



2017

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Recommended Citation

Leggins, Autumn S.; Grosseohme, Nick; and Stern, Matthew M. (2017) "Controlling Oct4 Expression Levels Using Invitrogen's GeneSwitch™ System," *The Winthrop McNair Research Bulletin*: Vol. 3 , Article 6.

Available at: <https://digitalcommons.winthrop.edu/wmrb/vol3/iss1/6>

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Controlling Oct4 Expression Levels Using Invitrogen's GeneSwitch™ System

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SC INBRE Grant

ABSTRACT

Oct4 is a protein that is involved in the retention of pluripotency in adipose derived stem cells (ADSCs). Despite this knowledge, Oct4's exact role in the complex system used in maintaining pluripotency is not known. One approach to explore Oct4's role would be through the use of cellular assays to control the expression of Oct4. This can possibly be accomplished by introducing a biological switch and the gene of interest into ADSCs. In this project, the GeneSwitch™ System is used to ultimately induce Oct4 expression. Before the GeneSwitch™ System can be used, the Oct4 gene is extracted from murine embryonic stem cell (ES) RNA. This ES RNA is then used as a template to create complimentary DNA (cDNA) that can then be used to create an insert with the Oct4 gene. In addition to the cDNA, recognition sites for endonucleases must be added on to fully create the Oct4 insert. This insert could then be placed into one of the GeneSwitch™ System plasmids that have the same recognition sites and placed into ADSCs along with the plasmid that will act as a biological switch. With this system put into ADSCs, it is expected that Oct4 levels will be successfully controlled. Once controlled, Oct4 expression can be tested and investigations can be completed to determine how Oct4 expression levels influence pluripotency of ADSCs. This may have significant impact on the creation of regenerative medicine.

INTRODUCTION

Cells are the most basic living component that make up an organism. They divide, communicate, metabolize, transport, and complete many other tasks that the human body requires in order to remain at homeostasis. Remarkably, cells grouped together as tissues, organs and organ systems can accomplish much more complex regulatory processes than individual cells. Since cells have such an important role in the body, losing large amounts of cells would make normal body functions more difficult. The body is often able to replace naturally lost cells, but may not be able to replace large amounts of cells lost due to injury or disease—at least not quickly. Regenerative medicine has made major scientific progress in procedures and tissue repair that can help the body to replace cells on a larger scale. For example, people who have been severely burned can replace the lost tissue through the use of regenerative medicine. Stem cells, immature

cells that have not differentiated (i.e. specialized into a specific type of cell), have many possibilities in terms of what kind of cell they can become; this ability or potential to become any type of cell is referred to as pluripotency. This characteristic makes them an important resource in the field of regenerative medicine and holds tremendous potential for the future of all medicine (Pan et al. 2002). There are quite a few sources from which stem cells can be attained, but the most commonly discussed origins of stem cells are bone marrow or embryos.

Despite the potential and major progress in the study of stem cells, there are still some ethical and procedural issues that come with stem cell retrieval. For example, the use of embryonic stem cells presents a serious dilemma for many U.S. citizens. Others raise concern about the procedure to gather bone marrow stem cells (BMSCs), a process that is relatively expensive, intrusive, and has a comparatively

low yield (Zhu et al. 2008). Adipose derived stem cells (ADSCs) have become an attractive alternative source; these cells are cheaper, easier to obtain, and can be harvested in a much less invasive manner. This can be attributed to the abundance of fat available for medicinal use from either the patient or willing donors (Zhu et al. 2008). Not only is the use of ADSCs cheaper and more efficient, it is also still capable of having specified differentiation induced. This can be seen through an experiment conducted by Zhu et. al (2008), where ADSCs were successfully induced to differentiate into fat cells, bone cells, cartilage cells, and heart muscle cells.

