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Harnessing Platelet-derived factors to reactivate Nucleus Pulposus cells in Intervertebral Disc Regeneration

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ABSTRACT

Introduction: Degenerative disc disease (DDD) is a significant healthcare challenge, particularly in the elderly population. Current treatment options range from conservative approaches to invasive surgical interventions. In intervertebral disc (IVD) disease, the degeneration of the Nucleus Pulposus (NP) is a key factor in the initiation of the pathology. Several tissue engineering strategies have been explored to restore the NP cell and matrix components, using adult mesenchymal stem cells (MSCs) and, more recently, induced pluripotent stem cells. However, these approaches have not been fully successful due to the acidic environment of the NP, which is harmful to implanted heterologous cells.

Recent studies have shown that platelet derivatives, particularly Platelet-Rich Plasma (PRP) and Platelet Lysate (PL), are effective in promoting connective tissue regeneration.

Methods: This study aimed to explore the potential of PL to stimulate NP cell proliferation and differentiation, either by directly activating resident NP cells or by expanding them in vitro for subsequent implantation, with the overarching goal of promoting IVD regeneration. IVD biopsies were processed using two methods to isolate NP cells: enzymatic digestion (NPd), which degrades the extracellular matrix to release the cells, and direct plating of biopsy fragments (NPwd), which isolates migrating cells from the tissue. Phenotypic characterization was performed using flow cytometry to assess mesenchymal marker expression and cell proliferation was quantified by cell doubling counts. Chondrogenic differentiation was evaluated in a 3D culture model, followed by immunohistochemical analysis.

Results: Our results demonstrate that NP cells cultured with PL show a progenitor-like state, with the ability to form cartilaginous pellets upon chondrogenic stimulation. Moreover, PL directly induces the release of progenitors from biopsy fragments, exhibiting mesenchymal stem cell-like properties and chondrogenic differentiation potential.

Conclusions: In conclusion, PL supports the maintenance of a progenitor state in NP cells and may contribute to the regeneration of damaged IVD tissue by activating resident quiescent stem/progenitor cells within the disc.

1. Introduction

Lower back pain (LBP) affects millions of people, significantly impacting their quality of life, making it a major healthcare concern and imposing high costs on patients and society. About 40% of chronic LBP is associated with the degeneration of the intervertebral disc (IVD) [1].

The IVD is a complex structure with biomechanical functioning depending on a balance between its three main tissues: (i) the Cartilaginous Endplates (CEP), which are composed of cartilage separating and anchoring the IVD to the vertebrae via the bony end plates, and are

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the gateway for nutrient transport into the IVD; (ii) the Nucleus Pulposus (NP), a central region which contains a glycosaminoglycan-rich gel-like tissue [1]. In most cases, the NP degeneration is the initiation site and the trigger for IVD degeneration. Indeed, the loss of NP viable cells and the shift to a catabolic phenotype is one of the main causes of IVD pathogenesis; (iii) the Annulus Fibrosus (AF), surrounding and enclosing the NP.

Present treatments to combat LBP are pharmacological (opioids, non-steroidal anti-inflammatory drugs, antidepressants), physical (exercise, manipulation, and massage), alternative (acupuncture, magnet therapy), or surgical (tissue removal, spine fusion) [1,2]. Although these therapies are potentially helpful in acute LBP, they have a limited effect in the treatment of chronic LBP since they can only provide alleviation of symptoms through pain relief, but do not promote repair or regeneration of IVD and in particular of NP. In principle, repair and regeneration of IVD and NP could be obtained by tissue engineering and regenerative medicine approaches [3]. The main goal for a tissue engineering approach should be to restore the appropriate cell and matrix content of the NP which would be beneficial for the function of the whole IVD. The canonical tissue engineering strategy would be the implant of a biomaterial seeded with cells capable to recreate the NP microenvironment [4,5]. Several natural or synthetic biomaterials suitable for an IVD implant are today available [6]. The choice of cells to be implanted is more complex and should consider the mesodermal origin of IVD. More specifically, NP cells originate from the notochord, whereas cells of other IVD connective tissue structures forming the vertebral column derive from the sclerotome [7]. Among cells of mesodermal origin, it was proposed to use adult stromal cells from bone-marrow or adipose tissue, chondrocytes, and notochordal cells. More recently it was also suggested the use of cells obtained from the expansion and differentiation of pluripotent stem cells (iPSC) [8]. However, regardless of the cells adopted, cell therapy of IVD is not a simple task because of the acidic environment and the low concentrations of oxygen and glucose characterizing the IVD. These are culture conditions to which cells adapt with great difficulty.

An alternative therapeutic option comes from a regenerative medicine approach. In this case, the stem and progenitor cells, reported to be present in the IVD are not expanded ex vivo and re-implanted [4,9]. Instead, their proliferation and redifferentiation is attempted by the local injection of specific inducers. In recent years, Platelet Rich Plasma (PRP) has received considerable attention as a cell proliferation inducer. PRP is a plasma fraction with a high concentration of platelets, obtained by centrifugation of whole blood to separate platelet-enriched plasma from red blood cells [10]. PRP contains a large variety of bioactive proteins, including growth factors, peptides, and cytokines that stimulate tissue healing. Because of its ability to promote cell proliferation, the PRP-derived product Platelet Lysate (PL), has been proposed as a substitute for bovine serum in cell culture media [11,12]. Although both PRP and PL are derived from platelets and are rich in growth factors, they differ significantly in their preparation, composition, and potential applications. PRP consists of intact platelets suspended in plasma, and requires activation (e.g., via calcium or thrombin) to release growth factors in situ, leading to a more sustained release over time. In contrast, PL is obtained by disrupting platelets—typically through freeze-thaw cycles—resulting in an acellular solution where growth factors are immediately available upon application. This distinction affects their biological activity, consistency and clinical utility. PL offers a more standardized and reproducible preparation, making it particularly suitable for in vitro and potentially clinical regenerative applications. PRP, while more physiological in its release pattern, may be more variable in content depending on the donor and preparation protocol. Understanding these differences is critical when considering their use in IVD regeneration.

