Characterization of KRT8^{+}/Brachyury^{-} Subsets in Human Fetal Intervertebral Disc Cells via a New Conditionally Immortalized System

Hanxing J He[1], Wai-Kit Tam[1], Vivian Tam[2], Kelvin S Cheung[2], Martin Cheung[2], Kathryn Cheah[2], Antoine A de Vries[3], and Victor Y Leung[1]
[1] Department of Orthopaedics and Traumatology, and [2] School of Biomedical Sciences, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China
[3]Leiden University Medical Center, Department of Cardiology, Leiden, Netherlands

Email of Presenting Author : jameshhx@connect.hku.hk

Disclosures: Hanxing J He (N), Wai-Kit Tam (N), Vivian Tam (N), Kelvin S Cheung (N), Martin Cheung (N), Kathryn Cheah (N), Antoine A de Vries (N), and Victor Y Leung (N)

INTRODUCTION: In foetal axial skeleton development, notochord undergoes condensation and segments to form the primitive nucleus pulposus (NP) composing of notochordal cells (NCs). *Albeit* putative NC molecular markers: brachyury (TBXT) and cytokeratin 8 (KRT8) have been reported to be depleted with age and intervertebral disc (IVD) degeneration, animal studies indicated that a proportion of notochordal-derived cells could persist in adulthood. By understanding the ontogenetic, cellular and molecular characteristics of NCs is pivotal to the successful development of cell replacement therapies and IVD regeneration However, the lack of an expandable NC model in the field has limited in-depth studies on human NP cell biology.. Recently, a conditional immortalization in relying on lentiviral vectors and the doxycycline-controlled expression of SV40LT in human foetal atrial myocytes was developed for immortalization of cells in generation of *in vitro* models of atrial myocytes. Here, we hypothesized that a primitive human NC cell line can be established by conditional immortalization of foetal NP cells. To this end, we aimed at conditionally immortalization of foetal human NCs with doxycycline-controlled expression of SV40LT and characterized the expression of phenotypic NCs markers: TBXT, KRT8 and chondrogenic marker: SOX9 in different clonal cell lines. Furthermore, we investigated if the different phenotypically distinct clonal cell lines representing different cellular subpopulations in foetal disc could be generated and maintained using immortalization strategies.

METHODS: Human foetal samples were obtained with ethical approval from the institutional review board (IRB, reference no. UW 21-680). IVD tissue was dissected from the trunk and separated from adjacent vertebral structures. Cells were then harvested by. enzymatic dissociation from the tissues using 0.025 mg/mL Collagenase P, 0.5% Collagenase II, and 0.5% Dispase and subsequently cultured on Matrigel. Lentiviral (LV) shuttle plasmid with a proprietary repressor-based lentiviral Tet-On system for the doxycycline-controlled expression of SV40LT and fusion green fluorescent protein (GFP): pLV.iEF1α.SV40LT.GFP was co-transfected with the packaging plasmids psPAX2 (Addgene) and pLP/VSVG (Thermo Fisher) at a molar ratio of 2:1:1 in 293T cells for LV production. LV supernatants were harvested post 48 hours of transfection and concentrated with Lenti-X™ concentrator. (Takara). Foetal IVD cell mixture was transduced with 15 ul concentrated LV.iEF1α.SV40LT.GFP with 10μg/ml polybrene (Millipore), following 1 day recovery with growth media: DMEM/F12 -10% FBS. Conditionally immortalization was achieved by addition of growth medium supplemented with 100ng/ml doxycycline (Sigma) every other day. Immunostaining of NC and chondrogenic markers was performed by using anti-TBXT antibody (Santa Cruze) in 1:100 dilution, anti-KRT8 antibody (Thermo Fisher) in 1:100 dilution and anti-Sox9 antibody (Abcam) in 1:100 dilution. Fluorescence-activated cell sorting (FACS) was conducted to collect GFP positive cells by BD FACSAria™ SORP Cell Sorter. Thereafter, GFP positive cells were subjected for clonal expansion.

RESULTS: To isolate successfully transduced foetal IVD cells with SV40LT.GFP from the cell mixture, transduced cells were sorted by FACs upon doxycycline addition. A significant proportion of 21.4% GFP positive population was identified in comparison with untransduced (3.05%) and transduced cells without doxycycline addition (3.12%) (Figure 1A). To characterize the proliferative capabilities of the isolated cell clones, growth curves of transduced foetal disc cell clones were evaluated. Strikingly, transduced cell clones exhibited nearly a three-fold increase, whereas transduced bulk cells with ? fold increase in proliferation rate compared to untransduced cells upon doxycycline treatment.. (Figure 1B). To characterize the phenotypic attributes of these clones, we detected partial expression of KRT8, but not TBXT in the subsequent expanded cell clones (Fig. 1E), whereas Sox9 was ubiquitously expressed in almost all clones (n= ?) (Fig. 1C). We also confirmed that KRT8 expression pattern was uniquely present in the NP region of the foetal spine. (Fig. 1B)

DISCUSSION: Our study represents a significant advance in the field of spinal biology, particularly in the context of human intervertebral disc (IVD) cells. We successfully established a conditionally immortalized notochordal cell (NC) lineage, leveraging the specificity of a doxycycline-inducible system to modulate the expression of the SV40 Large T antigen (SV40LT). The establishment of a conditionally immortalized NC cell line not only provides a powerful tool for future research but also uncovers a novel cell subpopulation, potentially altering our conceptual landscape of spinal disc biology.

SIGNIFICANCE/CLINICAL RELEVANCE: By unraveling specific markers and cellular behavior, the findings hold potential for advancing personalized treatments for degenerative disc diseases, thereby contributing to the broader pursuit of enhancing spinal health and patient well-being.

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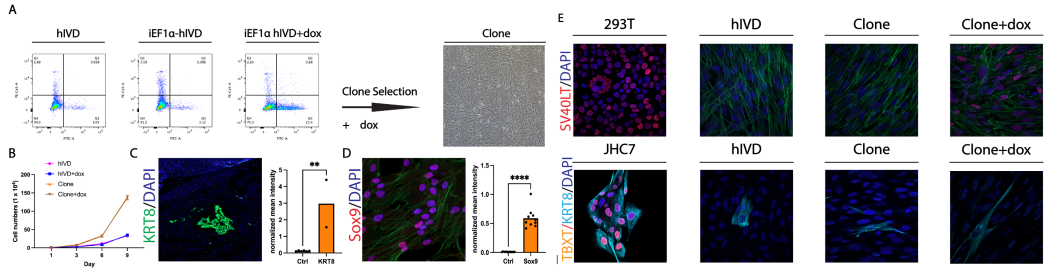

Figure Legend:
(A) Fluorescence-activated cell sorting (FACS) based selection of conditionally immortalized clones exhibiting GFP signal post-doxycycline induction. Clones were isolated for subsequent in-depth characterization. Abbreviations: hIVD, human intervertebral disc cells.
(B) Quantitative assessment of proliferative capacity in both native hIVD cells and isolated clones under doxycycline treatment. Data are represented as mean ± standard deviation (n=3).
(C) Immunohistochemical analysis revealing the specific expression of the notochordal marker, Cytokeratin 8 (KRT8), in tissue sections of human intervertebral discs.
(D) Immunofluorescence staining of Sox9, a chondrogenic transcription factor, in isolated clones derived from transduced human IVD cells.
(E) Co-immunostaining of SV40 Large T antigen (SV40LT), Brachyury (TBXT), and KRT8 in both native hIVD cells and doxycycline-treated clones. HEK293T cells serve as a positive control for SV40LT expression, while JHC7 cells act as a positive control for TBXT expression.