

Introduction

- SIT identified as potential bone mass regulator
 - SIT (SHP2-interacting transmembrane adaptor) phosphorylated by c-src family kinases
 - Previously only known to be involved with function of T-cells
 - Mice in which gene had been deleted (knockouts) have increased bone volume, trabecular number and connectivity
- Bone cells in culture useful in further study
 - CRISPR system used to delete SIT in rat osteosarcoma (ROS) cells
 - SIT RNA levels higher in cells in which SIT was deleted
- Further testing to be done on cells in culture
 - Growth rate, differentiation markers, SIT protein levels

The CRISPR gene editing system was used to delete the gene in rat osteosarcoma (ROS) cells in culture. A guide RNA for rat SIT predicted to have the best balance between efficacy and lack of off-target effects was chosen. Its coding DNA was cloned into an all-in-one CRISPR plasmid (PX458: both guide RNA cloning and Cas9) at the Bbs1 sites. This plasmid was introduced into the rat osteosarcoma cell line ROS 17/2.8 and transfected cells were selected for G418 resistance. Interestingly, we were unable to clone individual cell sublines. This suggests some critical role for SIT in regulating growth in response to cell density. In the G418-resistant SIT CRISPR pool, the levels of SIT RNA and were found to be higher than in cells transfected with the empty vector. We are determining the levels of SIT protein by Western blot as well as analyzing how Cas9 disrupted the SIT gene. The growth rate and expression of osteoblastic differentiation markers such as alkaline phosphatase, osteocalcin and matrix Gla protein genes are also being determined in these cells as compared to the control cells.

