CREATION OF MULTILINAGE ADULT STEM-LIKE CELLS FROM TERMINALLY DIFFERENTIATED FIBROBLASTS

John Moore
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CREATION OF MULTILINAGE ADULT STEM-LIKE CELLS FROM TERMINALLY DIFFERENTIATED FIBROBLASTS

John H. Moore II
Acknowledgements

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<th>Description</th>
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<tr>
<td>iPSc</td>
<td>Induced Pluripotent Stem Cell</td>
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<td>ESC</td>
<td>Embryonic Stem Cell</td>
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<td>Nanog</td>
<td>Homeobox protein NANOG</td>
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<tr>
<td>Oct4</td>
<td>Octamer-binding Transcription Factor 4</td>
</tr>
<tr>
<td>Sox2</td>
<td>SRY (sex determining region Y) box 2</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Myelocytomatosis viral oncogene homolog</td>
</tr>
<tr>
<td>N</td>
<td>Nanog adenovirus</td>
</tr>
<tr>
<td>OS</td>
<td>Oct4 and Sox2 adenoviruses</td>
</tr>
<tr>
<td>NOS</td>
<td>Nanog, Oct4, and Sox2 adenoviruses</td>
</tr>
<tr>
<td>CNOS</td>
<td>c-Myc, Nanog, Oct4, and Sox2 adenoviruses</td>
</tr>
<tr>
<td>SSEA1</td>
<td>Stage Specific Embryonic Antigen 1</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein Lipase</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
</tr>
<tr>
<td>JAK-STAT</td>
<td>Janus Kinase - Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>KLF4</td>
<td>Kruppel-Like Factor 4</td>
</tr>
<tr>
<td>LIN28</td>
<td>Lin-28 homolog A</td>
</tr>
<tr>
<td>abbreviation</td>
<td>full form</td>
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<td>-------------</td>
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<tr>
<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
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<tr>
<td>PD</td>
<td>Population doublings</td>
</tr>
<tr>
<td>ABAM</td>
<td>Antibiotic/Antimycotic Solution</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio-Immunoprecipitation Assay</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>PPAR γ</td>
<td>Peroxisome proliferator activated receptor gamma</td>
</tr>
<tr>
<td>C/EBP alpha</td>
<td>CCAAT/enhancer binding protein alpha</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
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ABSTRACT

CREATION OF MULTILINEAGE ADULT STEM-LIKE CELLS FROM TERMINALLY DIFFERENTIATED FIBROBLASTS

By John Moore, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University, Department of Human Genetics, Virginia Commonwealth University, 2011.

Induced Pluripotent Stem cells (iPScs) are artificially generated cells that demonstrate multilineage differentiation potential. These cells demonstrate similar morphology and high differentiation potential to Embryonic Stem Cells (ESCs). Generation of these cells from a terminally differentiated cell line requires activation of the core pluripotency genes Nanog, Oct4, and Sox2 as well as an oncogenic stimulus such as c-Myc.

Here we examine the effect of the Human Pappiloma Virus derived proteins E6 and E7 on the ability of a terminally differentiated fibroblast cell line to a more primitive state and examine its multilineage differentiation capacity. In this paper, we attempt to differentiate BJ hTERT fibroblasts into adipogenic and osteogenic lineages with and without the core pluripotency factors Nanog, Oct4, Sox2 and also c-Myc using non-integrative adenoviral infections. We review the potential mechanisms through which changes in differentiation capacity changes occur through examination of the effects of E6 on the tumor suppressor protein p53. We determined that the proteins E6 and E7 when stably infected into BJ hTERT fibroblasts increase induced differentiation into adipogenic and osteogenic lineages. E6 and E7 can be considered components for generating cells with multipotent capacity with the addition of as little as one core pluripotency factor.
Chapter 1: Background

The ultimate goal of this study is to determine the variation in differentiation potential between cells expressing the pluripotency factors Nanog, Oct4, and Sox2 with and without the HPV-derived proteins E6 and E7. In order to effectively examine and ultimately understand these differences, we first need to examine what constitutes a stem cell, how “stemness” is quantified, and the interplay between the proteins being used for our study.

What are stem cells and where are they found?

Stem cells play many vital roles both the development of the embryo, for the adult body to heal, and for overall cellular and organismal homeostasis. There are two basic classes of naturally occurring stem cells – embryonic stem cells and adult stem cells. During development, embryonic stem cells (ESCs) are found in the inner cell mass of the embryonic blastocyst, which eventually becomes all tissues that comprise the adult body. Adult stem cell niches (microenvironments that regulate the cells and determine the lineage that they will differentiate into) are located in several areas of the body – in adipose tissue, bone marrow, and in the subventricular region of the brain. Some examples of adult stem cells include (but are not limited to) hematopoietic stem cells, which are vital in maintaining the blood supply [1]; mesenchymal stem cells, which are located in bone marrow and adipose tissue and can differentiate into fat, bone, and cartilage [2]; neural stem cells, which can develop into new neurons [3]; and intestinal crypt cells, which maintain the epithelium of the small intestine [4].

Characteristics of Stem Cells

The vast majority of stem cells are classified as multipotent cells, meaning they can differentiate down multiple lineages and self-renewal. Stem cells have potency, which is a cell’s
ability to differentiate into a more specialized cell type. In addition to potency, stem cells have the ability of self-renewal, which is the cell’s ability to divide multiple times while remaining in an undifferentiated state [5]. The nomenclature for stem cell classification is generally based on the number of lineages the cell is capable of differentiating into:

**Totipotent/Pluripotent** – These stem cells are able to differentiate into all somatic cell types. Examples include embryonic stem cells and induced pluripotent stem cells, both of which are capable of differentiating into all 3 vertebrate germ layers: endoderm, mesoderm, and ectoderm.

**Multipotent** – These stem cells are able to differentiate into several distinct unrelated cell types, usually within the same germ layer lineage.

**Oligopotent** – These stem cells are able to differentiate into only a few, closely related cell types.

**Unipotent** – These stem cells are only able to produce a single cell type, likely a precursor to another cell type (e.g. myoblasts – pre-muscle cells, which differentiate into myocytes).

Stem cell potency can be demonstrated and quantified in a number of different ways. To test for pluripotency, there are several specific *in vivo* and *in vitro* tests that can be used, including embryoid bodies, teratomas and chimeras, and numerous markers of differentiation. *Embryoid body formation* is an *in vitro* assay which has the test cells dropped into solution in a container where the cells are unable to adhere to the sides. The cells are then allowed to grow and are then assessed for signs of an embryoid body, a hollow spherical structure that will form secondary structures such as a yolk sac and cardiomyocytes [6].
Teratoma formation is an in vivo assay used to determine if the test cells are pluripotent. Cells are injected into a host animal and allowed to differentiate for 2-3 weeks. Following this, the animals are sacrificed and the resulting tumors excised and examined to see if the cells have differentiated into the three vertebrate germ layers. The creation of a chimeric animal is another in vivo test of pluripotency [7]. A blastocyst (a stage in embryonic development that is a hollow ball of cells) is injected with the test cells and implanted into a surrogate mother. The resulting embryo is allowed to develop until adulthood; the animal is then sacrificed and examined to see if the test cells have been integrated into the animal’s three germ layers.

