PHARMACOLOGICAL RESCUE OF NONSENSE MUTATIONS IN RETT SYNDROME

by

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Abstract

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Rett syndrome is a neurological condition that affects primarily girls. Approximately 40% of Rett syndrome cases arise from nonsense mutations. Several studies have shown that certain aminoglycosides can suppress some types of nonsense mutations in a context dependent manner, and allow the generation of a full length protein. It remains mostly unclear whether different nonsense mutations of MECP2 will be responsive to aminoglycoside treatment. In this study I tested whether some nonsense mutations of MECP2 seen clinically in Rett syndrome girls can be partially suppressed by aminoglycoside administration. My results show that aminoglycosides allow different mutant forms of MECP2 to be overcome in transiently transfected HEK-293 cells, but with differing levels of efficiency. Furthermore, I also show that aminoglycosides increased the prevalence of full length MeCP2 protein in a lymphocyte cell line derived from a Rett girl with R255X mutation. This study establishes the “proof of principle” that some nonsense mutations causing Rett syndrome can be suppressed by drug treatment.
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Table of contents

Abstract ............................................................................................................................. ii
Acknowledgements ........................................................................................................ iii
Table of contents .......................................................................................................... iv
List of Tables ................................................................................................................ viii
List of Figures ............................................................................................................... ix
List of Abbreviations .................................................................................................... xi

1 Introduction .................................................................................................................. 1
1.1 Concept of epigenetics and the role of Methyl-CpG-binding proteins ....................... 1
1.2 Rett Syndrome ......................................................................................................... 5
   1.2.1 Pathology ........................................................................................................ 5
   1.2.2 Mutations in MECP2 are the predominant cause of Rett syndrome ................ 6
   1.2.3 The structure and function of MeCP2 ............................................................. 8
   1.2.4 BDNF is one gene regulated by MeCP2 .......................................................... 11
   1.2.5 MeCP2 is post-translationally regulated ......................................................... 11
   1.2.6 There are two isoforms of MECP2 with distinct N-termini ............................. 14
   1.2.7 Mutations that occur in Rett syndrome .......................................................... 15
   1.2.8 Nonsense mutations and NMD pathway ......................................................... 18
   1.2.9 Genotype/Phenotype analysis in Rett syndrome ............................................. 19
   1.2.10 Therapeutic approaches for Rett syndrome ................................................. 21
1.3 The molecular mechanism of premature stop mutations ........................................ 24
1.4 Aminoglycosides ................................................................. 25
  1.4.1 What are aminoglycosides? ........................................... 25
  1.4.2 Toxicity of aminoglycosides .......................................... 28
  1.4.3 Megalin receptor is important in the uptake of aminoglycosides in cells .... 29
  1.4.4 Potential of aminoglycosides to treat genetic diseases with nonsense mutations ................................................................. 30
  1.4.5 Proposed mechanism of aminoglycoside mediated read-through .......... 31
  1.4.6 Do aminoglycosides facilitate read-through at normal stop codons? .......... 38
1.5 Aims of my thesis and hypothesis ........................................... 41

2 Methods .............................................................................. 43
  2.1 Molecular Biological Techniques ........................................... 43
    2.1.1 Construction of mutant forms of MECP2 .......................... 43
    2.1.2 DNA transformation ................................................... 45
    2.1.3 DNA purification ....................................................... 48
    2.1.4 Preparation of cell lysates ............................................ 49
    2.1.5 Nuclear extraction ....................................................... 49
    2.1.6 Western blot analysis .................................................. 50
    2.1.7 Immunocytochemistry ............................................... 51
  2.2 Statistical analysis ............................................................. 52
  2.3 Aminoglycosides used in my study ........................................ 52
  2.4 Cell culture ..................................................................... 53
    2.4.1 HEK-293 cell culture and transfection ............................ 53
    2.4.2 Lymphocyte culture and drug treatment ........................... 54
3 Results.................................................................55
3.1 In vitro...............................................................55
  3.1.1 Nonsense mutations generating truncated forms of MECP2 are expressed in transiently transfected HEK-293 cells..........................................................55
  3.1.2 The aminoglycosides gentamicin and geneticin facilitate read-through of the R294X Rett syndrome causing mutation.........................................................60
  3.1.3 Amikacin and paromomycin are not effective in inducing read through of R294X mutation.................................................................................................63
  3.1.4 Aminoglycoside treatment induces read-through of Q170X mutation........66
  3.1.5 Aminoglycosides induce read-through of Y141X mutation with different efficiencies...........................................................................................................69
  3.1.6 Aminoglycosides are not effective in inducing read-through of E205X mutation..............................................................72
3.2 In vivo....................................................................75
  3.2.1 Acute aminoglycoside treatment increases the prevalence of full length MeCP2 in a R255X lymphocyte cell line...............................................................75
  3.2.2 Long-term treatment of R255X lymphocyte cells at clinically-relevant concentrations of aminoglycosides fails to increase the prevalence of full length MeCP2...........................................................................................................83
3.3 Summary of results..................................................86