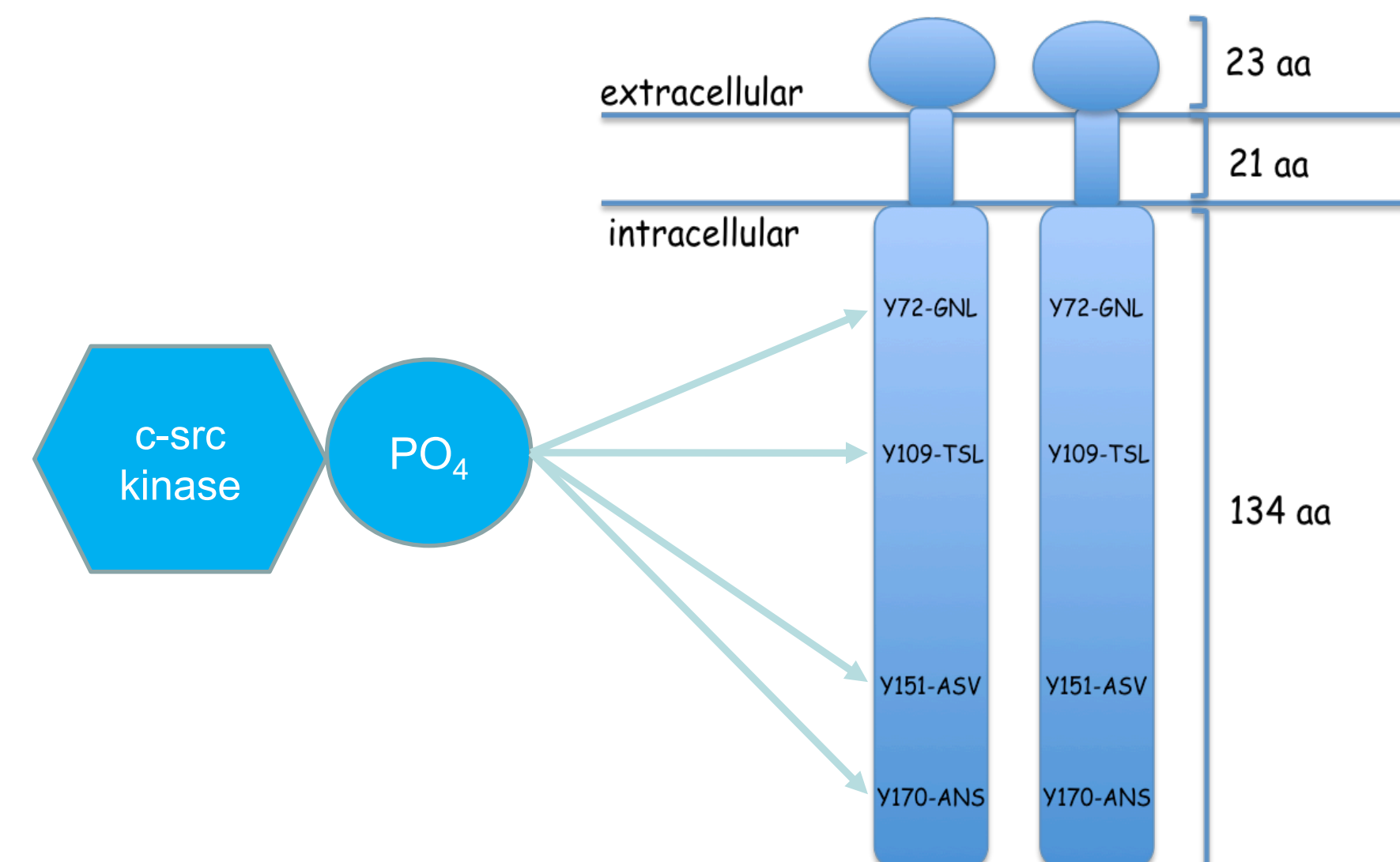


Figure 1
A diagram of the mechanism by which c-src kinase could phosphorylate the tyrosine targets on the intracellular portion of SIT.

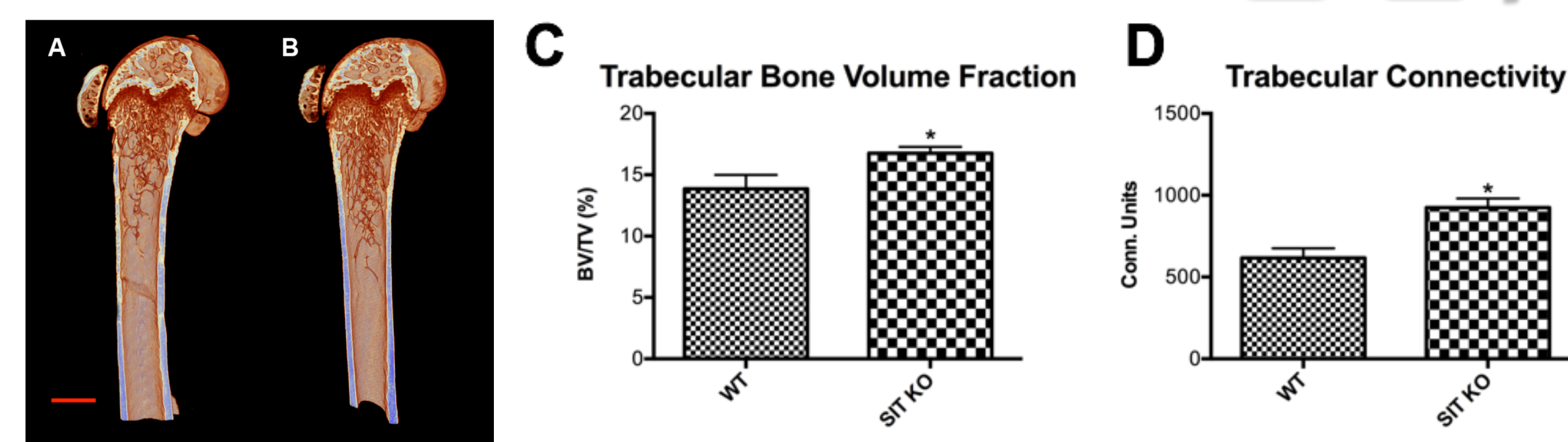


Figure 2
Our lab has shown that deletion of the SIT gene in mice results in changes in a number of trabecular bone parameters. MicroCT analysis of trabecular bone in femurs of wild type (WT; **Figure 2A**) and SIT knockout (KO; **Figure 2B**) reveals an increased amount of trabecular bone in the SIT KO bones compared with WT bones. Quantitation of two parameters (TBV; **Figure 2C** and Trabecular Connectivity; **Figure 2D**) demonstrates an increase in trabecular bone in the SIT KOs.

Citations and Acknowledgements

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- Tarr, J., et al. SIT (SHP2-interacting transmembrane adaptor) – a novel regulator of bone mass. *J. Bone Min. Res.* **30S**, MO0198. 2015.
- Xu, J., et al. Cloning of the full length cDNA for rat connective tissue growth factor (CTGF): implications for skeletal development. *J. Cell. Biochem.* **77**, 103-115. 2000.

