIT2, TNC |
| GO.0007409     | Axonogenesis                                              | 10                  | 1.57x10^{-6} | COL1A1, COL4A2, COL5A1, COL6A2, COL6A3, NGFR, NRP1, NRP2, RELN, SLIT2, TNC      |
| GO.003175      | Neuron projection development                             | 11                  | 1.57x10^{-6} | COL1A2, COL4A1, COL4A2, COL5A1, COL6A2, COL6A3, NGFR, NRP1, NRP2, RELN, SLIT2, TNC |
| GO.0006902     | Movement of cell or subcellular component                 | 14                  | 1.58x10^{-6} | COL1A1, COL4A1, COL4A2, COL5A1, COL6A2, COL6A3, FAT1, FLT1, NGFR, NRP1, NRP2, RELN, SLIT2, TNC |
| GO.0048468     | Cell development                                         | 15                  | 1.79x10^{-6} | APOA4, COL4A1, COL4A2, COL5A1, COL6A2, COL6A3, FOXO3, HEG1, MECOM, NGFR, NRP1, NRP2, PDGFRB, SLIT2, TNC |

The 20 best hits are shown. FDR, false discovery rate.
'Focal adhesion' and 'ECM-receptor interaction' (Table IV). The phosphoinositide 3-kinase (PI3K)-RAC serine/threonine-protein kinase (Akt) signaling pathway is likely to drive forward liver regeneration via hepatocyte growth factor stimulation, as observed on rat oval cells in vitro (30). Inhibition of the PI3K-Akt pathway disturbed liver regeneration in mice (31).

A more detailed examination of gene expression in specific pigs between day 0 and 9 revealed certain notable facts (only values with a log2 FC ≥ 3 with >4 normalized edgeR counts were taken into account). Only certain genes in pig nos. 4 and 6 (that received stem cell treatment) met these more stringent criteria (Table V). In pig no. 6, there was an extremely large increase in the expression of the RNA component of RNase P and 7S kinase (7SK) RNA. According to Reiner et al (32), RNase P may serve an important role in transcription of a number of non-coding RNAs that are transcribed by RNA polymerase III. 7SK RNA is one of the genes transcribed by RNA polymerase III. It is therefore likely that in pig no. 6 there was co-expression of these two genes, which are localized on the same chromosome (RNase P RNA component, chromosome 7:83, 579, 873-83, 580, 200 forward strand; 7SK RNA, chromosome 7:134, 400, 749-134, 401, 079 forward strand). There were also three overexpressed genes for Metazoan
signal recognition particle RNA (also transcribed by RNA polymerase III). Interleukin-13 receptor subunit α2 was also downregulated in pig no. 6. However, these results for individual pigs cannot conclusively inform on the mode of action of the applied stem cells, but serve as a source of hypotheses for subsequent studies.

**Discussion**

Although the liver has the ability to regenerate itself, the application of stem cells speeds up the process; this has been demonstrated in the present study via the presence of fewer differentially expressed genes in the presence of stem cells, indicating that the regeneration process is finished or is in the late phase. Timing is crucial in the ALPPS procedure, so faster liver regeneration between stages is highly beneficial. According to the experimental design, no significant changes to liver morphology were expected; as 9 days is too short a period to observe liver fibrosis (33-37), gene expression analyses were performed, which reliably identify expression changes in collagen and other fibrogenic factors before they become visible via microscopy. Previous animal studies demonstrated that microscopic changes to liver structure following intervention were not observed for several weeks (33-37).
Differentially expressed genes in the group of pigs that did not receive stem cell application (between day 0 and 9) encode proteins primarily involved in extracellular matrix remodeling, angiogenic and neurogenic processes. Owing to the fact that in the group that underwent the application of stem cells, there were no differentially expressed genes between day 0 and 9, the application of stem cells seemingly positively modulated the regenerative processes by accelerating regeneration, and preventing an unwanted fibrosis and inflammation processes. To provide more precise interpretation a larger number of biological replicates and more time-points are required (ideally on day 0, 3, 5, 7, 9, 11 and 20 to observe upward/downward trends in gene expression in broader time scale), although in a large animal model, such an approach is limited by financial costs.

