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Tissue-Engineered Intervertebral Disc and Chondrogenesis Using Human Nucleus Pulposus Regulated through TGF-β1 in Platelet-Rich Plasma

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Human intervertebral disc (IVD) degeneration often initiated from the human nucleus pulposus (hNP) with aging leading to IVD destruction and extracellular matrix (ECM) depletion. Previously, we have successfully employed transforming growth factor-β1 (TGF-β1) to promote chondrogenesis of mesenchymal progenitor cells (MPCs) and immortalized human mesenchymal stem cells. In this study, we examine the role of TGF-β1 in platelet-rich plasma (PRP) on disc regeneration, including proliferation, redifferentiation, and the reconstitution of tissue-engineered NP. hNP cells were isolated from volunteers with different ages and cultured in the presence of PRP. We found that the most effective concentration for hNP proliferation was 1 ng/ml TGF-β1 in PRP, which was further applied in the following experiments. hNP cell proliferation in all age groups were increased time-dependently by PRP and cell morphologies showed aggregation. The mRNA of *Sox9*, *type II collagen*, and *aggrecan* were all significantly upregulated by PRP through RT-PCR. Glycosaminoglycan (GAG) accumulation reached the highest value at day 7 and continued to day 9 culture. PRP promoted NP regeneration via the Smad pathway was also determined and highly activated p-Smad2/3 at 30 min and continuously sustained to 120 min. Immunostaining of type II collagen indicates that PRP participates in chondrogenesis of tissue-engineered NP with collagen scaffolds. We concluded that growth factors in PRP can effectively react as a growth factor cocktail to induce hNP proliferation and differentiation, and also promote tissue-engineered NP formation. These findings are the first to demonstrate that PRP might be a therapeutic candidate for prevention of disc degeneration. J. Cell. Physiol. 209: 744–754, 2006. © 2006 Wiley-Liss, Inc.

Intervertebral disc (IVD) has a specific load-bearing organization which restricts extrinsic stresses and provides flexibility in the spine. It consists of three different chondrocytic tissues (from outer to inner region): annulus fibrosus (AF), transition zone (TZ), and nucleus pulposus (NP). Among these three IVD structures, NP tissue is the most critical to IVD physiology because of its mechanical properties (Oegema, 2002; Hunter et al., 2003). Water, proteoglycans, and collagen in the extracellular matrix (ECM) of NP tissue provide fluidity and viscoelasticity to the structure, acting as a shock absorber, maintaining tensile function, and protecting against compressive loads in IVDs (Bibby et al., 2001; Sato et al., 2001; Oegema, 2002; Melrose et al., 2003).

Low-back pain caused by disc degeneration usually occurs in the central NP, leading to disc height reduction and damage of adjacent spinal structures. It is a common disease in adult populations from 20 to 50 years of ages (Biering-Sorensen, 1982; Cole et al., 1985; Miller et al., 1988; Buckwalter, 1995). In parallel, ECM's degradation is also increased in NP of aged individuals (Jahnke and McDevitt, 1988; Sztrolovics et al., 1997). A tight collagen network with high proportion of proteoglycan content is found in younger discs and provides fluid flow (Pearce et al., 1989; Krajickova et al., 1995).

The notochord is the transitional structure from which IVDs form. Its maturation results from the formation of AF and NP tissues, in which cell-matrix and cell–cell interactions occur throughout this developing tissue. Notochordal cells gradually decrease during skeletal maturation, making nuclei pulposi nearly undetectable in the mature skeleton, which also indicates the very low recovery rate of NP cells (Wang et al., 2001). IVD cells comprise only 1% of the volume of the IVD, through they have a fundamental role on production of the ECM of the

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discs (Bibby et al., 2001). NP cells easily lose their phenotypes during in vitro cultures, leading to apoptosis (Urban and Maroudas, 1981; Ichimura et al., 1991; Roughley, 2004). Various growth factors that participate in notochord development were proved to promote NP cell differentiation and phenotype stabilization. Studies on growth factors such as transforming growth factor-β (TGF-β) superfamily (including TGF-β1, TGF-β2, TGF-β3, and TGF-β4), Insulin-like growth factor (IGF), and basic fibroblast growth factors (bFGFs) were demonstrated to stimulate NP and chondrocyte proteoglycan in vitro synthesis (Osada et al., 1996; Konttinen et al., 1999; Okuda et al., 2001). TGF-β and FGF induced more ^{35}S-sulfate incorporation into proteoglycan in the NP and the TZ (Thompson et al., 1991). Recombinant human bone morphogenetic protein-2 (rhBMP-2) has also shown to enhance disc matrix production and the chondrocytic phenotype of cultured rat IVD cells (Tim Yoon et al., 2003). IGF-I is a peptide known to be synthesized in disc cells and that affects their biological functions such as proteoglycan synthesis and chondrogenic specific gene expression (Okuda et al., 2001). Human NP exposure to IGF-I and platelet-derived growth factor (PDGF) has shown to prevents cell from apoptosis (Gruber et al., 2000).

Respective single recombinant growth factor has been tested in many studies with its effect found to be limited. Platelet-rich plasma (PRP) is extracted from the whole blood and contains mixed growth factors such as TGF-β1 and TGF-β2, vascular epithelial growth factor (VEGF), PDGF, and IGF, all of which are naturally released from platelets after being activated by calcium or thrombin (Tozum and Demiralp, 2003; Arpornmaeklong et al., 2004). Many reports showed that PRP can regulate the proliferation and differentiation of mesenchymal cells, including osteoblasts and fibroblasts, and also can induce bone matrix formation (Liu et al., 2002; Lucarelli et al., 2003; Arpornmaeklong et al., 2004; Kanno et al., 2005). Among all of the naturally released growth factors, TGF-β1 exists in the highest concentration and shows more important in PRP (Wadhwa et al., 1996; Weibrich et al., 2002), indicating that TGF-β1 can be recognized as the core ingredient and the indicator for applying PRP in these studies.

Replacement of damaged NP tissue is ideal for regenerative repair. Various biomaterials have been used in 3-dimensional (3D) cultures to construct tissue-engineered NP tissue. NP-like tissue was formed with a porous calcium polyphosphate substrate, and the NP phenotype was maintained over 6-week cultures (Seguin et al., 2004). Kotani et al. (2002) used a 3D fabric for an artificial IVD and showed that both its biomechanical and interface histological properties were conserved. Higher levels of aggrecan and type II collagen were preserved in NP cells re-encapsulated in alginate (Wang et al., 2001). These studies suggested that a 3D culture environment can improve NP cell regeneration.

Growth factors required for notochordal development are also formed in PRP, suggesting that such components are applicable in promoting disc regeneration. In this study, we created an NP culture system to determine the effects of PRP on proliferation and differentiation of hNP, and a collagen/PRP scaffold was also used to provide tissue-engineered NP constructs. We show here that PRP acts on human NP cells physiology and leads to obvious disc regeneration in 3D cultures. Moreover, PRP promotes hNP redifferentiation through TGF-β signaling pathway.