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Deleting SIT Gene in Bone Cells in Culture

CRISPR–Cas9 Mechanism and Generation of Plasmid

- Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)
- CRISPR associated genes (Cas)
- Type II CRISPR based on mechanism of homology
 - Double-stranded breaks at target locus
 - Cas9 directs small RNAs to target
- Cell initiates Non-Homologous End Joining
 - Error prone – frameshift mutation

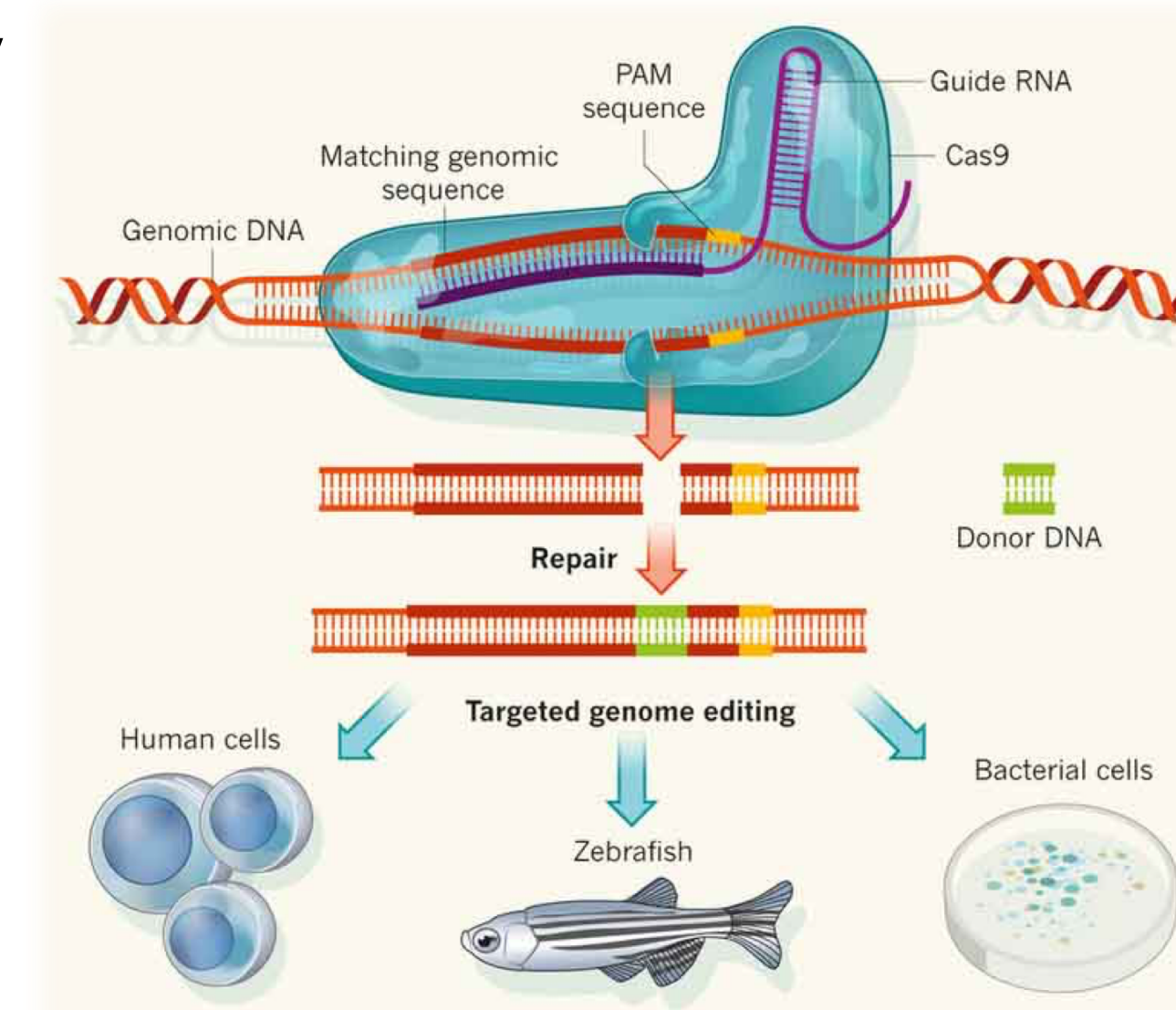


Figure 3A

all guides	guide #1	quality score: #1			
scored by inverse likelihood of off-target binding	guide sequence: TTCCTCCACAGGCTCCAC	90%			