Angiogenesis is a process that accompanies liver regeneration process and serves an important role in restoration of vascular networks in the place of liver damage. This process is driven by several pro-angiogenic growth factors. A number of the primary pro-angiogenic factors are vascular endothelial growth factors that bind to their membrane receptors, including Fms-related tyrosine kinase-1 (Flt-1), fetal liver kinase-1 or Flt-4. The present study observed the increased expression of Flt-1 receptor in the group without application of stem cells between day 0 and 9, which is in congruence of former study in a rat model, in which expression of Flt-1 was significantly increased between day 4 and 10 following 70% hepatectomy (38).

The process of axon guidance in liver regeneration may be mediated by secreted third class semaphorins (Sema3A-G), which bind to a membrane receptor complex whose main component is a transmembrane glycoprotein neuropillin 1 or neuropillin 2, or a heterodimer of the two (39). The interaction between the semaphorins 3A and neuropillin 1 is also notable in the angiogenic processes (40). The present study revealed increased expression of neuropillin 1 and neuropillin 2 in the group without application of stem cells between day 0 and 9.

The remodeling of extracellular matrix serves an important role in the process of liver regeneration. In the initiation stage of liver regeneration, the extracellular matrix is broken down to allow for the proliferation of hepatocytes. Subsequently, the extracellular matrix requires rebuilding to ensure physical support is provided to endothelial cells. Production of extracellular matrix is primarily provided by the population of stellar liver cells. Restoration of the extracellular matrix is manifested by an increased synthesis of collagen, structural glycoproteins and proteoglycans, which occurs mainly between day 3 and 5 following partial hepatectomy in a rat
The present study observed an elevated expression of a number of genes associated with extracellular matrix remodeling between day 0 and day 9 in the group without application of stem cells. The application of stem cells in pig no. 6 (that received the application of stem cells) likely decreased the expression of interleukin 13 receptor subunit α2 (IL13RA2). Functional IL13RA2 was overexpressed in activated hepatic stellate cells in rat livers (42). Activated hepatic stellate cells are associated with unwanted liver fibrosis (43). The anti-fibrotic effect of xenogeneic adipose mesenchymal stem cells was recently observed by Maria et al (44), whereby a mouse model of systemic sclerosis was used. It would be necessary to use more biological replicates than in the present study to determine more accurately the number of pigs in which this effect occurred.

In pig no. 6, rapid co-expression of RNAseP and 7SK functional RNAs (>16 times higher expression) was observed. It would be interesting to examine this observation in similar experiments in the future. However, owing to the limited number of biological replicates, clear interpretation cannot be performed. It is possible, that RNAseP may serve as a major inductor of 7SK RNA expression, as, according to Reiner et al (32), RNAseP activates the transcription of RNA polymerase III.

The change in gene expression in pig no. 4 that underwent application of stem cells likely demonstrates the termination of proliferative processes, characterized by the downregulation of Mdm4 p53 binding protein homolog (mouse) and LATS large tumor suppressor homolog 1 (Drosophila) and thereby stabilization of the p53 suppressor protein. This also reflects the decreased expression of other transcription factors, including one cut homeobox 1 (ONECUT1) or heart development protein with EGF like domains 1. The overexpression of ONECUT1 was observed in early stages of liver regeneration in a rat model (45). SH3 and PX domains 2A is apparently involved in the production of free radicals as a member of the NADPH oxidase complex complex (46). This finding indicates that the proliferative processes in pig no. 4 were accelerated owing to the application of stem cells and similarly, the formation of undesirable free radicals was limited.

RNA sequencing studies aided the evaluation of gene expression in animal models of variety human clinical conditions, including in the study by Arvaniti et al (47), which revealed numerous previously unknown genes associated with renal fibrosis using a mouse model (47). Although the present study encountered limitations including the mortality of one pig due to source contamination and also the corruption of one sequencing data file. These limitations resulted in decreased animal numbers; however, the results obtained may provide insight and could be validated by future studies that build on these findings. Certain differentially expressed genes identified in the present study may serve as molecular markers for monitoring the progress of liver regeneration generally, not only during ALPPS, in human patients. Analysis of differentially expressed genes indicates that the application of stem cells elicited a positive effect in the acceleration of regenerative processes; however, there is a requirement for further experiments to be conducted with more biological replicates and tissue sampling time-points.