There are several markers that are associated with potency and pluripotency. These markers can be examined by several different ways, depending on if they are protein, RNA, or mineral, and where they are located (inside the cell or adhered to its surface). Examples of these markers are listed below:

**Alkaline phosphatase** is a hydrolase enzyme that dephosphorylates proteins and nucleotides under alkaline conditions [8]. Elevated expression of this enzyme is associated with undifferentiated iPS cells and ESCs but not with adult stem cells.

**SSEA1** *(Stage Specific Embryonic Antigen 1)* is an adhesion molecule expressed on the cell surface that is thought to mediate phagocytosis and chemotaxis [9], and plays a role in the adhesion and migration of cells within the preimplantation embryo.

**Telomerase** is an enzyme that adds DNA sequence repeats to the end portion of the chromosome known as a telomere, which is a non-coding region of DNA that protects the coding regions of the chromosome from telomere loss (i.e. the end replication problem, where approximately 20-200 bases of DNA are lost from the end of the chromosome following mitosis).
Telomerase is important for stem cell self-renewal as the cell does not enter senescence following many population doublings.

**Nanog, Oct4, and Sox2** are transcription factors that are important for the induction of the *pluripotent ground state* \[10\], and are typically mentioned together as they regulate each other in an autoregulatory loop. These factors will be discussed later in more detail as they are critical components of this project.

In order to examine the potency of a cell line related to differentiation down a specific lineage, we can induce the cells to differentiate using a specific set of reagents for a given cell type \[11\]. There are two mesodermal lineages that are frequently used to gauge differentiation capacity – osteogenic (bone) and adipogenic (fat) differentiation. Cells are grown in a medium that contains specific growth and chemical factors in order to induce transcriptional regulation of lineage-specific genes. Relative expression of these genes is accomplished using *quantitative real-time PCR* (qPCR) and by Western to measure changes in protein expression.

**How is induced adipogenic differentiation achieved and what determines if a cell has been differentiated into an adipocyte?**

To induce adipogenic differentiation of stem cells, one can supplement their media with a cocktail of dexamethasone, insulin, and IBMX to direct fat cell production. *Dexamethasone* is a synthetic glucocorticoid that increases expression of PPAR γ (peroxisome proliferator activated receptor gamma – this is an adipocyte-specific surface protein) and C/EBP alpha (CCAAT/enhancer binding protein alpha) \[12\]. *Insulin* is a hormone that induces prenylation (addition of a hydrophobic molecule to a protein) of Ras to activate CREB-mediated adipogenic differentiation \[13\]. *IBMX* (3-isobutyl-1-methylxanthine) is a phosphodiesterase inhibitor that
raises intercellular cAMP and activates transcription factor C/EBPβ which induces transcription of itself and PPARγ [14].

In order to determine whether or not the cells have achieved adipogenic differentiation, there are a variety of markers associated with adipocytes that can be measured. These markers include *PPARγ* and *Lipoprotein Lipase (LPL)*. *PPARγ*, or peroxisome proliferator-activated receptor gamma, is a type II nuclear receptor protein that regulates fatty acid storage and glucose metabolism. Members of the PPAR family are ligand-dependent transcription factors. Activation of PPARγ transcriptionally regulates C/EBPα (CCAAT-enhancer-binding protein alpha), which is a transcription factor that is needed for adipogenesis to occur as well as for normal adipocyte function [15]. *Lipoprotein Lipase (LPL)* is an enzyme that hydrolyzes triglycerides into free fatty acids and monoglycerol, and is expressed most highly in adipocytes [16]. In addition to these two markers, cell staining with the dye Oil Red O can be used to stain lipid droplets in the cytoplasm that are characteristic of adipocytes.

**How is induced osteogenic differentiation achieved and what determines if a cell has been differentiated into an osteocyte?**

To induce osteogenic differentiation of stem cells, one can specifically supplement the media with dexamethasone, glycerophosphate, and ascorbic acid 2 phosphate. *Dexamethasone* is a synthetic glucocorticoid (as mentioned earlier) and plays a different role in osteogenesis than in adipogenesis; in particular through stimulation of Runx2 (an important early osteocyte transcription factor) phosphorylation via MKP-1 [17]. *Beta Glycerophosphate* is a substrate for alkaline phosphatase, and is used to create calcium phosphate, the primary mineral component of bone. *L-Ascorbic Acid 2 phosphate* is a compound that induces alkaline phosphatase expression.
In order to determine whether or not the cells have achieved osteogenic differentiation, there are a variety of osteocyte markers that we can assess. *Osteocalcin*, also known as bone gamma-carboxyglutamic acid-containing protein (BGLAP), is a protein secreted only by osteoblasts and is a biochemical marker for bone formation [18]. *Osterix* is a zinc-finger domain containing transcription factor which is essential for embryonic bone development and is a marker for differentiating bone.[19]

In general, a cell must have some degree of stemness in order to differentiate into multiple specific lineages, like adipogenic and osteogenic. Recent unpublished observations from our own lab suggest that classification of stem cells into specific categories (i.e. pluripotent vs. multipotent) is dependent on the cells’ ability to differentiate into a defined number of lineages rather than specific gene expression patterns or cell surface markers.

**Stem Cell Classification**

As mentioned above, there are three different basic stem cell types: embryonic stem cells, adult stem cells, and induced pluripotent stem cells. ESCs are derived from the inner mass of the blastocyst stage of an embryo [20]. These cells are pluripotent, meaning that they are able to differentiate into all three germ layers, and therefore all somatic cell types. ESCs can proliferate indefinitely *in vitro* [21] if cultured appropriately, as they have active telomerase when maintained in an undifferentiated state.

However, these ESCs are difficult to culture *in vitro*, as they require a feeder layer of mouse embryonic fibroblasts and are prone to spontaneous differentiation and karyotypic instability. They require LIF (Leukemia Inhibitory Factor) supplementation in their media to prevent spontaneous differentiation. LIF binds to the LIF receptor, resulting in the JAK/STAT
pathway and MAP kinase pathway cascades, which results in Nanog upregulation. Interestingly, LIF was not required to maintain ESC cultures in cells over-expressing the homeobox protein Nanog [22].

**What are IPS cells and how are they generated?**

Induced Pluripotent Stem cells (or iPS cells) are derived from terminally differentiated adult somatic cells that have been supplemented with various reprogramming factors, which ultimately transform them into cells that have characteristics similar to embryonic stem cells. Like ESCs, iPS cells can differentiate into all three germ layers. A research group headed by Shinya Yamanaka first demonstrated iPS generation using retroviruses to stably infect mouse fibroblasts with the genes Oct3/4, Sox2, KLF4, and c-Myc [23]. Yamanaka’s group, along with a group headed by James Thomson, simultaneously developed the first human iPS cell lines. Interestingly, Yamanaka’s protocol used a retroviral system to express Oct3/4, Sox2, KLF4, and c-Myc, while Thomson expressed the factors Oct4, Sox2, Nanog, and Lin28 using a lentiviral system [24]. iPS cells have been generated from many different cell types, even those using a transient (non-integrating) adenoviral system and mouse liver cells by a group headed by Kondrad Hochedlinger [25].

