

Human induced pluripotent stem cells (hiPSCs) are a true breakthrough in regenerative medicine with a potential to successfully treat many diseases, including orthopedic ones (e.g. cartilage lesions, osteoarthritis) that are unresponsive to current treatments. The development of human induced pluripotent stem cells (hiPSCs) has increased the potential of SCs for new treatments. Human articular cartilage (AC) has a poor regenerative capacity. Therefore, extensive studies on AC regeneration, including the cell-based tissue engineering research, are carried out. However, chondrogenic differentiation *in vitro* is a complex process and more research is needed in this field. Based on the novel chondrogenic differentiation protocol, this study has two main aims: a) to investigate gene expression profile *via* high-throughput analysis of obtained chondrocyte-like cells and b) to examine most markers characteristic of functional chondrocytes in those cells.

Numerous studies have described the main techniques to perform chondrogenic differentiation in hiPSCs, the most common being the formation of embryoid bodies (EBs). Other methods include micromass culture, pellet culture, and directed differentiation. Efficient *in vitro* chondrogenesis requires adding exogenous defined growth factors (GFs) from the transforming growth factor β superfamily to a chondrogenic medium. However, most of the aforementioned chondrogenesis methods require numerous steps that substantially lengthen the time needed to complete the *in vitro* differentiation process. Thus, the main aims of this study were to generate efficient chondrogenic protocol (1), to gain a close look at the nature of obtained chondrocyte-like cells at the molecular level (2), and to demonstrate the functionality of differentiated cells in the animal model (3). Firstly, we generated hiPS cell line from primary human dermal fibroblasts using a lentiviral system. Importantly, hiPSCs generated in our laboratory show the ability to form teratomas with derivatives of three primary germ layers (ecto-, endo- and mesoderm) in nude mice. hiPSCs cells were subsequently differentiated towards chondrocyte-like cells (ChiPS). Importantly, our group established a new chondrogenic protocol that includes a three-week monolayer culture using supplementation with relatively small amounts of GFs (FGF-2, BMP-4, PDGF, TGF- β 3, and IGF-1). Our protocol constitutes a novel approach because it obviates the need for additional steps (e.g., embryoid body formation), thus providing a notable cost and time advantage over other protocols. Next, we performed a global gene expression analysis using the Affymetrix platform—to understand the processes directing cell fate better. Our findings suggest that differentiated cells lose their pluripotent state. The key genes involved in maintaining the self-renewal and pluripotency pathways are significantly down-regulated. This finding suggests a lower risk of potentially uncontrolled proliferation and, consequently, a reduced risk of undesirable tumorigenesis. Selected gene sets were assigned to significantly enriched Gene Ontology (GO) analysis related to the regulation of differentiation and limb development. This analysis concerned the following biological processes: “embryonic limb morphogenesis”, “limb development”, “limb morphogenesis”, “embryonic skeletal system development”, “embryonic skeletal system morphogenesis”, “embryonic organ development” and “embryonic organ morphogenesis”. Based on that, we can assume that we obtained chondrocyte-like cells from early-stage chondrogenesis and these cells strongly activated signaling pathways engaged in limb formation and development. This finding was confirmed by RT-qPCR analysis, which demonstrated that the *ALX1*, *EYA1*, *HOXB6*, *HOXC11*, *HOXD13* and *RARB* genes were more highly expressed in the ChiPS versus both hiPSCs and adult chondrocytes. The microarray analysis provided valuable data on the global gene expression of the ChiPS, showing that the expression of genes belonging to the Hox cluster family was particularly high, thereby indicating that early-stage chondrocytes had been obtained. Our next findings allowed for the selection of crucial miRNAs engaged in both diminishing the pluripotency state (*inter alia* hsa-miR-302a-5p, hsa-miR-302c-5p, hsa-miR-302b, hsa-miR-302d) and chondrogenic process (*inter alia* hsa-miR-525-5p, hsa-miR-520c-3, hsa-miR-628-3p, hsa-miR-196b-star, hsa-miR-629-star, hsa-miR-517b, hsa-miR-187). These miRNAs regulate early chondrogenic genes such as *HOXD10*, *HOXA11*, *RARB*, *SEMA3C*. These results were appropriately visualized and confirmed by RT-qPCR analysis (hsa-miR-302a-star, hsa-miR-302c-star, hsa-miR-525-5p, hsa-miR-520c-3p, hsa-miR-628-3p, hsa-miR-196b-star, hsa-miR-629-star). As far as we know, it was the first report concerning the selection of key miRNAs responsible for regulating the first stages of chondrogenesis. In the next step, we demonstrated that ChiPS exhibit features characteristic of functional chondrocytes, *inter alia* high level of type II collagen, sex-determining region Y-box 5,6,9 (SOX5,6,9), and NK3Homeobox 2 (NKX3.2) expression. Those results were confirmed at the protein level using Immunofluorescence (Cartilage Oligomeric Matrix protein- COMP, type II and IX collagen, Aggrecan and SOX6/9) as well as flow cytometry (CD44 and CD151) analyses. Finally, we successfully performed Alcian blue staining to confirm the production of a high amount of desirable proteoglycans components of the extracellular matrix associated with chondrocytes during hiPSCs differentiation. This work provides a more precise understanding of the processes and mechanisms activated during *in vitro* chondrogenesis of hiPSCs.

