Apoptosis in Hind Limb Osteocytes of SKI-1 Conditional Knockout Mice

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BACKGROUND

What is SKI-1?

•is a serine protease
•required for normal skeletal development
•regulator of apoptosis through its action on ATF-4

Relation to Apoptosis:

•SKI-1 activates ATF-4
•ATF-4 leads to the activation of ubiquitin ligases and caspases in over-stressed cells
•ATF-4 and SKI-1 act as the first step in apoptosis
•We propose that elimination of SKI-1 will affect the rate of apoptosis in tissue samples

Elimination of SKI-1 leads to hind-limb paralysis, vertebral fusion, and kinky tails in mice

Hypothesis:

Does elimination of SKI-1 lead to a change in the rate of apoptosis in cortical osteocytes?

METHODS

- First step was breeding of conditional knockout (cKO) mice, which were killed at 10 days old
- Gross observation was done
- Then, microscopic examination to measure the rate of apoptosis was begun, using Roche's *In Situ* Cell Death Detection Kit and a TUNEL Staining Protocol* that was optimized for our tissue samples.

Overview of Staining Protocol:

1. Deparaffinization – slides of bone samples were deparaffinized using xylene
2. Proteinase K digestion – tissue samples were treated with Proteinase K to dissolve the extracellular matrix in order to allow stains to penetrate cells
3. DNAse treatment – the positive controls were treated with DNAse to break apart the DNA, which simulates fragmentation of DNA during apoptosis
4. TUNEL Stain – the slides were stained with TUNEL, which binds to the broken ends of DNA and therefore highlight all cells that have fragmented their DNA
5. DAPI Stain – the final step was to stain the samples with DAPI, which stains DNA and highlights all cell nuclei

However, initial staining using this protocol did not allow us to see punctate cells, making optimization of the assay conditions necessary.

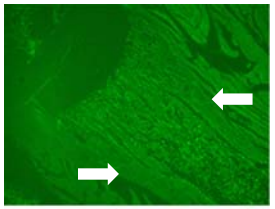
RESULTS

Optimization:

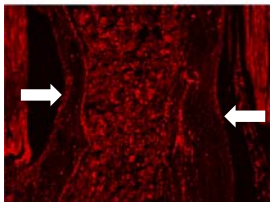
Phase I
•Deparaffinization time in xylene was optimized
•Optimal time was 5 min

Phase II
We switched from using a shorter wavelength TUNEL stain to a longer wavelength one to reduce background auto-fluorescence

Shorter Wavelength TUNEL



Longer Wavelength TUNEL



Higher background in samples that were treated with the shorter wavelength TUNEL stain

Phase III
•Proteinase K digestion time and temperature were optimized
- Optimal time and temperature were 60 min and 50 ^{0} C respectively

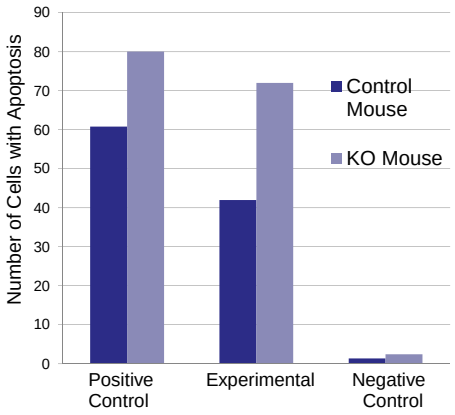
Phase IV
- DNAse digestion time and temperature were optimized
- Optimal time and temperature were 15 min and 37 ^{0} C respectively

Phase V
A heating step was added before the deparaffinization
New xylene and alcohol solutions were used for each experiment to better dissolve the xylene

Phase VI
Samples were washed with PBS twice after Protein-ase K digestion to remove residual enzyme

After making the aforementioned adjustments, we were able to observe punctate cells.

We divided the number of TUNEL positive osteocytes by the number of DAPI positive osteocytes to calculate the percentage of cells that have undergone apoptosis.

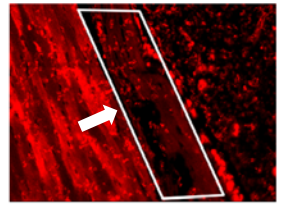


Apoptosis was greater in the SKI-1 cKO mice than in the control mice. Only 42 ± 10% of control mice cortical osteocytes had undergone apoptosis, as opposed to the 72 ± 14% of cKO mice.

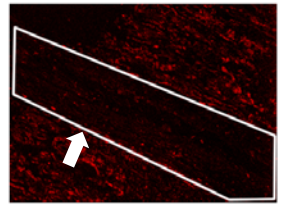
CONCLUSIONS

The higher rates in the cKO mice suggest that eliminating the SKI-1 gene did increase the apoptosis and very well may contribute to the physiological changes of hind limb paralysis and vertebral fusion we had observed grossly.

cKO Mouse



Normal Mouse



Rate of apoptosis in cortical osteocytes of cKO mouse is much greater than in Normal Mouse

Limitations:

•Small sample size because few cKO mice born

Further Experimentation:

•conduct further studies on other cKO mice
•we have now encountered the problem of the tissue samples coming off the slides, causing multiple depths of field and making focusing under the microscope very difficult
•Solution: 1. make protocol gentler
2. acquire a confocal microscope

References:

*University of Ljubljana, Slovenia, Biotechnical Faculty, Department of Biology
http://botanika.biologija.org/exp/

Jeff P Gorski; Nichole T Huffman et al. *Inhibition of proprotein convertase SKI-1 blocks transcription of key extracellular matrix genes regulating osteoblastic mineralization*. The Journal of biological chemistry 2011;286(3):1836-49