Quiescent human bone marrow- and adipose-derived Mesenchymal Stem Cells (MSC), chondrocytes and osteoblasts, when treated with PL, resume proliferation maintaining their capability to differentiate [13,14]. In this scenario, initial attention was given to studies that identified the role of PL in its ability to activate resident and quiescent cells in tissues during healing and regenerative processes [15]. Terminally differentiated osteoblasts and chondrocytes showed a burst of proliferation after PL stimulation and returned to a none or low dividing condition when the PL stimulus was removed. Furthermore, PL-stimulated osteoblasts maintained the capability to organize a bone matrix when transferred to in vitro and in vivo osteogenic conditions [16].

In the clinical setting, PRP has been used for IVD regeneration. In agreement with its anti-nociceptive effect [17], PRP injection could significantly relieve patient pain [18].

However, the results obtained regarding the IVD tissue regeneration are still controversial. A 2020 review [19] summarizes and critically analyzes the evidence regarding IVD treatment with PRP. Since then, few other clinical studies were completed and others are required to clarify the roles of PRP in IVD prevention and treatment. Here we investigated the possibility that PL could revert differentiated NP cells to a proliferative state followed by a subsequent re-differentiation, thus supporting the option of local PRP injection for a surgically less invasive IVD regeneration treatment.

2. Methods

2.1. Cell isolation

The biological samples used in this study derived from surgery discarded degenerated intervertebral disc of patients who underwent discectomy because of lumbar degenerative disc disease. All patients gave informed consent in accordance with ethical guidelines and the research was approved by the Ethic Committee CER Liguria (protocol registration number 372/2019). Patient-derived samples were collected from both male and female individuals with a mean age of 55 ± 16 years (range: 23-80 years).

The intervertebral disc biopsies obtained from the patients undergoing discectomy were fragmented with a scalpel and subjected to two different manipulations to obtain the cells: (i) an enzymatic digestion which degrades the extracellular matrix (*NPd cells*); (ii) the direct plating of biopsy fragments into Petri dishes, without any enzymatic digestion (*NPwd cells*).

To obtain cells by digestion, fragments approximately 100 mm^3 in volume were placed in a Falcon tube and digested with an equal volume of trypsin (Trypsin 2.5%, Gibco) for 30 min at 37 °C. After the trypsin removal, the processed tissue was additionally digested for 16 h with Collagenase II (75% in PBS Ca^{2+} Mg^{+}) in a 1:8 (ml of tissue suspension: ml of collagenase) ratio at 37 °C with continuous stirring. The sample was then subjected to centrifugation, resulting in the separation of an upper liquid phase, which was subsequently discarded, and a lower compact phase (pellet) containing the cells. Cells were resuspended in a small volume of culture medium and plated. Alternatively, fragments measuring approximately 30-50 mm^3, obtained from larger tissue pieces, were directly placed in 100 mm Ø Petri dishes. This process allowed NP cells to spontaneously emerge from the fragments. When the cells reached approximately 80% confluence, they were detached using trypsin/EDTA. The fragments contained in the plate that sprout the cells were transferred to a new plate to obtain a second wave of cells. The process was repeated until the pieces were able to release cells (2-3 times for the fetal bovine serum condition and up to 8 times for the platelet lysate condition). The obtained cells from each wave were maintained in culture for 5-10 passages. Residual cells from both kinds of cultures were frozen and stored in liquid nitrogen in medium containing 10% DMSO and supplemented with 50 % fetal bovine serum or 50 % plasma, according to the respective culture condition.

2.2. Culture conditions

For both *NPd cells* and *NPwd cells* the basic culture medium was

Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Where indicated, this medium was further supplemented with either 10% Fetal Bovine Serum (FBS) or 5% Platelet Lysate (PL) prepared according to the protocol published by Muraglia et al. (1% platelet concentrate + 4% Platelet Poor Plasma) [12]. To the cultures supplemented with PL, 5 IU/ml sodium heparin (Epsoclar, Biologici Italia Laboratories Srl, Masate, Milano, Italy) was added to avoid in vitro gel formation. Cells were kept at a temperature of 37 °C with humidified air and 5% carbon dioxide. The medium was changed approximately every 3 days. Once confluence was reached, the cells were detached using a trypsin/EDTA solution and used for subsequent experiments. In most cases experiments were repeated with at least three different cell populations obtained from biopsies of different IVDs.

2.3. Clonogenic assay

The Colony Forming Unit-fibroblast (CFU-f) assay was adopted to evaluate the colony-forming efficiency at passage 0 (P0). Briefly, a low density of cells, 100 μl of the digested derived cell suspension, was plated in 100-mm Ø Petri dishes in the basic medium supplemented with either 10% FBS or 5% PL. For cell expansion, since it was not possible to accurately determine the cell number in the digested suspension, cells were plated based on the volume of the digested tissue. In particular, we plated 1×10^3 μl (high-density seeding) of the digested-derived cell suspension. The medium was removed after 5 days from plating and replaced with fresh medium. Cultures were checked daily. After 10-15 days, depending on the culture, colonies were washed with PBS, fixed with 4% formalin in PBS, stained with 1% methylene blue for 45 min at room temperature, and then washed with distilled water. Images of the dishes were acquired with a scanner.