mouse over for details	on-target locus: unknown				
	number of off-target sites: 49 (7 are in genes)				
		top 20 genome-wide off-target sites			
Guide #1	91	TTCCTCCACAGGCTCCAC	90%		
Guide #2	91	AGTGGACCCCTGCTCCAG	85%		
Guide #3	90	TTCCTCCACAGGCTCCAC	90%		
Guide #4	84	CTGGCTCTCTCCACAGC	90%	11.4	3896 (51618)
Guide #5	83	CCCGTATCTCTCCACAG	90%	0.7	4196 (2141812)

Figure 3B

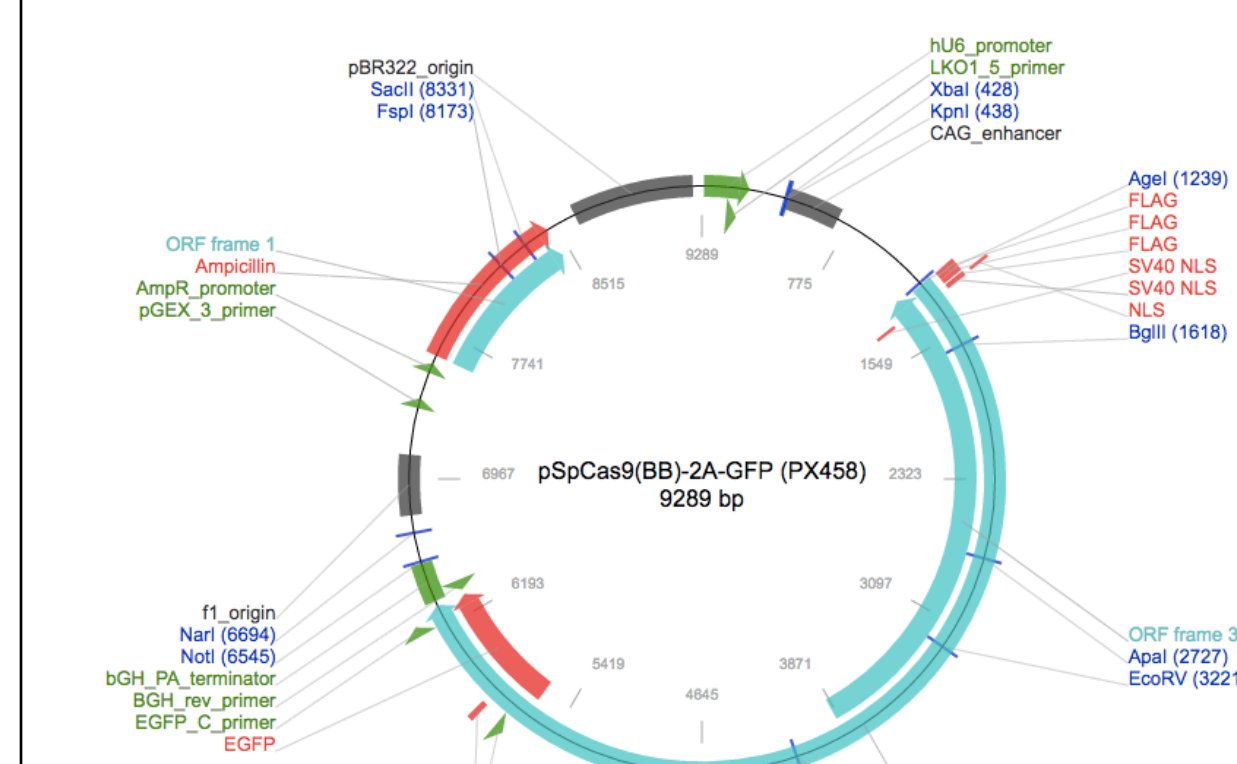


Figure 3C

1. 2-Log DNA Ladder
2. px458 cut with BbsI
3. px458 uncut

Figure 4

Diagram of the mechanism by which a genome is edited using CRISPR.

