Determination of optimized oxygen partial pressure to maximize the liver regenerative potential of the secretome obtained from adipose-derived stem cells

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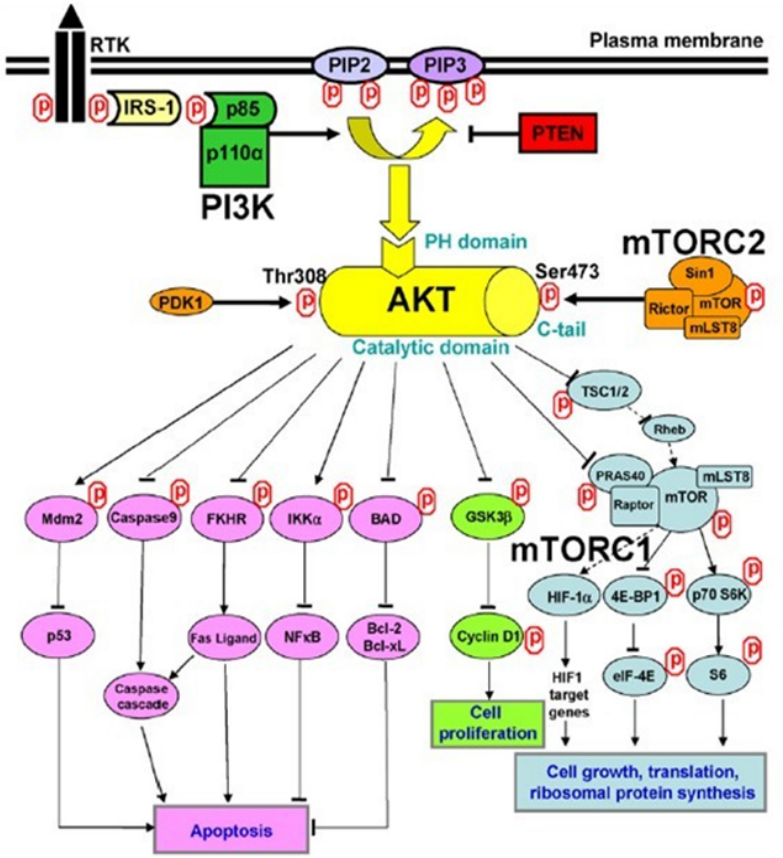
Liver regeneration

- Liver is the visceral organ with the capacity to regenerate
  - Regeneration after surgical removal or after chemical injury
- Liver regeneration involves the replication of liver cells, such as hepatocytes, biliary epithelial cells and sinusoidal endothelial cell
- Several signaling pathways are known to stimulate regeneraton in the liver:
  - Cytokines (TNF-α or IL-6)
  - Growth factors (VEGF, HGF)
  - PI3K pathway
  - IL-6/Sat 3 pathway

Liver regeneration

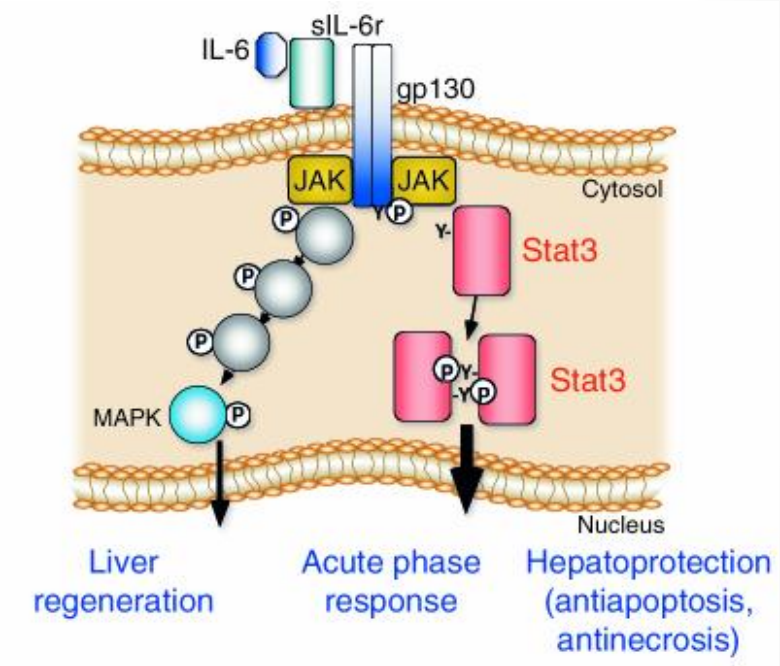
PI3K/Akt pathway



Workman P et al. Curr Opin Pharmacol. 2008 Aug;8(4):393-412

https://pharmaceuticalintelligence.com/tag/pi3kakt-pathway/
cited 06.01.2018

IL-6/Stat 3 pathway



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC198534/
cited 06.01.2018

Hypothesis

A hypoxic preconditioned secretome from stem cells shows a more effective functional and regenerative capacity of the liver