### Table IV. Kyoto Encyclopedia of Genes and Genomes pathway enrichment in the group without application of stem cells between day 0 and 9, sorted by lowest FDR value.

| Pathway ID | Observed gene count | Pathway description | FDR |
|------------|---------------------|---------------------|-----|
| 4151       | 13                  | PI3K-Akt signaling pathway | 9.81x10^{-13} |
| 4510       | 11                  | Focal adhesion       | 1.49x10^{-12} |
| 4512       | 9                   | ECM-receptor interaction | 3.52x10^{-7} |
| 4974       | 4                   | Protein digestion and absorption | 0.00149 |
| 5146       | 4                   | Apoptosis            | 0.00872 |
| 5200       | 4                   | Pathways in cancer   | 0.0144 |

FDR=0.05 was chosen as a threshold. FDR, false discovery rate.
Table V. Differentially expressed genes in pig nos. 4 and 6 (that received stem cell treatment) between the day 0 and 9, sorted by highest Log_{2}FC value.

| Identifier               | Symbol         | Gene name                                      | log_{2}FC |
|--------------------------|----------------|-----------------------------------------------|-----------|
| ENSSSCG00000019556       | 7SK            | 7SK RNA                                       | 4.11      |
| ENSSSCG00000020439       | RNaseP_nuc     | Nuclear RNase P                               | 4.02      |
| ENSSSCG00000024699       | Metazoa_SRPa   | Metazoan signal recognition particle RNA       | 3.48      |
| ENSSSCG00000029839       | Metazoa_SRPa   | Metazoan signal recognition particle RNA       | 3.47      |
| ENSSSCG00000029605       | Metazoa_SRPa   | Metazoan signal recognition particle RNA       | 3.06      |
| ENSSSCG00000012594       | IL13RA2        | Interleukin 13 receptor subunit α2            | -3.09     |
| ENSSSCG00000029023       | ARL5B          | ADP-ribosylation factor-like 5B               | -5.43     |
| ENSSSCG00000008595       | APOB           | Apolipoprotein B                              | -3.73     |
| ENSSSCG00000002387       | GAPATCH2L      | G patch domain containing 2-like              | -3.59     |
| ENSSSCG00000030247       | EPM2AIP1       | EPM2A (laforin) interacting protein 1         | -3.57     |
| ENSSSCG00000024674       | ABL2           | v-abl Abelson murine leukemia viral oncogene homolog2 | -3.48     |
| ENSSSCG00000030726       | CH242-150C11.4 | CH242-150C11.4                               | -3.46     |
| ENSSSCG0000005466        | ROD1           | PTBP3-polypyrimidine tract binding protein 3  | -3.23     |
| ENSSSCG0000016510        | UBN2           | Ubinuclein 2                                  | -3.19     |
| ENSSSCG0000008909        | CLOCK          | Clock homolog (mouse)                        | -3.17     |
| ENSSSCG0000004616        | ONECUT1        | One cut homeobox 1                           | -3.12     |
| ENSSSCG0000015284        | MDM4           | Mdm4 p53 binding protein homolog (mouse)      | -3.10     |
| ENSSSCG0000008292        | TET3           | Tet methylcytosine dioxygenase 3              | -3.09     |
| ENSSSCG0000016119        | RAPH1          | Ras association (RafGDS/AF-6) and pleckstrin homology domains 1 | -3.09     |
| ENSSSCG0000004106        | LATS1          | LATS, large tumor suppressor, homolog 1 (Drosophila) | -3.08     |
| ENSSSCG0000010604        | SH3PXD2A       | SH3 and PX domains 2A                        | -3.07     |
| ENSSSCG0000025182        | ELK4           | ELK4, ETS-domain protein (SRF accessory protein 1) | -3.04     |
| ENSSSCG0000002755        | NFAT5          | Nuclear factor of activated T-cells 5, tonicity-responsive | -3.03     |
| ENSSSCG0000016031        | CRLR           | Calcitonin receptor-like                      | -3.02     |
| ENSSSCG0000005285        | GNAQ           | Guanine nucleotide binding protein (G protein), q polypeptide | -3.02     |

Only values of log_{2}FC higher than ±3 with more than four normalized edgeR counts were taken into account. Log_{2}FC, log_{2} fold change.
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Availability of data and materials

Preprocessed RNA sequencing datasets generated during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

MB, JC, MP and PP designed the study, MP, PV, PZ and VP performed the experiments with animals. MB and JC performed molecular biology experiments. MB, JO, VB and PP analysed the data. MB, JO, VB and PP wrote the text.

Ethics approval and consent to participate

Approval from the Bioethical Committee from the Center for Cardiovascular Research and Development, American Heart of Poland S.A. (Ustroń, Poland) was obtained.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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