Figure 3

250 bp of sequence around the start codon of the rat SIT gene was submitted to the Crispr guide RNA design site at MIT (crispr.mit.edu). Guide 2 was chosen for use for its combination of high on-target and low off-target scores (above). Phosphorylated DNA oligos for guide 2 (top and bottom strand with the appropriate restriction site overhangs) were annealed, ligated into BbsI-cut and alkaline phosphatase-treated px458 plasmid (gel at the right), transformed into *E. coli*, and plated on LB-ampicillin.

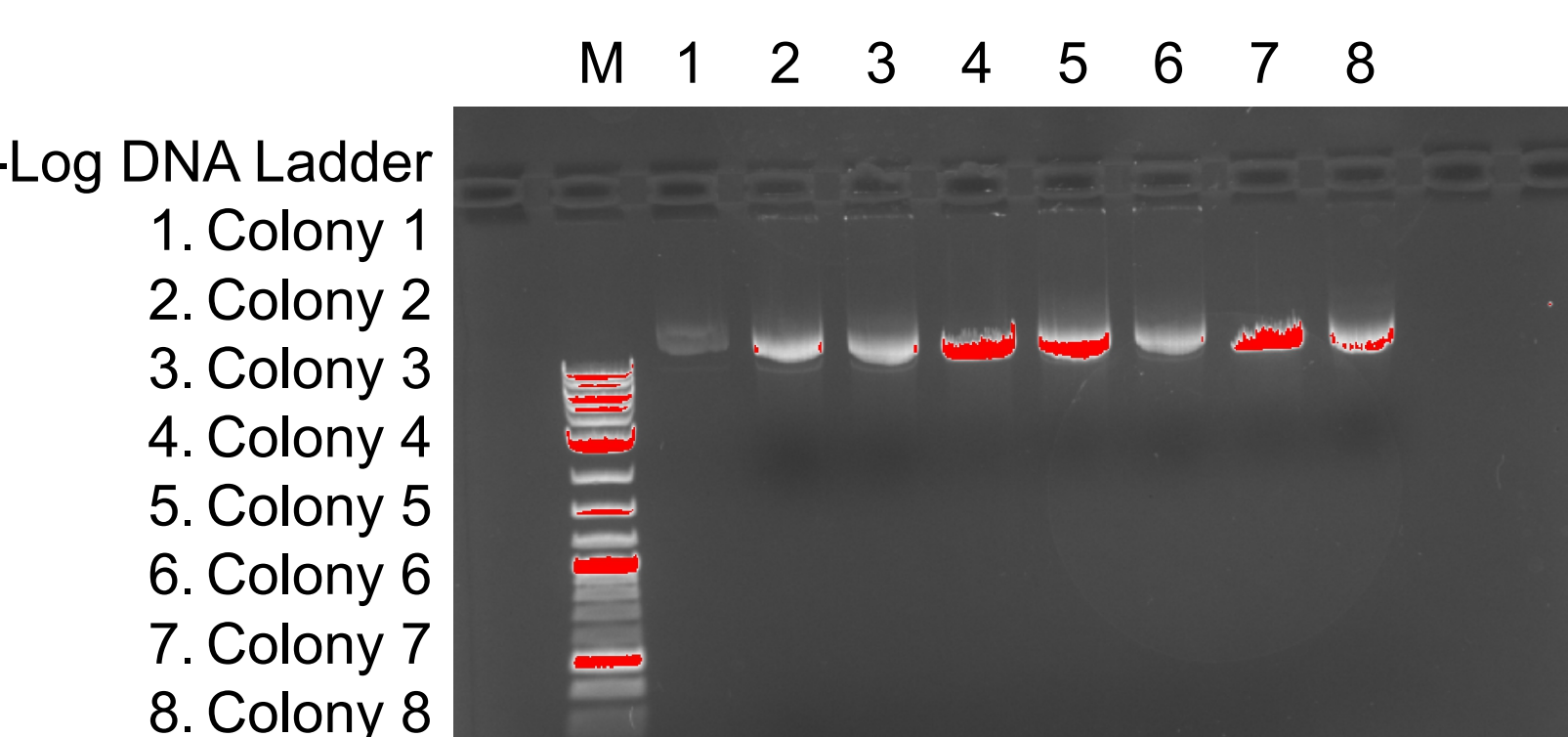


Figure 5

Example of transformation of *E. coli*. This technique was used to transform our colonies with px458 plasmid.