While stem cells are able to differentiate, inducing their differentiation into specific cells can be more difficult. Typically, epigenetics—the study of how signals from an environmental or external source affect genes—plays a large role in the differentiation of stem cells. Cells can respond to signals in different ways based on many factors including: what kind of signal is being sent, how strong the signal is, and where the signal originates (Weidgang et al. 2016). Certain signals are able to trigger the stem cells to specifically differentiate into a certain type of cell. Using this same notion, it is possible for scientists to create induced pluripotent stem cells (iPSCs) that allow stem cell differentiation to be controlled through many methods including the use of a signal that can be operated using a biological “*switch*” (Stadtfeld and Hochedlinger 2010). This “*switch*,” depending on what genes it controls and what kinds of signals that gene is in control of, would be used to control whether the signal is “on” or “off” and the intensity of the signal. In order to make use of a “*switch*” to control stem cell differentiation, the gene of interest would have to be a gene that plays a role in the differentiation of stem cells. One such gene could be Oct4, a transcription factor that plays an important role in retaining the pluripotency of stem cells (Kim et al. 2009). Oct4 is usually used alongside many other transcription factors such as Sox2, Nanog, Klf4, and c-*Myc* in order to regulate the differentiation and retention of pluripotency of a stem cell (Takahashi and Yamanaka 2006). Typically,

many transcription factors are needed to control pluripotency of stem cells as noted by the research of Boyer et al. (2005) with embryonic stem cells that are involved in the early stages of embryo development. Out of the transcription factors listed, Oct4 is the only one that is absolutely essential for self-renewal and pluripotency of a stem cell (Jerabek et al. 2014); this means that there is a chance that controlling the pluripotency of stem cells could possibly be done by Oct4 only.

The work described here focused on controlling Oct4 expression through the use of Invitrogen’s GeneSwitch™ System. An insert with Oct4 and two recognition sites had to be made and introduced to the pGene vector of the GeneSwitch System—which should have the same recognition sites—through the use of cloning. From there, further investigation can be done to study whether the expression of Oct4 alone can regulate the pluripotency of murine ADSCs. Once more studies have been completed using murine ADSCs, it will be possible to expect many of the same findings within human ADSCs due to the fact that the *Pou5f1* gene sequence that controls expression of Oct4 for humans is 87% identical to that of murine *Pou5f1* (Jerabek et al. 2014).

METHODS

Embryonic stem cell ribonucleic acid (ES RNA) was used as a template to create complementary deoxyribonucleic acid (cDNA) through the process of reverse transcription. Reverse transcription is the formation of cDNA using RNA as a template rather than the formation of RNA from DNA that is seen with normal transcription. An iScript™ Reverse Transcription Supermix for quantitative reverse transcription polymerase chain reaction (RT-qPCR) from Bio-Rad and thermocycler were used to create the cDNA. The reaction protocol used to create the cDNA was 5 minutes of priming at 25°C, 30 minutes of reverse transcription at 42°C, and 5 minutes of reverse transcriptase inactivation at 85°C. The cDNA acted as a template for the polymerase chain reaction (PCR) process used to amplify the Oct4 gene for molecular cloning. The cDNA, which already contained the Oct4 gene, was

reacted with oligonucleotide primers from Eurofins Genomics containing recognition sites for KpnI and NotI in order to make the Oct4 insert. The 5' primer (5-CTTGGTACCATGGCTGGACACCTGGCTTCAGACTTCGCC) contained the recognition site for KpnI and the 3' primer (5-CGAGCGGCCGCCCCGTTTGAATGCATGGGAGAGCCAGAGCAGTG) contained the recognition site for NotI. A 3' primer with a stop codon and the recognition site for NotI (5-CGAGCGGCCGCCCCCTCAGTTTGAATGCATGGGAGAGCCAGAGCAGTG) was also used during PCR. The reaction protocol used for PCR was 2 minutes of denaturing at 94°C, 30 seconds of denaturing at 94°C, 30 seconds of annealing at 50°C, 3 minutes of elongation at 72°C, 1 minute of elongation at 72°C, and storage of PCR reaction at 4°C until the PCR reaction is retrieved. The PCR reaction went through 41 cycles of 30 second-denaturing, 30 second-annealing, and 3 minute-elongation before moving on to the step of 1 minute-elongation. Gel electrophoresis was completed and the gel was viewed through a Gel Doc™ XR+ System to ensure that the insert was the size it was expected to be. The insert was removed from the gel and gel DNA extraction was completed to purify the insert. The insert and pGene vectors—from Invitrogen's GeneSwitch™ System—were then digested in KpnI and NotI endonucleases at 37°C in a thermocycler to create the correct cuts on both the insert and vector. The DNA of the insert and vector was purified using gel electrophoresis. Each sample was loaded on to a 1% agarose gel and separated for 30 minutes at 115V. The agarose gel containing the samples was viewed under a Gel Doc™ XR+ System from Bio-Rad using ethidium bromide to invoke DNA fluorescence. The DNA was carefully extracted from the gel and further purified using the Gel DNA Extraction protocol.