**How do these “factors” get into the cell and how are they expressed?**

Lentiviruses are a member of the retrovirus family. A retrovirus is an RNA virus (coated by a protein sheath) that is replicated in a host cell using the host’s reverse transcriptase to transcribe its RNA genome into DNA. The retrovirus DNA is then integrated into the host’s genome using an integrase enzyme. Lentiviruses are unique to the family of retroviruses as they
are able to infect and replicate in non-dividing cells. The prefix *lenti*- means “slow” in latin, as these viruses have a long incubation period.

In order for the retro-/lenti-virus to have a cell express a certain gene product, the gene of interest must be cloned into the viral DNA downstream of a promoter sequence that will allow gene expression. Furthermore, the virus must be loaded with a selectable marker that will allow for the determination of which cells have become infected with the virus expressing the desired gene product. The virus may be carrying a gene for antibiotic resistance to a specific drug, which when applied will kill only the cells not carrying the gene (hence, resistance gene). Examples of antibiotic resistance genes include those for the drugs *puromycin* and *G418* (*geneticin/neomycin*).

Adenoviruses are composed of a nucleocapsid and double-stranded DNA but lack an envelope. These viruses are non-integrating viruses, meaning that they do not integrate into the host cell’s genome. Adenoviral gene expression is generally considered transient, where the gene or genes of interest are expressed for a short period of time (less than 2 weeks), eliminating viral DNA integration and the concerns with genomic instability. Here, we use both retroviruses and Adenoviruses to express specific gene products in our test cells.

**What are the key genes that have been found to induce and mediate pluripotency?**

There are a few genes that have been heavily implicated in the establishment of pluripotency, many of which we will discuss here. **Nanog** is a key transcription factor associated with the development of pluripotency and self-renewal in embryonic stem cells and induced pluripotent stem cells. According to *Silva et al [10]*, Nanog is necessary for the development of the so-called “pluripotent ground state”. For our purposes in the current study, we are defining
the effects of oncogenes that suppress anti-tumor proteins and their effect on mediating potency in normal fibroblast cells. One specific gene we are interested in is the \textit{p53} protein, which is a tumor suppressor and cell cycle regulator, as well as a known inhibitor of Nanog. \textit{Lin et al} found that \textit{p53} binds directly to the Nanog promoter and suppresses its transcription \cite{26}. \textit{p53} is also found to be inhibited in many different cancer types.

\textbf{Oct3/4} – is part of the family of Octamer transcription factors. Oct4’s protein product heterodimerizes with the protein product of Sox2, both of which are needed to stimulate Nanog production. Oct4 is important for maintaining pluripotency, as it has been shown that ESCs that have had Oct4 knocked out will spontaneously differentiate \cite{27}. \textbf{Sox2}, also known as SRY box 2, is a transcription factor that is critically involved with the mediation of pluripotency.

\textbf{Lin28} is used as a marker for undifferentiated stem cells. The protein encoded by Lin28 is an mRNA binding protein that stabilizes IGF2 mRNA, thereby increasing its ability to be translated. Lin28 is one of the factors used by the Thomson group in their generation of iPS cells.

\textbf{KLF4}, which stands for Kruppel-Like Factor 4, is a member of the Kruppel-Like family of transcription factors, which have been studied for their role in cell proliferation and differentiation; particularly their role in cancer. Structurally, they are characterized on their three zinc-finger domains at the C-terminus of the protein. KLF4 inhibits \textit{p53} transcription through interaction with the C-terminal domain of MUC1 \cite{27}.

\textbf{c-Myc} is a proto-oncogene transcription factor that can also regulate chromatin structure by changing histone acetylation \cite{26}.

In order to induce pluripotency, a combination of the factors \textit{Nanog, Sox2, and Oct3/4} must be introduced, along with one or more of the oncogenes \textit{Lin 28, KLF4, and/or c-Myc}. Because of the commonalities between \textit{p53} suppression and the ability of \textit{Lin 28, KLF4, and c-}
Myc, this project is set to assess the effects of introducing E6/E7 which suppresses p53 without the ectopic expression of any of these 3 oncogene proteins. The model we are using to summarize how these core pluripotency factors are working with each other is summarized in Figure 1.

What are the genes we are introducing and what are their known effects?

p53 is a tumor-suppressor gene, the so-called “guardian of the genome”. The number 53 in its name refers to its molecular weight of ~53 kilodaltons and is activated in response to DNA damage and oncogenic stressors. After its activation, there are three primary outcomes: DNA repair, cell cycle arrest, or apoptosis. One of its primary downstream targets is the cyclin-dependent kinase inhibitor p21 [28], which controls cell cycle progression at the G1 phase. p53 regulates Nanog expression by binding to its promoter and repressing transcription.

E6 is a Human Papilloma Virus type 16 (HPV-16) derived protein that hijacks the protein E6AP, an ubiquitin ligase protein. Because HPV-16 E6 is able to associate with p53, E6AP specifically targets p53 for ubiquitination and subsequent proteosomal degradation [28]. There have been a few studies that have implicated p53 in iPS reprogramming. Kawamura et al were able to generate murine iPS cells by suppressing p53 and by infecting with the factors Oct4 and Sox2 [29]. The induction of oncogenic stimuli such as c-Myc induces apoptosis or cell cycle arrest in the presence of p53, thereby reducing reprogramming efficiency. This, along with the fact that p53 suppresses Nanog transcription, implicates p53 in reprogramming suppression.
Figure 1: The transcription factors Nanog, Oct4, and Sox2 work together in an autoregulatory loop to establish pluripotency in terminally differentiated somatic cells. Expression of these factors is controlled upstream by Klf4. Activation of c-Myc is required to allow expression of the factors that make up the pluripotency network.
Other factors that may influence pluripotency

\textbf{pRb} is the protein product for the Retinoblastoma gene. Retinoblastoma is a tumor-suppressor protein and is a member of the pocket protein family, which interacts with other proteins. pRb binds to and inhibits transcription factors in the \textit{E2F} family, ultimately preventing cells from transitioning from G1 into the S phase of the cell cycle. Inactivated pRb, which occurs through hyperphosphorylation, encourages cell cycle progression by blocking its ability to bind to and inhibit the E2F family, allowing for transcription of DNA replication- and proliferation-specific genes. \textbf{E7} is another HPV-16 associated protein that is associated with cancer. E7 binds pRb and sequesters it so it may not act on its substrates (e.g. the E2F transcription factor), resulting in an increase in cell cycle progression and unregulated cell growth.