2.4. Cell proliferation rate

For both cell types obtained from the vertebral tissue (*NPd cells* and *NPwd cells*) the proliferation rate was determined by measuring the number of doublings performed by the cells in the time unit. Briefly, *NPd* and *NPwd cells* at passage 1 (P1) were plated at a density of 1×10^5 cells in duplicate in 60-mm Ø Petri dishes. Culture conditions were as above. Media were changed twice a week. When the cells reached 70-80% confluence, they were detached with trypsin/EDTA, counted and replated at the same cell density (1×10^5 cells) in new dishes. The process was repeated each time the cells reached 70-80% confluence. Proliferation rate was determined for 4 different primary cultures ($n = 4$) with medium supplemented with 5% PL and for 3 primary cultures ($n = 3$) with medium supplemented with 10% FBS.

2.5. Flow cytometric analysis

Adherent cells isolated from digested (*NPd cells*) and undigested samples (*NPwd cells*) were harvested and phenotypically characterized by flow cytometry. In compliance with the standards proposed by the International Society for Cellular Therapy (ISCT), cell immunophenotyping was performed with the use of mouse anti-human antibodies: CD 34 (clone 4H11), CD 44 (clone IM7), CD 73 (clone AD2), CD 90 (clone 5E10) and CD 105 (clone SN6) (all from eBioscience) revealed by fluorescein isothiocyanate (FITC) or phycoerythrin (PE) staining. Control samples were stained with isotype-matched control antibodies. At 70% confluence, cells were detached from the tissue culture plates and $2{,}5 \times 10^5$ cells were suspended in PBS for the treatment with each antibody. Two different antigens were investigated at the same time thanks to the availability of antibodies carrying two different dyes (FITC, PE). Hybridized cells were incubated in the dark for 20 min at room temperature. After the incubation, to remove the excess of labeled antibodies, the cells were subjected to centrifugation and resuspended in PBS. The process was repeated twice. After the last centrifugation, the labeled cell pellet was resuspended in 0.5 mL of PBS and subjected to flow cytometric analysis using Cyan ADP cytofluorimeter (Beckman-Coulter). Data were analyzed with the FlowJo 10.8.1 software. The results were expressed as percentage of positivity. We summarized, to better understand the results, a table with ± marks representing the mean of different positivity of the cells. The meaning of the marks reported in the table had represented a range of positivity: (−) 0%-25%; (+) 26%-50%; (++) 51%-75%; (+++) 76%-100%.

2.6. In vitro chondrogenic differentiation

The chondrogenic potential of NP cells was checked at passage 1 by micromass pellet cultures in vitro ($n = 3$) according to the procedures described by Johnstone and colleagues [20] with minor modifications. About $2{,}5 \times 10^5$ cells were pelleted in conical tubes and cultured for 3 weeks in chondrogenic medium containing 10 ng/ml human TGF-β1 (PeproTech, Rocky Hill, NJ, USA), 10^{-7} M dexamethasone, and 0.3 mM ascorbic acid (both from Sigma-Aldrich, St. Louis, MO, USA). Chondrogenic differentiation was investigated by histology after staining of the pellets with toluidine blue and by immunohistochemistry with antibodies against aggrecan, type I and type II collagens.

2.7. Statistical analysis

Differences between cell culture conditions in proliferation experiments were assessed by the Student's t-test and two-way ANOVA; for flow cytometric analysis experiments two-way ANOVA was used. Statistical significance was accepted for any p value ≤ 0.05. Analyses were performed using GraphPad Prism 9.4.0 software.

3. Results

3.1. Colony Forming Unit - fibroblast assay (CFU-f)

To determine, by the CFU-f assay, the ability of NP cells cultured in different conditions to form colonies, a population of cells obtained by enzymatic digestion of IVD was split in two sub-populations in Petri dishes in medium supplemented with 5% PL and 10% FBS respectively. After 4–5 days from plating, in both groups, we observed the initial formation of colonies adherent to the bottom of the Petri dishes (Fig. 1). The colony growth was monitored daily. After 10–15 days from plating, the colonies were stained with methylene blue. The quantitative results of colony formation, expressed as colony counts, are summarized in Table 1 (for three representative cultures). A high degree of variability in colony numbers was observed among the different primaries; however, despite this variability, the difference in the number of colonies between the two treatments remained statistically significant. In general, most colonies in the control condition were less than 1 mm in diameter and only about 3% had a diameter of about 3 mm. Interestingly, in the PL culture condition, a higher number of colonies had a diameter superior to 1 mm and about 15-20% of colonies had a diameter around 3-6 mm (Fig. 1 panels A-B). The cells showed a fibroblastic phenotype, but in the presence of PL they had a more pronounced spindle shape than cells cultured in medium containing FBS (Fig. 1 panels C-D, bar 50 μm).

3.2. Proliferation rate

The life span of a *NPd cell* population expanded in vitro in the two culture conditions was monitored by determining the number of cell doublings in a period of 35 days (Fig. 2). The proliferation rate of cells cultured with PL was statistically higher than cells cultured in FBS. Essentially, *NPd cells* did not proliferate in the presence of FBS (about 2 doublings in 35 days) whereas they underwent 11-12 doublings when they were cultured in the presence of PL (Fig. 2 A).

