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# REGULATION OF SOX 4 AND SOX 11-MEDIATED GENE TRANSCRIPTION BY MIR-30-BASED RNAi

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MQP-BIO-DSA-8533

# REGULATION OF SOX 4 AND SOX 11-MEDIATED GENE TRANSCRIPTION BY MIR-30-BASED RNAi

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by


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April 30, 2009

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# ABSTRACT

This project's goal was to use a plasmid previously cloned in our laboratory encoding miR30 to determine its effectiveness to knock down the expression of Sox4 and Sox11, two genes closely associated with tumor initiation and progress. Western blots and luciferase reporter assays showed that miR30-based shRNAs resulted in strong knock-down of Sox4 and Sox11 expression and a decrease in their induction of Sox-mediated gene transcription in 3T3 cells. Our results suggest that miR30-based shRNAs might be used as an effective cellular tool to investigate the role of Sox4/11 in tumorigenesis.

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# ACKNOWLEDGEMENTS

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# BACKGROUND

## Hedgehog Signal Transduction Pathway

### *Discovery*

The hedgehog (Hh) signal transduction pathway was first discovered as a major pathway regulating human and animal development, but has since been shown to participate in stem-cell maintenance, carcinogenesis and tumorigenesis (Bürglin, 2008). Hh was first discovered in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980), but Hh family members have now been identified in most metazoans, deuterostomes, and protostomes (Bürglin, 2008). The Hh pathway was first linked to human cancers in the Gorlin Syndrome, while looking at germline mutations of Patched1 (Ptc1) (Mao, 2008 Personal Communication). Today, the exact roles of the Hh pathway in development, growth, and metastasis remain unknown (Mao, 2008 Personal Communication).

### *Main Hh Pathway Members*

The Hh signaling pathway has many members (see **Figure-1**). Hh proteins themselves act as ligands for receptor Ptc1. The Hh family has three distinct members, Sonic (Shh), Indian (Ihh), and Desert (Dhh) (Mao, 2008). First reported in 1993, members of the family have been shown to be key mediators of fundamental embryonic processes. Dhh is most similar to the Hh proteins found in *Drosophila*, and Shh and Ihh are very similar to each other. Hh proteins are similar to each other, but differ in their own way. Specific roles of Hh proteins remain widely unknown, but these proteins can produce different responses depending on when and where the signal operates (Ingham

and McMahon, 2001). All Hh proteins bind to Ptc1, a twelve transmembrane receptor known to exist in epithelial cells (Ingham and McMahon, 2001). Even though Ptc1 is known to exist in epithelial cells, this project focused on Ptc1 within stomach and pancreatic cells. Smoothened (Smo) is a seven transmembrane protein associated with the Hh pathway. Smo inhibition is relieved by the binding of proteins to Ptc1. Once Smo is activated it signals through an intracellular signaling pathway to control the activities of three members of the Gli family (Gli1, Gli2, and Gli3). Smo activation eventually activates a negative regulator Suppressor of Fused (Sufu) which diminishes Hh signaling. Smo is an important factor in Hh signaling, as it is the center of the signaling (Rohatgi et al, 2008).

Hh proteins are composed of two domains, HhN and HhC. HhN is an amino-terminal domain that retains the biological signal activity. HhC is a carboxy-terminal autocatalytic domain which cleaves Hh into two parts in an intramolecular reaction. The HhC also adds moiety to HhN and has a sequence similar to self-splicing inteins with a shared region called Hint (Bürglin, 2008).