→ Determination of the optimal oxygen concentration

Hypoxic preconditioning is known to acitivate a number of signaling pathways prerequisite for survival, proliferation and release of proinflammatory cytokines, such as the phosphytidylinositol-3,4-5-triphosphate (PIP3)/Akt, p38 mitogen-activated protein kinase (p38MAPK) and ERK pathways

Methods

Cell culture

- AML12 mouse hepatocyte cell line (obtained from American Type Culture Collection and maintained in Dulbecco‘s Modified Eagle Medium/Ham‘s F-12) was supplemented with 10% fetal bovine serum, 1% antibiotics, 1x ITS supplement and 40 ng/ml dexamethasone at 37 °C.
- TCMK-1 (purchased from korean cell line bank)
- TCMK-1 and HK-2 cells were maintained in DMEM (Thermo)
- The medium was supplemented with 10% fetal bovine serum and 1% antibiotics at 37°C
- Adipose tissue derived stem cells (ASCs) were cultured in MesenPro RS basal medium and supplemented with antibiotics at 37°C (Antibiotic-Antimycotic-Invitrogen)
- ASCs expressed the MSC marker (CD 90) and did not express hematopoetic markers (CD31 and CD34)

Methods

Establishment of ischemia-reperfusion injury in cell culture

- AML12, TCMK-1 and HK2 cell lines were incubated in a Krebs-Henseleit buffer with 10 µM antimycin A and 1nM 2-deoxyglucose for 1h.
- Reperfusion was achieved by washing cells in Krebs—Henseleit buffer and incubation in complete growth medium (DMEM/F12) for 1h

Preparation of secretome obtained under the different cultural pO_2

ASCs were re-fed with serum-free low-glucose DMEM and cultured under either normoxic (21% pO_2) or hypoxic (10%, 5%, 1%) conditions for 24h

  - Usage of hypoxic chamber at 37°C. Concentration of CM 25-fold with ultrafiltration units with a 3-kDa cutoff.
  - Storage at -80°C
- Expression of signaling intermediates during HP (see results)

Methods

Cell proliferation assay

- Evaluation of cell proliferation with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium assay using the EZ-Cytox Cell Proliferation
- AML12 cells were cultured overnight (1x 10^4 cells per well) in 96-well plates. 96-well plates were washed with phosphate-buffered saline (PBS) twice and incubated with the secretome of various cultural pO_2. Reagent from the EZ-Cytox Cell Proliferation assay kit was applied to each well and absorbance was measured at 450 nm using a microplate reader

Quantitative real-time PCR

- Extraction of the total RNA of ASCs using Tri-RNA reagent. Performance of reverse transcription with 1 ug of RNA, random primes and M-MLV reverse transcriptase.
- The primers used for SYBR Green reverse-transcription qPCR were for the following parameters: IL-6, VEGF, hepatocyte growth factor (HGF), NAD-dependent deacetylase sirtuin-1 (SIRT1), GAPDH.
- Measurement + Calculation of the expression levels for each target gene by comparative threshold cycle method.

Methods

Western blotting analysis

- Lysis of AML12 cells and liver specimens of hepatectomized mice via EzRIPA Lysis kit and quantification by Bradford reagent. Proteins were visualized by western blot analysis with primary antibodies against **PCNA, p-STAT3, STAT3, HGF. VEGF, SIRT1, p-AKT, AKT, p-ERK, ERK, Mcl-1, Bax, HIF-1α, ß-actin.**
- **HRP-conjugated secondary antibodies** were used and specific immune complexes were detected using the Western Blotting Plus Chemiluminescence Reagent

Injection of secretome with differrent cultural pO2 into the partially hepatectomized mice