MATERIALS AND METHODS

Culture of human nucleus pulposus cells

Human nucleus pulposus (hNP) cells were collected from six healthy IVD donors aged 24, 33, 47, 50, 68, and 76 years and designated as 24hNP, 33hNP, 47hNP, 50hNP, 68hNP, and 76hNP, respectively. These volunteers provided informed consent for the use of their nucleus pulposus cells, as required by the Ethics Committee of Taipei Medical University Hospital. Normal NP tissue harvested aseptically from donors was minced into pieces in Hank's balanced salt solution (HBSS) (Gibco BRL, Grand Island, NY) with antibiotics. NP cells were then isolated from these slices in an enzymatic solution (0.4% collagenase and 0.04% pronase, purchased from Sigma, St. Louis, MO) for 4 h at 37°C. The cell suspension in the enzyme solution was filtered through a 40-μm nylon mesh (Falcon, NY), and then centrifuged at 1,800 rpm for 10 min, resuspended in Dulbecco's modified Eagle's medium (DMEM/F-12) (Gibco BRL) with 10% fetal bovine serum (FBS). Isolated NP cells were seeded into 10-cm tissue culture dishes and incubated at 37°C in 5% CO_2 before subsequent experiments. All expanded cells were stored in liquid nitrogen.

PRP preparation and TGF-β1 concentration evaluation

Human total blood was purchased from Taipei Blood Center and driven into MCS blood cell separation system (Haemonetics Corp., Braintree, MA). Bovine thrombin (100 IU bovine thrombin/150 ml PRP) was then added to the solution, shakes to remove the aggregated fibrin, and centrifuged for 6 min, 3,000 rpm at room temperature. Human PRP was prepared and stored at −20°C.

To confirm the consistency of PRP for in vitro utilization and to determine the most appropriate concentration for study, TGF-β1 was quantitatively analyzed using a Quantikine enzyme-linked immunosorbent assay (ELISA) kit (#DB100, R&D Diagnostics, Wiesbaden, Germany), and used as the core ingredient of PRP. A dilution series of TGF-β1 standards (#890207) was prepared in 100-μl volumes in 96-well microtiter plates coated with TGF-β1-receptor II. Since a large proportion of TGF-β1 is often present in a latent form, conversion of TGF-β1 to its active form was necessary to estimate total TGF-β1. The 0.1 ml PRP solution was then mixed with 0.1 ml 2.5 N

TABLE 1. Primer sequences for PCR products and their expected sizes

Specific gene	Primer sequence (5′ → 3′)	Product size (bp)
GAPDH	Sense: GCT CTC CAG AAC ATC ATC CCT GCC Antisense: CGT TGT CAT ACC AGG AAA TGA GCT T	346
SOX9	Sense: GGC AGC TGT GAA CTG GCC A Antisense: GCA CAC GGG GAA CTT GTC C	408
Type II collagen	Sense: CAC GCA GAA GTT CAC CAA GAA Antisense: CCT TGC TCC AGG GCC AGC	501
Aggrecan	Sense: TGA GAA GGG CTG GAA CAA GTA CC Antisense: GGA GGT GGT AAT TGC AGG GAA CA	350
Type I collagen	Sense: AGC GCT GGT TTC GAC TTC AGC TTC C Antisense: CAT CGG CAG GGT CGG AGC CCT	466

GAPDH, glyceraldehyde phosphate dehydrogenase.

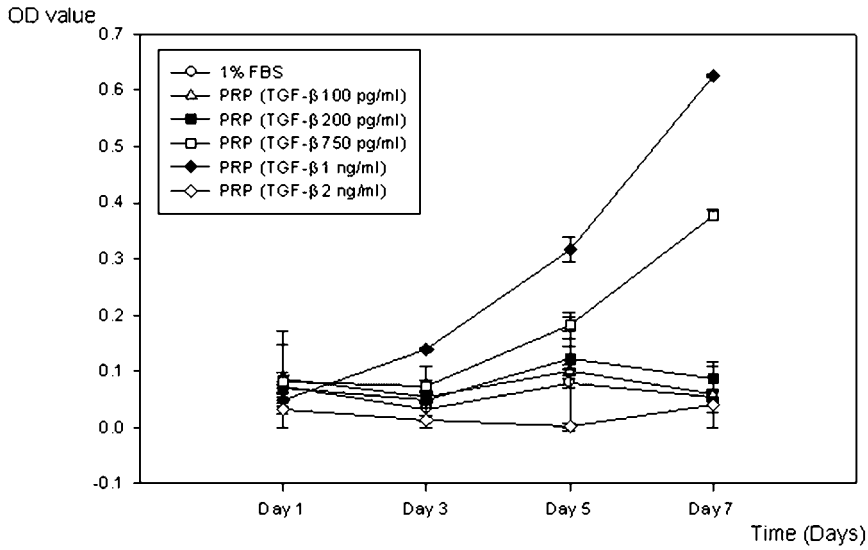

Fig. 1. Proliferation of 24hNP (human nucleus pulposus from a 24-year-old subject) cells in platelet-rich plasma (PRP) conditional medium. Cells were grown in various concentrations of PRP medium, and cell proliferation was determined by the MTT assay to obtain the optimal concentration of transforming growth factor-β1 (TGF-β1) in PRP. Cells were cultured for 7 days, and the results are shown as the mean ± SD for three separate replicates.

acetic acid/10 M urea, incubated at room temperature for 10 min, and neutralized by an addition of 0.1 ml of 2.7 N NaOH/1 M HEPES (*N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]; Sigma) (#H-7523).

Conditional culture medium preparation

Various PRP concentrations according to ELISA results of TGF-β1 were dissolved in 1% FBS containing DMEM/F12 medium (for basal cell maintenance) and filtered through a 0.22-μm-pore filter. PRP-containing media were changed every 2 days for the proliferation assays to detect the optimal PRP concentration.

MTT assay

Cell proliferation was determined using the cell proliferation reagent tetrazolium salt MTT [(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), Roche, Mannheim, Germany)] assay. hNP cells from different-aged subjects were seeded into 96-well plate at a density of 2×10^4 cells/ml and treated with or without PRP, while the experimental controls were cultured in 1% FBS containing DMEM/F-12. The MTT reagent was added into each well on days 1, 3, 5, and 7. OD values (OD 595 ~ OD 690) were analyzed 4 h after the MTT reaction using Multiskan PC (Thermo Labsystem, Franklin, MA) and cell survival curves were then plotted against time.