The same proliferation behavior was observed in *NPwd cells*

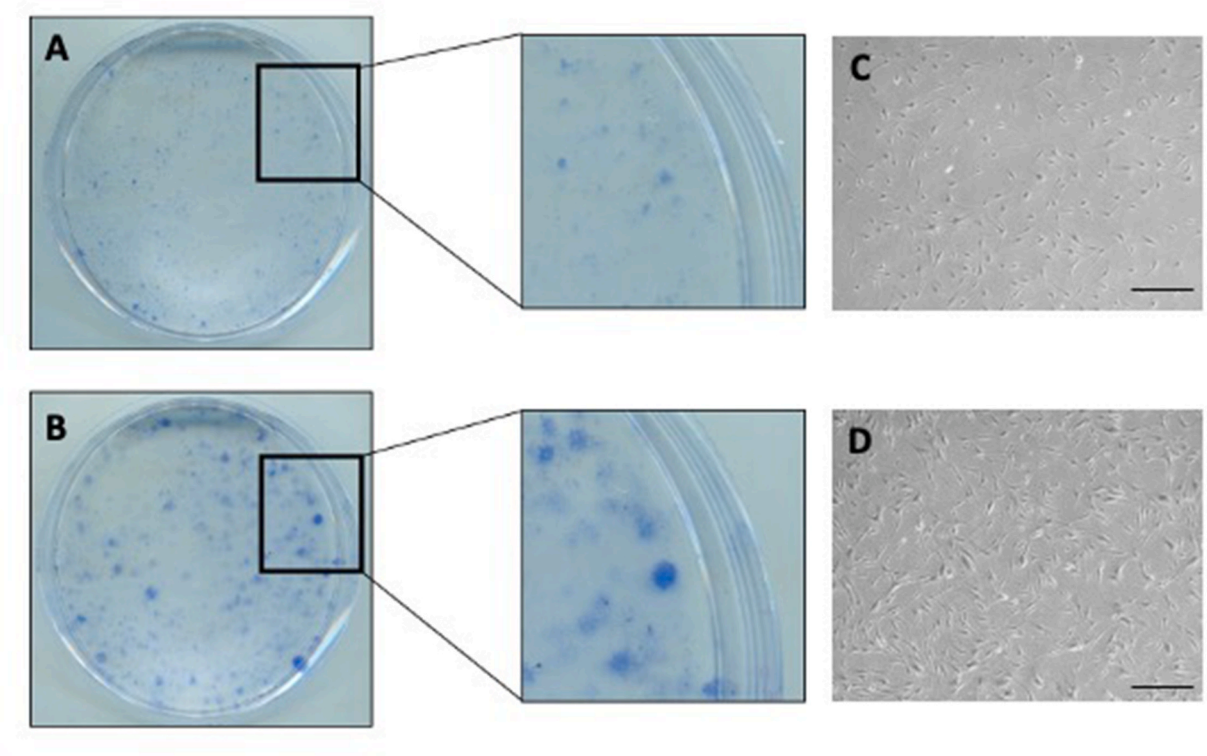


Fig. 1. Colony-Forming Unit-fibroblast assay of NP cells plated and cultured in FBS (A) and PL supplemented medium (B). After 2 weeks, colonies were stained with methylene blue (MB) to identify all colonies. (C) and (D) represent the morphology of cells grown in FBS and PL culture conditions respectively (Bar: 50 μm).

Table 1
CFU-f efficiency in primary cultures of human *NPd* cells.

Culture ID	Cell culture condition	Colony number/ dish (2 dishes for each condition)		Mean colony number±SEM*
# 1	FBS	165	150	
	PL	180	190	
# 2	FBS	80	83	*FBS 119,7 ± 15,0*
	PL	213	200	*PL 227,2 ± 20,5*
# 3	FBS	100	140	
	PL	300	280	

Colony number obtained under FBS and PL culture conditions. * statistically significant difference (*t*-test, $p = 0{,}0121$).

outgrown from the IVD fragments (Fig. 2 B). For this cell population, the proliferation rate was determined for the 3 initial cell outgrowths obtained transferring the fragments in new Petri dishes and maintaining as medium supplement either PL or FBS and for the eighth outgrowth for cells only expanded with PL. In fact, in the case of cells cultured in the presence of FBS, it was impossible to obtain additional waves of cells from the fragments after the third transferring of pieces. Outgrowing cells from the tissue fragments cultured in PL-supplemented media underwent a statistically significant number of cell doublings compared to those obtained from FBS-derived outgrowths, as shown in the table associated to Fig. 2 A.