4 Discussion...............................................................88
4.1 Principal findings of my study........................................88
4.2 Wild-type and mutant forms of MECP2 migrate at higher sizes than expected.......89
4.3 Different Rett syndrome causing mutations respond differently to aminoglycoside treatment........................................................................................................89
4.4 Different aminoglycosides suppress nonsense mutations with different efficiencies in transfected HEK-293 cells...........................................................................91
4.5 Possible reasons of the context dependence effects of aminoglycosides.............93
4.6 NMD pathway and aminoglycoside mediated read-through.................................94
4.7 Aminoglycoside treatment in a lymphocyte cell line having R255X mutation (CGA A >TGA A)........................................................................................................97
4.8 Possible reasons for the difference in aminoglycoside mediated read-through in lymphocytes vs. transfected HEK-293 cells.................................................................99
4.9 Related study........................................................................................................101
4.10 Aminoglycosides may be able to facilitate read-through at premature stop codons and not at normal stop codons.................................................................102
4.11 Future directions and potential clinical implications...........................................104

5 Summary ..................................................................................................................113

6 References ...............................................................................................................116
List of Tables

1. Percentage of girls with mutations in MECP2 that retain functional ability..................20
2. Cycling Parameters for the Site-Directed Mutagenesis Method........................................44
3. Effect of 48 hours treatment of aminoglycosides on HEK-293 cells transfected with the mutant forms of MeCP2.........................................................................................................................86
4. Effect of 4 days aminoglycoside treatment on a lymphocyte cell line..............................87
5. Effect of 12 days aminoglycoside treatment on a lymphocyte cell line..............................87
# List of Figures

## Introduction

- Figure 1: Structure of MBD proteins and Kaiso.................................................................4
- Figure 2: Structure and function of MeCP2........................................................................13
- Figure 3: The two isoforms of MeCP2 and the type and frequency of mutations that occur on MECP2 in Rett syndrome.................................................................17
- Figure 4: The structures of aminoglycosides used in my study........................................27
- Figure 5: The mechanism of aminoglycoside interaction with ribosomal protein synthesis..................................................................................................................34
- Figure 6: The structures of ribosomal decoding sites of prokaryotes and eukaryotes.........37
- Figure 7: The molecular mechanism of the aminoglycoside mediated read-through.......40

## Methods

- Figure 8: The truncated forms of MeCP2 that I used in my study.....................................47

## Results

- Figure 9: The mutant forms of MeCP2 are expressed in transiently transfected HEK-293 cells........................................................................................................................................57
- Figure 10: Transfection efficiency in HEK-293 cells determined by immunocytochemistry.59
- Figure 11: Gentamicin and geneticin induce read-through of the R294X mutation in a dose response manner........................................................................................................62
- Figure 12: Amikacin and paromomycin do not facilitate read-through of the R294X mutation.................................................................................................................................65
- Figure 13: Aminoglycoside treatment induces read-through of Q170X mutation..............68
Figure 14: Gentamicin and geneticin induce read-through of Y141X mutation with different efficiencies ................................................................. 71

Figure 15: Aminoglycosides fail to increase the prevalence of full length MeCP2 from E205X mutation ........................................................................ 74

Figure 16: Geneticin induces the prevalence of full length MeCP2 protein in a dose response manner in the lymphocyte cell line with R255X mutation .......................................................... 78

Figure 17: Gentamicin induces the prevalence of full length MeCP2 protein in a dose response manner in the lymphocyte cell line with R255X mutation ...................................................... 80

Figure 18: Amikacin is effective in restoring the full length MeCP2 protein at a high concentration in the lymphocyte cell line with R255X mutation .................................................... 82

Figure 19: Long term culture of R255X lymphocytes in clinically-relevant concentrations of aminoglycosides does not induce a significant increase in full length MeCP2 protein ........................................................................ 85

Discussion

Figure 20: The chemical structures of PTC124 and NB54 ........................................................................ 112

Figure 21: Model of aminoglycoside mediated read-through ...................................................................... 115
List of Abbreviations