Future Directions

- The levels of SIT RNA and protein expression in clonal ROS sublines will be compared to those of cells transfected with the empty vector.
- ROS sublines where SIT expression is very low will be analyzed for markers of osteoblastic differentiation over a 12 day time course. These markers include cell proliferation, alkaline phosphatase enzyme activity, and the expression of the osteocalcin and matrix Gla protein genes.
- Another group of students is validating CRISPR reagents for deleting the c-src gene. We will use these reagents to delete c-src in ROS sublines that have reduced SIT expression to identify additive effects on osteoblast markers.

Growth and Transfection of ROS Cells

Growing ROS Cells

- Rat osteosarcoma cells (ROS 17/2.8) cells were plated at 1 x 10⁴ cells/cm² in DMEM + 10% Fetal Clone III
- Cells were harvested at Days 3, 6, 9 and 11. For isolation of RNA and membrane proteins, cells were pelleted by centrifugation and frozen in liquid nitrogen. For analysis of alkaline phosphatase enzyme activity, cells were lysed in an alkaline buffer containing 1% Triton X-100 detergent and frozen at -70° C until assayed.
- While osteosarcoma cells do not completely differentiate into functional osteoblasts, they do exhibit increases in osteoblast phenotype marker gene expression and alkaline phosphatase enzyme activity over time.

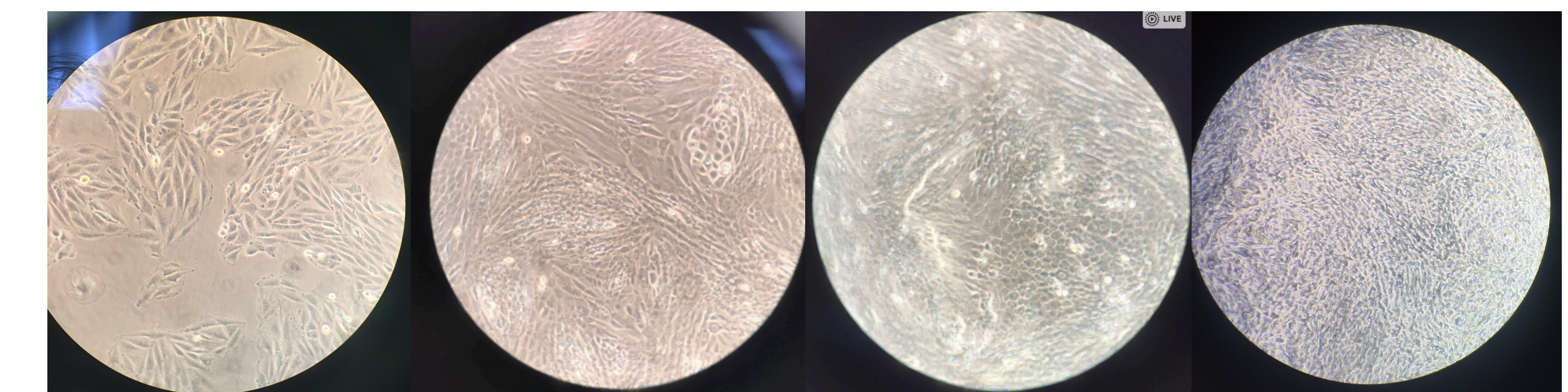


Figure 7
Growth of ROS cells over the course of 11 days. (phase contrast 150x)

Transfecting ROS Cells

Rat osteosarcoma cells (ROS 17/2.8) cells were plated at 2 x 10⁴ cells/cm² in DMEM + 10% Fetal Clone III

The next day, cells were transfected using Lipofectamine 3000

The first group of cells was cotransfected with 1 µg/well of a 20:1 mixture of the ligated rat SIT guide 2-px458 DNA and the pcDNA3.1 plasmid (G418 antibiotic resistance).