RNA extraction and semiquantificative reverse-transcription PCR

Total RNA harvested from subconfluent monolayer cultures was extracted using the TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and subjected to reverse transcription followed by PCR amplification of specific expressed genes. Reverse transcription was performed with SuperScript™ III (Invitrogen Life Technologies) and an Oligo $d(T)_{12-18}$ primer. Four micrograms of RNA was added into a final volume of 21-μl solution containing 10 mM dNTP mix, 10× RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNase inhibitor and RNase H. Six micrograms of RT product was used to the PCR amplification in a final volume of 50 μl containing 2.5 mM dNTP, 25 mM $MgCl_2$, upstream/downstream primers (see Table 1), and *Taq* DNA polymerase (Invitrogen Life Technologies). Following an initial denaturation at 95°C for 5 min, the DNA was amplified in the Touchgene Gradient (Techne, Cambridge, UK) using 35 cycles of 1 min at 94°C for denaturation and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 5 min. The annealing temperatures differed depending on the specific genes. PCR products were then run on 1% agarose gels (Agarose I, AMRESCO, Solon, OH) and visualized with EtBr staining. Images were analyzed using FloGel-I (Fluorescent Gel Image System, TOP BIO Co., Taipei, Taiwan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

GAG accumulation analysis

Confluent cells were fixed in 3.7% formaldehyde and stained with 0.05% Alcian blue in 0.018 M acetic acid containing guanidine-HCl and Triton X-100 for 3 h. The fixed cells were then washed in 0.018 M acetic acid, followed by gradual elution

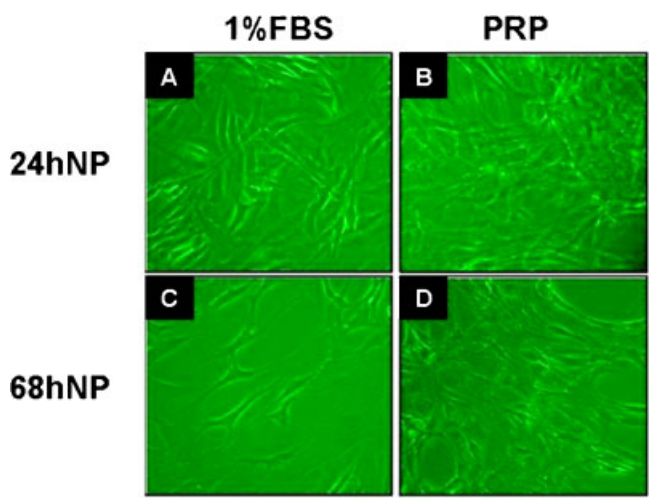

Fig. 2. Cell morphologies of 24hNP and 68hNP (human nucleus pulposus from a 68-year-old subject) cells cultured in conditional medium. Phase-contrast photomicrographs showed morphological changes of human nucleus pulposus (hNP) from different-aged subjects grown in a monolayer after 5 days of cultivation. Subparts **A** and **B** are 24hNP cells and Subparts **C** and **D** are 68hNP cells cultured in 1% FBS without and with PRP containing medium, respectively (×200). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of the bound dye by dissociated solution. Then the eluted solutions were examined using a spectrophotometer (Thermo Labsystem, Finland). The dissociated solution was used as the blank group.

Western blotting

Cells were trypsinized and dissolved in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% DOC, 1% NP-40, and 0.1% SDS), and then centrifuged for 15 min at 12,000 rpm and 4°C. The upper fluid, containing total protein, was extracted. The extracted protein was denatured for 5 min at 95°C and loaded on a 7% SDS–PAGE gel. The membrane was blocked overnight in blocking-buffer and incubated with anti-Phospho-Smad2/3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by washing with PBS-T. The secondary antibody (peroxidase-conjugated affinpure goat anti-mouse IgG, Jackson ImmunoResearch, West Grove, PA) was added for 1 h. Bands were made visible on film (Hyperfilm ECL, Amersham Pharmacia, Braunschweig, Germany) using the ECL plus-kit (Amersham Pharmacia).

Three-dimensional (3D) constructs in collagen/PRP scaffolds

hNP cells were expanded and then embedded at a density of 1×10^6 cells/ml in 3D matrices suspended in DMEM/F12 containing 2 mg/ml purified type I collagen, 2 mg/ml purified type I + type II collagen mixture, or purified collagens mixed with PRP, respectively. Types I and II collagens were prepared

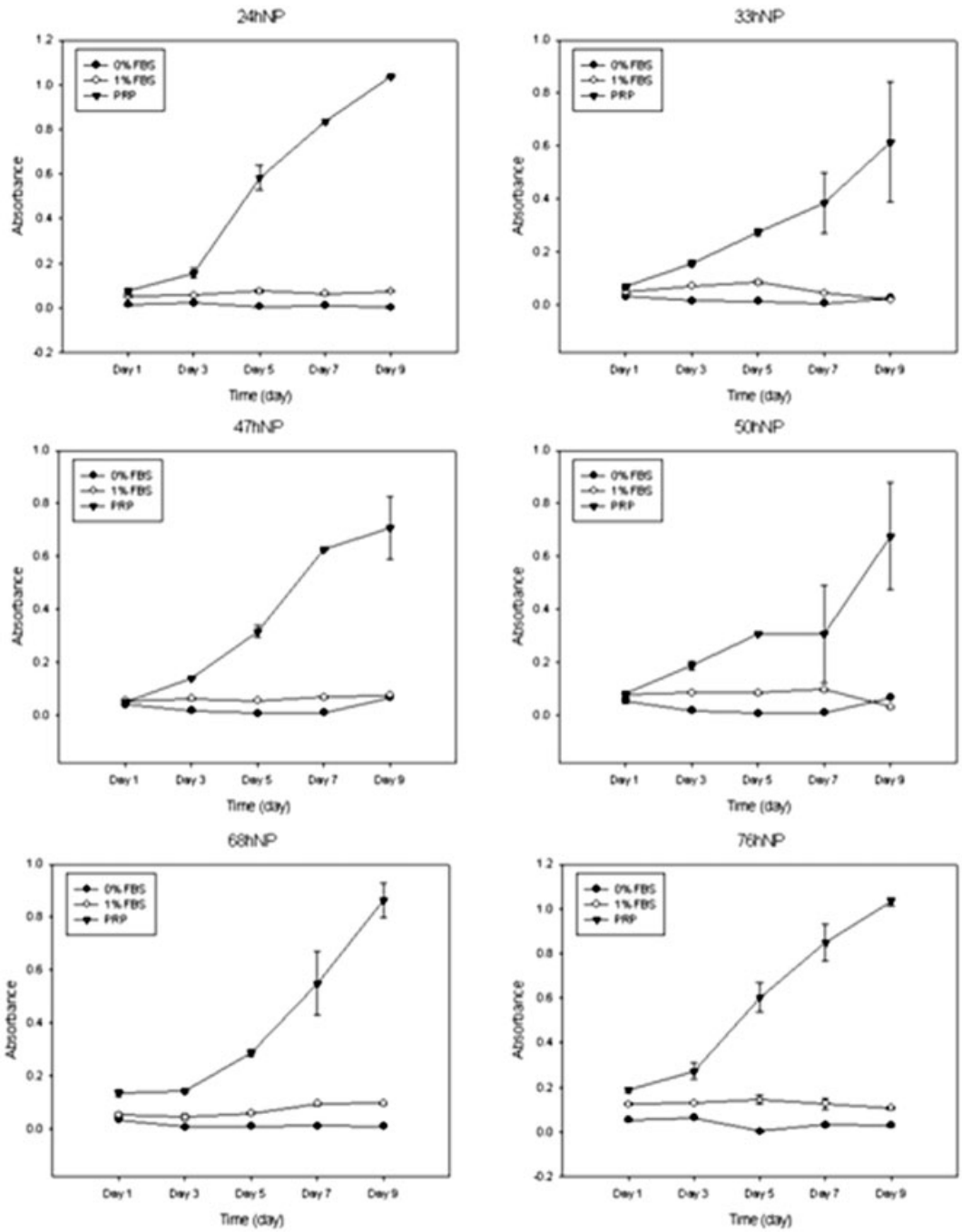

Fig. 3. Effects of PRP on hNP cell proliferation from different-aged subjects in monolayer cultures. Induction of proliferation was obvious for all ages, and results are given as the mean ± SD for three independently repeated experiments.