We obtained cells with features characteristic of cells at early-stages of chondrogenesis. This finding suggests that these cells are likely to undergo terminal, spontaneous chondrogenic differentiation *in vivo* in a favorable microenvironment. However, early chondrocyte-like cells may not be fully functional and do not possess regenerative properties. To investigate these issues, *in vivo* experiments in the animal models was needed. Hence, we decided to verify the capacity of our cells to repair cartilage damage in an animal rabbit model. For this purpose, we examine the repair of “full thickness” cartilage damage which penetrates subchondral bone. A 3x3 mm loss on rabbit's articular surface of the knee joint is performed. Two hind limbs are operated simultaneously. The knee joints are opened with a single parapatellar cut. Patella is dislocated laterally, next “full thickness” lesion, exceeding subchondral bone, is performed by a drill with an appropriate diameter. Then, previously cultured ChiPS suspended in a scaffold are implanted in the site of the lesion. Subsequently, the same procedure is performed on the second joint, implanting a scaffold without cells. Knee joints are closed with a layered suture. After the surgery of limbs, no immobilization is applied. Animals placed in separate cages are allowed to move freely and fully weight-bear their operated joints. Cartilage repair is assessed at 8, 12 and 24 weeks. Bone and cartilage blocks, which are used to microscopic evaluation, are taken from knee joints. Bone and cartilage blocks are fixed in buffered solution of formalin and stained with appropriate antibodies (e.g. type X collagen) and techniques: Hematoxylin and Eosine, Safranin O, Toluidine Blue and Masson's Trichrome Staining. Microscopic evaluation with the use of O'Driscoll point scale is performed. Point scale, according to Brittberg, is used to macroscopic evaluation of repair filling. The scale is modified to adjust the sizes of the rabbit's knee articular cartilage surface and the size of the performed lesion. Our preliminary results indicate that the cells obtained from hiPSCs are functional chondrocyte-like cells with notable repair capacities. It is important to mention that repair induced by the presence of ChiPS is characterized by the presence of hyaline but not fibrous cartilage. However, more data is needed to reach definitive conclusions.