\textbf{Significance/Impact of Research}

Understanding the mechanisms behind the interplay between pluripotency factors and the suppression of antitumor proteins may help us better understand the role of stem cells in certain cancers. For example, osteosarcoma cell lines derived from a murine model are associated with the suppression of \textit{p53} and \textit{pRb} and were shown to differentiate into adipogenic and osteogenic lineages \textit{in vitro} \cite{30}. Because p53 inhibition has been investigated only in murine iPS models, we wanted to investigate the role that p53 plays in the suppression of differentiation capacity in normal cells after expression of the pluripotency genes, Oct4, Sox2, and Nanog. Specifically, our study addresses the hypothesis that decreasing p53 function using E6/E7 allows terminally differentiated normal fibroblasts to de-differentiate into a more primitive mesenchymal stem cell, thereby increasing the cells’ ability differentiate down adipocyte and osteocyte pathways.
Chapter 2. Materials and Methods

Cell Lines and Culturing

BJ fibroblast cells isolated from human foreskin that were stably infected with the human telomerase reverse transcriptase (hTERT) were used as our primary test cell line. They had undergone 100 population doublings (PD) at the beginning of the experiment. BJ-hTERT fibroblasts were grown in high glucose Dulbecco's modified eagle's medium (DMEM; Invitrogen) supplemented with 10% cosmic calf serum (Hyclone) and 1% Antibiotic/Antimycotic Solution (ABAM; Sigma-Aldrich). PA317 cells were cultured in the same media as BJ cells.

Adipose-derived stem cells 8 (ASC 8) at PD10 were used as our positive controls and were previously isolated in our laboratory from lipoaspirate obtained from the VCU Surgery Department in accordance with VCU IRB procedures for medical waste. ASC 8 cells were grown in low glucose DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% ABAM.

Retroviral Infection

In order to determine sensitivity to G418, we performed a kill curve by exposing BJ-hTERT fibroblasts to varying concentrations of G418 ranging from 200 ng/mL to 1000 ng/mL in 200 ng/mL increments. We found that 100% cell death occurred at a minimum concentration of 600 ng/mL, which was the concentration used for selection following infection. PA317 cells with stable integration of pLXSN (empty vector), pLXSN-HPV-16 E6, or pLXSN-HPV-16 E6/E7 were cultured in BJ-hTERT media for 24 hours, followed by harvesting the media with productive virus. The virus-containing media was filtered (0.45μm) and either frozen (-80°C) or
used for infection. The BJ fibroblasts were retrovirally infected with the viral stocks and subjected to 600 ng/mL of G418 for selection for 10-14 days, until mock-infected plates were 100% dead upon selection. Stable selection of BJ-hTERT cells created the BJ-hTERT/pLXSN, BJ-hTERT/E6, and BJ-hTERT/E6/E7 cell lines, which were used for subsequent experiments.

**Western Analysis**

To confirm p53 knockdown in these cells, we performed Western analysis for p53 and β-actin (as an internal control) after treating the cells with the DNA damaging agent, Adriamycin 1μM for 1 hour). Cells were trypsinized and then lysed with Radio-Immunoprecipitation Assay (RIPA) buffer supplemented with protease inhibitor (Sigma) for 30 minutes on ice. The lysate was transferred to a 1.5-mL tube and centrifuged at 11,000 x g for 20 minutes. The resulting supernatant was collected and analyzed for total protein content using a Biorad Protein Assay kit and a spectrophotometer.

Protein samples were denatured in 4x sample buffer at 85°C for 10 minutes and then loaded (30μg total protein per sample) on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) followed by electrophoresis for 1.5 hours. Following SDS-PAGE, the gel was transferred to nitrocellulose membrane by electroblotting at 100 volts for 1 hour. The membrane was blocked using 5% non-fat milk for 1 hour at room temperature, followed by multiple washes with PBS+1% Tween-20 (PBS-T). The blot was incubated with primary antibodies (anti-p53, at 1:1000 dilution; BD Biosciences; anti-β-actin at 1:5000 dilution; Sigma) for 1 hour at room temperature, followed by multiple PBS-T washes. Secondary antibody (goat anti-mouse conjugated to horseradish peroxidase, at 1:5000 dilution; KPL, Inc., Gaithersburg, MD) was used to probe the blot for 1 hour at room temperature, followed by extensive washing with PBS-T.
Pierce SuperSignal (luminol and peroxide solutions) was used for detection, and the blot was exposed to Kodak OMAT film and developed.

**Adenoviral Infections**

Adenoviral vectors and stocks were obtained from the VCU Core Laboratories after cloning each gene into the shuttling vector, pZeroTg-CMV. Productive Adenovirus carrying the genes *Nanog, Oct4, Sox2,* and *c-Myc* were used to transiently infect BJ-hTERT cells with an initial concentration of 100,000 PFU (plaque forming units). We then exposed our BJ-hTERT cell lines (infections done based on Table 1) to 25-50μL per vector (2.5-5 million PFU total for 3 million cells per dish) for a total volume of 100μl per dish (50 for Nanog only). This procedure was done 2X, separated by a 1 week recovery phase between each infection.

**Differentiation Media Conditions**

For adipogenesis induction, low glucose DMEM with 10% FBS and 1% ABAM media was supplemented with the following final solution concentrations: 0.5mM isobutyl-methylxanthine (IBMX), 1μM dexamethasone, 0.01mM insulin, and 200μM indomethacin. Cells were exposed to adipogenic media for up to 2 weeks, replacing the media every 72 hours. At time 0 (control) and two weeks following exposure, cells were trypsinized, suspended in DMEM, and centrifuged at 300 x g for 3 minutes. Dry cell pellets were stored at -80°C for later analysis.

For osteogenic induction, high glucose DMEM with 10% FBS and 1% ABAM media was supplemented with 10mM β-glycerophosphate, 50μM Ascorbate-2-Phosphate, and 0.1μM dexamethasone. Cells were exposed to this media for a period of 2 weeks, replacing the media
<table>
<thead>
<tr>
<th>Gene</th>
<th>Method of Infection</th>
<th>Role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-16 E6</td>
<td>Lentivirus</td>
<td>p53 inhibitor, hijacks E6AP which then in turn ubiquitinates p53 targeting it for proteosomal destruction</td>
<td>el-Deiry et al, 1993</td>
</tr>
<tr>
<td>HPV-16 E7</td>
<td>Lentivirus</td>
<td>pRb inhibitor, binds pRB so that it may not work on its substrate p16^INK4a</td>
<td>Giarre et al, 2001</td>
</tr>
<tr>
<td>Nanog</td>
<td>Adenovirus</td>
<td>Transcription factor associated with the induction of pluripotency</td>
<td>Johnson et al, 2008</td>
</tr>
<tr>
<td>Oct4</td>
<td>Adenovirus</td>
<td>Transcription factor associated with the induction of pluripotency, heterodimerizes with Sox2 to upregulate Nanog</td>
<td>Johnson et al, 2008</td>
</tr>
<tr>
<td>Sox2</td>
<td>Adenovirus</td>
<td>Transcription factor associated with the induction of pluripotency, heterodimerizes with Oct4 to upregulate Nanog</td>
<td>Johnson et al, 2008</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Adenovirus</td>
<td>Transcription factor, creates global changes that effect cell proliferation, differentiation, and survival. Recruits MAD complex which in turn can make several global epigenetic changes.</td>
<td>Grandori et al, 2000</td>
</tr>
</tbody>
</table>
every 72 hours. At time 0 (control) and 2 weeks following exposure, cells were trypsinized, suspended in DMEM, centrifuged at 300 x g for 3 minutes, and stored at -80°C for later analysis.