A: Adenine
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AT: Ataxia-Telangiectasia
AVPR2: V2 vasopressin receptor
BDNF: Brain-derived neurotrophic factor
BSA: Bovine serum albumin
C: Cytosine
C-terminus: Carboxy-terminus
CDKL5: Cyclin-dependent kinase like 5
CF: Cystic Fibrosis
CFTR: Cystic fibrosis transmembrane receptor protein
CHX inhibitor: Cycloheximide inhibitor
CNS: Central nervous system
CO2: Carbon dioxide
CREB1: CAMP responsive element binding protein 1
DAPI: 4,6-Diamidino-2-phenylindole dihydrochloride
DHCR7: 7-Dehydrocholesterol reductase
DMD: Duchenne Muscular Dystrophy
DMEM: Dulbecco’s Modified Eagle’s Medium
DNA: Deoxyribonucleic acid
dNTP: Deoxyribonucleotides triphosphate
2–DOS: 2-Deoxystreptamine
Dpn: Diplococcus pneumonia
dsDNA: double stranded DNA
DTT: Dithiothreitol
E: Glutamate or Glutamic acid
EB: Elution buffer
EDTA: Ethylenediaminetetraacetic acid
eRF1: Eukaryotic release factor 1
eRF3: Eukaryotic release factor 3
FBS: Fetal bovine serum
FoxG1: Forkhead box protein G1
G: Guanine
H1: Histone 1
H2: Histone 2
HA: Hemagglutinin
HDAC: Histone deacetylase
HEK: Human Embryonic Kidney cells
HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HS: Hurler Syndrome
ID: Interdomain region
IDUA: Iduronidase Alpha-L
IRSF: International Rett Syndrome Foundation
KCl: Potassium chloride
KdA: Kilodalton
LB: Luria-Bertani
LTD: Long term depression
LTP: Long term potentiation
MBD: Methyl-CpG binding domain
MeCP2: Methyl CpG binding protein 2
MeCP2_e1: Methyl CpG binding protein 2 isoform 1
MeCP2_e2: Methyl CpG binding protein 2 isoform 2
MECP2: gene encoding methyl CpG binding protein 2 in humans
MECP2_e1: gene encoding methyl CpG binding protein 2 in humans isoform 1
MECP2_e2: gene encoding methyl CpG binding protein 2 in humans isoform 2
MeCP2: gene encoding methyl CpG binding protein 2 in mouse
mg: Milligram
MgCl$_2$: Magnesium Chloride
mM: Millimolar
mRNA: Messenger ribonucleic acid
N-terminus: Amino terminus
NaCl: Sodium chloride
NaOH: Sodium hydroxide
NLS: Nuclear Localization Signal
NMD: Nonsense-mediated mRNA decay
NMDA: N-methyl-D-aspartate
NuRD: Nucleosome remodelling and histone deacetylase activity
OD: Optical densitometry
PBS: Phosphate buffered saline
PCDH15: Protocadherin 15
PCR: Polymerase chain reaction
PTC: Premature termination codon
PTC124: (3-[5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl]benzoic acid)
Q: Glutamine
R: Arginine
rRNA: ribosomal ribonucleic acid
RT-PCR: Real time polymerase chain reaction
RTT-Rett syndrome
S: serine
SDS: Sodium dodecyl-sulphate
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM: Standard error of the mean
Sin3A: SIN3 homolog A, transcriptional regulator
siRNA: small interfering RNA
SMN: Survival Motor Neuron
T: Thymine
TRD: Transcriptional repressor domain
TRIS: Tris(hydroxymethyl)aminomethane
tRNA: Transfer ribonucleic acid
U: Uracil
μg: microgram
USH1: Usher Syndrome
UTR: Untranslated region
V: volt
W: Tryptophan
WT: Wild-type
WW: Two tryptophan residues
Y: Tyrosine
YB-1: Y box-binding protein 1
1 Introduction

1.1 Concept of epigenetics and the role of Methyl-CpG-binding proteins

One of the most important epigenetic modification in mammalian genomes is the addition of methyl groups to position five of cytosine bases. The major target site for DNA methylation is on the cytosine residues in CpG dinucleotides. Most CpG sites are methylated at a frequency of 60%-90% (Bird, 1980). However, distinct regions with a very high CpG content called CpG islands, which are found in promoters of highly expressed genes are not methylated (Cross and Bird, 1995). Proper DNA methylation is important for normal development in mammals (Okano et al., 1999; Bird, 2002). The primary effect of DNA methylation is to repress transcription; active genes are generally non-methylated, whereas non-transcribed genes are heavily methylated (Bestor and Tycko, 1996).

DNA methylation-mediated transcriptional repression is achieved through a mechanism in which a protein containing a methyl-CpG binding domain (MBD) binds to methylated CpG nucleotides to repress transcription. Currently, five family members have been described: MeCP2, MBD1, MBD2, MBD3, and MBD4 (Hendrich and Bird, 1998). The MECP2 gene is X-linked (Amir et al., 1999), whereas the other MBD proteins map to autosomal loci (Hendrich et al., 1999 a). The MBD sequence is well conserved between these family members (Figure 1) having between 45%-75% overall amino acid identity (Hendrich and Bird, 1998). Among them, MeCP2, MBD1, and MBD2 are able to bind methylated DNA and are involved in transcriptional repression (Hendrich and Bird, 1998). Kaiso is a protein that uses zinc fingers to bind both methylated and non-methylated DNA.
(Prokhortchouk et al., 2001; Filion et al., 2006). During deamination, methylated cytosines become converted to thymines (Bestor and Tycko, 1996). MBD4 is a thymine glycosylase which binds G-T (guanine-thymine) mismatches at methylated CpG sites to prevent such mutations thus MBD4 is a mismatched repair protein (Hendrich et al., 1999 b). MBD2 and MBD3 are more closely related to each other than to the other MBD proteins having 75% similarity (Hendrich and Bird, 1998). MBD2 is present in the MeCP1 complex and is associated with histone deacetylases (HDAC) to repress transcription. Despite the sequence similarity, MBD3 is different from MBD2 because it does not bind methylated DNA (Hendrich and Bird, 1998) since it has a mutation in the MBD domain (Bogdanovic and Veenstra, 2009). MBD3 is an integral component of the NuRD (Nucleosome remodelling and histone deacetylase activity) co-repressor complex that contains histone deacetylases which are implicated in silencing genes as well (Wade et al., 1999; Zhang et al., 1999). Loss of MBD3 in mice is associated with embryonic lethality; thus, MBD3 is important for development (Hendrich et al., 2001). MeCP2 is the most extensively studied of the MBD proteins since mutations in this gene are responsible for a majority (up to 90%) of Rett syndrome cases (Smeets et al., 2009; Neul et al., 2008).
Figure 1. Structure of MBD proteins and Kaiso. MBD proteins display homology within their MBD domains. The transcriptional repression domains (TRD) in MeCP2, MBD1 and MBD2 are non-homologous and are important for interaction with various co-repressor complexes. Other motifs shown are cysteine-rich regions (CxC) in MBD1 and the glycosylase domain of MBD4. Kaiso is in a different family than MBD proteins, but also binds methylated DNA through its zinc finger (zf) domains.

