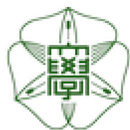


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## Summary of the dissertation

### Disease Modifying Osteoarthritic Drug, Pentosan Polysulfate Sodium Modulates Cytokine-Induced Osteoarthritic Changes and Promotes Articular Cartilage Tissue Regeneration *in vitro*

（変形性関節症病態修飾薬である多硫酸ペントサンは、関節軟骨におけるサイトカイン誘導性関節症病態を変化させ、培養関節軟骨の再生を刺激する）

Pentosan polysulfate (PPS) is a semi-synthetic sulfated polysaccharide derived from wood of beech plant, *Fagus sylvatica* that is available for the relief of various medical conditions including thrombi and interstitial cystitis in humans, and osteoarthritis (OA) in dogs and horses. Although the mechanism of action of PPS remains to be fully elucidated, it has been demonstrated to have a positive anabolic effect on articular chondrocytes, reduce cartilage destruction by inhibiting the synthesis and activity of cytokine-driven catabolic mediators of OA, and to significantly increase some anti-inflammatory cytokines, and was recently shown to promote chondrogenic differentiation and proliferation of human bone marrow-derived mesenchymal precursor cells (MPCs). Based on these attributes, PPS has long been considered as a prospective disease modifying osteoarthritic drug (DMOAD) although until the findings of the studies presented in this dissertation, its effects on some novel therapeutic targets of OA such as inducible nitric oxide synthase (iNOS), c-Jun and hypoxia inducible factor-alpha (HIF-α) isoforms remained unknown. On the other hand, bone marrow-derived mesenchymal stem cells (BMSCs) are considered a potential alternative source of differentiated chondrocytes for cartilage tissue regeneration and repair of small OA cartilage defects. Defining *in vitro* culture conditions to differentiate BMSCs as well as redifferentiating the phenotype of dedifferentiated

articular chondrocytes into functional and phenotypically stable chondrogenic cells prior to implantation is the current focus for successful cartilage tissue engineering. Therefore, the present dissertation investigated the effects of PPS on iNOS, c-Jun and HIF-α isoforms recently identified as novel potential targets for the treatment of OA, and to evaluate its chondroinductive potential to promote the redifferentiation of dedifferentiated monolayer expanded articular chondrocytes as well as chondrogenic differentiation of cBMSCs intended for cartilage tissue regeneration *in vitro*. The major findings of the dissertation can be summarized as follows;

1. Interleukin-1 beta (IL-1β)-induced iNOS, c-Jun, HIF-1α and HIF-2α mRNA expression in first passage (P1) canine articular chondrocytes (CACs). However, the preincubation of P1 CACs with PPS significantly inhibited ($P < 0.05$) IL-1β-induced iNOS, c-Jun and HIF-1α mRNA upregulation with minimal inhibitory effect observed on IL-1β-induced HIF-2α mRNA upregulation. Furthermore, normal CACs demonstrated recalcitrance to single IL-1β, tumor necrosis factor-alpha (TNF-α) or lipopolysaccharide (LPS)-induction of iNOS protein expression including to a combination of IL-1β + TNF-α, IL-1β + LPS except for TNF-α + LPS and IL-1β + TNF-α + LPS suggestive of a protective mechanism from iNOS detrimental effects on OA progression. Preincubation of CACs with PPS significantly abrogated IL-1β + TNF-α + LPS-induced iNOS protein expression. The results also confirmed that PPS colocalized with the transcriptional factors, NF-κB p65 and c-Jun reducing their nuclear translocation and localization activity in IL-1-induced CACs. These results demonstrated for the first time that PPS is a novel inhibitor of IL-1β-induced iNOS, c-Jun, and HIF-1α mRNA upregulation, and iNOS protein-induction and thus may translate to its beneficial effects in

treatment OA and prevention of disease progression.

