Validating a Quantitative Readout of ER-stress Suitable for High Throughput Drug Screening

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INTRODUCTION

Genetic skeletal diseases (GSDs) affect the development and homeostasis of the skeleton (Figure 1.). They are individually rare but affect ~1 in 4,000 children a year[1]. Studies show that endoplasmic reticulum (ER) stress in response to an accumulation of mutant protein within the ER of chondrocytes is the primary cause of the several GSDs[1,2,3].

There are very few therapeutic interventions are available which prevent, halt or modify disease progression. Recently studies show that the anti-epileptic drug Carbamazepine restores cell homeostasis and bone growth in Metaphyseal chondrodysplasia type Schmid (MCDS) caused by mutations in Collagen X[1].

However, carbamazepine cannot be used to treat all GSDs that result from ER stress, particularly those caused by mutations in the extracellular matrix protein Matrilin-3 (Personal Communication). This study focuses on generating a high throughput screening assay to monitor ER stress in response to drugs to assist in finding treatments for GSDs that are currently incurable.

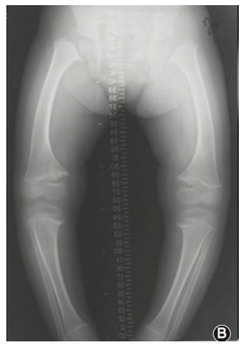


Figure 1. X-Ray of Metaphyseal chondrodysplasia type Schmid (MCDS) patient[4].
Patient's with MCDS display shortened irregular femora resulting in short stature, genu varum (bow legs) and painful joints.

AIM

- To generate a quantitative readout of ER-stress that will be used for high-throughput drug screening. We aim to produce an assay that will measure a reduction in ER stress monitored by Luciferase technology in response to drug treatment.
  1. Validate a Luciferase ER-stress reporter plasmid by stimulation with known chemical inducers of ER-stress.
  2. Use this reporter plasmid to monitor ER stress in cells expressing wild type and mutant collagen X before and after carbamazepine treatment.

METHOD

1. **Transfection:** Lipofectamine 2000 was used to transiently transfect HELA cells with:
   1. ERSE1 luciferase reporter plasmid (Addgene). (Figure 3.)
   2. Wild-type/Mutant (N617K) Collagen X (COL X) in pcDNA3.1(+). (Figure 4.)
   3. ERSE1 luciferase reporter plasmid and wild-type/mutant Collagen X in pcDNA3.1(+)

24 hours after transfection, cells were stimulated with either: 100 nm Thapsigarin, 10 µg/mL Tunicamycin, 20 µM Carbamazepine. Cells were harvested after 24 hours.

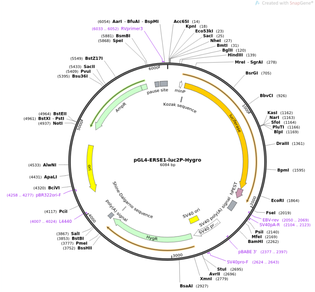


Figure 2. ERSE1 Luciferase reporter plasmid.
The promoter region in the ERSE1 luciferase reporter plasmid contains tandem repeats of ER stress response elements that is activated by transcription factors upon ER stress. This then stimulates the transcription of the *Luciferase* gene providing a measurable read out for ER stress in ERSE1 transfected cells.

2. **Protein Analysis:** Cells were lysed in RIPA buffer and proteins were analysed by western blotting.
3. **Gene Expression Analysis:** RNA was extracted using Trizol, converted into cDNA by reverse transcription and gene levels were analysed by quantitative real-time PCR.
4. **Luciferase Expression Analysis:** Cells were lysed in passive lysis buffer (Promega) and Luciferase production was analysed using the Promega Luciferase Assay System.

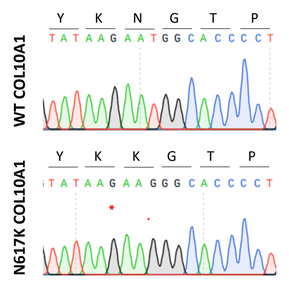


Figure 4. Base change in N617K COL10A1.
WT and N617K COL10A1 was previously cloned into pcDNA3.1(+). Plasmid DNA was sent to GATC Biotech for sequencing. The N617K COL10A1 had a T>G base change that resulted in a aspartate to lysine mutation at position 617 in the protein. Base change highlighted by *.