- Six-week-old male BALB/c mice were partially hepatectomized under tiletaminezolazepam sedation. The left lateral lobe (30% liver mass) and the whole median lobe (40% liver mass) were resected. → Total reduction of 70% liver mass.
- Infusion of the CM with various cultural pO_2 tensions. Therefore mice were divided into two subgroups according to the materials administered (saline, secretome with 21%,10%, 5%, 1%) with 25 mice per group. Further devision into 2 subgroups according to the manner of specimen collection.
- One subgroup (n=5 per group) was for obtaining continuous data (levels of serum transaminases, sera and liver specimens) after euthanization of mice on day 1,2,3,7. Estimation of liver regeneration by weighting excised livers. LW/BW = ratio of liver weight to body weight

Methods

Immunohistochemical analysis

- Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in alcohol. The antigen was retrieved with 0.01 M citrate buffer (pH 6.0).
- The tissue sections were placed in 3% hydrogen peroxide for 5 min to inactivate the endogenous peroxidase and blocked with normal horse serum for 30 min.
- Antibodies: **Ki-67 rabbit polyclonal antibody** (1:300), **VEGF & SIRT1 mouse polyclonal antibodies** (1:150), **cleaved caspase-3 rabbit polyclonal antibody** (1:300), **Bcl-xL mouse monoclonal antibodies** (1:100)
- Application of prediluted primary antibodies overnight at 4°C. Treatment with biotinylated secondary antibody for 30 min at room temperature followed by applying streptavidin-HRP and 3,3‘-diaminobenzidine solution for 10 min.
- Counterstaining with hematoxylin

Enzyme-linked immunosorbent assay

- Serum levels of IL-6 and TNF-α of partially hepatectomized mice were determined by an ELISA kit on day 7 after injection

Methods

Assessment of liver functions

- Blood samples were centrifuged for 10 min at 10000 rpm and serum was collected on day 1,2,3,7 after injection. Measurement of liver injury with AST and ALT

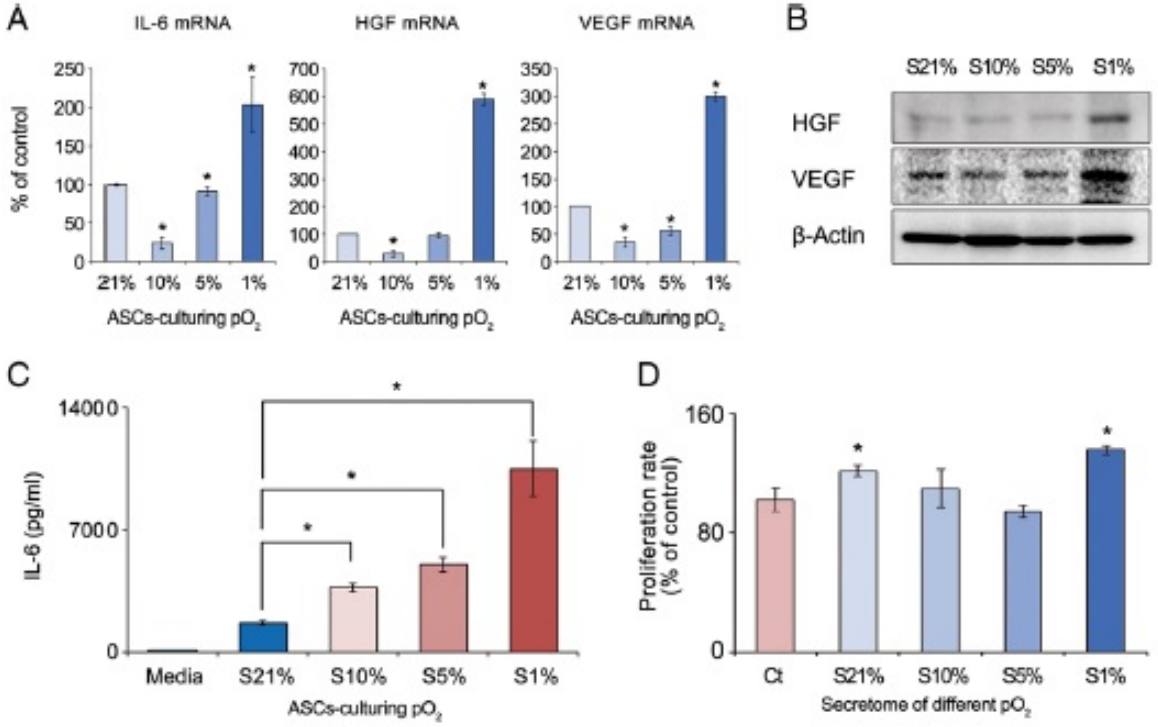
Statistical analysis

- Analysation with SPSS 11.0 software
- Mann-Whitney U test for mean comparison of two groups
- Kruskal-Wallis test for comparision of 3 or more groups
- $P<0.05$ is considered statistically significant