**Adipogenic Characterization**

To assess adipogenic differentiation, cells were fixed with a 10% formalin solution for 30 minutes, followed by washing the dish with 60% isopropanol. Cells were then stained with a working solution of Oil Red O (100mg powdered Oil Red O (Sigma), 30mL 99% ethanol, 20mL deionized water) for 5 minutes. The stain was rinsed off with water and counterstained with 2mL hematoxylin for 2 minutes. Images were obtained using phase-contrast microscopy at 40x magnification, and image analysis was performed using Adobe Photoshop CS3. Three fields were selected and cells that contained Oil Red O stained lipid droplets were counted as well as a total cell count for the field. Differentiation was measured as percentage of cells containing Oil Red O stained lipid droplets divided by the total number of cells counted averaged over the three fields.

**Osteogenic Characterization**

To assess osteogenic differentiation, cells were fixed with an iced-cold 70% ethanol solution for 1 hour and washed twice with water. The cells were then stained with Alizarin Red for 30 minutes, and then washed multiple times with water. Images were obtained using phase-contrast microscopy at 40x magnification. Image analysis was performed using ImageJ version 1.44. Three fields of each cell type and infection schedule were selected and we split each image into its constituent red, green, and blue channels. The red channel was selected and converted to grayscale, and a threshold was established (1/2 max). All areas displaying a minimum of ½ max
red intensity were then selected and quantified compared to the entire area, giving us a percentage of the field that positively stained for Alizarin Red. Results were then graphed and compared to baseline red intensity (which we defined as BJ hTERT no factors).

**RNA Isolation and Reverse Transcription**

RNA was isolated from whole cells using materials and protocol from the GenElute Mammalian Total RNA Miniprep Kit. RNA concentration and purity was quantified using a NanoDrop spectrophotometer (Thermo Scientific). We then performed a reverse-transcriptase reaction to create a cDNA library. The reaction consisted of 2μL RT buffer, 0.8μL dNTP mix (100mM), 2μL 10x RT random primers, 1μL HotStarTaq, 1μL RNase inhibitor, and nuclease-free water with 2ng total RNA for our sample for a total volume of 20μL. The following program was used to create the cDNA library: 25 °c for 10 minutes, 37 °c for 120 minutes, 85 °c for 5 minutes, and 4 °c for 23 hours (storage temp).

**Quantitative Real-Time RT-PCR**

We performed a Quantitative Real-Time PCR (qPCR) using the generated cDNA library to examine the expression levels of genes using primer sets as follows:

- **Pluripotency markers**: Oct4 (forward 5’-CAGTGCCGAAACCCCACAC-3’, reverse 5’-GGAGACCCAGCAGCCTCAAA-3’), Sox2 (forward 5’-TACCTCTTCCTCTCCACTCCA-3’, reverse 5’-GGTAGTGCTGGGACATGTGA-3’), Nanog (forward 5’-TTTGGAAAGCTGCTGCTGGGGAAG-3’, reverse 5’-GATGGGAGGAGGGAGGAGGA-3’), and c-Myc (forward 5’-GCGTCTGGGAAGGAGATCCGAGC-3’, reverse 5’-TTAGGGGCGATCGTCGGCGGGAGGCTG-3’).
Adipogenic markers: PPARγ (forward 5’-TCTCTCGTAATGGAAGACC-3’, reverse 5’-GCATTATGAGACATCCACCAC-3’), LPL (forward 5’-TCAGCTGTGCTCTGCAGGGG-3’, reverse 5’-CTCCAGAGTCTGACCGCCT-3’)

Osteogenic markers: Osteocalcin (forward 5’-AGCAAGGTCAGCCTTTGT-3’, reverse 5’-GCGCCTGGGTCTCTTCCTC-3’), Osterix (forward 5’-GGGACTGGAGCCATAGTGAA-3’, reverse 5’-CTCAGCTCCTCTCCATCTGCC-3’)

Control (housekeeping gene): Cyclophilin A (forward – 5’-GCATGATCGGGAGGGTTTACT-3’, reverse – 5’-TCCTTGCCACTCCTATTCTTT)

An ABI PRISM 7900 (Life Technologies) was used to perform the gene expression analysis. We used the following reagents per reaction – 10μL ABI SYBR Green 2x master mix, 2μL cDNA (~20ng total), 1μL of each primer set (~10pmoles of each), 6μL DEPC treated water for a total of 20μL per reaction.

**Statistical Methods**

The software used to perform the qPCR was and extract the raw data was SDS 2.2. Microsoft Office Excel 2007 was used to perform statistical analysis. A Student’s T-test was used to compare differences between samples and our significance level for all differences was set at P<0.05.
Chapter 3. Results

Specific Aim: Examine the effects of E6/E7 on induced differentiation in BJ fibroblast cells expressing with the pluripotency factors Oct4, Nanog, Sox2, and c-Myc, focusing specifically on osteogenic and adipogenic lineages. Cells will be infected with various combinations of the pluripotency genes, based on the schedule in Table 1, and differentiation potential and gene expression changes will be assessed for at least 3 weeks.

Hypothesis: We propose that by introducing E6/E7 (and the resultant downregulation of p53 and Rb), normal fibroblast cells will be more able to differentiate down mesenchymal lineages when expressing various combinations of the genes of pluripotency – Nanog, Oct4, Sox2, and/or c-Myc. We also predict that while these cells have de-differentiation potential (i.e. from terminally differentiated cells to more multipotent), they will likely not be pluripotent or show iPS morphology.

Rationale: Because p53 inhibits Nanog, and because reprogramming has been achieved with p53 suppression and stable infection of the iPS factors Oct4 and Sox2 without oncogenes in mice, we expect that infection with iPS factors will result in cells that are able to differentiate down adipogenic and/or osteogenic lineages. While we are hopeful that oncogenic expression (c-Myc) will not be necessary to provide this de-differentiation effect in the absence of functional p53 and pRB, our expectation is that c-Myc will be required to provide the necessary conversion of BJ fibroblasts to a more primitive state. As controls, cells infected with an empty vector (and therefore retain functional p53 and pRB) in the presence of iPS factors, with or without c-Myc, are expected to remain as terminally differentiated skin fibroblasts when exposed to
differentiation-inducing media. In order to test our hypothesis, our various BJ-infected cells will be subjected to osteogenic or adipogenic differentiation cocktails for 3 weeks, and differentiation will be quantified by multiple methods, including staining and counting versus controls and RT-PCR for lineage specific gene expression.

**Creation of a p53 deficient cell line**

In order to investigate the role of p53 as an inhibitor, we created a three stably infected BJ hTERT cell lines via the use of three lentiviral vectors: a pLXSN vector containing the gene for the human papillomavirus-derived E6 protein, a pLXSN vector containing both the HPV-derived E6 and E7 proteins, and an empty pLXSN vector. Three separate virus-generating PA317 cell lines containing each of the three previously described lentiviral vectors (E6, E6/E7, and empty pLXSN) were used to generate viral-particle containing media which was used to perform the infections to generate three stably-infected BJ hTERT cell lines. These cells were selected using G418 for a period of 10-14 days.