(Figure modified from the review paper by Bogdanovic and Veenstra, 2009).
Figure 1

MeCP2
MBD1
MBD2
MBD3
MBD4

Kaiso
1.2 Rett syndrome

1.2.1 Pathology

Rett syndrome, initially described by Andreas Rett in 1966, is an X-linked progressive, neurodevelopmental disorder that affects almost exclusively girls. The prevalence of this disease is 1/10,000 to 1/15,000 girls worldwide, making it one of the most common genetic cause of severe mental retardation in girls (Hagberg and Hagberg, 1997). Rett syndrome is characterized by normal development for the first 6 to 18 months of age, followed by a period of regression in which the girls lose language and motor skills (Dunn and MacLeod, 2001). Purposeful hand use is replaced by repetitive stereotyped hand movements. Decelerating head growth and autistic features such as diminished eye contact and emotional withdrawal also occur. Additional characteristics include anxiety, respiratory dysfunctions, impairment of sleeping patterns, cardiac abnormalities, seizures, loss of locomotion and bone density deficits. Furthermore, girls with Rett syndrome tend to be growth retarded, and have a reduced life span. There is some stabilization of the disease at 4 to 7 years of age and girls may recover some of the skills (Hagberg et al., 1985).

Rett syndrome is believed to be a disease of arrested neuronal development, rather than neurodegeneration, as there is no evidence of neuronal loss (Armstrong, 2001 a). The brain is the organ most affected in Rett Syndrome and is typically underweight. The average size of a mature Rett brain is approximately the same as a 12-month child (Johnston et al., 2001; Glaze, 2005). Neurons within the Rett brain display significant decreases in dendritic branching and somal size and elevated neuronal packing density (Belichenko et al., 1994).
Also, Blue et al (1999 a, b) have demonstrated abnormalities in receptor densities: AMPA and NMDA receptors are increased in the young Rett brain (<8 years old) compared to controls, while in older Rett brain these receptors are dramatically reduced. This suggests that disturbances in excitatory neurotransmitter levels might be found in Rett syndrome girls (Blue et al., 1999 a, b). A study conducted by our group has demonstrated that NMDA-receptor-dependent long term potentiation (LTP) and long term depression (LTD) in the hippocampus from symptomatic Mecp2-null mice are significantly reduced compared to controls of the same age (Asaka et al., 2006). Thus, Rett syndrome is believed to be a disorder that results from an impairment in synaptic plasticity (Johnston, 2004).

1.2.2 Mutations in MECP2 are the predominant cause of Rett syndrome.

Rett Syndrome is an X-linked neurological disorder. The genetic defect was mapped to Xq28 (Sirianni et al., 1998) and 90% of mutations were identified in the gene MECP2 which encodes the transcriptional regulator MeCP2 (methyl CpG-binding protein 2) (Amir et al, 1999; Smeets et al., 2009; Neul et al., 2008). A small number of Rett Syndrome cases are caused by mutations in cyclin-dependent kinase like 5 (CDKL5), an X-linked gene (Weaving et al., 2004; Bertani et al., 2006; Tao et al., 2004), which is a kinase for MeCP2 and may play a role in regulation and phosphorylation of MeCP2 (Mari et al., 2005). Furthermore some Rett syndrome cases arise from mutations in FoxG1 gene, which encodes a brain-specific transcriptional repressor which is important for early development of the brain (Ariani et al., 2008).
In 99.5% of all cases, Rett syndrome is sporadic and due to de novo mutations in the MECP2 gene. Since affected females have a reproductive disadvantage, familial cases of Rett syndrome are rare and are due to inheritance from a carrier mother (mother that has a MECP2 mutation but does not express the disease due to skewed X inactivation) (Trappe et al., 2001; Orrico et al., 2000). The majority of MECP2 mutations are due to cytosine-to-thymine transitions in CpG dinucleotides (Dragich et al., 2000). This is most likely due to deamination of methylated cytosine to thymine, which is not easily recognized by DNA repair processes. DNA in sperm is much more highly methylated than the same sequences in oocytes due to the need for greater nuclear compaction (Morgan et al., 2004) therefore the DNA in sperm is more susceptible to mutations in a CG rich gene such as MECP2 (LaSalle, 2004). Thus, de novo mutations in Rett syndrome occur predominantly on the paternal X-chromosome, which is inherited only by the female offspring, and this is the most probable cause of high female: male ratio observed in patients with Rett syndrome (Girard et al., 2001; Trappe et al., 2001).