as previously described in this laboratory (Lai et al., 2003). Cells/collagen constructs were then cultured in DMEM/F12 medium containing 10% FBS as the control group while cells/collagen/PRP constructs were cultured in medium containing PRP to detect the induced differentiation effects of PRP. Constructs were cultured in a 37°C, 5% CO_2 incubator for 4 weeks and then histologically analyzed with hemotoxylin and eosin (H&E) staining, immunohistochemistry staining (IHC) of type II collagen, and Alcian blue staining. Mouse anti-human type II collagen monoclonal antibody (Chemicon International, Temecula, CA) used in IHC only reacts with human and bovine type II collagen.

Apoptosis assay

The apoptosis assay was performed to measure apoptotic cells using FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). hNP cells were treated with IL-1β (10 ng/ml) for 48 h, and then PRP was added for 24 h. The fixed hNP cells were incubated at 37°C for 1 h in PBS containing 50 μg/ml RNase A and 20 μg/ml propidium iodide. A minimum of 10,000 cells was analyzed using Cellquest Software (Becton Dickinson Immunocytometry Systems). Results were evaluated and plotted to detect the cell cycle phases.

Statistical analysis

The results of all experiments are shown as the mean ± standard deviation (SD). Differences of mRNA expression and GAG accumulation in the response to PRP were compared to 1% FBS medium and statistically evaluated using the paired *t*-test in each group. Flow cytometric quantification was compared by using Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

PRP extracted from total blood contains various growth factors, in which TGF-β1 is the highest content and has been recognized as the core ingredient for PRP (Landesberg et al., 2000; Weibrich et al., 2002). TGF-β1 has also been reported to be the major growth factor participating in the chondrogenic process of mesenchymal stem cells (MSCs) for chondrogenesis (O'Driscoll and Fitzsimmons, 2001) and used as an indicator for applying PRP in the following study. We therefore investigated the effects of PRP, as a growth factor cocktail, on tissue engineering and chondrogenesis of the hNP. hNP cells were cultured in DMEM/F12 with 1% FBS and various concentration of PRP designated by the concentration of TGF-β1. In parallel, in all time-course studies, cells were also maintained in DMEM/F12 with 1% FBS only (basal medium) and used as a control group. Using these conditions, we determined whether PRP treatment induced redifferentiation of hNP cells by characterizing cell proliferation and chondrogenic phenotype, such as chondrogenic-specific mRNA expressions and proteoglycan synthesis.

Proliferation assay of hNP cells for optimal PRP

To determine the effect of PRP on cell proliferation, a time-course experiment of NP cell proliferation was performed using the MTT assay in 96-well plates. The hNP cells were isolated aseptically from the 24-year-old volunteer (24hNP). The time-response results in

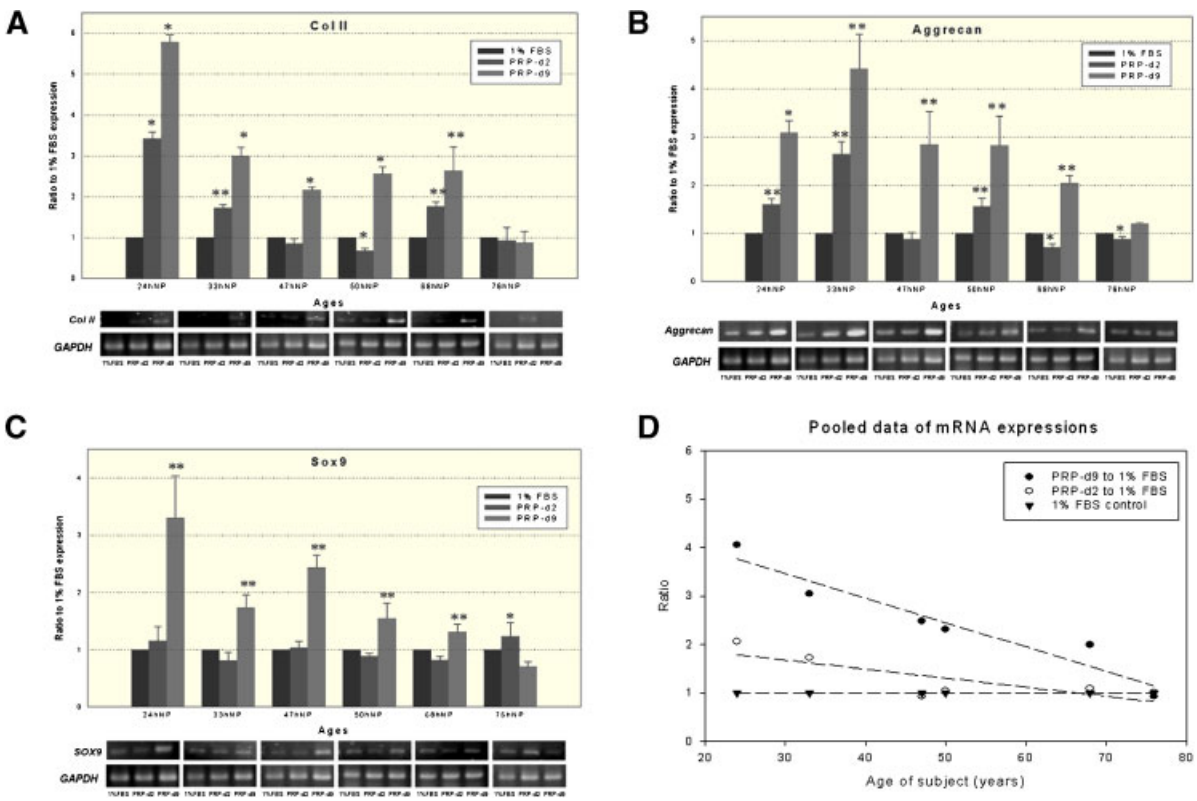

Fig. 4. Chondrogenic-specific mRNA expression enhancement of hNP cells from different-aged subjects with PRP treatment. Semi-quantitative RT-PCR results of *type II collagen* (*ColII*) mRNA expression (**A**); *aggrecan* mRNA expression (**B**); and *Sox9* mRNA expressions (**C**). Respective expressions were normalized to glyceraldehyde phosphate dehydrogenase (*GAPDH*) mRNA levels. The normalized values of PRP-d2 and PRP-d9 were then normalized to their respective 1% FBS (1% FBS was set as ratio = 1) to compare the differences between groups and results are shown as the mean ± SD for three independent experimental cultures. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the value in cells cultured in 1% FBS medium in the independent group using paired *t*-test. Response effect of specific mRNA expressions during 2- and 9-day PRP treatments from pooling together the RT-PCR results (**D**). The ratio of PRP-d9 to 1% FBS expressed a significant increase in all ages.