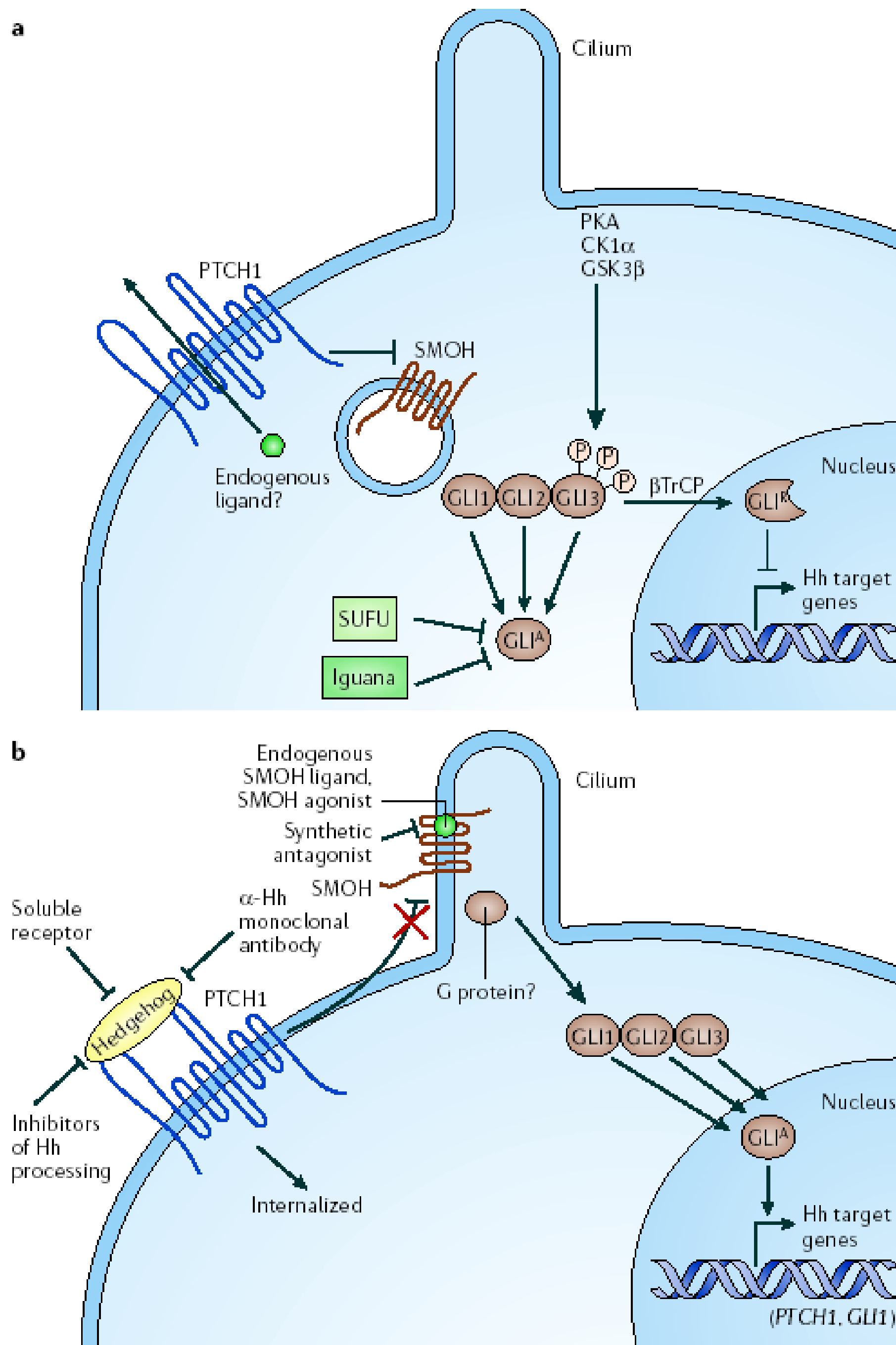


**Figure-1 Diagram of the Hedgehog Signaling Pathway in the presence and absence of Hh Ligands.** The Hedgehog pathway shown here depicts how it is activated with Ptc1 and Smo. Ptc1 (denoted as PTCH1 in the diagram) binds to the outside of the cell and takes a piece of the cell with it inside to the cell center. Once this particle enters the cell, Smo (denoted as SMOH in the diagram) is activated. After activation, Smo binds to the membrane of the cell. This particle, bound to the membrane, attracts SuFu, PKA, and finally Gli. After Gli binds to those particles it is activated and released into the double-helix inside the nucleus to promote cell growth (Rubin and de Sauvage, 2006).

The Hh pathway and its correct activation are vital for embryo development. During embryo development the Hh pathway regulates proliferation and differentiation. The Hh pathway regulates those functions in order for tissues to develop and reach their correct size and have the correct degrees of innervations and vascularization. If the pathway is mutated or deregulated, it can play a role in tumorigenesis (see **Figure-2**). Mutations in the Hh pathway, such as deactivating Smo, have been shown to cause certain types of cancer such as basal cell carcinoma (BCC) and medulloblastomas. The Hh pathway can also play a role in tumorigenesis when it is reactivated within adult tissues. BCC can arise from the Hh pathway being abnormally activated (Rubin and de Sauvage, 2006).