The tumor-suppressor protein p53 is not normally expressed in healthy cells in levels detectable by western blot analysis. In order demonstrate suppression of p53 in our three stably-infected cell lines, we exposed the cells to Adriamycin for a period of 1 hour then harvested the cells for western blot analysis. We performed a western blot and found that Adriamycin did cause an upregulation of the p53 protein in the non-infected BJ hTERT line as well as the E6, and pLXSN stably infected BJ hTERT fibroblast cell lines (Figure 2). p53 was not upregulated in our E6/E7 stably infected BJ hTERT cell line following Adriamycin treatment (demonstrating functional p53 suppression in this line) and was used as our positive comparison line for p53
suppression in further experiments. Beta actin was used as our endogenous loading control and was consistent across all lines and treatments (Figure 2).
Figure 2: BJ fibroblasts previously stably infected with the hTERT catalytic subunit were infected with lentiviral E6, E7 and E7, and pLXSN (an empty vector construct). Adriamycin (a p53 inducer) was then applied to each of the groups of cells for a period of 1 hour. A western blot for p53 was performed to demonstrate upregulation of p53 following Adriamycin treatment.
Adenoviral infection and examination of pluripotency factor expression

Infections were performed carrying the genes Nanog, Oct4, Sox2, and c-Myc according to the infection schedule in Table 2 in duplicate to ensure expression, each infection 1 week apart to allow the cells to recover in between. We then extracted the RNA from each line, converted it to cDNA via reverse transcription PCR, and quantified expression of each factor relative to an endogenous cyclophilin A control using Quantitative Real-Time Polymerase Chain Reaction (qPCR). Results of this experiment are shown in Table 3. All expression is measured relative to the “baseline” expression of BJ hTERT fibroblasts without any adenoviral vectors added; baseline expression being equal to 1. A logarithmic y-axis (base 10) was used to show differences in expression that would not have been seen on a non-logarithmic scale. A sample of Induced Pluripotent Stem Cell (iPSc) RNA was used as a control for core pluripotency genes was included in Figure 3 a-c.

We found that Nanog was expressed highly (at least to the level of the comparative iPSc line) in every cell line/infection combination that we had introduced Nanog into except for the BJ hTERT stably infected E6/E7 cell line with all 4 introduced adenoviral vectors (Nanog, Oct4, Sox2, and cMyc) (Figure 3a). We also found that there was minimal Nanog expression in comparison with baseline in each cell line infected with the adenoviral vectors Oct4 and Sox2, suggesting that Oct4 and Sox2 alone are not enough to stimulate Nanog expression even in the p53 suppressed E6/E7 infected BJ hTERT fibroblast cell line.

We found that Oct4 was expressed highly in each cell line that we had introduced it into, except for the BJ hTERT stably infected pLXSN cell line with Oct4 and Sox2 added (showed no difference when compared to baseline), and to a lesser extent the E6/E7 stably infected Bj
Table 2: Adenoviral infection combinations

<table>
<thead>
<tr>
<th>Cell Types (stable infection)</th>
<th>Nanog</th>
<th>Oct4</th>
<th>Sox2</th>
<th>cMyc</th>
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</thead>
<tbody>
<tr>
<td>BJ hTERT pLXSN</td>
<td>XX</td>
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<td>XX</td>
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<tr>
<td>BJ hTERT E6/E7</td>
<td>XX</td>
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<tr>
<td></td>
<td>XX</td>
<td>XX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BJ hTERT</td>
<td>XX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Vector Added</td>
<td>XX</td>
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</tbody>
</table>
A  Relative Expression of Nanog to Baseline

B  Relative Expression of Oct4 to Baseline

C  Relative Expression of Sox2 to Baseline
Figure 3

A. Comparison of expression of the core pluripotency factor Nanog across the different infection combinations and iPS cells using Quantitative Real-Time PCR. Cyclophlin A was used as an endogenous comparison. Relative expression is in comparison to BJ fibroblast expression, no factors added.

B. Comparison of expression of the core pluripotency factor Oct4 across the different infection combinations and iPS cells using Quantitative Real-Time PCR. Cyclophlin A was used as an endogenous comparison. Relative expression is in comparison to BJ fibroblast expression, no factors added.

C. Comparison of expression of the core pluripotency factor Sox2 across the different infection combinations and iPS cells using Quantitative Real-Time PCR. Cyclophlin A was used as an endogenous comparison. Relative expression is in comparison to BJ fibroblast expression, no factors added.
hTERT line with Nanog, Oct4, and Sox2 added (showed increased expression over baseline but less than 3-fold).

We found that Sox2 was expressed highly in each cell line that it was introduced into as well. Interestingly, we also found Sox2 expression in a 2.6 fold increase over baseline in the E6/E7 stably infected BJ hTERT cells without Nanog infection. Overall this shows the nearly all of our adenoviral factor infections led to expression of those factors, with some potential synergy between factors in expressing Oct4 and Sox2 in the E6/E7 stably infected BJ hTERT cells (expression of Oct4 and Sox2 is much higher with the addition of all 4 factors in comparison with none, one, two, or three factors).

**Comparison of adipogenic differentiation between the infected groups**

Each of the infected cell lines (referenced in the Table 2 infection schedule) plus an adipose-derived stem cell positive control were subjected to an adipogenic differentiation media (LG DMEM with 10% FBS, 1% ABAM, 0.5mM isobutyl-methylxanthine (IBMX), 1μM dexamethasone, 0.01mM insulin, and 200μM indomethacin) for a period of two weeks. The cells were then fixed with a 10% formalin solution, washed with 60% isopropanol, and stained with Oil Red O to detect any lipid droplets that had formed in the cytoplasm as a result of adipogenic differentiation. Cells were counterstained with Gills Hematoxilin.

The cells were then photographed under phase-contrast microscopy at 40x. Three fields for each of the infection combinations were photographed and analyzed for Oil Red O staining. Representative images of each of the infection combinations are seen in Figure 4, part A. The different combinations of BJ hTERT and BJ hTERT +pLXSN fibroblasts grew to confluence and tended to flatten out a bit, while the BJ hTERT +E6/E7 fibroblasts continued to grow and
Figure 4: Adipogenic Differentiation

A. Following adenoviral infection, cells were exposed to adipogenic differentiation media for 2 weeks. The cells were then stained with Oil Red O and examined under bright field microscopy. Representative images of the different cell types (BJ hTERT, BJ hTERT +pLXSN, BJ hTERT +E6/E7) and adenoviral infection subsets (no infections; +Nanog; +Oct4 and Sox2; +Nanog, Oct4, and Sox2, and +Nanog, Oct4, Sox2, and cMyc) are shown at 40x magnification.

B. Cells containing any stain were counted as positively staining for Oil Red O. Positively stained cells were counted in 3 fields for each infection group then divided by the total number of cells in a field. Percentages were then recorded and graphed (1 = 100%).
become superconfluent, suggesting possible suppression of a growth inhibitor or overexpression of a growth promoter.