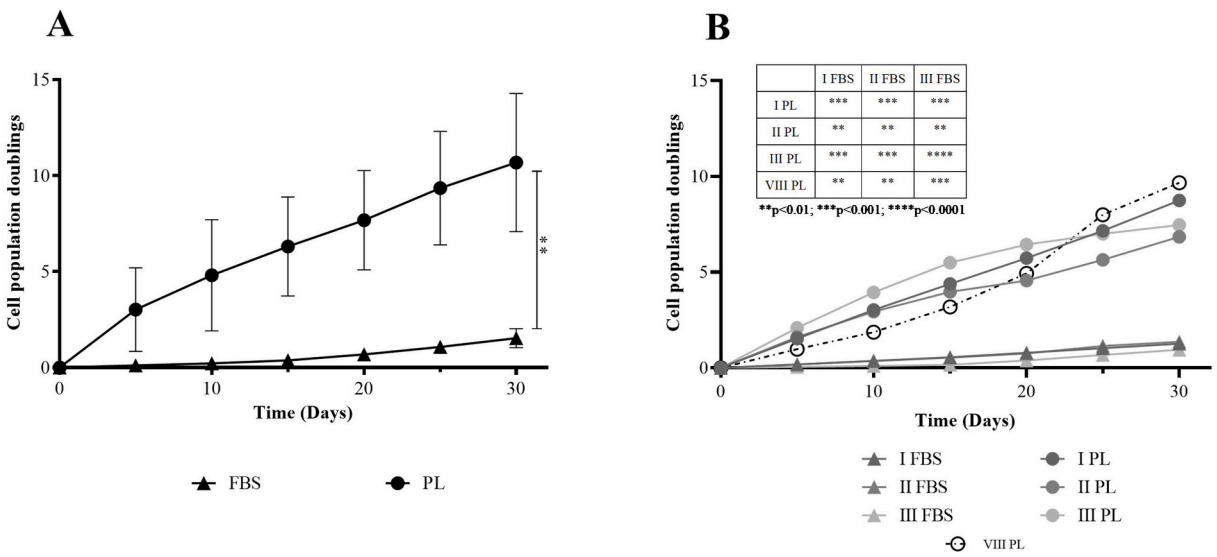
3.3. Flow cytometric characterization

The phenotypic profile of the cells derived from IVD digestion, was obtained by flow cytometric analysis for some antigens characteristic of stem/progenitor cells of mesodermal origin. The analysis was performed at passage 1 for *NPd cells* cultured either in FBS or in PL supplemented medium when more cells were obtained. All *NPd cells* were negative for the haemopoietic stem cell marker CD34 and expressed, with a high positivity, CD44, CD73, CD90 and CD105. No significant differences in marker expression, were observed between cells cultivated in the presence of FBS or PL, thus confirming the maintenance of a mesenchymal phenotype in both culture conditions (Fig. 3).

When flow cytometric analysis was performed on the various outgrowths of *NPwd cells* cultured under FBS and PL conditions, distinct behavioral differences were observed between the two culture environments and across successive sproutings. Cells isolated and expanded in the presence of FBS exhibited a fluctuating mesenchymal profile across the different outgrowths (Fig. 4 A–C). This variation was evident when comparing *NPwd cells* obtained directly from the initial explant to those recovered from the subsequent three outgrowths. In contrast, *NPwd cells* cultured in PL maintained a more stable mesenchymal phenotype, with consistent results observed from the initial explant (0) through all three subsequent outgrowths (I, II, III) (Fig. 4 B–D). Raw percentage data are shown in the Supplementary Table I.

3.4. Chondrogenic differentiation

NP cells expanded in vitro in the presence of PL were tested for their ability to form cartilage tissue using the pellet culture assay, developed by Johnstone et al. [20]. Cell pellets were maintained in culture in the presence of TGFβ1, ascorbic acid and dexamethasone for 3 weeks. During this time the cell aggregates grew reaching a 1-2 mm in diameter at the end of the 3 weeks. Histological analysis following Toluidine Blue staining, revealed an organized, metachromatic cartilaginous matrix where chondrocytes were evident and clearly embedded in lacunae (Fig. 5). The presence of a cartilaginous matrix was confirmed by immunohistochemistry with antibodies against the aggrecan, a cartilage-specific proteoglycan (Fig. 5). The immunohistochemistry with antibodies directed against type I and type II collagens revealed the



	I FBS	II FBS	III FBS
I PL	***	***	***
II PL	**	**	**
III PL	***	***	****
VIII PL	**	**	***

Fig. 2. Cumulative cell doublings of (A) *NPd cells* population derived by enzymatic digestion; (B) *NPwd cells* from the three initial outgrowths, obtained by transferring tissue fragments to new Petri dishes, were isolated and expanded in vitro under FBS and PL culture conditions. The graph represents the results of a representative primary culture. The table associated to the graph contains the results of the statistical comparison between the different cultures (n = 3; numbers represent mean ± SEM).

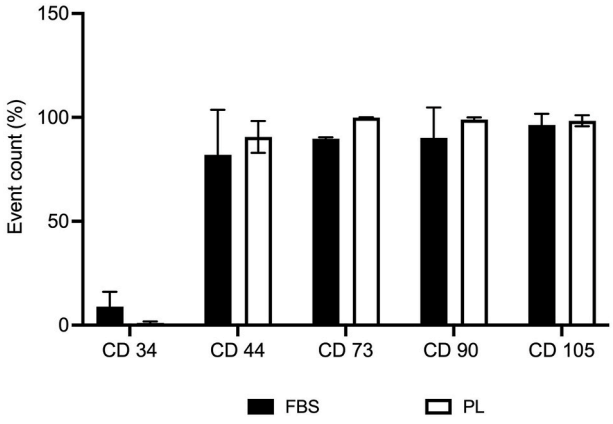


Fig. 3. Phenotypic profile of *NPd cells*, assessed by flow cytometry analysis for characteristic antigens of mesodermal stem/progenitor cells, selected under FBS and PL culture conditions, respectively.

presence of some type I collagen at the periphery of the cell aggregates and of type II in the central aggregate nucleus (Fig. 5).

