Adipose Tissue from Retinoblastoma Patients: A Potential Source for Disease Carrying Stem Cells

Analiese DiConti, Jennifer Aparicio, PhD, and Thomas Lee, MD[1,2]

[1]*Director, Retina Institute,* Children's Hospital Los Angeles

[2]*Associatete Professor of Ophthalmology, Keck School of Medicine, University of Southern California*

Introduction

Retinoblastoma is a rare cancer of the eye affecting 300 children annually in the US. This tumor is a result of loss of the retinoblastoma gene (*Rb*) with approximately half of affected children being heterozygous for *Rb* and at risk for both multiple retinal tumors as well as non-ocular sarcomas.

There is no accurate animal model for retinoblastoma due to the unique differences between human and mouse retinal development. In this study we explore the feasibility of modeling retinoblastoma using mutation harboring stem cells derived from retinoblastoma patients. Children's Hospital Los Angeles is one of the nation's largest referral centers for retinoblastoma with over 60 new patient referrals a year and is in a unique position to address this issue.

Here, we present our initial results from 20 patient samples in which we have harvested orbital fat tissue as a source of stem cells. We successfully isolated mesenchymal stem cells (MSCs) from these adipose samples and have reprogrammed them with Oct4, Sox2, Klf4, and c-Myc in an attempt to generate induced pluripotent stem cells (iPSCs)

Methods

Harvesting and Maintenance of MSCs

Orbital adipose tissue samples are surgically harvested during an enucleation of the eye in patients with retinoblastoma. Tissue samples are digested with collagenase then cultured in MSC Media (IMDM, 20% FBS, 1x ITS, 5mM Glutamax). Adherent cells were saved and cultured further.

Differentiation of MSCs

Assays were carried out as previously reported (Lee MW). In brief, MSCs were grow in Adipogenic, Osteogenic of Chondrogenic Induction Media for three weeks then stained with Oil Red O, Alizarin Red S, or embedded in paraffin and stained with Toluidine Blue, respectively.

Flow Cytometry

With the help of the third floor FACS Core, the following antibodies were chosen for flow cytometry: FITC-CD90, APC-CD106, PE-CD34 (Biolengend), PerCP-Cy5.5-CD105, APC-H7-CD44 (BD Pharmigen), and Pacific Orange-CD45 (Invitrogen).

RT-PCR Analysis

RNA was isolated from MSCs using Qiagen RNeasy Mini Kit, and Reverse Transcription was carried out using iScript Reverse Transcription Kit (BioRad). PCR was then carried out with appropriate primers as described (Yamanaka et al, Sugii et al).

iPSC Formation

MSCs were infected with a lentiviral vector containing Oct4, Sox2, Klf4, and c-Myc (Somers), and transferred to irradiated MEFs seven days post-infection, and placed in previously defined hES media (Yamanaka) with 200uM NaButyrate. Around day 24, iPSC-like colonies appeared, and individual colonies were isolated and expanded.

IRB

This study is approved under IRB Protocols:

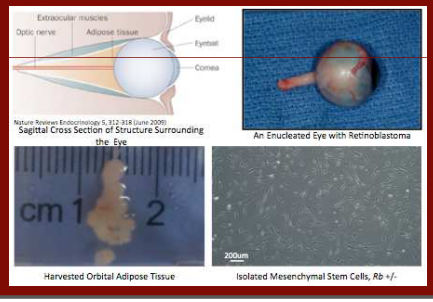
CCI-09-00322

CCI-10-00149

Results

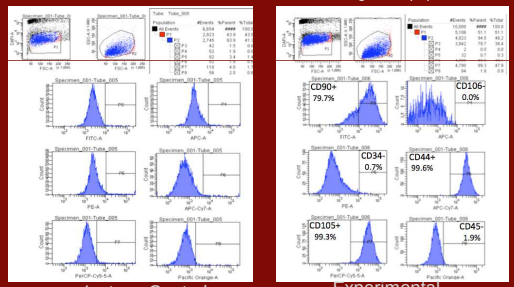
Orbital Adipose Tissue Can Be Harvested from Retinoblastoma Patients during Enucleation

A small pocket of adipose tissue is found above the eye (top left panel), which can be harvested during enucleation (top right panel). These small samples are harvested in culture (bottom left panel, and the adherent cells, the MSCs, are isolated (bottom right panel). So far, 95% of tissue samples have produced adherent cells. So far, these cells have been grown out to passage 10 without any indication of senescence, but the full growth potential of these cells have yet to be explored.