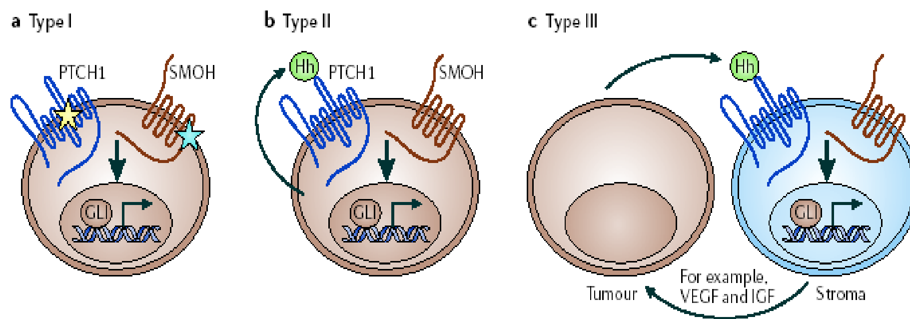


**Figure-2: Diagram of the Hh Pathway and Its Activation in Cancer.** Type 1 depicts the loss-of-function mutations in PTCH1 and gain-of-function mutation in SMOH. These mutations are shown to lead to constitutive Hh pathway activation. Type 2 shows an autocrine model where tumor cells are produced and respond to Hh ligand. The pathway activation can either occur in all tumor cells, or in a smaller number of tumor stem cells. Type 3 is a paracrine model. The tumor cells produce the Hh ligand and the stroma cells surrounding the cell respond to this process by producing growth factors that allow the tumor cell to keep growing (Rubin and de Sauvage, 2006).

**Sox Genes**

*Discovery*

Sox proteins were first discovered in *Drosophila* as key players in tissue growth, tumorigenesis, and tissue patterning during embryonic development (Bergsland et al., 2006).

*Types*

There are over twenty different types of Sox genes in mammals. Sox proteins are divided into families A-H. Sox proteins are similar to the other proteins within their family, and are not closely related to proteins within other families. HMG box proteins bind to DNA directly, generally at the minor groove of the DNA helix. This binding normally takes place at the sequence A/TA/T CAA A/T G. In addition to the HMG box domain, many Sox proteins have one or two other functional domains, including transactivation, transrepression, or homodimerization domains. With these extra domains Sox proteins are able to control transcription in several ways (Dy et al., 2008).

Sox genes get their name from the sex determining region of the Y chromosome High Mobility Group (HMG) box (Sox), with the box number corresponding to the Sox number. Group C of the sex determining region of the Y chromosome (Sry) has three critical members within its HMG, Sox4, Sox11, and Sox12. Sox4 and Sox11 are closely related, and show a high degree of homology within the HMG DNA binding domain and the C-terminal transactivation domain. Sox4 and Sox11 also have highly conserved overlapping expression patterns (Dy et al., 2008). All the members of Group C are closely related.

*Function*

Sox proteins have two characteristics that separate them from other transcriptional factors. First, they bind to the minor groove of DNA causing a strong bed in the DNA. Also, Sox proteins require other transcription factors for efficient target gene activation. Within the twenty different types of Sox proteins, there are certain groups labeled A through H. Group E, also known as SoxE contains Sox8, Sox9, and Sox10. During embryogenesis, SoxE proteins can exhibit partially overlapping expression patterns. Also, it has been shown that when SoxE proteins are co-expressed they exhibit similar functions (Wissmüller et al., 2006).

The two Sox genes investigated in this MQP, Sox4 and Sox11, are closely structurally related, and play a role in neuronal, cardiac, and other major developmental processes. Sox11 has a higher efficiency of activating transcription than Sox4, leading to a more stable α-helical structure of its transactivation domain (TAD) (Dy et al., 2008).

*Sox and Tumors*

Expression of Sox4 and Sox11 has been shown to increase in many different types of human cancers, including basal cell carcinomas (BCC) and medulloblastomas (Mao, 2008 Personal Communication). The carcinomas can attack various places in the human body, including the liver, prostate, ovary, lung, and colon. Sox4 has been shown to regulate cell proliferation and apoptosis, and its upregulation has been shown to induce these processes. Sox4 has also been identified as one of the key mediators in *miR335*-controlled breast cancer metastasis (Mao, 2008 Personal Communication). Although Sox4/11 have been shown to help induce cell proliferation, experiments demonstrating a

potential role of Sox4/11 in tumorigenesis and the Hh pathway have yet to be performed, and are the basis of this project.

