Extracellular Matrix Reprograms Adipose Stem Cells from Infrapatellar Fat Pad toward Chondrogenesis
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Introduction
Once damaged, articular cartilage has a limited capacity for self-repair. Autologous chondrocyte transplantation is one approach for cartilage defect repair, but donor source limitation and in vitro expansion senescence can be problematic. Compared with stem cells derived from bone marrow (BMSCs) and synovial membrane (SDSCs), adipose stem cells (ASCs) are easier to obtain, have relatively-low donor site morbidity and a high yield at harvest, can be expanded more rapidly in vitro, and can be a potential candidate for autologous stem cell-based therapy. However, ASCs have less capacity for chondrogenic differentiation. Our recent study indicated that SDSC-derived extracellular matrix (ECM) can serve as an in vitro three-dimensional niche, greatly enhancing SDSC propagation and allowing restoration of stem cell stemness toward chondrogenesis [1]. In this study, we hypothesized that ASCs can be epigenetically regulated toward chondrogenesis when they are expanded on ECM deposited by SDSCs; an ECM deposited by SDSCs yielded expanded ASCs with higher chondrogenic differentiation capacity than ECM deposited by ASCs.

Methods
Three-month-old porcine synovial tissue and infrapatellar fat pad were used to isolate SDSCs and ASCs for ECM preparation [1], referred to as S-ECM and A-ECM. ASCs were expanded on S-ECM and A-ECM for two passages. Monolayer expansion on plastic flasks served as a control. The expanded cells were assessed for proliferation capacity and intracellular reactive oxygen species (ROS). Pellets from the expanded ASCs were incubated in a serum-free chondrogenic medium with either 10 ng/mL transforming growth factor beta 3 (TGF-β3) alone or combined with 10 ng/mL bone morphogenetic protein 6 (BMP-6). The pellets were collected at days 0, 7, and 14 for chondrogenic evaluation using histology (Safranin O and alcian blue staining for sulfated glycosaminoglycan (GAGs), immunostaining for type I and II collagen); biochemical analysis (DNA amount for cell proliferation and GAG amount for chondrogenic differentiation), and real-time PCR (type II collagen and aggrecan mRNAs for chondrogenic differentiation, type X collagen and matrix metalloproteinase 13 (MMP-13) mRNAs for hypertrophy). The adipogenic potential of expanded cells was also evaluated using Oil Red O staining and real-time PCR for adipogenic gene expression (LPL, PPARγ, and C/EBPα).

Results
ECM expansion enhanced ASC proliferation and maintained stem cell morphology
Passage 1 ASCs were expanded on Plastic, AECM, or SECM for two passages. In regard to cell morphology, ASCs grown on Plastic appeared large and flat while ASCs plated on ECM were smaller and spindle-like and, with time, overlapped in a 3D format. Intriguingly, ASCs grown on AECM or SECM exhibited similar cell morphology and underwent directional migration along the ECM fibrils whereas the cells on Plastic had a random direction. During a seven-day incubation, the proliferative rate of ASCs on Plastic dropped with each passage, with a 4.53-fold increase at passage 2 and a 45.47-fold increase at passage 3 compared to a 15.47-fold increase at passage 1. On the contrary, ASCs plated on AECM and SECM exhibited a robust and stable proliferative ability, with a 43.73-fold increase at passage 2 and a 44.8-fold increase at passage 3 for ASCs expanded on AECM and a 43.73-fold increase at passage 2 and a 48.8-fold increase at passage 3 for ASCs expanded on SECM. There was no significant difference in the cell yielding number on the different ECMs. In order to determine whether ECM expansion could decrease cell stress, we examined the intracellular levels of ROS accumulation in ASCs cultured in Plastic, AECM, or SECM by flow cytometry. The ASCs cultured in Plastic indeed showed a higher level of DCF fluorescence compared with those expanded in AECM or SECM. The mean fluorescent intensity (MFI) of ROS in ASCs in Plastic was 148 ± 6, which was 2.4-fold that in AECM and 3.5-fold that in SECM, indicating that ECM effectively reduced the generation or accumulation of intracellular ROS in ASCs.

ECM expansion enhanced ASC chondrogenic capacity
After expansion on Plastic, AECM, and SECM, ASCs were cultured in a serum-free pellet system supplemented with 10 ng/mL of TGF-β3 to evaluate the effect of expansion approach on ASC chondrogenic capacity. Compared to nondetectable staining at day 0, ASC pellets from P1.Plastic or P3.Plastic were barely positive for sulfated GAGs and type II collagen at day 7 despite gradually positive staining on the periphery of pellets at day 14. The pellets from P3.AECM and P3.SECM, however, were intensively positive for sulfated GAGs and type II collagen even at day 7 and dramatically improved in range and density after incubation in chondrogenic medium for 14 days (Figure 1). At the protein level, ASCs from P3.Plastic yielded pellets with lower chondrogenic index (GAG/DNA) compared with that from P1.Plastic. In contrast, ASCs from P3.AECM and P3.SECM yielded pellets with not only maintained cell number but also greatly enhanced chondrogenic index. There was no significant difference in chondrogenic index for ASCs expanded on AECM or SECM. At the mRNA level, ASCs from P3.AECM or P3.SECM yielded pellets with higher mRNA levels in type II collagen (at days 7 and 14), aggrecan (at days 7 and 14), and Sox9 (at day 7) than that from P1.Plastic and P3.Plastic. Similar to protein expression, there was no significant difference in the above chondrogenic marker genes for ASCs expanded on AECM or SECM.

Conclusion
In summary, our study proved that the utilization of ECM on ASC ex vivo expansion was able to dramatically increase the cell proliferative rate and improve the chondrogenic differentiation potential. The combination of TGF-β3 and BMP-6 promoted chondrogenic induction but suppressed hypertrophic gene expression. The large-scale cell number of ASCs with highly chondrogenic potential will benefit cartilage repair and tissue regeneration in the future.