Males with classic Rett syndrome have been described in a few familial cases. Male patients fall into two categories: boys with classic Rett syndrome and boys with a severe neonatal encephalopathy that leads to death within the first year of life (Hofbuhr et al., 2001). The boys with Rett Syndrome carry the same mutations in MECP2 gene as those that cause Rett syndrome in girls. In some cases the boys are mosaics and have a mixed cellular population of mutated and wild-type MECP2 (Clayton-Smith et al., 2000; Armstrong et al., 2001 b; Topcu et al., 2002). In other cases, the males have a 46, XXY karyotype associated with Kleinfelter’s syndrome. Since they have an extra X chromosome (and thus one normal
copy of MECP2), they reproduce the Rett phenotype (Leonard et al., 2001; Schwartzman et al., 2001). Several studies have found boys with classic Rett syndrome with mutations in MECP2 but no evidence of Kleinfelter’s syndrome or mosaicism (Budden et al., 2005; Dayer et al., 2007; Masuyama et al., 2005; Ravn et al., 2003). This suggests that additional genetic factors may influence the clinical features of Rett syndrome in boys.

1.2.3 The structure and function of MeCP2

MeCP2 is a 53 kDa nuclear protein which is part of a family of methyl-CpG-binding domain proteins (MBD) (Lewis et al., 1992). It has four exons, and its protein sequence has six domains: MBD, transcriptional repressor domain (TRD), nuclear localization signal (NLS), interdomain region (ID), Carboxy-terminal domain (CTD) and N-terminal domain (NTD) (Figure 2 a) (Kumar et al., 2008). MeCP2 is a highly disordered protein (Ghosh et al., 2008). The distinct domains of MeCP2 are organized into a tertiary structure that is 60% unstructured and has coil-like properties (Adams et al., 2007). The MBD is 85 amino acids in length encoded within exons 3 and 4 and is essential for binding of MeCP2 to methylated DNA (Lewis et al., 1992; Nan et al., 1993). When this domain binds methylated DNA, it blocks other transcription factors from associating. The TRD encoded within exon 4 (residues 207 – 310) recruits histone deacetylases and Sin3A transcriptional co-repressor to repress transcription (Figure 2 b). HDAC1 and HDAC2 are histone deacetylases that combine with transcriptional repressor Sin3A to form a co-repressor system. Interaction between TRD and the transcriptional co-repressor complex results in deacetylation of
histones H3 and H4 by histone deacetylases leading to compaction of the chromatin, making it inaccessible for the components of the transcriptional machinery to bind, thus repressing transcription (Nan et al., 1998; Jones et al., 1998). NLS is important for targeting the protein to the nucleus. One NLS is embedded within the ID region (residues 174-190) and a second NLS is found between residues 255 and 271 in TRD region of MeCP2 (Nan et al, 1996; Kumar et al., 2008) (Figure 2 a). Carboxy-terminus may be involved in RNA-mediated functions as it has been shown to interact with WW domain splicing factors (Buschdorf and Stratling, 2004) and with the RNA-binding protein YB-1 (Y box-binding protein 1) (Young et al., 2005). It has also been shown that Carboxy-terminus may be involved in facilitating the binding of MeCP2 to the nucleosome core (Chandler et al., 1999). Furthermore, interdomain region has been shown to be important in stabilizing the interactions of MBD (Kumar et al., 2008). It is not yet known what the function of N-terminus is.

MeCP2 also binds to non-methylated DNA, but with lower affinity (Koch and Stratling, 2004). MeCP2 has been shown to bind to chromatin fibers and compact them. The genes used in these studies were non-methylated, suggesting a potential role for MeCP2 in modulating chromatin structure independent of methylation status (Georgel et al., 2003; Nikitina et al., 2007 a, b). Thus, MeCP2 influences chromatin structure and inappropriately regulated chromatin structure is proposed to be a mechanism for the development of the pathophysiology of Rett syndrome.

Until recently, it was believed that MeCP2 can only act as a transcriptional inhibitor. However, Ben-Shachar and his group (2009) have found that MeCP2 can also act as a transcriptional activator by associating with the CREB1 transcriptional factor at an activated
promoter (Figure 2 b) (Ben-Shachar et al., 2009). Moreover, a study by Yasui et al., (2007) has shown that the majority of MeCP2 bound promoters are on highly expressed genes. These results suggest that MeCP2 is a key transcriptional regulator that has dual functions on gene expression. However, it is not known whether Rett syndrome is due to MeCP2 loss of transcriptional activation, repression, or both.