2. For successful cartilage tissue regeneration and repair of OA defects by autologous chondrocyte transplantation (ACT), redifferentiation of dedifferentiated articular chondrocytes following *in vitro* monolayer expansion has long been proposed as the best hope for returning chondrocytes to their native articular cartilage mode of expression prior to implantation. Dedifferentiated monolayer P1 CACs cultured for 18 days by encapsulation in alginate beads under normoxia condition in 20% DMEM supplemented with only PPS demonstrated a full retain to their 'native' cartilage phenotype as verified by enhanced synthesis of cartilage-specific gene markers, type II collagen and aggrecan mRNA with complete suppression of type I and X collagen. PG deposition, a marker of articular cartilage phenotype was also significantly enhanced by PPS compared to the control. Alginate encapsulation of CACs resulted in Sox-9 suppression, which was associated to the reduced cell-to-cell interactions in alginate beads cultured cells and the binding of TGF-β to alginate. HIF-1α and HIF-2α proteins were detected at protein level for the first time under normoxia condition an indication that culture of chondrocytes in alginate beads may stabilize the HIF-α isoforms at the protein level leading to promotion of a chondrocyte phenotype. The results demonstrated that the phenotype of dedifferentiated articular chondrocytes may be restored by combining alginate encapsulation with culture in standard medium supplemented with only PPS without the addition of known chondrocytic growth factors. Therefore, this study verifies that PPS is a novel alternative chondroinductive factor with the potential

to offer a solution to the major challenges that exist in cartilage tissue engineering efforts.

3. Although chondroinductive growth factors such as TGF-β are considered necessary for chondrogenic differentiation of BMSCs, independent and spontaneous chondrogenic differentiation has been previously demonstrated in adult horses, bovine calves and adult human BMSCs but not in canine BMSCs (cBMSCs). This study was undertaken to evaluate the independent chondrogenic potential of cBMSCs sourced from young dogs in monolayer expansion cultures in the absence of known chondroinductive factors. The results showed for the first time that cBMSCs exhibit independent chondrogenic differentiation in the absence of dexamethasone and TGF-β1 previously shown to be necessary as verified by the expression of Sox-9, type II collagen and aggrecan. Sox-9 was significantly downregulated ($P < 0.05$) from P1-P3 compared to P0 while type II and X collagen, and aggrecan were significantly downregulated at P3 compared to P0. Sox-9, type II collagen and aggrecan gene expression showed a significant negative correlation ($P < 0.05$) with passaging, which may explain why after extensive passaging cBMSCs fail to exhibit independent chondrogenesis. Type I collagen was highly expressed after P0 indicating a phenotype drift to a fibroblastic-like phenotype. HIF-2α mRNA was expressed in all cBMSCs passages, which may reflect its important regulatory role in the chondrogenesis process. The results demonstrated that independent chondrogenic potential and phenotype retention of cBMSCs is passage-dependent and decreases with extensive passaging in standard medium, thus caution must be exercised when

evaluating the chondrogenic potential of BMSCs intended for cartilage tissue engineering and repair of OA cartilage lesions.

4. Mesenchymal stem cells (MSCs) are a potential alternative source of differentiated chondrocytes for cartilage tissue regeneration and repair of small OA cartilage defects. Both PPS and polysulfated glycosaminoglycan (PSGAG) had no significant effect on type II collagen, aggrecan and HIF-2α mRNA expression in alginate cultured cBMSCs indicating that both failed to promote chondrogenesis in this culture model. In addition, PPS had no effect on type I collagen whereas PSGAG upregulated type I collagen mRNA expression. PPS had a dose-dependent inhibitory effect on type X collagen mRNA while PSGAG showed an inverse effect with increased concentration correspondingly increasing type X collagen mRNA expression. In contrast to alginate beads culture, PPS enhanced chondrogenesis and PG deposition in cBMSCs in micromass culture (MMC) whereas PSGAG inhibited chondrogenesis and promoted a fibrocartilage-like phenotype with reduced PG deposition. The result verified PPS as a chondroinductive factor that may enhance chondrogenesis of cBMSCs in MMC but appears to have no effect in alginate beads culture. This finding has serious implications in cartilage tissue engineering as it indicates that the response of MSCs to chondroinductive factors is culture system-dependent and varies significantly between alginate and MMC.

The findings in this dissertation will contribute in a positive way to the body of knowledge on the effects and possible mechanism of action of PPS on novel therapeutic targets for OA treatment and prevention of disease progression. Furthermore, by demonstrating and

verifying the positive *in vitro* chondroinductive effects of PPS on articular chondrocytes and BMSCs, the findings of this dissertation will surely have a positive impact in influencing the future focus for cartilage tissue regeneration and repair efforts of OA joint defects in both humans and companion animals.