4. Discussion

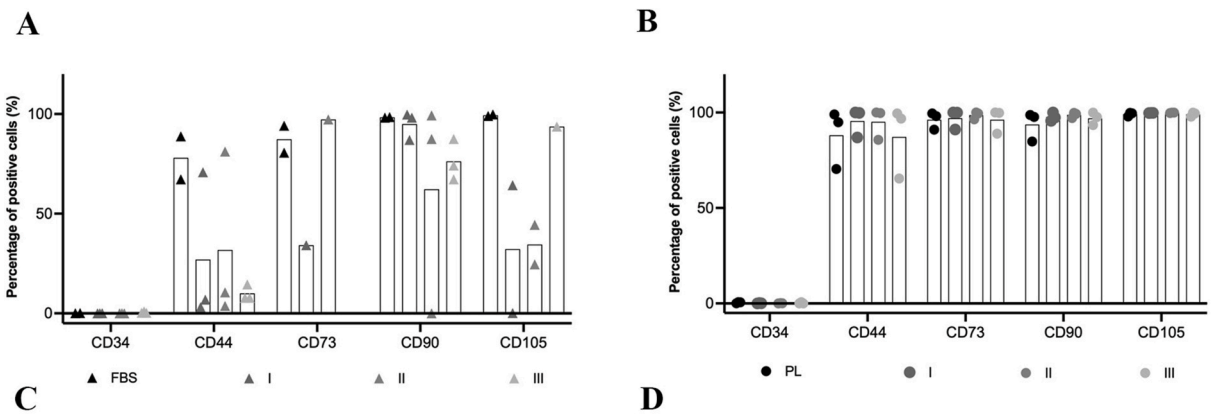
Lower back pain affects a very large percentage of humans, and in about 40% of these patients, the pain is due to the degeneration of the intervertebral discs (IVD). Several types of treatments, both physical and pharmacological, have been proposed and are being adopted to reduce pain and possibly restore the IVD damaged structure. Unfortunately, almost all these treatments have some effect on pain relief but fail to remove the cause of the pain by promoting repair or regeneration of the degenerated IVD. Regenerative medicine aims to replace tissues or organs damaged by disease, trauma, or age using stem/progenitor cells or their derivatives, vs. other clinical strategies mainly focused on symptom treatment. Stem and progenitor cells, the major players in the process, can be activated or reactivated either through an ex vivo expansion in vitro and a re-implantation or by the local injection of specific inducer molecules. In the last case, stem/progenitor cells are somehow recruited to the damaged site where differentiate to restore complex structures of tissues and organs. Alternatively, cells close to the damaged site are induced to de-differentiate, proliferate, and re-differentiate to restore and repair the tissue.

For the repair of degenerated IVD by a regenerative medicine approach, the re-implantation of a variety of stem/progenitor cells of mesodermal origin has been proposed [21,22]. A key challenge of IVD cell therapy is to choose the correct cell source that can not only survive within the natural difficult environment of the IVD, including avascularity and acidic pH, but that are safe to be used and can produce the extracellular matrix needed to restore the biological function of the disc. In an open label pilot study, 26 patients received autologous bone marrow concentrate disc injections [23]. Subjects presented a substantial reduction in pain indicating an effect of mesenchymal cell concentration on discogenic pain reduction. Other mesodermal stem/progenitor cells which were cultured ex vivo and re-implanted include MSC, induced Pluripotent Stem Cells (iPSCs), embryonic stem cells, muscle-derived stem cells, haematopoietic stem cells and olfactory mucosa stem cells [24–26].

However, although there were significant improvements in the patients' pain scores, again there were not clear results about the disc regeneration. Still the lack of relevant evidence regarding safety aspects, effectiveness and surgical cost effectiveness are main concerns.

The microenvironment in which cells operate plays a crucial role. Recent studies have shown that PL, thanks to its high concentration of growth factors, significantly enhances the proliferative and differentiation potential of mesenchymal cells. Compared to FBS, PL offers several key advantages: it is human-derived, reducing xenogeneic risks and making it more suitable for clinical use; it ensures better batch consistency due to more standardized and controlled production processes, especially when pooled from multiple certified donors; and it often supports faster and more efficient cell proliferation [27,28]. Overall, PL more closely mimics the human physiological environment, which is essential for translational and GMP-compliant applications.

In this context, NPd cells should be the favored ones, because they can maintain their phenotypic and differentiative characteristics in the IVD microenvironment. However, low cell viability has restricted the therapeutic potential of NP cells and access to healthy cells, especially autologous cells, is limited in clinical settings. For this reason, bone marrow- or adipose-derived MSC are preferred to be used in the IVD regeneration for their ability to differentiate into NP-like cells and for



C

FBS	0	I	II	III
CD34	-	-	-	-
CD44	+++	-	+	-
CD73	+++	+	+++	
CD90	+++	+++	++	+++
CD105	+++	+	+	+++

D

PL	0	I	II	III
CD34	-	-	-	-
CD44	+++	+++	+++	+++
CD73	+++	+++	+++	+++
CD90	+++	+++	+++	+++
CD105	+++	+++	+++	+++

Fig. 4. Flow cytometric characterization of *NPwd cells* derived from three consecutive waves of outgrowth from tissue fragments cultured in (A) FBS and (B) PL conditions. Panels (C) and (D) provide a summarized graphical representation of the data shown in (A) and (B), respectively. The tables show the average marker expression levels across the three cultures, using a qualitative scale: (−) = 0–25% positive cells; (+) = 26–50% positive cells; (++) = 51–75% positive cells; (+++) = 76–100% positive cells.