*Mao Lab Purpose*

The main purpose of the Mao lab is to study the effects of the Hedgehog pathway on tumorigenesis. The goal of the research is to observe and understand the mechanics underlying the molecular and cellular control of Hh signaling. The emphasis is placed on stem and progenitor cell function. There are currently two projects underway in the Mao laboratory. The first is the study of the involvement of Hh signaling in rhabdomyosarcoma. Rhabdomyosarcoma is a very malignant and fast growing skeletal muscle tumor and is the most common soft tissue sarcoma found in children. A novel inducible somatic mouse model of Hh related rhabdomyosarcoma has been created to study the effect of Hh signaling in tumorigenesis. The involvement of Hh signaling in muscle stem cell transformation and tumor initiation is being studied through the use of a combination of biological and mouse transgenic approaches. The second project is researching the role of Hh signaling in gastrointestinal tract tumors including those of the stomach, colon and pancreas. Although recent studies have revealed an extensive significance of Hh signaling in gastrointestinal carcinomas, the exact role remains unclear. The genetic and cellular interactions between the Hh pathway and other pathways critical in gastrointestinal mesenchymal cell growth are being studied. The study of these pathway interactions may provide a better understanding of Hh participation in gastrointestinal tumorigenesis.

# PROJECT PURPOSE

As discussed in the Background section, Sox4 and Sox11 are two proteins of the hedgehog signaling pathway whose expression and activity are closely linked to tumor initiation and progression. The purpose of this MQP was to use a previously designed and cloned plasmid encoding an shRNA for microRNA-30 (miR30), designed by our lab to bind to the 3' UTR of Sox4/11 mRNAs, to determine its effectiveness to decrease Sox expression. By knocking down cellular levels of Sox4/11, this methodology could be used in the future as a tool for investigating the role of Sox proteins in tumorigenesis. Western blots were used to assay cellular levels of Sox proteins, and a luciferase assay was used to assay the cellular levels of functional Sox proteins and their ability to transactivate mRNA.

# METHODS

## FLAG-Tagged Sox Plasmids

To clone a plasmid encoding FLAG-tagged Sox proteins, a plasmid encoding a *3X*-Flag tag was fused to the c-terminus of mouse Sox4 and Sox11 cDNAs (made by Junhao Mao).

## shRNA miR-30 Plasmid

A plasmid encoding miR-30 shRNA was provided by Junhao Mao. Using bioinformatics, Dr Mao determined that the miR30 sequence in theory could base pair with the 3' UTR of Sox4 and Sox11 mRNAs, so might act as a reagent for controlling Sox expression. miR30 is synthetically made using bioinformatics. The miR30 serves as a vector for transfections. The miR30-encoding unit was extracted and made into a unique construct for this project (see **Figure-3**). The shRNA plasmid was cloned by the authors of this report.

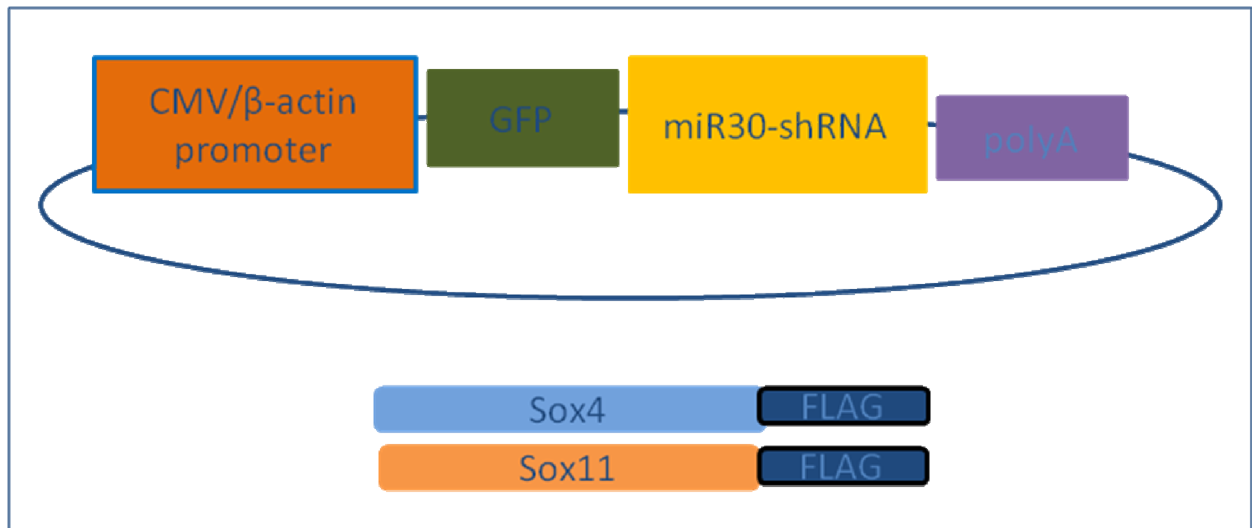


**Figure-3: Diagram of Plasmid Encoding miR30.** The shRNA construct shown is made of a CMV/B-actin promoter, Green Fluorescent Protein (GFP), miR30-shRNA, and a polyA tail. The GFP expression is used to validate shRNA expression. miR30 is a small non-coding RNA that regulates gene expression by binding to the 3' UTR of Sox4 or Sox11 mRNAs to decrease their expression.