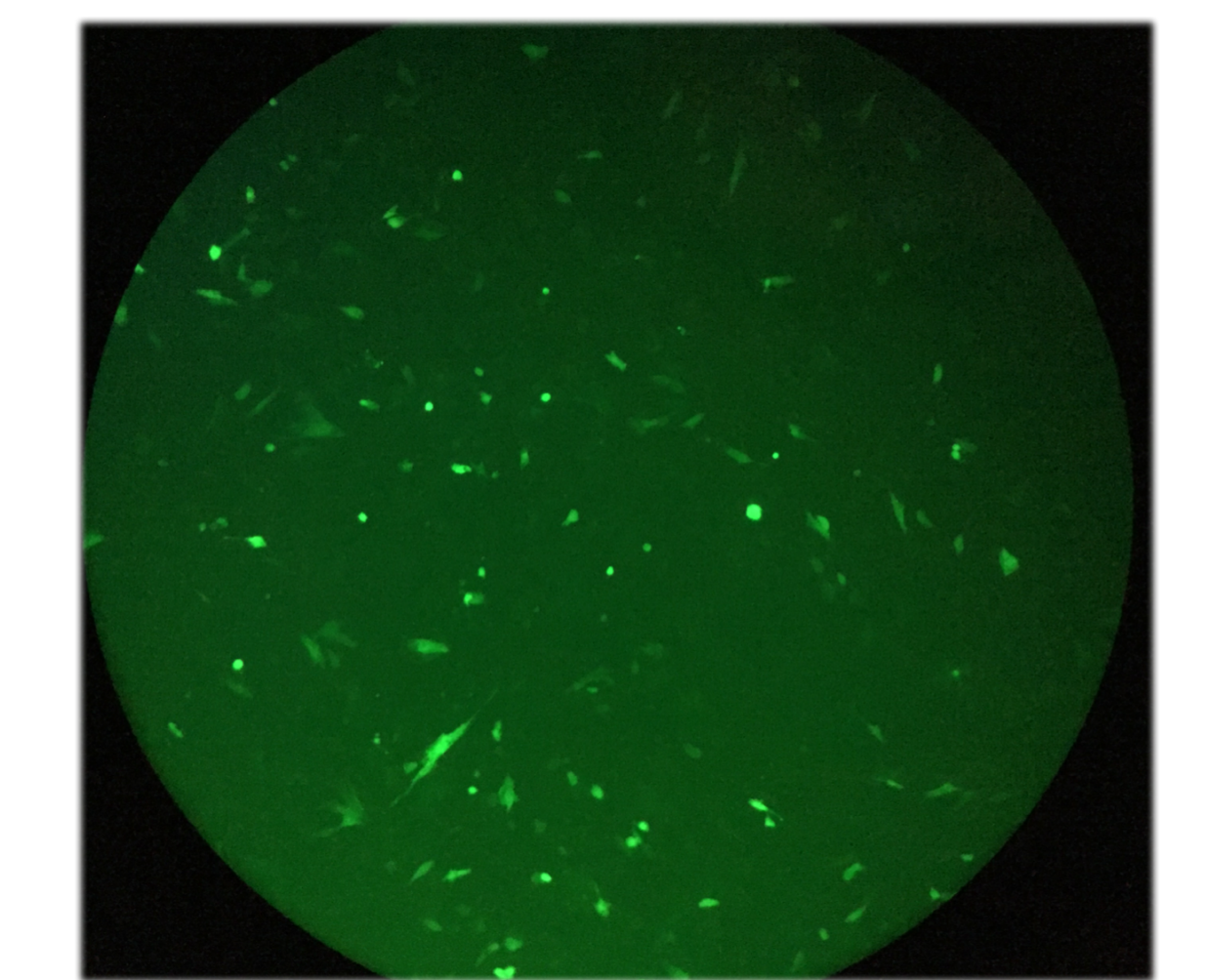
The second group of cells was transfected with the pcDNA3.1 plasmid alone (G418 antibiotic resistance). A third group of cells was transfected with the pGreen Lantern (Green Fluorescent protein) plasmid as a positive control for transfection.

Three days after transfection the cells were transferred to T75 flasks in DMEM + 10% Fetal Clone III with 250 µg/ml G418 antibiotic to kill any cells that were not successfully transfected.

Figure 8

Successful transfection of the ROS cells. The green fluorescent signal in this photograph is from the GFP gene also present on the px458 plasmid.

Photographed at 100x total magnification.



Ethical Considerations of Research and Drug Development

\$2.55 billion to develop a new drug (DiMasi et al. 2016)

Why is it worth investigating the mechanisms behind bone loss when treatments already exist?

Four Pillars of Medical Ethics

1. Autonomy – Give patients more choice and information
2. Beneficence – Increase quality of life
3. Non-Maleficence – Lack of research considered negligent, omission of better care
4. Justice – Equalize treatment options. Cost must be shared, benefits distributed according to need. (Childress and Beauchamp 1978)

Osteoporosis is increasingly prevalent in our aging population. Today, it is the second most disruptive affliction according to the Institute of Bone Health. Research and a potential new treatment will give patients more options and information, better quality of life, and commit to reducing harm of bone loss. The distribution of the advancements will be just if given according to need.