Figure 1 show that on day 7, a substantial concentration effect of TGF-β1 was dose-dependently evident among the concentrations of 100, 200, and 750 pg/ml, and 1 ng/ml. However, a significant inverse concentration effect was observed when TGF-β1 reached 2 ng/ml, in which the cell numbers showed no increase in the 7-day culture, nearly similar to those cultured in 1% FBS-containing medium. The results indicated that 1 ng/ml TGF-β1 in PRP appeared to be the optimal concentration for hNP proliferation and was applied in the following experiments.

Distinct differences in cell morphologies were also determined for different culture conditions. hNP cells from the 24-year-old (24hNP) and 68-year-old (68hNP) volunteers were seeded on a monolayer at 5×10^5 cells per dish and photographed on day 5. When cultured in control medium (1% FBS in DMEM/F12), both 24hNP (Fig. 2A) and 68hNP (Fig. 2C) cells became elongated and irregularly ridged, and both types of cell also showed lower survival rates. However, when cultured in PRP-conditioned medium (Fig. 2B,D), the 24hNP and 68hNP cell morphologies appeared smaller and more spindle-like and were organized into aggregates.

Proliferation of human nucleus pulposus from different-aged subjects by PRP

Based on the results in Figure 1 showing that 1 ng/ml TGF-β1 in PRP was the optimal concentration for cell proliferation of 24hNP, the cell proliferation of hNP from volunteers of other ages of 33, 47, 50, 68, and 76 years was examined in this PRP condition. Figure 3 shows that at all ages, the proliferation of hNP cells obviously increased with PRP (1 ng/ml TGF-β1) and was about 7–11 times higher than those cultured in 1%FBS or serum-free medium. The induction of cell proliferation was obvious from day 3 and reached a peak on day 9.

Induction of chondrogenic-specific genes in hNP cells by PRP

To determine the chondrogenic-specific gene expression of hNP in response to PRP, different ages of hNP cells cultured in PRP medium for 2 (PRP-d2) and 9 days

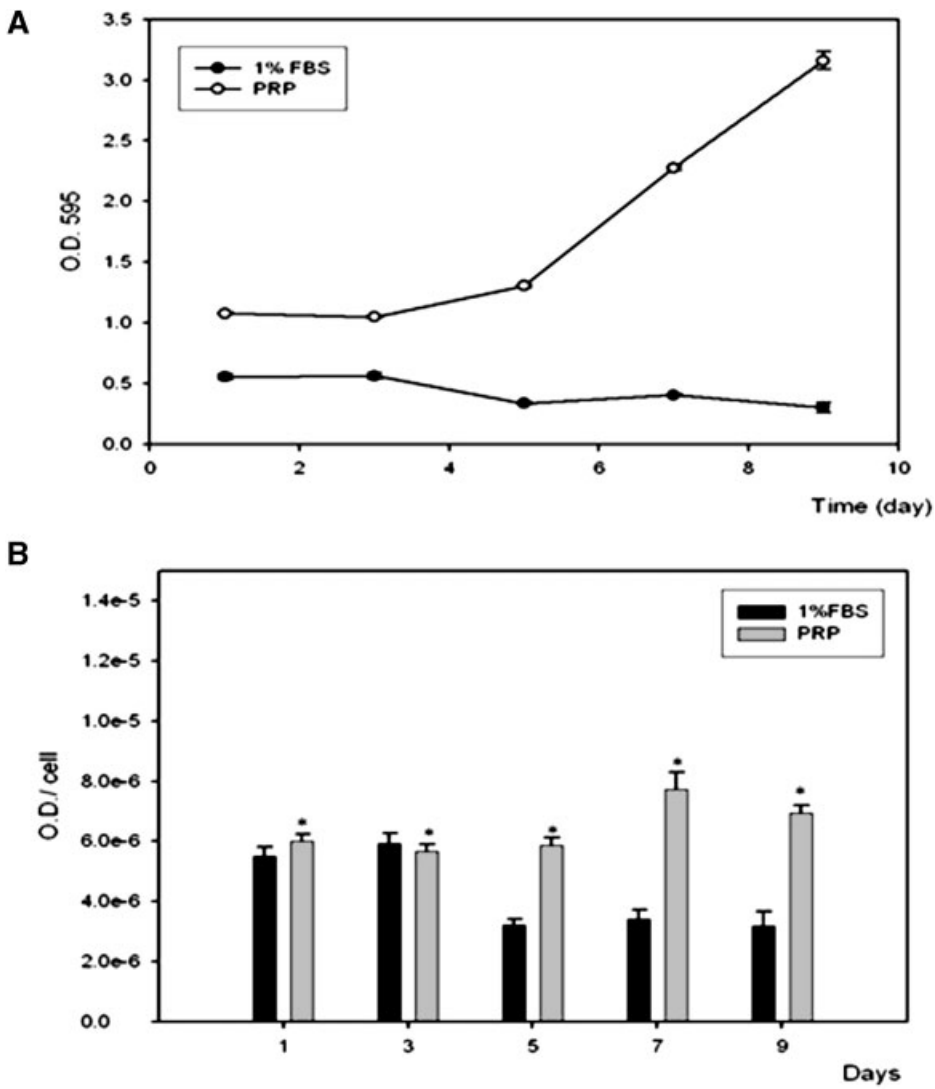

Fig. 5. GAG accumulation of hNP cells in the presence of 1% FBS or PRP for 9 days. **A**: Time-course assay of GAG accumulation of 24hNP was preserved by incubating with PRP (TGF-β1 1 ng/ml). **B**: OD value of respective 24hNP cell. Values were measured using a spectrophotometer, and results were normalized to total cell numbers and shown as three independent experimental cultures. $^*P < 0.05$ as PRP group compared to 1% FBS control group at the same time point, using paired *t*-test.

(PRP-d9) were compared with those cultured in 1% FBS medium. RT-PCR results showed that the expressions of chondrogenic genes, *type II collagen*, and *aggrecan* were all significantly increased in the 9-day treatment with PRP (Fig. 4A,B). The transcription factor of *type II collagen*, *Sox9*, was also apparently induced (Fig. 4C). After normalization with the gene expression in 1% FBS of the respective groups, the expressions of the three marker genes were pooled together and showed an inverse function with the volunteer's age (Fig. 4D). The induction rate of the mRNA level was higher in cells cultured in PRP for 9 days.