**3T3 Cell Culture and Transfections**

Human NIH3T3 cells (see **Figure-4**) were grown in DMEM with penicillin/streptomycin supplemented with 10% FCS and penicillin/streptomycin. Cells were typically seeded on the plates at a concentration of 0.1 million/mL, and grown for about 3-5 days. Cells were transfected with FLAG-tagged plasmids, or shRNA plasmid, by adding 0.6 µg of plasmid with 2 µl per ml of Lipofectamine 2000 (Invitrogen), and the cells were grown for 1 day.

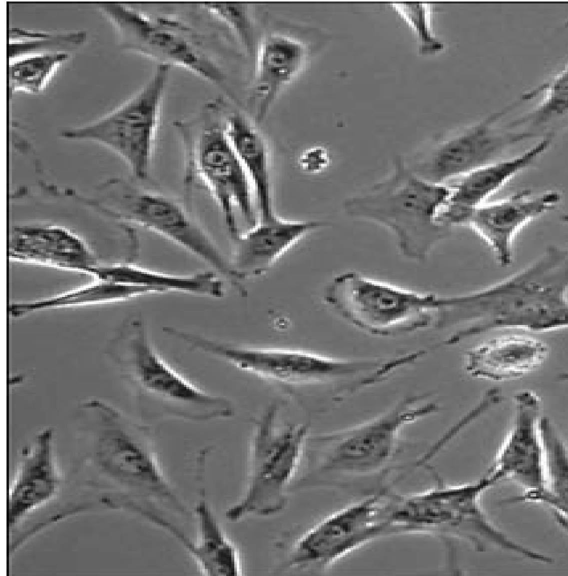

**Figure-4: Micrograph of NIH3T3 Cells.**
Cells are shown at 100X magnification.

**Western Blots**

This technique was the major methodology used in this project, used to detect cellular levels of Sox4 and Sox11 proteins, and Sox4/11 FLAG-tagged proteins. Proteins were separated on a 10% polyacrylamide gel, then blotted to nitrocellulose membrane using transfer buffer. The membrane was blocked in a solution containing 10% FCS in PBS-T, at 25°C for 1 hour. The anti-FLAG used as primary antibody was purchased from Open BioSystems, and used 1:1000 at a final concentration of 1.0 µg per ml. Membranes were washed in PBS-T buffer, and then incubated with Anti-rabbit IgG secondary antibody (purchased from Invitrogen) at 5 µg/ 5 ml for 1 hour at 25°C, prior to chemiluminescence detection.

**Luciferase Assay**

The luciferase assay was used in this project to quantitate the cellular levels of active Sox proteins in 3T3 fibroblast cells. The luciferase construct contained 6X Sox-binding sites upstream from the luciferase gene (see **Figure-5**). In this assay, the levels of functional Sox protein are directly related to the levels of measured luciferase activity.

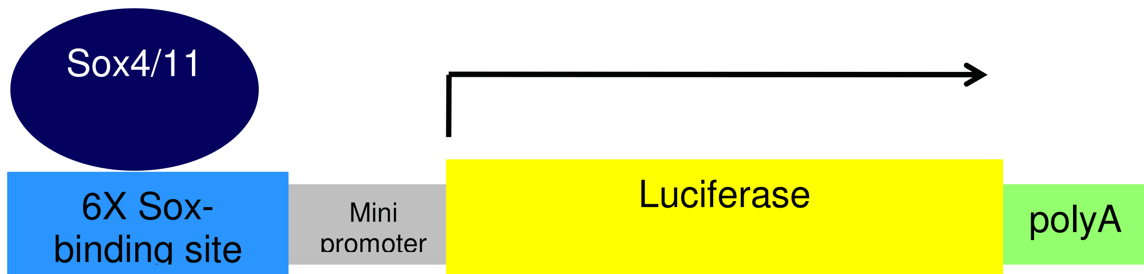


**Figure-5: Diagram of the Luciferase Reporter Construct Used to Assay Sox4/11-Mediated Gene Transcription in 3T3 Cells**. The promoter (blue) contains 6X Sox binding sites upstream from a luciferase reporter (yellow). The transcriptional stop is shown in green.

After the cells were transfected with the Sox-encoding plasmid and or luciferase construct, a luciferase reporter gene assay was performed. Cells were lysed in the 24 well plate using 120 µl of lysis buffer, and then 30 µl was transferred to a 96 well plate. For a luciferase assay, 30 µl of cell lysate was mixed with 30 µl of luciferase substrate solution (purchased from Invitrogen). The reactions were read in a luminometer for luminescence, zeroed against a blank containing no extract.