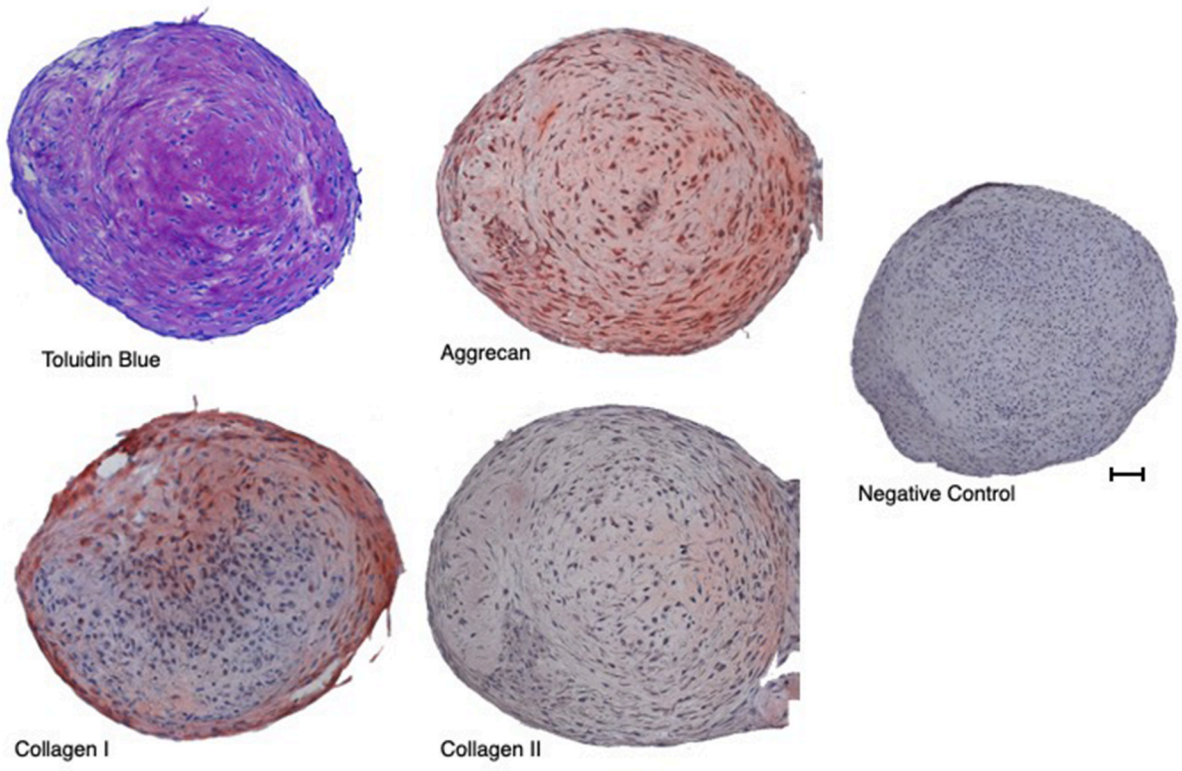


Fig. 5. Chondrogenesis in pellet cultures. Cartilage beads formed in vitro by NP cells selected in the presence of PL and cultured under chondrogenic conditions. Overall morphology and metachromatic staining with toluidine blue are typical of cartilage. Aggrecan, type II Collagen positivity and type I Collagen positivity confirm the chondrogenic differentiation (Bar: 100 μm).

their easier access site [29]. When NPd cells were cultured in the same culture condition of MSC and other stem/progenitor cells of mesodermal origin, they come up against the demand for the large number of cells needed to regenerate even a few millimeters of damaged disc.

In this article we reported that *NPd cells* obtained by a standard procedure from a tissue biopsy, i.e. by proteolytic digestion of tissue fragments, could be successfully expanded in vitro in PL supplemented medium to obtain a relatively large number of cells maintaining their

capacity of organizing a cartilaginous matrix when exposed to a chondrogenic environment. In a conventional culture medium supplemented with FBS, *NPd cells* had a very limited life span and could at most perform two duplications in one month, whereas the same cells cultured in PL-supplemented medium could perform 11-12 duplications in one month. This is raising the possibility of using *NPd cells* not only for autologous cell reimplantation but also for allogenic treatments. In fact, because of its avascular nature, IVD is an immune-privileged tissue and should tolerate the implantation of allogeneic cells [30]. In cells cultured in the presence of bovine serum the chondrogenic differentiation did not occur. The result is extremely interesting because, as reported in literature, it is easier to differentiate mesenchymal stem cells from bone marrow or adipose tissue into NP cells, but few articles discuss the direct differentiation of NP isolated from the intervertebral disc into chondrocytes [31]. Arana et al. showed that NP cells in coculture with cartilage end-plate tissue induced an increase in the production of proteoglycans and collagen type II. This means that a favorable environment, in this case a cartilage tissue, is able to induce NP cells into chondrocytes [32]. In our experimental conditions, the *NPd cells* have characteristics similar to bone marrow-derived MSC for their capability to form colonies and to express the phenotypical mesenchymal markers. Besides only *NPd cells* cultured in PL condition and induced with chondrogenic factors such as TGF-β, ascorbic acid and dexamethasone, which create a favorable environment, were able to differentiate into chondrocytes, as confirmed by histological and immunohistochemical analyses.