RESULTS

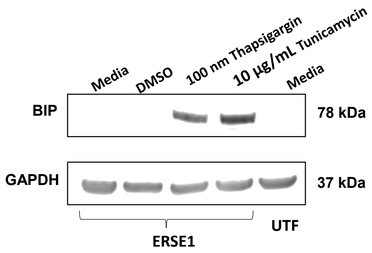


Figure 6. ER stress stimulates *Luciferase* expression in ERSE1 transfected HELA cells.
ER stress was stimulated in using tunicamycin and thapsigargin. ER stress successfully stimulated the expression of *Luciferase* in the cells transfected with the ERSE1 Luciferase reporter plasmid. (Expression levels normalised to GAPDH. n=3. *=p<0.05)

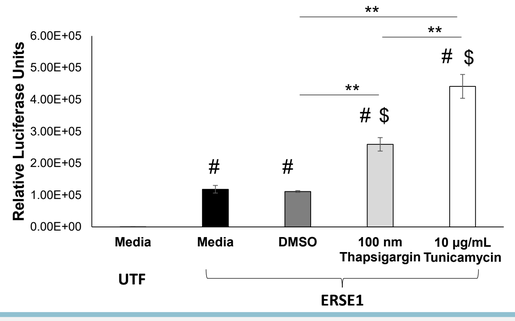


Figure 5. Tunicamycin and thapsigargin treatment results in an upregulation of the ER stress marker BIP.
ERSE1 transfected HELA cells were stimulated for 24 hours with two known ER stress inducers, tunicamycin and thapsigargin. BIP expression was undetectable in cells grown in complete media or DMSO but was upregulated upon stimulation with tunicamycin and thapsigargin.

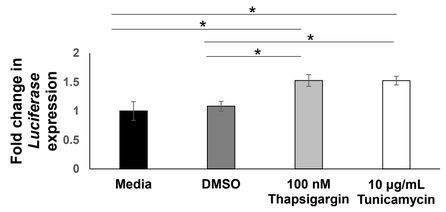
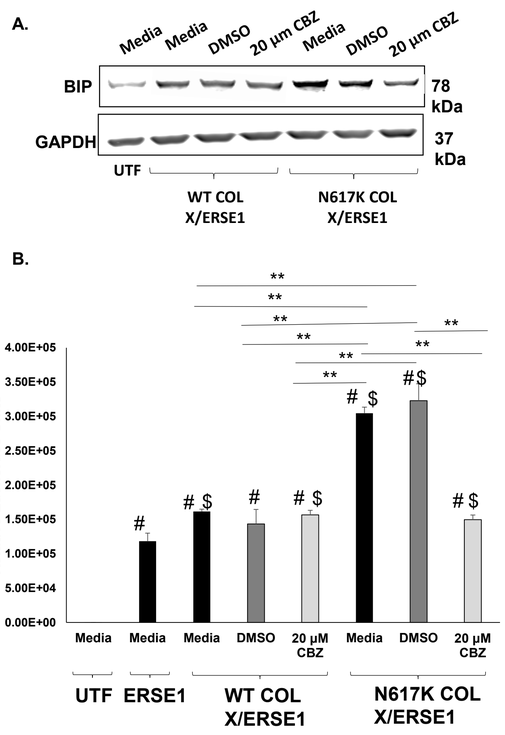


Figure 7. ER-stress inducing chemicals stimulated Luciferase activity in ERSEI transfected HELA cells.
ER stress was stimulated using tunicamycin and thapsigargin. ER stress successfully stimulated Luciferase activity in the cells transfected with the ERSE 1 Luciferase reporter plasmid as analysed using the Promega Luciferase assay. (UTF= Untransfected. n=3. #=significant to UTF, $=significant to ERSE1, **=p<0.005)

Figure 8. The Luciferase reporter assay can be used to identify drug treatments that reduce ER stress.
HELA cells were co-transfected with ERSE1 and either Wildtype (WT) or N617K (MUT) Collagen X. Cells were then treated with 20 µM CBZ for 24 hours prior to collection. Mutant collagen X induced ER stress as BIP was upregulated at the protein level (A.). This ER stress response stimulated Luciferase activity in N617K COL X/ERSE1 cells transfected with both mutant Collagen X and the ERSEI Luciferase reporter plasmid (B.). This stress was returned to normal levels (A.) upon treatment of CBZ resulting in a reduction in luciferase activity (B.) in the N617K Collagen X/ERSE1 expressing cells. (UTF= Untransfected. n=3. #=significant to UTF, $=significant to ERSE1, **=p<0.005)



CONCLUSION

- The ERSE1 luciferase reporter plasmid expresses Luciferase in response to ER-stress.
- The N617K mutation in Collagen X results in pathological ER stress that can be monitored using the ERSE1 plasmid.
- Previous studies have shown that carbamazepine treatment significantly reduces ER stress in cells expressing mutant collagen X and we show here that this reduction in ER-stress can also be monitored using the ERSE1 reporter plasmid.
- Therefore, the ERSE1 reporter plasmid can be successfully used as a quantative readout for ER-stress and can be used as a reporter assay to find potential drug therapies for other diseases that are caused by ER-stress, such as Multiple epiphyseal dysplasia (MED) that results from mutations in Matrilin-3.

References

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4. Al Kaissi A et al. Orthopaedic Surgery. 2019 Aug 17;10:241-246.