# RESULTS

The purpose of this project was to test the ability of a previously designed plasmid encoding an shRNA for microRNA-30 (miR30) (designed by our lab to bind to the 3' UTR of Sox4/11 mRNAs) to functionally decrease Sox expression.

### Ability of miR30 to Knockdown Sox Protein Levels in 3T3 Cells

Human fibroblast 3T3 cells were transfected with plasmid encoding FLAG-Sox4 or FLAG-Sox11 proteins, then co-transfected with plasmid encoding miR30 shRNA or parental plasmid (control). Many experiments were performed to optimize the plasmid transfections. The results indicate that 0.6 μg of plasmid worked best in plasmid optimization. Western blots were used to assay cellular levels of Sox proteins (see **Figure-6 to Figure-8**) to determine the effectiveness of the miR30 construct to knockdown Sox protein levels. Three colonies were picked and tested to see if the construct was transfected into the cells. As an example, colony #2 is shown in the figures below, as this colony had the best representation of the construct. The immunoblot data indicated a strong knockdown (quantitated in **Figure-9** from band ODs determined by Scion Image Sofware, NIH).

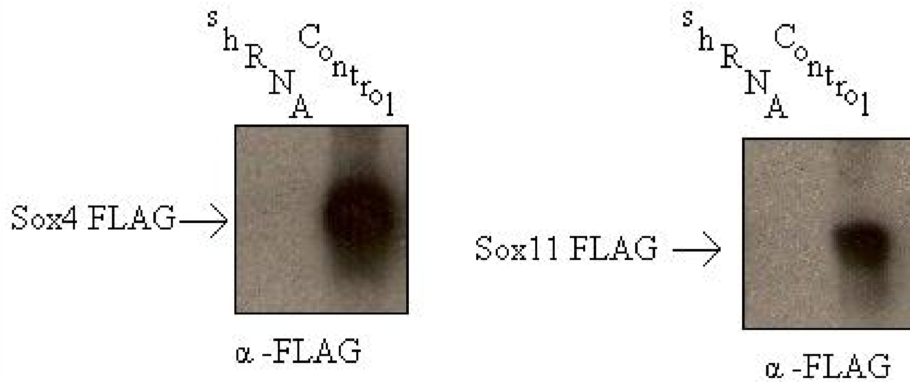


**Figure-6: FLAG Western Blot of Sox4 and Sox11 Protein Levels in Transfected 3T3 Cells.** Human 3T3 fibroblast cells were transfected with plasmid encoding miR30 shRNA (left lanes) or control plasmid (right lanes), and co-transfected with plasmids encoding FLAG-tagged Sox4 (left panel) or FLAG-tagged Sox11 (right panel). Transfected cells were grown for 1 day, and then protein lysates were analyzed by Western blots using FLAG-antibodies.

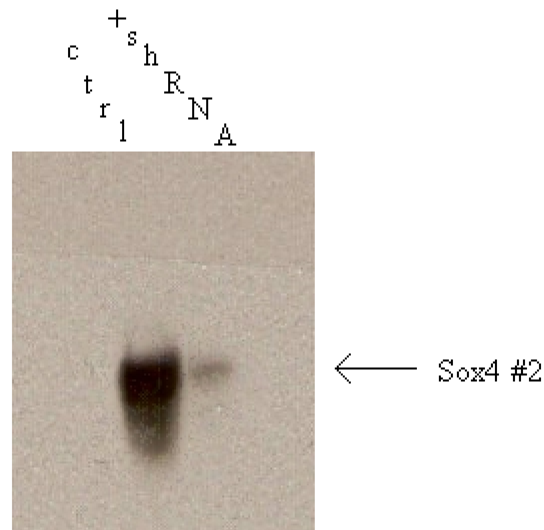


**Figure-7: FLAG-tagged Sox4 #2 Western Blot.** This figure depicts the western blot performed on Sox4 #2, as this was the premier colony chosen with the construct transfected, shown as a knockdown in this figure. The left lane is the control and the right lane is the Sox4 colony #2 transfected with the shRNA.