MECP2 is expressed in many tissues of the body, however it is expressed at higher levels in the brain (Shahbazian et al., 2002 a). Within the brain, MECP2 is expressed at high levels in mature neurons (Kishi and Macklis, 2004). The timing of MECP2 expression in mouse and human correlates with the maturation of the central nervous system (LaSalle et al., 2001). The initial period of normal development in Rett syndrome suggests that MECP2 expression is not essential in the developing brain, but becomes critical in mature brain. Kishi and Macklis (2004) demonstrated that MeCP2 maintains the mature neuronal state, rather than play a role in cell fate decisions. In agreement with this idea, a study by Giacometti et al., (2007) has demonstrated that expression of a Mecp2 transgene in postmitotic neurons of Mecp2-null mice reversed some symptoms of the mutant mice. Also, Chen and his group (2001) have shown that deletion of Mecp2 gene specifically in neurons leads to a Rett-like phenotype in mice. These data suggest that Rett syndrome may be caused by a deficiency of MeCP2 in central nervous system.

MeCP2 is believed to be important in the maturation of neurons and synapses. There is a reduced neuronal size and dendrites are underdeveloped in patients with Rett syndrome (LaSalle et al., 2001; Shahbazian et al., 2002 a). Furthermore, over-expressing MeCP2 in transgenic mice results in enhanced synaptic plasticity as observed by an increase in LTP
(Collins et al., 2004). A study by our group has shown that in cortical neurons overexpressing MeCP2, there is an increased dendritic complexity and axonal length (Jugloff et al., 2005). These results indicate that MeCP2 may play an important role in regulating synaptic function and plasticity.

1.2.4 BDNF is one gene regulated by MeCP2.

One gene that is regulated by MeCP2 is the brain-derived neurotrophic factor (BDNF) (Chen et al., 2003). This gene encodes a neurotrophic factor important for neuronal survival (Bonni et al., 1999), differentiation (Ghosh et al., 1994) and synaptic plasticity (Kuczewski et al., 2009; Poo, 2001). Several studies have shown that BDNF protein is reduced in MeCP2 mutant mice but is increased in transgenic mice that over-express MeCP2 (Chahrour et al., 2008; Chang et al., 2006). Also, Chang et al., (2006) have shown that BDNF mutant mice displayed many features of a Rett syndrome mouse model and introducing BDNF in MeCP2 mutant brain extended the lifespan, and reversed some electrophysiological deficits observed in MeCP2 mutant mice. This suggests that the pathophysiology of Rett Syndrome may be due to altered BDNF levels.

1.2.5 MeCP2 is post-translationally regulated.

Using mass spectrometry analysis, a study by Zhou et al., (2006) has shown that there are three major sites of phosphorylation on MeCP2: serine 80 (S80), serine 229 (S229) and
Figure 2. Structure and function of MeCP2. A) Structure of MeCP2_e2 (Abbreviations: NLS-nuclear localization factor, S-serine, MBD-methyl-CpG-binding domain, TRD-transcriptional repressor domain, N-amino terminus domain, C-carboxy terminus domain). Phosphorylation sites exist at S80, S229 and S421.

B) MeCP2 acts as a transcriptional repressor by binding methylated DNA and through the Sin3A co-repressor recruits histone deacetylases (HDAC), leading to deacetylation of histones and thus compacted chromatin. MeCP2 also acts as a transcriptional activator by associating with CREB1 transcriptional factor.

(Figure 2 A-modified from IRSF database, Australia - http://mecp2.chw.edu.au/mecp2)
Figure 2

A

B
serine 421 (S421) (Figure 2 a). This study showed that neuronal depolarization triggers the phosphorylation of MeCP2 at the amino acid residue S421 in the nervous system and mutation of MECP2 at this site blocks the ability of MeCP2 to allow proper dendritic maturation and activation of BDNF. These results suggest that phosphorylation of MeCP2 at S421 mediates dendritic patterning, dendritic spine development and activation of BDNF. However, phosphorylation at this site exhibits reduced binding to methylated DNA, raising the possibility that it might inactivate the repressor function of MeCP2 (Chen et al., 2003). Furthermore, a study by Tao et al., (2009) has shown that phosphorylation of MeCP2 at S80 is negatively regulated by neuronal activity. The opposing regulation of S421 and S80 phosphorylation by neuronal activity may suggest that S80 phosphorylation is associated with MeCP2 function in resting neurons, however, S421 phosphorylation might be associated with a role of MeCP2 in depolarized neurons. The disruption of this process in individuals with mutations in MECP2 may underlie the pathology of Rett syndrome (Tao et al., 2009).