In order to determine the amount of differentiation between the groups, we analyzed each of the three fields we photographed for each viral combination (Figure 4, Part B). Cells that contained any lipid droplets were considered “differentiated”; these were counted and then divided by the total number of cells counted in a field to give us a percentage of cells that had undergone some amount of differentiation. We performed a series of t-tests (two-tailed t-test assuming unequal variance) in order to compare the differences between the amount of differentiation in the PLXSN infected fibroblasts (our control group) to the E6/E7 infected fibroblasts (our test group).

We found significant differences (p<0.05) between the following groups: PLXSN infected fibroblasts with Nanog and E6/E7 fibroblasts infected with Nanog (p=0.014); PLXSN infected fibroblasts with Oct4 and Sox2 and E6/E7 fibroblasts infected with Oct4 and Sox2 (p=0.010). We did not find significant differences between the following groups: PLXSN infected fibroblasts with no adenoviral factors and E6/E7 fibroblasts with no adenoviral factors (p=0.086), PLXSN infected fibroblasts with Nanog, Oct4, and Sox2 and E6/E7 fibroblasts with Nanog, Oct4, and Sox2 (p=0.061); and PLXSN infected fibroblasts with Nanog, Oct4, Sox2, and cMyc and E6/E7 infected fibroblasts with Nanog, Oct4, Sox2, and cMyc (p=0.321).

In order to make a further comparison of the cells to compare differentiation capacity, we looked at two factors that are known markers of adipogenic differentiation: lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor gamma (PPAR-γ). After differentiating the same groups of cells for two weeks, we extracted the RNA using a GenElute Mammalian Total RNA Miniprep Kit, reverse-transcribed the RNA into cDNA, and performed a quantitative
real-time PCR of each of the factors separately to a cyclophilin A endogenous control. Baseline expression of each factor is compared with the relative expression found in BJ hTERT cells, no factors added. Results of this experiment are shown in Figure 5.

**Comparison of osteogenic differentiation between the infected groups**

Each of the infected cell lines (referenced in the Table 2 infection schedule) plus an adipose-derived stem cell positive control were again subjected to an osteogenic differentiation media (HG DMEM with 10% FBS and 1% ABAM media supplemented with 10mM β-glycerophosphate, 50μM Ascorbate-2-Phosphate, and 0.1μM dexamethasone) for a period of two weeks. The cells were then fixed with a 70% ethanol solution for 1 hour and washed twice with water. The cells were then stained with Alizarin Red for 30 minutes, and then washed multiple times with water. Cells were counterstained with Gills Hematoxilin.

The cells were then photographed under phase-contrast microscopy at 40x. Three fields for each of the infection combinations were photographed and analyzed for Alizarin staining. Representative images of each of the infection combinations are seen in Figure 6, part A.

The images were then analyzed for total red image intensity to determine the amount of Alizarin Red staining (and therefore osteogenic differentiation) they had undergone. Image analysis was performed using ImageJ version 1.44. Three fields of each cell type and infection schedule were selected and we split each image into its constituent red, green, and blue channels. The red channel was selected and converted to grayscale, and a threshold was established (1/2 max). All areas displaying a minimum of ½ max red intensity were then selected and quantified compared to the entire area, giving us a percentage of the field that positively stained for Alizarin Red. Results were then graphed and compared to baseline red intensity (which we defined as BJ hTERT no adenoviral factors added). Results are seen in Figure 6, part B.
Figure 5: Adipogenic Differentiation Markers

A. Comparison of expression of the adipogenic differentiation marker Lipoprotein Lipase (LPL) across the different infection combinations and iPS cells using Quantitative Real-Time PCR. Cyclophlin A was used as an endogenous comparison. Cells were exposed to adipogenic differentiation media for two weeks before RNA extraction, reverse transcription, and quantification. Relative expression is in comparison to BJ fibroblast expression, no factors added. Relative fold differentiation (Y-axis) is on a logarithmic scale (base 10).

B. Comparison of expression of the adipogenic differentiation marker Peroxisome proliferator-activated receptor gamma (PPAR-γ) across the different infection combinations and iPS cells using Quantitative Real-Time PCR. Cyclophlin A was used as an endogenous comparison. Cells were exposed to adipogenic differentiation media for two weeks before RNA extraction, reverse transcription, and quantification. Relative expression is in comparison to BJ fibroblast expression, no factors added.
We performed a series of t-tests (two-tailed t-test assuming unequal variance) in order to compare the differences between the amount of differentiation in the PLXSN infected fibroblasts (our control group) to the E6/E7 infected fibroblasts (our test group). We found significant differences between the following groups: PLXSN infected fibroblasts with Nanog and E6/E7 fibroblasts infected with Nanog (p=0.007); PLXSN infected fibroblasts with Oct4 and Sox2 and E6/E7 fibroblasts infected with Oct4 and Sox2 (p=0.019), PLXSN infected fibroblasts with Nanog, Oct4, and Sox2 and E6/E7 fibroblasts with Nanog, Oct4, and Sox2 (p=0.011); and PLXSN infected fibroblasts with Nanog, Oct4, Sox2, and cMyc and E6/E7 infected fibroblasts with Nanog, Oct4, Sox2, and cMyc (p=0.030). We did not find significant differences between the following groups: PLXSN infected fibroblasts with no adenoviral factors and E6/E7 fibroblasts with no adenoviral factors (p=0.360).

In order to make a further comparison of the cells to compare differentiation capacity, we looked at two factors that are known markers of osteogenic differentiation: Osteocalcin and Osterix. After differentiating the same groups of cells for two weeks, we extracted the RNA using a GenElute Mammalian Total RNA Miniprep Kit, reverse-transcribed the RNA into cDNA, and performed a quantitative real-time PCR of each of the factors separately to a cyclophilin A endogenous control. Baseline expression of each factor is compared with the relative expression found in BJ hTERT cells, no factors added. Results of this experiment are shown in Figure 7.
A

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<td>BJ hTERT E6/E7</td>
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B

Average Percentage of Stained Red Area over Threshold
Figure 6: Osteogenic Differentiation

A. Following adenoviral infection, cells were exposed to osteogenic differentiation media for 2 weeks. The cells were then stained with Alizarin Red and examined under bright field microscopy. Representative images of the different cell types (BJ hTERT, BJ hTERT +pLXSN, BJ hTERT +E6/E7) and adenoviral infection subsets (no infections; +Nanog; +Oct4 and Sox2; +Nanog, Oct4, and Sox2, and +Nanog, Oct4, Sox2, and cMyc) are shown at 40x magnification.