PRP accelerates proteoglycan matrix accumulation in cultured cells

To determine the proteoglycan accumulation in hNP cells, we next examined the effect of PRP on GAG deposition by Alcian blue staining. The GAG is the most abundant proteoglycan matrix synthesized from hNP cells. Alcian blue-positive staining appeared at day 5, whereas in 1% FBS groups it did not appeared during 9-day cultivation (Fig. 5A). To determine whether hNP cells lose their proteoglycan synthetic abilities during extended cultures, a time-dependent effect of PRP on NP cells was detected. As shown in Figure 5B, ratio of secreted GAG decreased after day 5 while cells cultured in 1% FBS medium. Relative GAG accumulation of human NP cells cultured in PRP reached the highest value at day 7 and continued to day 9 compared to 1% FBS medium. GAG synthesized from cells showed a significantly induction with 9-day PRP treatment.

PRP activates phosphorylation of Smad2/3 in hNP cells

Smad2/3 has been presumed to be a key regulator protein specific to TGF-β1 activation affecting chondrogenic genes (Furumatsu et al., 2005). To assess how PRP stimulates human NP chondrogenesis via the Smad pathway, phosphorylated Smad2/3 (p-Smad2/3) was detected through Western Blot analysis. TGF-β1 (10 ng/ml, as a positive control) only treatment activated Smad2/3 phosphorylation after 30 min, but diminished the phosphorylation after 120 min, while PRP (1 ng/ml TGF-β1) highly activated p-Smad2/3 at 30 min and continuously sustained to 120 min (Fig. 6A). Expressions of p-Smad2/3 were normalized and compared to the control group (Fig. 6B). Those results are consistent with our hypothesis that TGF-β1 of PRP plays the major role of promoting hNP chondrogenesis.

Gross morphology and histological analysis of engineered NP tissue

To construct tissue-engineered NP, the purified type I and type II collagens (from rat tail tendon and porcine cartilage, respectively) were mixed with PRP to perform the 3D cultures (Fig. 7I). Following 4 weeks of 3D cultures, chondrogenic markers were histologically characterized by hematoxylin and eosin (H&E) staining, type II collagen by immunostaining, and secreted proteoglycans by Alcian blue staining (Fig. 7II). hNP cells were maintained in purified type I collagen (Fig. 7II-A), type I + type II collagen (Fig. 7II-B), type I collagen + PRP (Fig. 7II-C), and type I + type II collagen + PRP (Fig. 7II-D), respectively. Constructs with PRP (Fig. 7II-C, -D) showed a shiny appearance and contributed to the significantly increase in size compared to the collagen-only groups (Fig. 7II-A, -B). Interestingly, H&E-stained constructs showed chondrocyte-like rounded cells which were evenly distributed in matrices in all groups (Fig. 7II-E to -H).

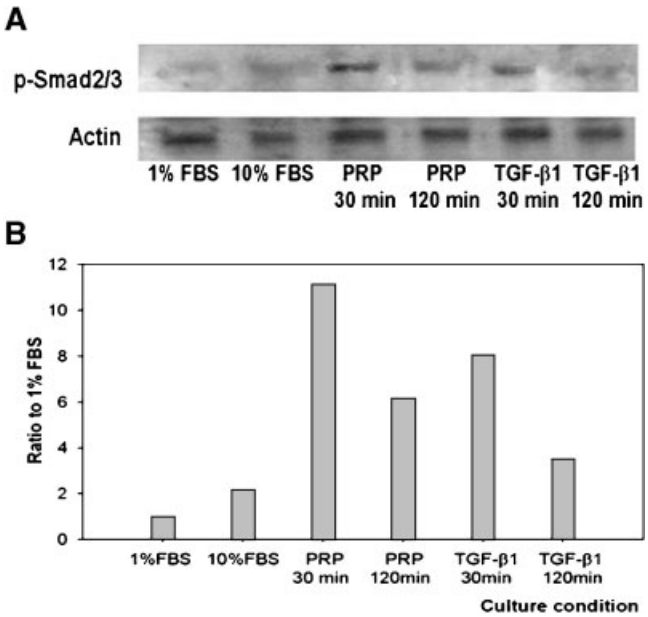

Fig. 6. Phosphorylation of Smad2/3 by PRP in hNP cells. **A**: Phosphorylated Smad2/3 (p-Smad2/3) was activated with 30-min treatment with PRP, and expression continued to 120 min. p-Smad2/3 was also detected in TGF-β1 treatment group which was used as a positive control. **B**: The p-Smad2/3 was first normalized with their internal control actin in individual group. The expression values of p-Smad2/3 to actin of last five groups were then normalized to their respective 1% FBS (1% FBS was set as ratio = 1).

Anti-human type II collagen mAb (Chemicon International) used in IHC staining reacts only with human and bovine type II collagen but not cross reacts with that from porcine cartilage in scaffold. To detect the major chondrogenic marker, type II collagen, IHC staining was performed, and positive staining was more intense and extensive in hNP cells cultured in PRP-treated cultures (Fig. 7II-K, -L), compared to their collagen control groups (Fig. 7II-I, -J). PRP-treated cultures revealed more positive stained regions in the matrix nearby cells at higher magnifications of IHC staining (Fig. 7O,P). Additionally, Alcian blue staining was used to confirm results of *aggrecan* mRNA expression and GAG accumulations in monolayer cultures. Histological results showed that abundant proteoglycan matrix accumulated in PRP constructs (Fig. 7S,T). Slight signals were observed in their respective controls (Fig. 7Q,R). Compared to the collagen groups (Fig. 7U,V), PRP groups exhibited a marked upregulation in the level of positive staining intensity, especially of cells surrounding the matrix (Fig. 7W,X), which indicated cartilage-specific proteoglycan production. These results were supported by their mRNA expressions and Alcian blue staining of monolayer cultures, which indicate that PRP also participates in chondrogenic differentiation in 3D cultures.

Anti-apoptic effect of PRP

To determine the anti-apoptotic effects by PRP, the hNP cells were treated with 10 ng/ml IL-1β for 24 h and then with PRP (TGF-β1 1 ng/ml) for 24 h. Apoptotic cells were defined as cells percentages at the sub-G1 stage by examining cell cycle phase using flow cytometry. The results showed that apoptotic cell numbers were