Interestingly, PL also supported the in vitro proliferation of NP cells outgrown from IVD fragments, thus suggesting that an activation or reactivation of stem/progenitor cells present in the NP could be possible with a local stimulation by a PL injection. Because there are several profound clinical problems associated with transplantation of autologous cells to regenerate disc structure, a more direct approach is to stimulate the resident cells to repopulate the damaged tissue. Previous data of our group have been reported on the PL capability to sustain the release and proliferation of chondroprogenitor cells from cartilage explants for more than one year, maintaining their differentiation potential [14,16]. Similar evidence was reported also for osteoblasts treated with PL that induced reactivation of proliferation of quiescent or very slow dividing osteoblasts isolated from bone of old patients [33]. Many authors have wondered whether the adult disc contains a replicating progenitor cell population and if this population persists during disc disease. Risbud et al. showed that both NP and AF tissues contained a heterogeneous population of proliferating, plastic adherent cells [9]. These cells expressed a repertoire of surface proteins that are characteristic of marrow stromal stem cells that are committed to the osteogenic, adipogenic and chondrogenic lineages. They hypothesize that AF is a source of stem cells that supply the NP during disc degeneration and because chondrocyte-like cell clusters form in the degenerate NP, the possibility exists that local conditions promote cell commitment to the cartilage phenotype [9]. Our data are consistent with what has been demonstrated by these authors because when disc tissue fragments are treated with PL, the cells come out adhering to the plastic of the plate. Subsequent releases allowed the collection of many cells with the characteristics of mesenchymal cells capable of forming cartilage tissue. The *NPwd cells* presented a stable mesenchymal phenotype with a comparable result between *NPwd cells* obtained directly from the initial explant to all 3 subsequent waves of cells cultured with PL. On the contrary, the same cells isolated and expanded in the presence of FBS, presented a fluctuation in the mesenchymal profile that was not maintained comparing the *NPwd cells* obtained directly from the initial explant to the cells recovered in the subsequent three waves. Based on previous experimental evidence, the maintenance of stemness features is better preserved when cells are cultured in human platelet lysate. This may be due to the higher compatibility between human-derived cells and human plasma (in which platelet lysate is obtained), compared to supplements of animal origin. Additionally, the enrichment of the culture medium with platelet-derived growth factors plays a key role. Among these, FGF-2 appears particularly important, as we previously demonstrated in studies on Bone Marrow-derived Mesenchymal Stem Cells, where the addition of FGF-2 to bovine serum led to the selection of a cell population enriched in multipotent mesenchymal progenitors [34,35].

By closely mimicking the native intervertebral disc (IVD) microenvironment, it is possible to suggest that *NPd cells* can effectively maintain their mesenchymal phenotype. This biomimetic environment not only supports the preservation of their stemness characteristics but also enhances their capacity for chondrogenic differentiation. As a result, PL-cultured *NPd cells* exhibit improved proliferative ability, and an increased potential to synthesize extracellular matrix components typical of healthy cartilaginous tissue. These findings underline the critical importance of replicating the IVD microenvironment in vitro to optimize NPd cell-based regenerative strategies aimed at treating degenerative disc diseases.

This could have a highly relevant impact regarding the number of patients that could benefit of a regenerative medicine approach. In fact, the high cost of the cultures and the difficult logistics make very challenging to extend the ex vivo expansion and re-implantation approach available to many patients. PRP injection therapy is based on platelet growth factors support to the three phases occurring during wound healing: inflammation, proliferation, and remodeling. The effect of a mixture of platelet growth factors as therapeutic agents for the repair of damaged tissues has been well documented in skin chronic ulcers and other tissues.

Although the in vitro approach provides useful insights into intervertebral disc cell behavior, it has several limitations. In fact the culture conditions do not fully replicate the native disc environment, such as low pH, hypoxia, high osmolarity and mechanical stress. Also standard culture systems, such as monolayers, may also lead to loss of the native cell phenotype coupled to the lack of interaction with other cell types and the natural extracellular matrix. For these reasons the biological relevance of the findings is reduced and more physiologically representative models are needed to overcome these limitations.

Based on our experimental results, a dual therapeutic approach could be explored in a clinical setting. The first strategy involves harnessing the regenerative potential of platelet-derived factors delivered as PL to promote the in vitro isolation and proliferation of Nucleus Pulposus-derived (NPd) cells obtained through enzymatic digestion. This method could also support the development of an allogeneic cell therapy for IVD regeneration. The second approach focuses on the direct in situ application of PRP, providing a simple and effective means to release platelet-derived bioactive molecules in vivo, to re-activate quiescent endogenous cells within the degenerated disc. This follows the fact that, although PRP can be used in settings, it represents a less controlled system, as it contains cellular debris and platelet aggregates that may interfere with cell culture conditions and data interpretation.

Moreover, under in vitro conditions, platelets are not activated in the same manner as in vivo. Therefore, platelet lysate is used in experiments to provide readily available platelet-derived factors, whereas for in vivo applications the use of PRP appears more appropriate, as it contains intact platelets capable of gradually releasing growth factors within the lesion microenvironment. This is supported by our in vitro findings, where platelet bioactive molecules induced the release of viable NP-derived (*NPwd*) cells from disc tissue fragments, suggesting a potential local biological treatment aimed at enhancing intrinsic repair mechanisms.

Declaration of competing interest

The authors declare that there are no conflicts of interest regarding the publication of this paper. No financial, personal, or professional relationships influenced the work described herein.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2026.101082.

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