1.2.6 There are two isoforms of MECP2 with distinct N-termini.

There are two isoforms of MECP2, which differ in their N-terminus domains. The more recently identified MECP2
e1 isoform has a longer and more acidic N-terminus compared with the MECP2
e2 isoform. MECP2
e2 has a translation start site in exon 2, whereas the start site for MECP2
e1 is in exon 1 (Figure 3 A) (Mnatzakanian et al., 2004). The functional distinction for the two protein isoforms is not known. The MECP2
e2 is more
abundant than \textit{MECP2e}1 in most tissues with the exception of the mature brain. In the adult brain, the expression of \textit{MECP2e}1 is approximately 10 times higher than \textit{MECP2e}2 (Mnatzakanian \textit{et al.}, 2004). The two isoforms are nuclear and colocalize with heterochromatin, thus it was suggested that the functions of the two isoforms may overlap significantly (Kriaucionis and Bird, 2004; Kumar \textit{et al.}, 2008).

1.2.7 Mutations that occur in Rett syndrome.

About 67\% of all \textit{MECP2} mutations are caused by cytosine to thymine transitions in CpG dinucleotides and are located in the third and fourth exon of \textit{MECP2} (Figure 3 B) (Dragich \textit{et al.}, 2000). Missense mutations (single amino acid substitutions) cluster in the MBD, whereas frameshift mutations (deletions or insertions) occur in the C-terminal domain (Weaving \textit{et al.}, 2003). Nonsense mutations (single nucleotide substitutions that introduce a premature stop codon) generate truncated MeCP2 proteins and account for approximately 40\% of Rett syndrome cases. Most nonsense mutations are located on the interdomain and TRD (Percy \textit{et al.}, 2007; Weaving \textit{et al.}, 2003). Very few nonsense mutations are found in the MBD. One of the nonsense mutations found in MBD is Y141X (IRSF database, http://mecp2.chw.edu.au/mecp2). To date, no mutations in exon 2 have been identified in individuals with Rett syndrome. It is possible that mutations in exon 2 do not cause Rett syndrome as a result of compensation of \textit{MeCP2}_e1 isoform which is much more abundant in central nervous system compared to \textit{MeCP2}_e2. However, a few mutations have been
Figure 3. The isoforms of MeCP2 and the mutations that occur on MECP2 in Rett syndrome.

A) MeCP2_e1 and MeCP2_e2 isoforms.
   (Figure modified from a review paper by Chahrour and Zoghbi, 2007).

B) The type and frequency of mutations that occur on MECP2 in Rett syndrome.
   (Figure modified from IRSF database, Australia - http://mecp2.chw.edu.au/mecp2).
Figure 3

A

noncoding sequence
coding sequence

MeCP2e1

MeCP2e2

B

Frequency(%)
reported in exon 1 of MECP2e1 (Figure 3 B) (Mnatzakanian et al., 2004; Fichou et al., 2009).

1.2.8 Nonsense mutations and NMD pathway.

Nonsense mediated decay pathway (NMD) is a mechanism by which eukaryotic cells eliminate mRNA that contains a premature stop codon arising from nonsense or frameshift mutations in order to prevent the synthesis of truncated proteins that might be non-functional or deleterious (Mendell and Dietz, 2001; Holbrook et al., 2004; Amrani et al., 2006). The current understanding of NMD is not completely understood. Not all transcripts containing premature termination codons (PTCs) are targeted by NMD pathway (Holbrook et al., 2004; Linde et al., 2007) and it is not known why some transcripts escape NMD pathway while others are degraded. It has been proposed that this pathway might vary depending where on the gene the mutation is found (Holbrook et al., 2004). Mutations near the 3`end of the open reading frame usually escape nonsense-mediated mRNA decay (Kerr et al., 2001) while nonsense mutations found more upstream in the open reading frame of mRNA are usually degraded by NMD (Holbrook et al., 2004). To date no studies have determined whether truncated proteins are detected in Rett cell lines, thus it is not known whether the nonsense transcripts that cause Rett syndrome are degraded by NMD. If NMD degrades these nonsense transcripts, then they may act as loss-of-function alleles, giving a very severe phenotype.
1.2.9 Genotype/Phenotype analysis in Rett syndrome.

The severity of Rett syndrome varies from mild to more severe phenotypes. This may depend on the type and location of mutations in the MECP2 gene (Table 1). Several studies have found that truncating mutations are more severe than missense mutations with early truncations being more severe than late truncations (Cheadle et al., 2000, Monros et al., 2001, Huppke et al., 2002; Neul et al., 2008; Bebbington et al., 2008). Also, mutations affecting nuclear localization factor (NLS) are more severe than mutations that preserve NLS (Huppke et al., 2002). For example, individuals with R133C missense mutation are less severely affected than those with the nonsense mutation R168X (Neul et al., 2008). The R133C genotype causes very mild Rett syndrome. No breathing irregularities or other signs of autonomic dysfunction were observed in girls with this mutation. These individuals did not experience any seizures and they preserve some hand use and speech ability (Smeets et al., 2009). The missense mutation R306C adversely affects only language (Neul et al., 2008). Individuals with this mutation seldom develop epilepsy (Smeets et al., 2009). Individuals with T158M missense mutation usually preserve ambulation. They all have epilepsy in some period of their lives and/or breathing irregularities (Smeets et al., 2009). Individuals with the R168X nonsense mutation are more severely affected than those with R294X and late C-terminal truncating mutations. Girls with R168X mutation have the greatest severity score and are less likely to walk, speak and retain hand use (Horska et al., 2009; Neul et al., 2008). However, individuals with C-terminal truncations are the least severe and are more likely to walk and use words (Neul et al., 2008; Huppke et al., 2002). A study by Bebbington et al., (2008) has shown that the nonsense mutations R270X and R255X which lose the NLS, are
Table 1: Percentage of girls with mutations in MECP2 that retain functional ability