B. Images were then analyzed for Alizarin Red staining using the program ImageJ. Red intensity over ½ max over the area of the image was quantified (as a percentage of the image) and recorded over 3 separate fields. This was averaged and then plotted on the chart above.
Figure 7: Osteogenic Differentiation Markers

a. Comparison of expression of the osteogenic differentiation marker Osterix across the different infection combinations and iPS cells using Quantitative Real-Time PCR. Cyclophlin A was used as an endogenous comparison. Cells were exposed to osteogenic differentiation media for two weeks before RNA extraction, reverse transcription, and quantification. Relative expression is in comparison to BJ fibroblast expression, no factors added.

b. Comparison of expression of the osteogenic differentiation marker Osteocalcin across the different infection combinations and iPS cells using Quantitative Real-Time PCR. Cyclophlin A was used as an endogenous comparison. Cells were exposed to osteogenic differentiation media for two weeks before RNA extraction, reverse transcription, and quantification. Relative expression is in comparison to BJ fibroblast expression, no factors added.
Chapter 4. Discussion

Our understanding of the network of pathways underlying the ability of terminally differentiated cells to become reprogrammed has increased significantly in the past five years, beginning with the generation of Induced Pluripotent Stem cells by Yamanaka et al. in 2006 [31]. Since then, much research has been performed to increase the efficiency of reprogramming and decrease the number of factors needed to achieve reprogramming. The ultimate goal of iPSc generation is to be able to reprogram a patient’s own cells using non-integrative factors with a high level of reprogramming efficiency. Understanding of the underlying networks which can increase or hinder reprogramming allows researchers to use this information to improve iPSc generation or partial reprogramming of terminally differentiated cells.

We wanted to examine the effects E6/E7 on BJ fibroblasts and if we could achieve partial reprogramming without the use of cMyc, since the E6/E7 proteins have been shown to suppress p53 (also suppressed by cMyc) and pRb. The addition of E6/E7 into our BJ hTERT fibroblast cell line did not appear to increase transcription of Nanog, Oct4, or Sox2 when compared to BJ hTERT cells or pLXSN infected cells (Figure 3), suggesting that any increases in differentiation capacity may not just be in response to transcriptional regulation of those core pluripotency factors.

We found that expressing the HPV derived E6 and E7 proteins can increase differentiation capacity when differentiating fibroblasts into adipocytes and osteoblasts. Going back to our t-tests to determine if there was a statistically significant difference between each of our pLXSN and E6/E7 infection combinations in the adipogenic differentiations (using a significance level of 0.05), we determined that there were significant differences between the groups that were infected with Nanog only, and infected with Oct4 and Sox2, with there being a
higher percentage of cells in the E6/E7 group showing adipogenic differentiation. Without factoring in significance, the E6/E7 group showed more differentiation across all infection combinations than the pLXSN infected groups.

When looking at osteogenic differentiation we find similar results. We found statistically significant differences between the E6/E7 group and the pLXSN group over all adenoviral infection combinations except for the groups that had no adenovirus added. In each of these instances, the E6/E7 groups had a greater percentage of Alizarin Red staining over the areas photographed when compared with the pLXSN groups, suggesting that there was increased osteogenic differentiation in the E6/E7 infected fibroblasts.

In order to examine whether or not the role of E6/E7 is similar to that of cMyc, we conducted t-tests comparing the percentage of adipogenic and osteogenic differentiation within a stably infected group between adenoviral infections. Our expectation, based on our knowledge of cMyc and E6/E7, is that we should see a significant difference between the Nanog, Oct4, and Sox2 infected group and the Nanog, Oct4, Sox2, and cMyc infected group with pLXSN but not with E6/E7, because both E6/E7 and cMyc have been shown to interfere with the normal function of p53. However, in the adipogenic differentiation group there were no significant differences in differentiation percentage with or without cMyc in either the pLXSN stably infected cells (p=0.205) or the E6/E7 infected cells (p=0.247). The same was true for osteogenic differentiation group, as there was no significant differentiation with or without cMyc in the pLXSN stably infected cells (p=0.294) or the E6/E7 stably infected cells (p=0.747). However in each case we did find increased differentiation with the addition of cMyc in all cases, if not significant.
It was surprising that we didn’t see statistically significant increased differentiation with the addition of the cMyc adenoviral vector across our different cell lines. Based on previous research that suggested the necessity of cMyc [31], it’s possible our vector didn’t work, or that the effect on differentiation was not enough to be statistically significant. Based on the increases in the amount of differentiation seen when E6/E7 is introduced in comparison with the empty vector pLXSN and the addition of core pluripotency factors, we have revised our model from Figure 1. Our new model is explained in Figure 8.

Supporting our assessment that we increased differentiation in the E6/E7 stably infected adipogenic lineages, we found increased expression levels of adipogenic markers lipoprotein lipase and peroxisome proliferator-activated receptor gamma in the E6/E7 infected cell lines in comparison with the pLXSN infected cell lines. We also found evidence for increased osteoblastic differentiation in E6/E7 infected cell lines in comparison with the empty vector pLXSN infected cell lines. Osteocalcin expression was increased up to 10 fold over baseline expression in the E6/E7 infected lines. However, there did not seem to be an increase in the osteogenic marker Osterix in any of our cell lines when compared to baseline expression.

There are some important limitations to this study that need to be addressed. First, our E6 only lentiviral vector failed to suppress p53 in BJ hTERT fibroblasts, so we ended up using the combination E6/E7 lentiviral vector. We would have liked to examine the effects of each protein separately on differentiation capacity. Suggested future studies include the examination of each of the proteins E6 and E7 separately, and examining the specific role of p53 and Rb suppression, perhaps with a stable infection of a factor expressing siRNA. We also would have liked to examine more lineages than just those of a mesodermal lineage to fully quantify differentiation.
There seemed to be a problem with the Nanog, Oct4, and Sox2 infected pLXSN BJ fibroblasts as well as the Nanog, Oct4, Sox2, and cMyc infected fibroblasts in that they did not grow back to confluence like our other cell lines following adenoviral infection. This may have been due to the amount of stress placed on them by the added adenoviral vectors. Due to this, we had much fewer cells to count for adipogenic and osteogenic differentiation, which resulted in increases in our standard error. It would have helped the accuracy experiment if we were able to have these cells remain confluent.

Overall we demonstrated that stable infection with fibroblasts with E6/E7 did have a positive effect on BJ hTERT fibroblasts ability to differentiate into an adipogenic or osteogenic lineage with the addition of core pluripotency factors. Based on what we already know about the mechanisms of E6 and E7 and their ability to suppress certain antitumor proteins, further study of suppressing p53 and Rb individually in fibroblast cell lines and inducing differentiation with a differential number of factors could help us determine their exact roles. Furthermore, methodologies could be employed to temporarily suppress p53, perhaps using siRNA or other techniques to increase reprogramming efficiency.
Figure 8: Oncogenic c-Myc is required for de-differentiation of terminally differentiated fibroblasts because of its ability to suppress p53. E6 infected cells showed the ability to de-differentiate fibroblasts with as little as the addition of a single pluripotency factor (Nanog) while cells infected with an empty vector (pLXSN) did not demonstrate the same level of differentiation, even when infected with the core pluripotency factors plus c-Myc.
CHAPTER 5. REFERENCES


Vita

John Hudson Moore II was born in the city of Stevens Point, Wisconsin. He finished his B.S. in Biology at the University of Wisconsin – Stevens Point in 2006. He came to Richmond, VA with his partner Nicole Bertrand to attend graduate school at Virginia Commonwealth University in 2008. He plans to continue biomedical research following the completion of his Master’s degree.