Introduction: Intervertebral disc (IVD) is the unique tissue that separates adjoining vertebrae and absorbs biomechanical forces on the spine. Magnetic resonance imaging has shown that 40% of patients with back pain have associated IVD degeneration. Mature nucleus pulposus cells (NPCs) form the gelatinous inner core of the IVD and are mainly composed of collagen II and aggrecan, which resembles chondrocytes. However, degenerated NPCs acquire an altered phenotype with dysregulated expression of senescent markers, matrix catabolic enzymes, and matrix component factors which produces an unhealthy matrix that is responsible for IVD degeneration. Synovium-derived stem cells (SDSCs) have been recently characterized as tissue-specific mesenchymal stem cells (MSCs) for chondrogenesis [1]. Our previous study has shown that decellularized stem cell matrix (DSCM) deposited by SDSCs rejuvenated the proliferative and chondrogenic potentials of recellularized porcine SDSCs and NPCs [2, 3]. Fetal SDSC deposited DSCM could reduce the percentage of senescent cells [4]. In this study, we hypothesized that DSCM deposited by fetal SDSCs is better than by adult SDSCs at rejuvenating human NPCs with higher proliferative and chondrogenic potentials.

Methods: Human NP tissues were collected from three patients (ages 32 to 38 years old) with disc herniation and NPCs were extracted. Briefly, the NP tissues were individually digested with 0.1% collagenase A and 10 U/mL hyaluronidase at 37°C for 4h. NPCs were collected from the filtrate by centrifugation and plated in αMEM with 10% fetal bovine serum and considered passage 1. Passage 3 fetal SDSCs (FSDSCs) and adult SDSCs (ASDSCs) were used to prepare DSCM as described in our previous work [4], referred to as “FE” and “AE”. Passage 3 NPCs were expanded on these two DSCMs or conventional plastic flasks (“PL”) for one passage. Cell numbers were counted. The CellVue® Claret kit was used to assess cell proliferation during expansion. MSC surface markers including CD90, CD29, CD105, and stage specific embryonic antigen 4 (SSEA4) were also analyzed by flow cytometry after expansion. For chondrogenesis, expanded cells (0.3×106 cells) were centrifuged to form a pellet and evaluated for chondrogenic potential after a 21-day incubation in TGF-β3-containing serum-free chondrogenic medium. Alcian blue staining was used for sulfated glycosaminoglycans (GAGs) and immunostaining was for types I, II, and X collagen; biochemical and real time-PCR (RT-PCR) analysis was used to assess chondrogenic differentiation. Proteomic analysis of FE and AE was performed using 1D SDS-PAGE and tandem mass spectrometry. Immunofluorescence staining of collagen I, fibronectin and laminin was performed to confirm proteomic data. Statistical significance was assessed using single factor ANOVA (p <0.05).

Results: DSCMs significantly promoted NPC proliferation and decreased MSC surface marker expression except SSEA4, especially FE expanded NPCs

As shown in the representative picture in Figure 1A, passage 2 human NPCs were seeded on three different substrates including PL, FE and AE initially at the same density and expanded for two passages. NPCs exhibited different sizes and formed aggregates spreading out randomly on the plastic flasks. NPCs expanded on AE and FE were more organized and uniform in size. Cell number data from all three NPCs showed that both the FE and AE groups resulted in higher NPC proliferation, especially FE (Figure 1B). Proliferation index results were also consistent with cell number data (Figure 1C).

MSC surface marker expression also showed an interesting trend in all three samples (Figure 2). Though the percentage of positive cells was similar between groups, the median fluorescence intensity (MFI) of all MSC markers was lower in FE and AE expanded NPCs, especially the FE groups. However, both percentage and MFI of SSEA4 expression were higher in the FE and AE groups (data not shown).

Chondrogenic potential of SDSC deposited DSCM expanded NPC is not enhanced

After 6 days of expansion, all groups of NPCs underwent chondrogenic differentiation for up to 21 days. DNA content of the pellets was measured at day 0, 10, and 21 to indicate cell viability (Figure 3A). GAG contents and chondrogenic index (ratio of GAG to DNA) indicated chondrogenesis (Figure 3B/C). We also included passage 2 NPCs (PL2) to observe the influence of passaging on NPCs expanded on plastic flasks. All three NPCs expanded on FE and AE did not exhibit enhanced chondrogenesis. Pellet sizes were also smaller from these groups. Alcian blue staining for GAG and immunostaining for collagen II also showed less staining in those pellets; RT-PCR data for chondrogenic marker gene expression was consistent with biochemistry data (data not shown).

Laminin possibly mediated DSCM mediated NPC rejuvenation of redifferentiation potential

We further analyzed the components of FE and AE to explore the potential mechanisms. As shown in Figure 4, our immunofluorescence data suggested that fibronectin and collagen I were strongly stained in both FE and AE; however, laminin
was missing in both DSCMs. Our proteomics data also supported this finding (data not shown).

**Discussion:** This is the first time that human SDSC derived DSCM was used to rejuvenate human NPCs. As we expected, the three-dimensional DSCM served as an expansion substrate for NPCs to proliferate, especially the fetal SDSC deposited DSCM. However, the chondrogenic potentials of NPCs expanded on DSCMs were not improved. We suspect that laminin, an important player in NPC biology, is responsible for maintaining and rejuvenating the chondrogenic potential of NPCs. Chen et al reported that laminin is unique to NPCs in immature IVD. With aging, laminin expression decreased in both human and porcine NPCs. [5] The detailed mechanisms are under investigation.

**Significance:** Decellularized stem cell matrix based expansion system significantly enhanced the proliferative potentials of human NPCs.

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**References:**
Fig. 2

Surface marker

A

NF2

B

NF3

C

NF4
Fig.4

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immunofluorescence