Results

Effects of various concentrations of culture pO_2 on the microenvironment of ASCs

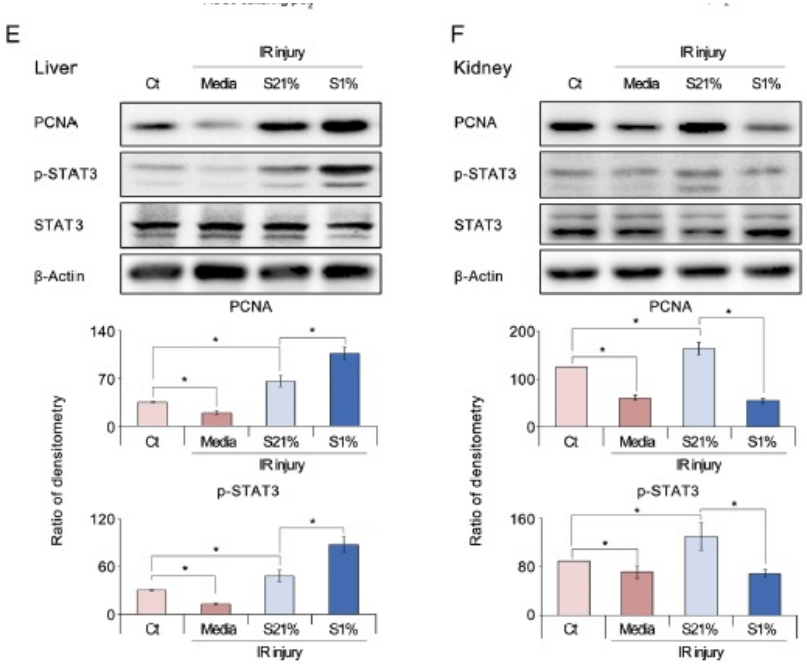
- mRNA expression of IL-6, HGF and VEGF was significantly higher in AML12 cells cultured under 1% pO_2 than under other pO_2
- Highest expression of HGF and VEGF in western blot analysis and highest concentration of IL-6 tested by ELISA under 1% pO_2
- Highest cell proliferation under 1% pO_2



Results

Effects of the secretome with culture 1% pO_2 on injured hepatocytes or renal cells

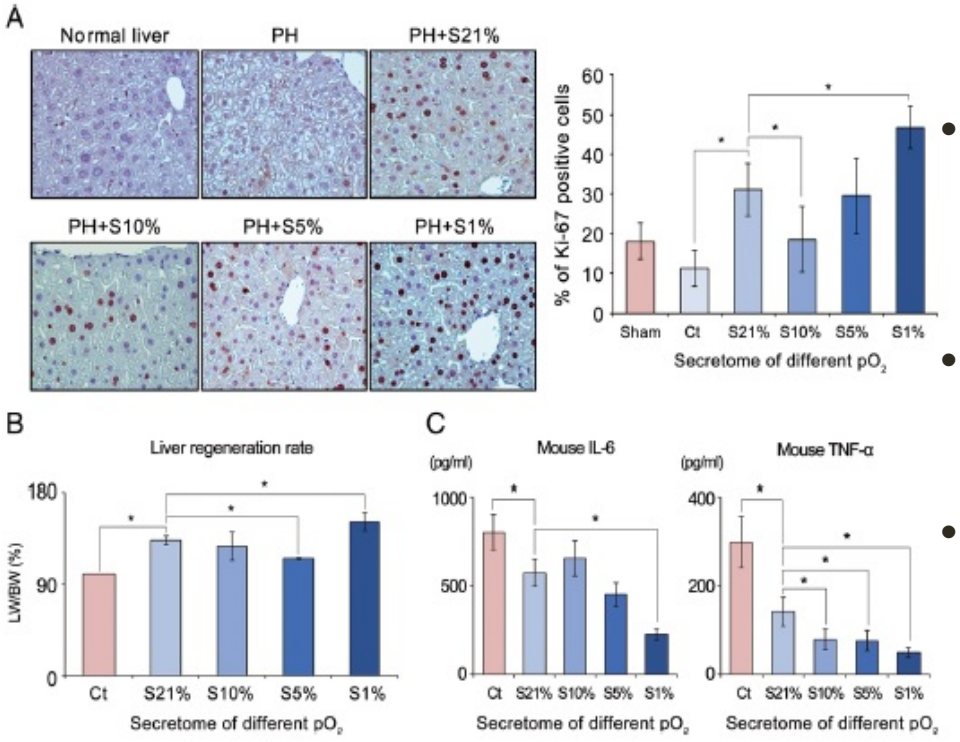
- Previously established in-vitro ischemia-reperfusion (IR)-injured AML12 hepatocytes or HK2 renal cells + secretome with culture 1% pO_2 were tested on its expression of proliferation markers (STAT3, PCNA)
- IR-injured AML12 hepatocytes supplemented with 1% pO_2 showed an increased expression of the markers
- IR-injured HK2 renal cells supplemented with 1% pO_2 had no significant increase, but a decrease of the expression of the markers