Orbital Adipose-derived adherent cells have typical surface markers of Adipose-derived MSCs

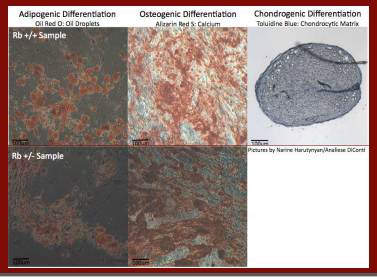
FACS Analysis was completed on three *Rb* +/+ MSC cells lines, and one Rb +/- MSC cell line. Each of these displayed the same surface markers, CD90+ CD106- CD34- CD44+ CD105+ and CD45-. This is consistent with adipose-derived MSCs described in Gimble et al. Below, both isotype controls and experimental FACS results are shown for an *Rb* +/+ MSC cell line. Four of twenty cell lines have been tested, including a *Rb* +/- line, and all have a consistent marker characterization and show a homogenous population.



Isotype Control Experimental

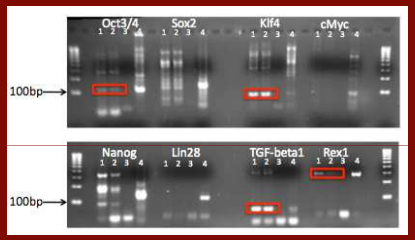
Orbital Adipose-derived adherent cells can differentiate into adipogenic, osteogenic, and chondrogenic lineage

All three cell lines tested were shown to have differentiation capability, demonstrating their pluripotency, characteristic of mesenchymal stem cells. Below, differentiation of two *Rb* +/+ MSC cell lines, patient 8 (Adipogenic and osteogenic) and patient 9 (chondrogenic) are shown, as well as differentiation in *Rb* +/- patient 10.



RT-PCR Analysis shows up-regulation of Klf4 pluripotency transcription Factor

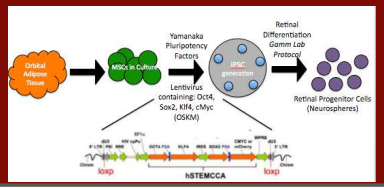
Orbital Adipose-derived adherent cells were found to have a minor expression of Oct3/4, TGFβ1, and Rex1 pluripotency transcription factors, but these levels are lower than embryonic stem cells. Levels of Klf4 are much higher than embryonic stem cells, which is consistent with adipose-derived MSCs (Gimble et al).

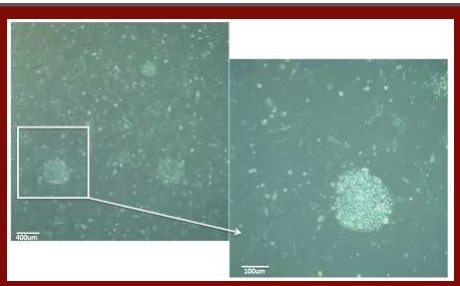


1: F1#9 (*Rb* +/+) 2: F1#10 (*Rb* +/-)
3: H_20 control 4: hESC control

Orbital Adipose-derived MSCs from Retinoblastoma patients can by reprogrammed into iPSC-like colonies

Using a lentiviral vector containing the four pluripotency factors described by Yamanaka in her landmark 2006 paper, we reprogrammed one cell line of *Rb* +/+ MSCs into "iPSC-like" colonies (right panel). We are in the process of testing these colonies for true pluripotency. As approximately half of patients have a germ line mutation in the Rb gene, we are looking to generate mutation harboring iPSCs which can then be differentiated into retinal progenitor cells (bottom panel). From this stage, we can watch the development of retinoblastoma as it would occur in utero.





iPSC-like colonies reprogrammed from *Rb* +/+ orbital adipose-derived MSCs. Testing of the true nature of these cells is underway.

Conclusions

The large number of retinoblastoma patients that come to Children's Hospital Los Angeles gives our lab the unique ability to study this disease and the implications of *Rb* gene mutation that occurs in so many other cancers. From 20 sample of orbital adipose tissue, we are the first to characterize an isolated population of adherent cells, which display surface markers, transcriptional up-regulation, and differentiation capability characteristic of mesenchymal cells. This relatively small orbital adipose tissue store has shown to be a consistent source of MSCs that are easy to grow and expand, even with germ line *Rb* mutations, as *Rb* +/- lines show normal RB protein levels.

Preliminary data suggests the ability to reprogram these cells to iPSCs, which opens many opportunities for discerning the developmental pathways in retinoblastoma. The *Rb*+/- MSCs will be extremely important in the study of both primary and secondary cancers that affect children with germ line mutations, due to the multipotent differentiation capability, ease of access, and robust viability of these cells.

Future Directions

The next step in this study is to determine the true nature of the iPSC-like colonies and determine their differentiation capability, through surface marker characterization, transcription factor up-regulation analysis, embryoid body formation in vitro, and teratoma formation in nude mice.

Once the identity of these colonies is confirmed, we plan to generate iPSCs from *Rb* +/- cell lines and differentiate these cells into mutation-harboring retinal progenitor cells, which, as they develop into different cell types, can be studied as they undergo tumorigenesis.

This institution has the opportunity provide the scientific community with the first comprehensive retinoblastoma model, as the home the premiere retinoblastoma team in the Western US.

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