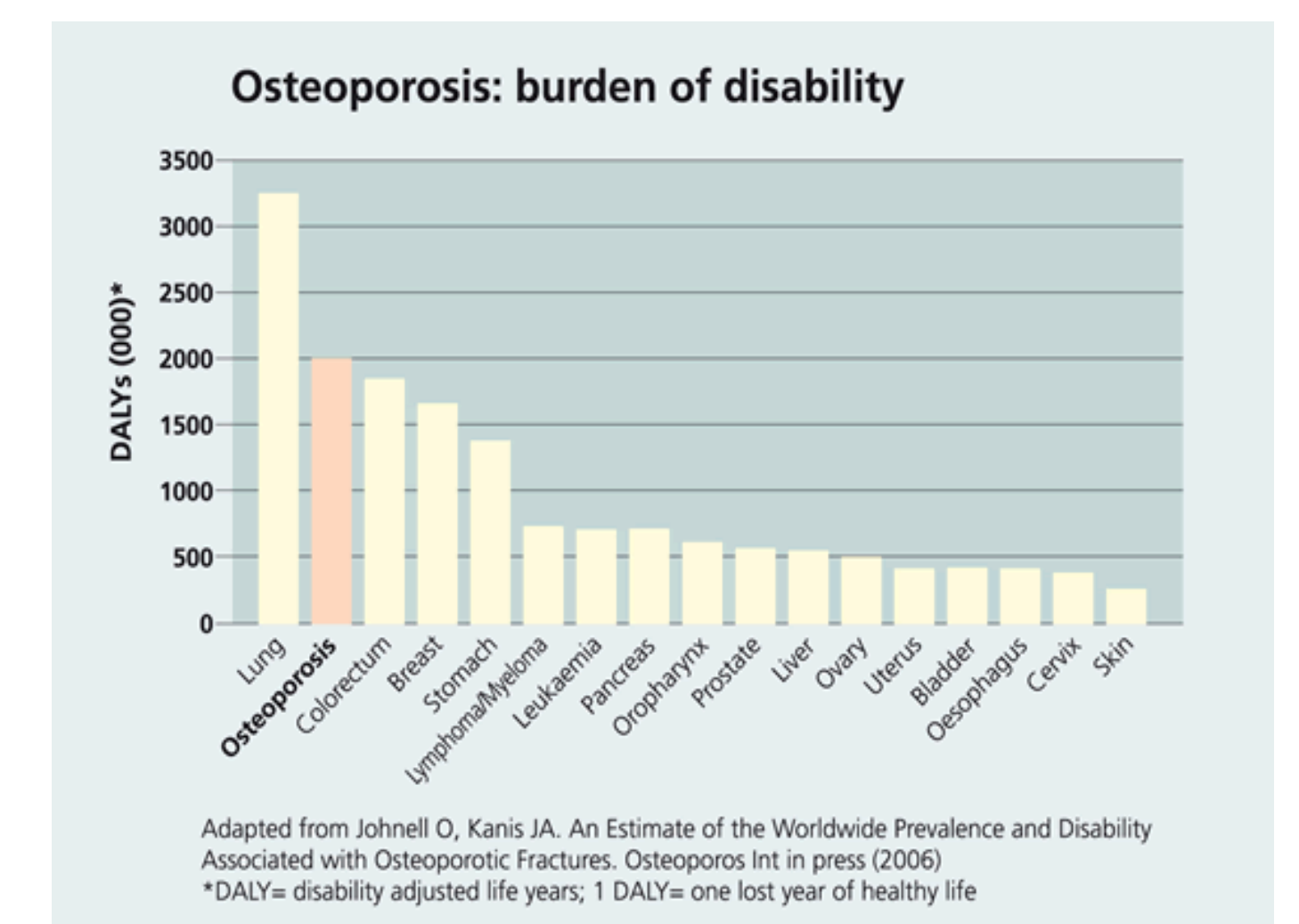


Figure 9
Chart of productivity lost to various diseases in units of Disability Adjusted Life Years. Osteoporosis costs the second most years of healthy life. <https://www.ictbonehealth.org/impact-osteoporosis>