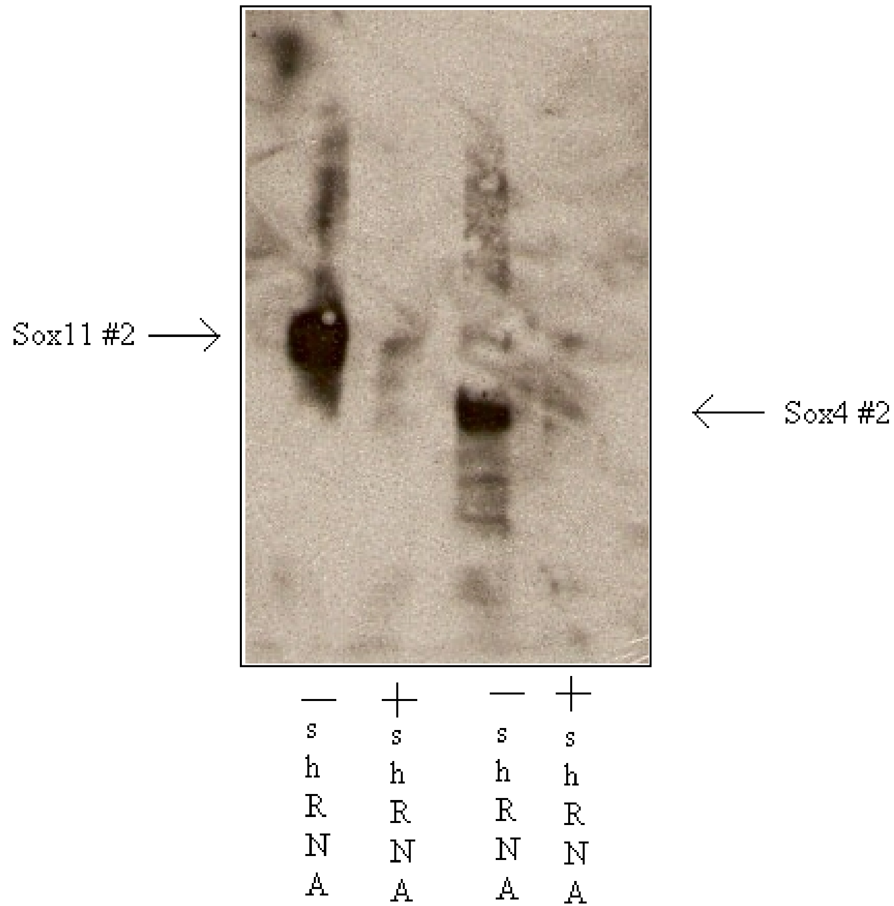


**Figure-8: FLAG Western Blot of Sox4 #2 and Sox11 #2 Protein Levels in Transfected 3T3 Cells.** Due to the Sox4 #2 colony being the premier colony, the Sox11 colony #2 was also chosen as a test. This is shown as a knockdown in the figure, and is comparable to the Sox4 #2 lanes.

| Sample | Band OD | Fold Down Regulation |
|---|---|---|
| **Sox 4** | **N=1** | |
| *shRNA* | 2.12 | |
| *Ctrl* | 738.45 | 346.7 |
| | | |
| **Sox4** | **N=2** | |
| *shRNA* | 37.6 | |
| *Ctrl* | 414.81 | 11.03 |
| | | |
| **Sox4** | **N=3** | |
| *shRNA* | 15.02 | |
| *Ctrl* | 722.17 | 48.1 |
| | | |
| **Sox11** | **N=1** | |
| *shRNA* | 1.05 | |
| *Ctrl* | 408.33 | 388.9 |
| | | |
| **Sox11** | **N=2** | |
| *shRNA* | 65.95 | |
| *Ctrl* | 796.45 | 12.1 |

**Figure-9: Table of Western Blot Band Densities (OD) and Down Regulation (fold).** This statistical analysis of the OD readings shows that the shRNA construct significantly knocked down the gene expression level of Sox4 and Sox11.

### Measurement of Sox Activity in 3T3 Cells

In order to determine whether the miR30-induced knockdown of Sox4/11 proteins also altered their ability to transactivate mRNA, a luciferase reporter assay was performed with a plasmid containing a promoter with 6X Sox4/11-binding sites. The levels of luciferase activity in this assay are directly proportional to the biological activity of the Sox proteins to transactivate the promoter. 3T3 cells were co-transfected with plasmid encoding miR30 shRNA or control plasmid, and the luciferase reporter plasmid.

Lysates were assayed for luminescence using a luminometer (see **Figure-10**). The results indicated that the transactivational activity of both Sox proteins was high in cells not receiving shRNA plasmid (blue histobars in the figure), and was lower in the cells receiving the shRNA plasmid (red histobars in the figure).