Results

Effects of the secretome with different concentrations of culture pO_2 on hepatic recovery in partially hepatectomized mice

- After 70% PH mice were injected with the secretome containing different concentrations of culture pO_2 . Determination of the effect of the secretome on liver regeneration using Ki67 immunohistochemistry [A] and liver weight measurement (LW/BW) [B]
- Effects of preconditioned secretome on reduction of systemic inflammation [C]

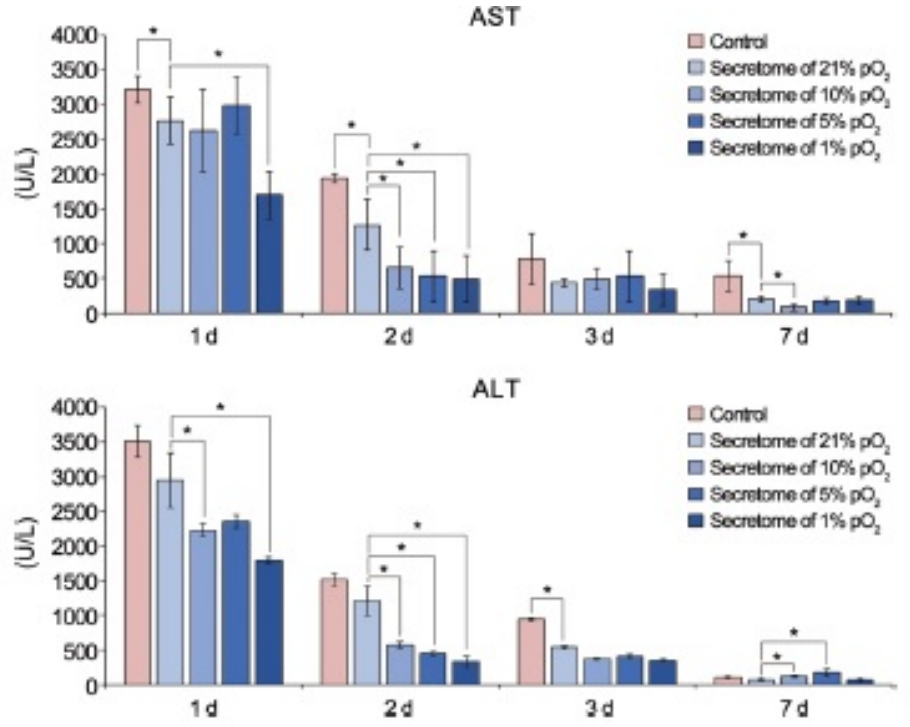


- Higher number of Ki67-positive cells in secretome injected groups, but highest in the 1% pO_2 group
- Hightest LW/BW in the secretome of 1% pO_2 followed by 21%, 10% and 5%
- Serum levels of IL-6 and TNF-a were significantly decreased in all secretome-injected groups, but mostly in 1% pO_2 group

Results

Effects of the secretome with different concentrations of culture pO_2 on hepatic function in partially hepatectomized mice

