INTRODUCTION
The anterior cruciate ligament (ACL) is the most commonly injured ligament of the knee. Unlike other major ligaments of the knee joint, the biological properties of the ligament and its intra-articular environment contribute to poor healing of the ligament after injury. Current materials used in ACL reconstruction including autografts, allografts and synthetic grafts. However, all these graft materials have unique disadvantages and limitations. Recently, mesenchymal stem cells (MSCs) have been used for various tissue regeneration because of their proliferation and differentiation potentials, and these cells have offered hope on tissue engineering approach of ACL reconstruction. In addition to bone marrow, there are increasing reports that MSCs can be isolated from various adult somatic tissues such as adipose tissue, peristem, synovium, ligament and meniscus. However, it is not clear whether stem cells can be isolated from the cruciate ligaments. The aim of our study is to isolate and characterize stem cells from human cruciate ligaments.

METHODS:
Stem cells were harvested from human ACL and posterior cruciate ligament (PCL) of patients receiving total knee arthroplasty by an explant culture technique. The ligaments were cut into small pieces and placed in culture dish containing growth medium (alpha-MEM + 10% FBS). Once the confluence of the cell colonies was reached, cells were treated with 0.25% trypsin and subcultivated as passage 1 at 50 cells/cm². After an additional 14 days of growth, the cells were harvested and cryopreserved in liquid nitrogen in FBS with 10% DMSO (passage 2). To expand the cells, a vial of cryopreserved cells was thawed and plated at a concentration of 5,000 cells/cm². Serial passage was performed at the same concentration. After initial outgrowth of ligament-derived cells from explant cultures, a portion of the passage 0 cells are clonogenic.

RESULTS:
Isolation and expansion of cells: ACL- and PCL-derived stem cells (LSCs) could be extensively sub-cultivated in standard culture condition. The morphology of the cells was plastic adherent and spindle-shaped (Fig. 1a ACL; fig.1b PCL). The doubling time of ACL- and PCL-derived cells was estimated to be 48 to 56 hours (Fig. 2). Ligament-derived cells are clonogenic. After staining with crystal violet, the colonies on each plate were counted (Fig. 3a: ACL-upper 4 plates; PCL-lower 4 plates). The average colony-forming efficiency was determined as 20.18 ±/− 6.06% for ACL and 21.68 ±/− 5.99% for PCL. There were no significant differences (Fig 3b). Mesodermal differentiation: under osteogenic induction, stem cells isolated from human ACL and PCL were positive for alkaline-phosphatase (ALK-P) and alizarin red S stain (data not shown). They express CD105, CD73, and CD90 and lack the expression of CD45 and HLA-DPQR. The cells were positive for Oil red-O stain (ACL-fig. 5a, c; PCL-fig. 5b, d). Under chondrogenic induction, typical fat droplet appeared one week after induction and was positive for Oil red-O stain (ACL-fig. 5a, c; PCL-fig. 5b, d). Under chondrogenic induction, the cells aggregated to form pellet (fig 6a, upper 4 pellets from ACL, lower 4 pellets from PCL). The size and weight of the pellets were higher in the PCL group (Fig. 6 b). The pellet was highly positive for Alcian blue stain (Fig. 6 c). RT-PCR analysis of the expression of bone, fat, and cartilage-specific genes in cells from ACL and PCL were performed. Specific PCR products of bone (Fig. 4d), fat (Fig. 5e), and cartilage (Fig. 6d) were expressed in cells treated with three weeks of osteogenic, adipogenic and chondrogenic induction medium. Anchorage-independent growth: LSCs from ACL and PCL began proliferate to form colonies from single cells in suspension conditions at about five days after inoculation. Multiple colonies of cells were present after 14 days of culture in soft agar (Fig. 7a-ACL; Fig. 7b-PCL). Characterization of surface immune-phenotype: Flow cytometry analyses indicated that phenotypic characteristics of the LSCs were consistently negative for hematopoietic cells markers such as CD34, CD133, CD14 and HLA-DPQR. The cells were positive for CD90, CD13, CD44, CD73, CD90, CD105, CD166 and HLA-ABC (data not shown). Karyotyping: LSCs after serial passage under standard culture medium (passage 10) were sent for karyotyping to evaluate the chromosome stability, there was no definite chromosomal abnormality noted (Fig. 8).

DISCUSSION:
LSCs are plastic-adherent when maintained in standard culture condition. They express CD105, CD73, and lack the expression of CD45, CD34, CD14, and HLA-DPQR. They can differentiate to osteoblasts, adipocytes, and chondrocytes in vitro. All these characteristics fulfill the minimal criteria for defining multipotent mesenchymal stem cells. In summary, our study demonstrates that human multipotent stem cells can be isolated and expanded from human ACL and PCL, which are easily obtained from patients following total knee replacement or arthroscopic surgeries. The high proliferation and multilineage differentiation potential of these cells make them a viable alternative cell source for regenerative medicine. Future efforts to apply LSCs to tissue engineered fabrication of cruciate ligaments are warranted.