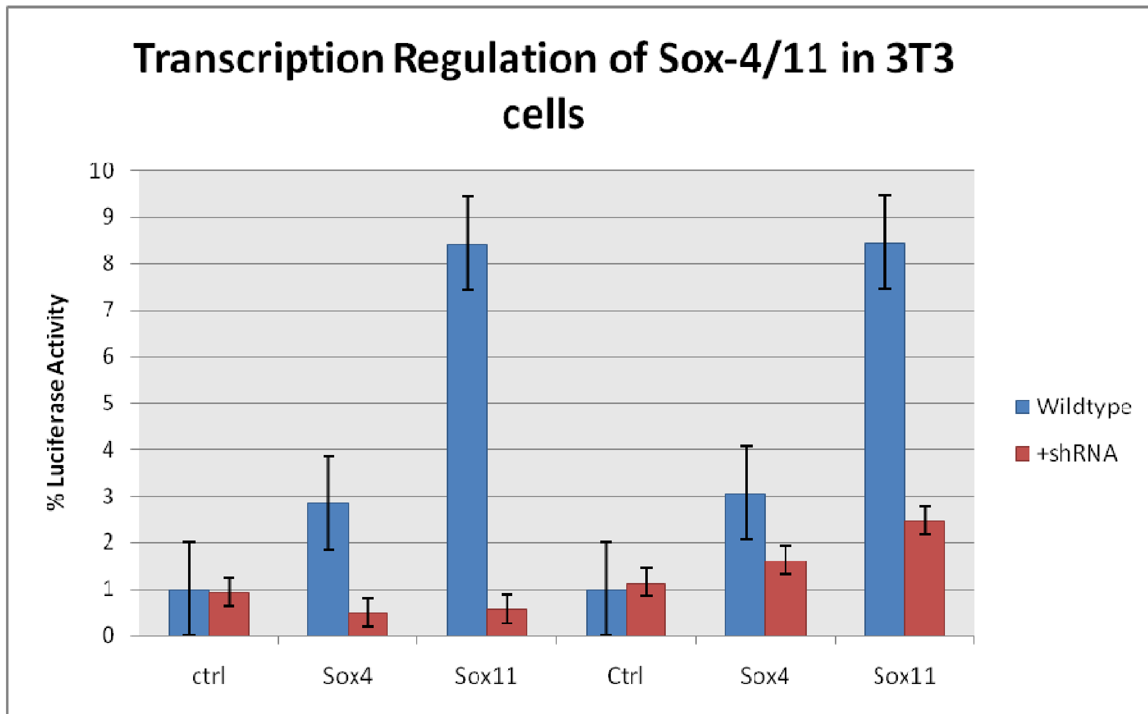


**Figure-10: Luciferase Assays on Transfected 3T3 Cells**. Cells were co-transfected with plasmid encoding miR30 shRNA (red) or control plasmid (blue), the luciferase reporter plasmid, and FLAG-Sox4 or FLAG-Sox11. Cell lysates were assayed for luminescence using a luminometer. Each bar represents the mean of triplicate sample determinations. The first trial is on the left, and the second trial is on the right.

# DISCUSSION

The purpose of this project was to test the effectiveness of a previously constructed plasmid encoding miR30 for its ability to knock down Sox4/11 protein levels in transfected cells, and to determine whether this knockdown diminishes Sox4/11-mediated transactivation. Bioinformatics performed previously in our lab identified miR30 as a microRNA potentially capable of binding the 3' UTR of Sox4/11 mRNAs, so the Mao lab constructed a plasmid encoding an shRNA for miR30. Transfections were performed using human 3T3 cells with miR30 plasmid or plasmids encoding FLAG-tagged Sox proteins. To determine whether the miR30 construct was effective in knocking down the Sox protein levels, western blots were used to assay the cellular levels of Sox proteins. The immunoblot data indicated a strong knockdown of both FLAG-tagged Sox proteins in cells transfected with miR30 plasmid, but not the control cells. This demonstrates strong effectiveness of this knockdown procedure *in vitro*.

A luciferase assay was performed using a plasmid containing 6X Sox-binding sites upstream from a luciferase reporter gene, to assay the cellular levels of *functional* Sox proteins and their ability to transactivate mRNA. The results of the luciferase assay indicated that the transactivational activity of both Sox proteins was lower in the cells receiving shRNA plasmid compared to control cells not receiving the plasmid. These results show that the shRNA miR30 construct was very effective in knocking down Sox4/11 protein levels and their activities.

The knockdown approach developed in our laboratory will be used in future experiments to identify the specific role of Sox4/11 proteins in tumorigenesis, since the role of Sox4 and Sox11 within tumorigenesis is currently unknown. By decreasing the

expression of the Sox4/11 proteins previously demonstrated to induce carcinogenesis, the construct can be used in the future to determine whether tumor induction is reduced in cells expressing the miR30 shRNA.

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