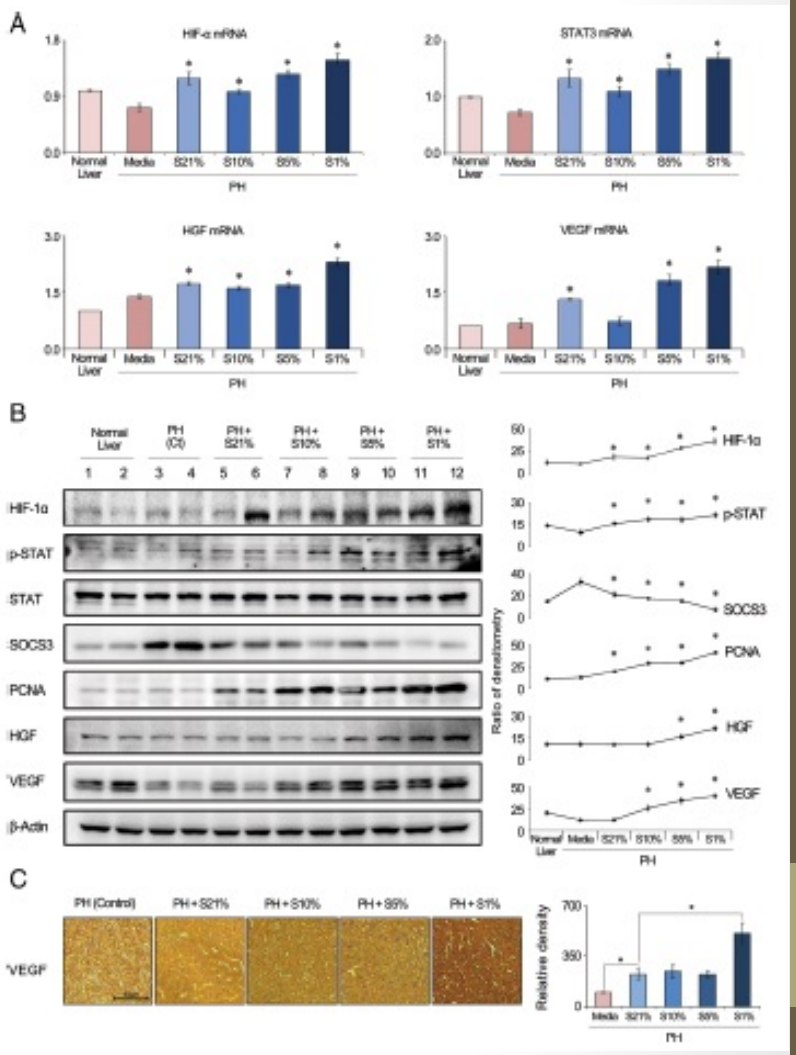
- Effects of the secretome with different pO_2 levels in serum levels AST and ALT on day 1,2,3,7 after injection
- Secretome-injected groups showed lower levels of AST and ALT with the group of pO_2 at 1% exhibiting the lowest levels of all



Results

Effects of the secretome with different concentrations of culture pO_2 on the signaling pathways essential for liver regeneration in the mouse liver

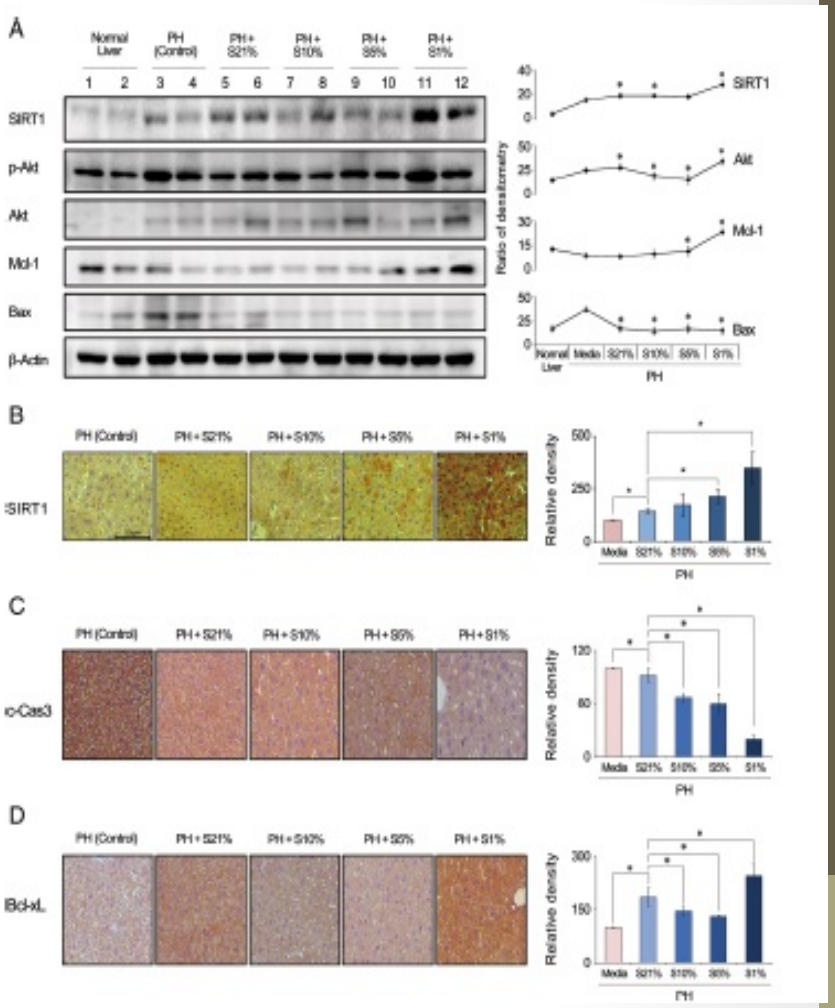
- Effects of the secretome with different pO_2 levels on the markers for cell proliferation in mouse liver on day 2 after injection
- RT-PCR: 1% pO_2 group showed highest expression of HIF-a, STAT3, HGF and VEGF
- Western blot analysis: 1% pO_2 group exhibited highest expression of the markers for liver cell proliferation (PCNA, HGF, VEGF, HIF-a)
- 1% pO_2 group showed also lowest expression of SOCS3