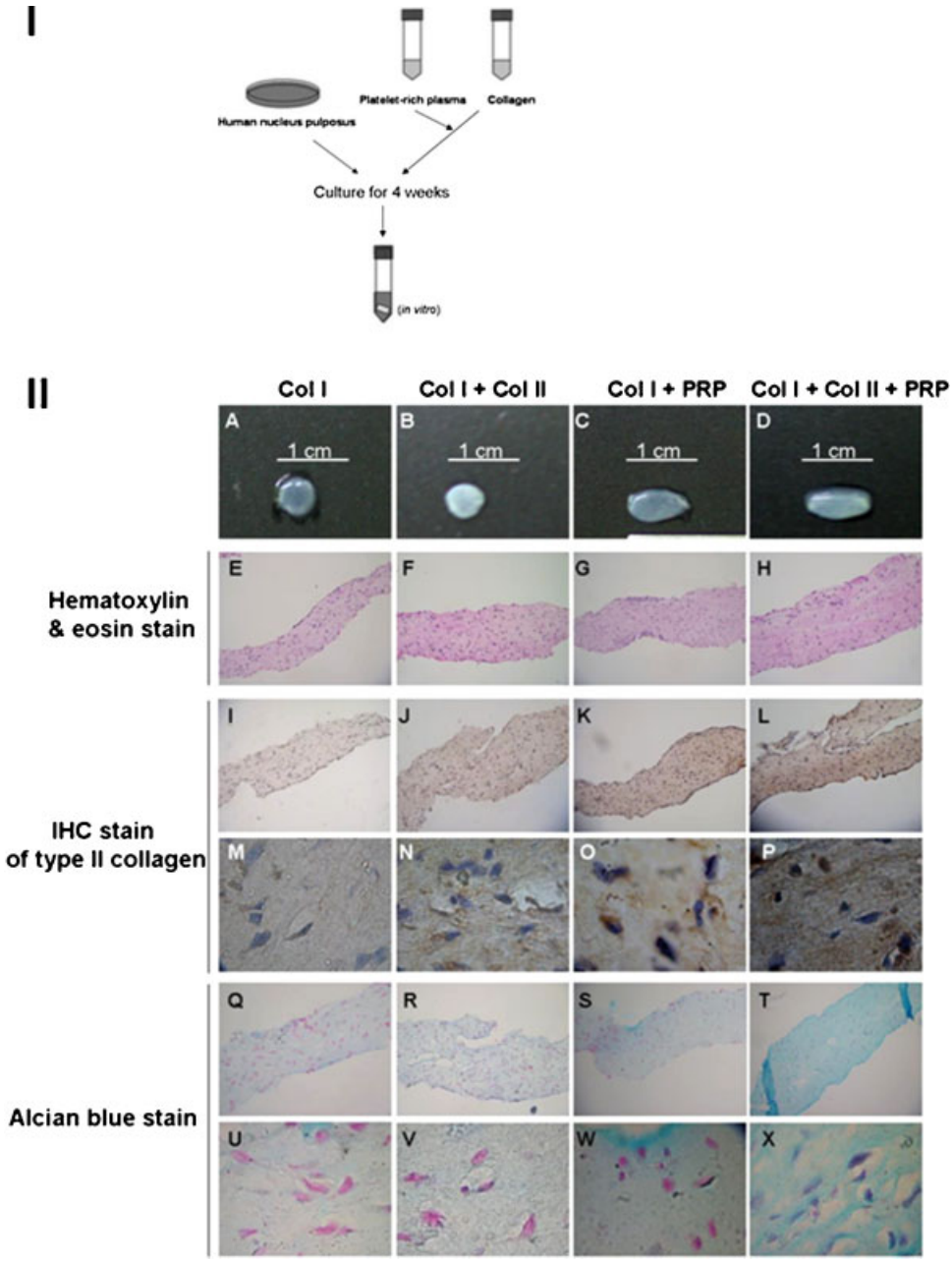

Fig. 7. Immunohistochemistry (IHC) of three-dimensional (3D) constructs of hNP cells cultured in purified collagen/PRP scaffolds. All constructs were examined and histologically analyzed in week 4. (I) Schematic diagram of in vitro 3D culture. (II) Morphologies of 3D cultures of 24hNP cells grown in purified (**A**) type I collagen, (**B**) type I + type II collagen, (**C**) type I collagen mixed with PRP, and (**D**) type I + type II collagen mixed with PRP scaffolds, respectively. **E**–**H**: hematoxylin and eosin staining (100×); **I**–**L** (100×) and **M**–**P** (1,000×), IHC staining of type II collagen; **Q**–**T** (100×) and **U**–**X** (1,000×), Alcian blue staining of secreted proteoglycans. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

dramatically decreased in PRP group, compared with the IL-1β group ($^{*}P < 0.05$) (Fig. 8).

DISCUSSION

The aim of this study was to examine the role of TGF-β1 in PRP in disc regeneration, including proliferation, redifferentiation, and the reconstitution of tissue-engineered NP. In our previous studies, we have successfully employed rhTGF-β1 to promote chondrogenic differentiation of primary mesenchymal progenitors cells (MPCs) and immortalized human mesenchymal stem cells (hMSCs) (Hung et al., 2004; Chen et al., 2005a). Human recombinant growth factor triggered-disc regeneration was limited to be applied in clinical treatments even though the effective results have been detected during in vitro studies (Konttinen et al., 1999; Gruber et al., 2000; Tim Yoon et al., 2003). In contrast to commercial recombinant growth factors, PRP can be viewed as a good natural source of growth factors since it can be easily purified from total blood avoiding immune responses and is convenient for clinical therapy. Hence, it is important to define the naturally released growth factors from PRP through TGF-β1 in reconstitution of tissue-engineered NP.

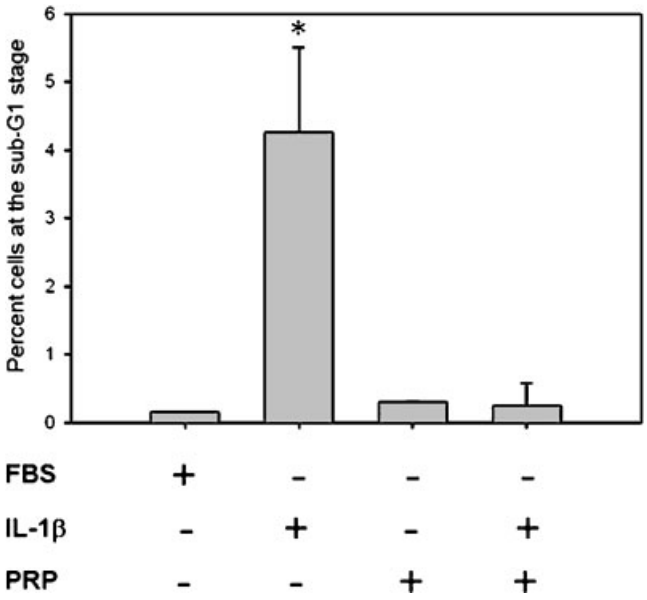

Fig. 8. Flow cytometric quantification of sub-G1 percentages of hNP cells in PRP culture. Cells were incubated with FBS, IL-1β (10 ng/ml), and PRP, respectively. Following incubation for 72 h, the proportion of sub-G1 was measured by flow cytometry with PI staining and values are representative of three independent experimental cultures. *$P < 0.05$ as compared to the FBS control group, using Student's t-test.