RESULTS & DISCUSSION

ES RNA went through reverse transcription to create cDNA with the desired Oct4 gene. The presence of Oct4 in the cDNA was confirmed by running a gel electrophoresis

on a PCR reaction that incorporated cDNA and Oct4 primers that could be used to identify Oct4 using only a small segment of the Oct4 gene. It was expected that a band around 0.1kB would appear in the agarose gel and a band was present in the expected location as seen in Figure 1.

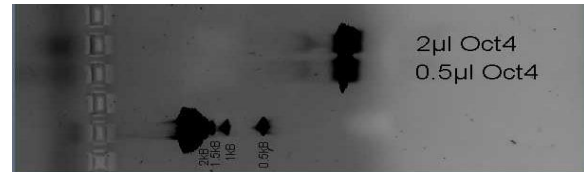


Figure 1. Gel Electrophoresis results for the PCR reaction including cDNA as the template and primers to identify a short sequence of Oct4. The lowest lane with bands contains the base pair ladder. The middle lane with bands contained the PCR product for the reaction using Oct4 primers and 0.5µL of cDNA. The top lane with bands contained the PCR product for the reaction using Oct4 primers and 2µL of cDNA. The expected band size for this PCR reaction was 100 base pairs or 0.1kB.

Once the cDNA was made, a PCR was completed using a 5' primer and 3' primer to add on recognition sites to the cDNA, but the PCR product did not appear in the region it was expected to be when it was viewed after running a gel electrophoresis. It was expected to appear around the 1kB region, but the band was found well below the 0.5kB region as seen in Figure 2. PCR reactions using various concentrations of MgCl₂ and DMSO were completed, but neither reaction resulted in bands within the 1kB region, as seen in Figure 3 and Figure 4; all reactions resulted in bands well below the 0.5kB region. A PCR reaction incorporating one of the Oct4 primers used to confirm the successful creation of cDNA and the 5' primer was completed along with a PCR reaction with the other Oct4 primer and the 3' primer. The resulting gel, shown in Figure 5, indicates that the reaction with the 3' primer worked, but the reaction with the 5' primer did not.

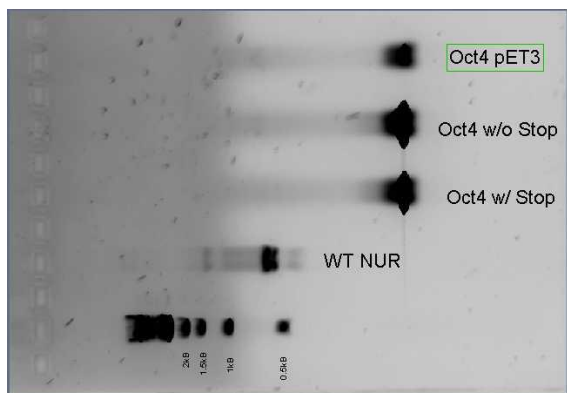


Figure 2. Gel Electrophoresis results for the PCR reaction including cDNA as the template, 5' primer with a recognition site for KpnI, and 3' primer with recognition site for NotI. The lowest lane with bands contains the base pair ladder. The second lowest lane contains the control PCR reaction using WT NUR, T7 promoter primer, and T7 terminator primer. The middle lane contains the PCR product for the reaction with cDNA as the template, a 5' primer with a KpnI recognition site, and a 3' primer with a NotI recognition site. The second highest lane with bands contains the PCR product for the reaction with cDNA as the template, a 5' primer with a KpnI recognition site, and a 3' primer with a NotI recognition site and a stop codon. The highest lane with bands contains the PCR product for the reaction with cDNA as the template, a 5' primer with a NdeI recognition site, and a 3' primer with a BamHI recognition site. The expected band size for the PCR reactions (excluding the control) were 1000 base pairs or 1kB.