Results

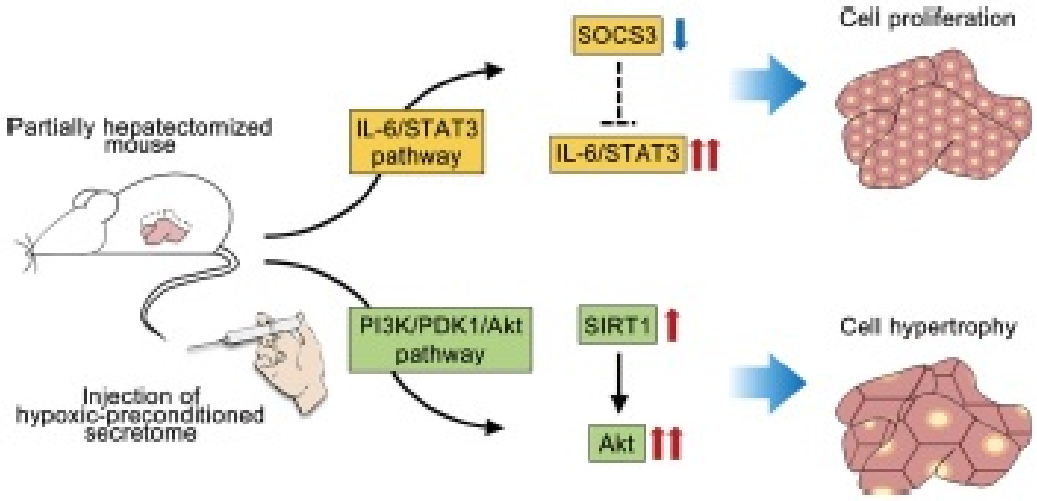
Effects of the secretome with different concentrations of culture pO_2 on the signaling pathways essential for liver regeneration in the mouse liver

- Effects of the secretome in the marker of hypertrophy in mouse liver on day 2 after injection
- Western blot analysis: 1% pO_2 group exhibited the highest expression of SIRT1 and Akt and the lowest expression of Bax and the highest expression of Mcl-1
- SIRT-1 Immunohistochemistry: 1% pO_2 group showed highest expression of SIRT1
- Resume that the 1% pO_2 group has the highest liver regenerative and anti-apoptotic potential among all secretomes tested



Conclusion

- ASCs cultured under 1% pO_2 showed the highest expression of proliferation-associated markers (Il-6, HGF, VEGF, PCNA) and the highest cell proliferation in AML12 cells cultered with ASCs of 1% pO_2
- Injection of 1% pO_2 secretome showed
  - Increased liver regneration
  - Reduction of serum levels of proinflammatory mediators (IL-6, TNF-a)
  - Reduction of serum levels of transaminases
  - Maximized expressioin of the PIP3/Akt and IL-6/STAT3 signaling pathways



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THANK YOU FOR YOUR ATTENTION!