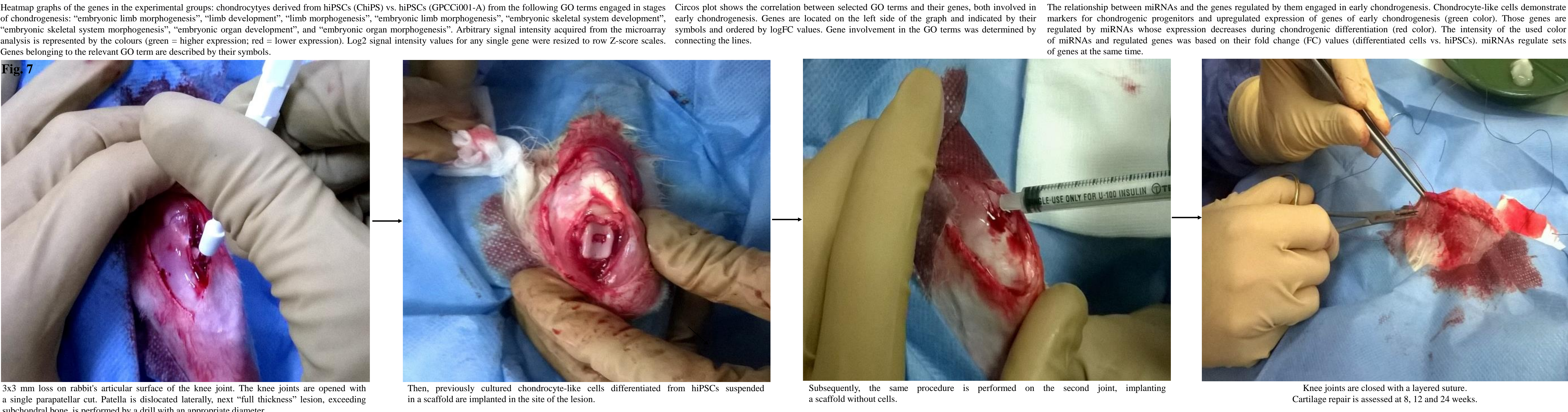
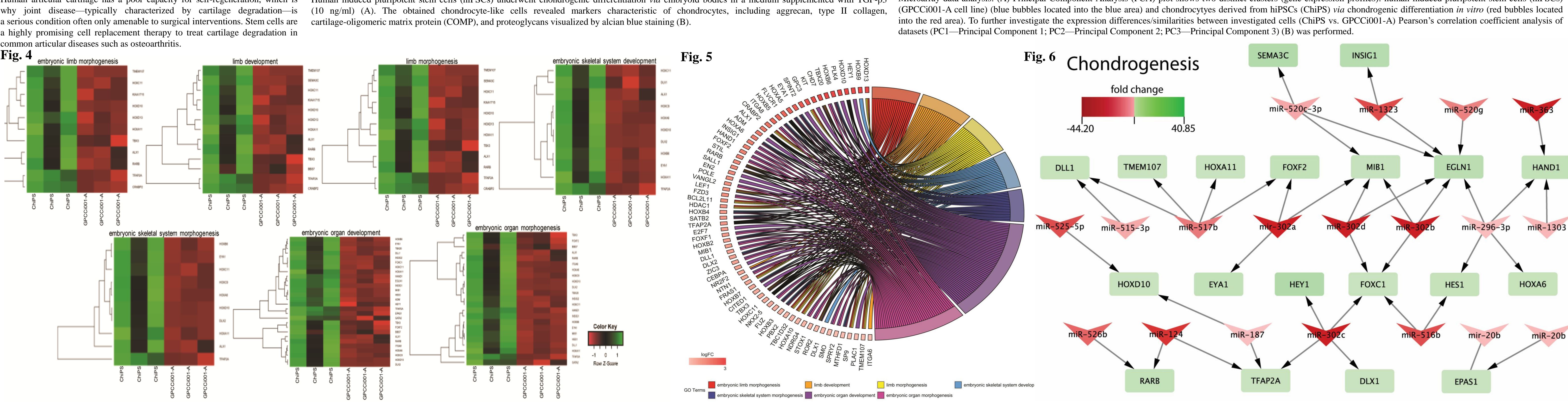
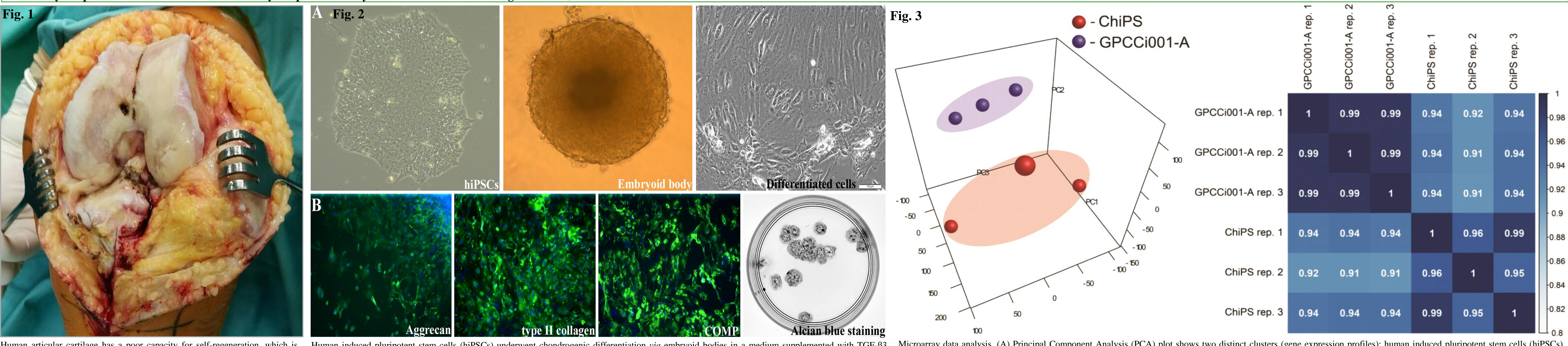


Fig. 8 Bone and cartilage blocks are stained with appropriate antibodies (e.g. type X collagen) and techniques: Hematoxylin and Eosine, Safranin O, Toluidine Blue and Masson's Trichrome Staining.

Repair of “full thickness” cartilage damage which penetrates subchondral bone. Bone and cartilage blocks, which are used to microscopic evaluation, are taken from knee joints and fixed.

In summary, our experimental setup forms a coherent entirety enabling execution of cross-cutting research:

- from performing a novel chondrogenic differentiation of hiPSCs to the analysis of *in vitro* generated cells (*via* high-throughput analysis such as microarrays) and demonstration the hiPS cells-derived chondrocytes in the regeneration of damaged cartilage using the experimental rabbit model. **Such experiments have not been performed so far.** In conclusion, we obtained chondrocyte-like cells from stem cells which are likely to constitute a potentially unlimited source of cells for regeneration of articular cartilage.

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