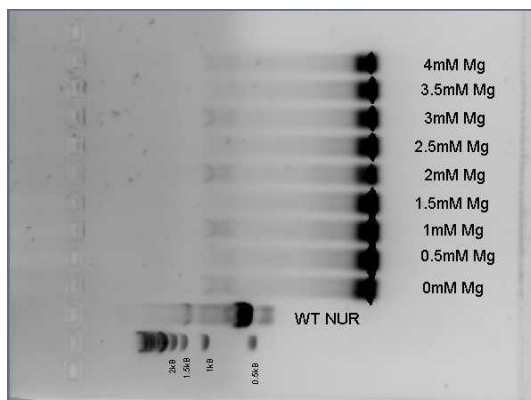


Figure 3. Gel electrophoresis results for PCR reactions with varying $MgCl_2$ concentrations. Each PCR reaction made use of cDNA as the template, a 5' primer with a KpnI recognition site, a 3' primer with a NdeI recognition site, and a specific concentration of $MgCl_2$. The $MgCl_2$ concentrations ranged from 0mM to 4mM and increased in increments of 0.5mM.

There are two possible reasons why the PCR of the 5' primer failed. One reason is that the 5' primer was not made correctly. Since the primer was designed while looking specifically at the expected cDNA sequence, there is very little reason to believe that primer design is the cause for this PCR to fail. The other possible reason is that the reverse transcription of ES RNA to make the cDNA did not completely work. When reverse transcription happens, the primer used during the process will find the template's poly-A tail in order to anneal to the 3' end of the mRNA. Once annealed, the primer will be used to copy the mRNA to cDNA, but it may not copy the entire template. This means that some of the 5' end of the cDNA is ultimately missing. Without this 5' end, the designed 5' primer will not have an overlapping template sequence to bind to and will not be able to amplify the cDNA.

A PCR to figure out where the 5' end of the cDNA started was completed. Primers that started at various intervals of the 5' end of the cDNA were made before being used in separate PCR reactions. Each reaction also used the 3' primer. A gel electrophoresis was completed after the PCR and bands were seen just below the desired region of 1kB, as seen in Figure 6. The bands were also sequentially increasing—which is what was expected—because of the fact that each primer was closer to the 5' end of the cDNA than the last. With this PCR, it was now known that the cDNA was completed at least to the 50th nucleotide of the expected cDNA sequence. It was decided that another PCR would be completed to manually build on the first 49 nucleotides. This PCR was completed using two primers that would build onto one another to form the remaining nucleotides needed for the cDNA. A gel electrophoresis was completed to see if the appropriate bands could be found after each build was added on to the cDNA and 3' primer. Bands around the 1kB marker were expected and found after viewing the agarose gel as seen in Figure 7.

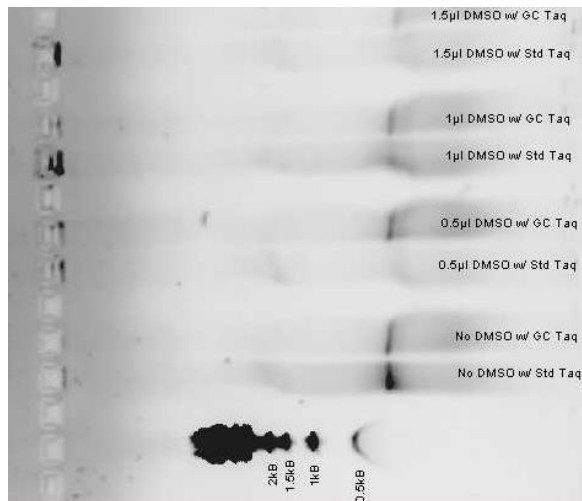


Figure 4. Gel electrophoresis results for PCR reactions with varying DMSO percentages. Each PCR reaction made use of cDNA as the template, a 5' primer with a KpnI recognition site, a 3' primer with a NdeI recognition site, and a specific concentration of DMSO. The DMSO percentages ranged from zero percent (0µL) to 3 percent (1.5µL).