We first defined the TGF-β1 concentration in purified PRP using an ELISA kit that was more accurately for confirming the PRP quantity. It also helped to control the stable reproductive results of repeated experiment groups. One nanograms per milliliter of TGF-β1 in PRP was selected as the optimal concentration for hNP proliferation. The increased proliferation rates of hNP by PRP were noted in a wide range of subject ages as a time-dependent manner (Fig. 3). Our results suggested that PRP might be a mitogen for hNP cells, and were supported by other studies from different cell types (Liu et al., 2002; Lucarelli et al., 2003; Arpornmaeklong et al., 2004; Gaissmaier et al., 2005). Previous studies showed that the decreasing number of disc cells in an aging disc is due to cell apoptosis (Gruber et al., 2000), however, the PRP could significantly rescue IL-1β-induced hNP cell apoptosis (Fig. 8), and DNA contents of the S and G2/M phases were also increased in PRP-treated groups (data not shown). Similar growth factor approach was applied in several recent studies for antiapoptotic pathways of IVD cell with PDGF and IGF-I (Romashkova and Makarov, 1999; Gruber et al., 2000).

Disc degeneration occurs from the chondrocytic cells of NP with increasing age while the chondrogenic mRNA also decreased with aging (Hunter et al., 2003; Roughley, 2004). mRNA expression levels of specific genes such as *type II collagen* and *aggrecan* were compared between cells from 24- and 68-year-old volunteers, and the expressions of both genes were significantly decreased in the hNP cells from older subjects (data not shown). It was reported that proteoglycan synthesis in disc cells also decreased in old rabbits compared to young ones (Sato et al., 2001). Proteinase and aggrecanase cleavage of the aggrecan core protein during aging was reported to result in fragments enriched in the CS1 domain or the G1 domain, causing ECM depletion in aging disc cells (Sztrolovics et al., 1997; Roughley et al., 2002).

As the initial step of our studies, we assessed whether the PRP defined by TGF-β1 is critical to cause cell physiological reactions. In the presence of PRP, hNP expressed morphological changes and formed aggregates (Fig. 2), indicating the upregulated chondrocyte differentiation phenotypes (Tim Yoon et al., 2003). Subsequently, quantitative RT-PCR was performed to detect the differentiation-associated mRNA expression. The transcription factor, Sox9, plays an important role in regulating *type II collagen* and *aggrecan* by binding their promoters which leads to chondrogenesis (Bell et al., 1997; Lefebvre et al., 1997; Sekiya et al., 2000). Our results showed that PRP stimulated a significant increase in *Sox9* mRNA in all ages of donors, especially in the 24hNP (Fig. 4C). Similar tendencies for *Sox9* were also observed for the downstream *type II collagen* and *aggrecan* mRNA expressions (Fig. 4A,B). Cells in response to PRP showed significant upregulation in time-dependent manner. It was noted that PRP showed less influence on chondrogenic mRNA of cells from the 76-year-old donor (Fig. 4D).

Proteoglycan synthesis in disc cells decreasing with aging has also been reported (Sztrolovics et al., 1997; Roughley et al., 2002; Roughley, 2004). The Alcian blue staining results showed that GAG expressions in hNP cells exposed to PRP were significantly upregulated in a time-dependent manner (Fig. 5A). This also suggested that PRP maintained a stable proteoglycan synthetic potential of respective hNP cells (Fig. 5B). Activation of phosphorylated Smad2/3 was specified as the TGF-β1-induced pathway in development, tissue homeostasis, and to promote chondrogenic gene upregulation, such as *Sox9*, *type II collagen*, and *aggrecan* (Fig. 9) (Miyazawa et al., 2002; Furumatsu et al., 2005; Lin et al., 2005). Results of Western blotting were consistent with our hypothesis that p-Smad2/3 was activated after PRP treatment even for 120 min (Fig. 6A,B), and that PRP could induce greater Smad2/3 phosphorylation than TGF-β1 only.

Previously, it was shown that 3D culture was used to maintain chondrocyte phenotypes and physiology (Stern et al., 2000; Sato et al., 2001; Sun et al., 2001; Yung Lee et al., 2001), and biocompatible matrices were critical for cell survival in tissue-engineered constructs. In mature hNP tissues, type I and type II collagen were the major constituents to form abundant ECM (Antoniou et al., 1996; Alini et al., 2003). In our previous studies, purified type I collagen was introduced in tissue-engineered cartilage with immortalized chondrocytes, and purified type I and type II collagens were also

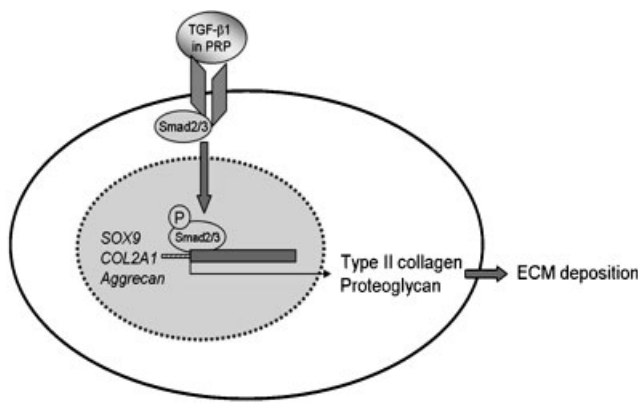

Fig. 9. Schematic model illustrating PRP-induced hNP differentiation via the Smad regulator protein. The Smad pathway was activated, and chondrogenic genes and synthetic proteins were also upregulated with PRP treatments. Therefore, TGF-β1 in PRP plays a potential role in regulating hNP chondrogenesis.

demonstrated to induce chondrogenesis of MPCs (Chen et al., 2005a,b). While PRP mixed with collagen scaffolds, the secreted type II collagen was obviously positively stained by IHC, demonstrating that PRP promoted specific protein synthesis in collagen scaffold (Fig. 7II). GAG accumulations were also upregulated in 3D cultures of PRP groups. Histological results were supported by cellular assays of RT-PCR and Alcian blue staining, and chondrocytic phenotype expressions in 4 weeks of PRP cultivation.

Growth factors such as TGF-β1 and IGF-I are main constituents of PRP, and their effects on disc physiology have been previously reported (Osada et al., 1996; Konttinen et al., 1999; Matsunaga et al., 2003). However, membrane-bounded receptors related to aging of the health of donors are also another factor of concern that may interfere with cellular responses. Alterations of growth factor receptors on cell membrane may be involved in aging. TGF-β1 and its receptors are abundant in young SAM mice but are depleted during the aging process (Matsunaga et al., 2003). The IGF-I receptor in the rabbit NP is downregulated in the late stages of aging (Okuda et al., 2001). Therefore, receptors decreasing with aging might be very important in elucidating the limitations of the influence growth factors of PRP on NP cells from the elder subjects in our studies. Since onset age for disc degeneration in human is around 30–50 years (Hunter et al., 2003), PRP may be a therapeutic substrate with great potential for preventing further disc destruction.

In conclusion, we believe that the current study is the first to apply PRP for chondrogenic differentiation in disc regeneration. Our results demonstrated a schematic model of PRP promoting mRNA expression, type II collagen, and ECM synthesis via the Smad signaling pathway, which was specifically activated by TGF-β1 in PRP (Fig. 9), promoting the proliferate rate of the hNP cells. It is also conceivable that growth factors included in PRP react cooperatively in a growth factor cocktail to preserve hNP differentiation, which is essential for the clinical application of IVD regeneration in the future.

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