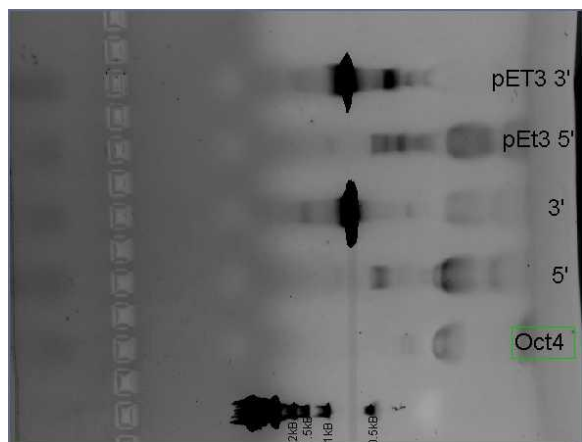


Figure 5. Gel electrophoresis results for PCR reactions using cDNA as the template, one of the Oct4 identifying primers, and either the 5' primer or 3' primer. The lowest lane with bands contained the base pair ladder. The second lowest lane with bands contained the PCR product for cDNA and the Oct4 identifying primers. The third lowest lane with bands contained the PCR product for the cDNA, 5' primer with a KpnI recognition site, and one of the Oct4 identifying primers. The third highest lane with bands contained the PCR product for cDNA, 3' primer with a NotI recognition site, and one of the Oct4 identifying primers. The second highest lane with bands contained the PCR product for cDNA, 5' primer with NdeI, and one of the Oct4 identifying primers. The highest lane with bands contained the PCR product for cDNA, 3' primer with BamHI, and one of the Oct4 identifying primers.

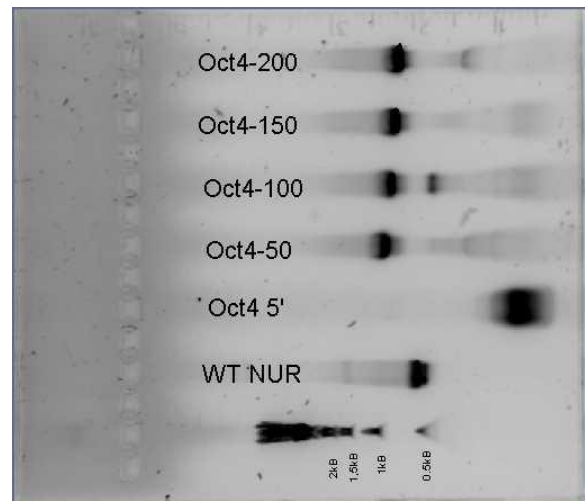


Figure 6. Gel electrophoresis results for PCR reactions with cDNA as the template, a 3' primer with a NotI recognition site, and of the primers starting at various intervals on the 5' end of the cDNA.

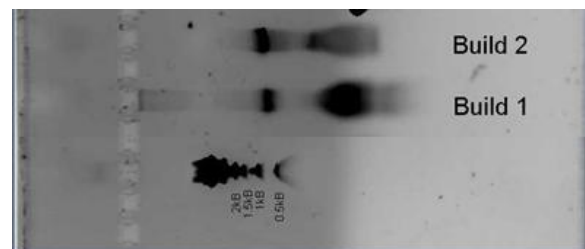


Figure 7. Gel electrophoresis results for PCR reactions with cDNA as the template, a 3' primer with a NotI recognition site, and either one or both of the Build primers.

After the cDNA received the needed recognition site from the 5' primer, the newly made Oct4 insert just needed to be cloned into the pGene plasmid before the pGene and pSwitch plasmids could then be transfected into the murine ADSCs and Oct4 expression levels could be investigated.

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