<table>
<thead>
<tr>
<th>Mutation</th>
<th>N</th>
<th>Walks alone (%)</th>
<th>Uses hands (%)</th>
<th>Uses words (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R106W</td>
<td>9</td>
<td>33</td>
<td>56</td>
<td>33</td>
</tr>
<tr>
<td>R133C</td>
<td>12</td>
<td>75</td>
<td>92</td>
<td>50</td>
</tr>
<tr>
<td>T158M</td>
<td>30</td>
<td>60</td>
<td>50</td>
<td>27</td>
</tr>
<tr>
<td>R168X</td>
<td>29</td>
<td>28</td>
<td>38</td>
<td>3</td>
</tr>
<tr>
<td>R255X</td>
<td>32</td>
<td>38</td>
<td>59</td>
<td>28</td>
</tr>
<tr>
<td>R270X</td>
<td>18</td>
<td>44</td>
<td>67</td>
<td>22</td>
</tr>
<tr>
<td>R294X</td>
<td>14</td>
<td>86</td>
<td>86</td>
<td>50</td>
</tr>
<tr>
<td>R306C</td>
<td>21</td>
<td>67</td>
<td>52</td>
<td>10</td>
</tr>
<tr>
<td>C-terminal truncations</td>
<td>17</td>
<td>82</td>
<td>88</td>
<td>71</td>
</tr>
<tr>
<td>Large deletions</td>
<td>17</td>
<td>41</td>
<td>53</td>
<td>12</td>
</tr>
</tbody>
</table>

Neul et al., 2008
more severe than R133C and R294X. Thus, specific mutations in *MECP2* confer different severity in Rett syndrome patients.

Furthermore, the phenotypic variability may also be the result of distinct patterns of X-chromosome inactivation. Although normal females inherit two copies of the X-chromosome, one copy is randomly silenced during early embryogenesis (Lyon, 1989). Since *MECP2* is located on the X-chromosome, it is subjected to X-chromosome inactivation. Normally, X-inactivation is random, with approximately half of the cells expressing the normal *MECP2* and half expressing the defective *MECP2* gene. If this process is non-random (skewed), then the X-chromosome with either the defective or the normal *MECP2* gene may be preferentially active in most cells of the body. This non-random X-inactivation can influence phenotype, resulting in a variety of clinical severity ranging from the absence of phenotype if normal *MECP2* gene is active to a more severe Rett syndrome case when the mutated *MECP2* gene is active (Archer et al., 2007). Rett syndrome girls usually show a random pattern of X-inactivation; however, a few studies have shown skewed patterns of X-inactivation (Archer et al., 2007; Amir et al., 2000). The skewing is toward the normal X-chromosome and is correlated with less severe phenotypes (Archer et al., 2007).

1.2.10 Therapeutic approaches for Rett syndrome.

Currently, there is no cure for Rett syndrome. At the moment, treatments of Rett syndrome focus on the management of symptoms. Genetic manipulations of mouse models
have been useful in determining whether Rett syndrome can be reversed when the function of MeCP2 is restored. However, the techniques used so far cannot be used in a clinical setting.

Mouse models of Rett syndrome have been generated using gene-targeting approaches. They recapitulate several of the clinical features of Rett syndrome: a period of normal development followed by a severe neurological dysfunction that includes tremors, hypoactivity, irregular breathing, abnormal gait and movements, social impairment, seizures, anxiety-like behaviour, learning and memory deficits. They also demonstrate impaired synaptic plasticity, and males usually die by 8-12 weeks of age (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002 b; Moretti et al., 2005; Asaka et al., 2005; Moretti et al., 2006).

Initially it was believed that Rett syndrome is an irremediable condition, and that MeCP2 is essential for neuronal development during a critical time window when synapses start to form. However, Guy et al., (2007) have created a mouse model in which endogenous Mecp2 was silenced but could be conditionally activated by its promoter. This study has demonstrated that re-activation of Mecp2 expression led to reversal of Rett phenotype in symptomatic mice. Also, Giacometti and his group (2007) have shown that reactivation of Mecp2 in Mecp2 knockout mice improved life-span and rescued some Rett-like symptoms of mutant mice. The Rett-like behaviour of Mecp2-deficient female mice was improved by re-introduction of a MeCP2 transgene in the forebrain of these mice (Jugloff et al., 2008). These results strongly suggest that the absence of MeCP2 does not irreversibly damage neurons and thus, the neurological defects seen in Rett syndrome can be reversible by restoring MeCP2 function.
However, MeCP2 levels have to be tightly regulated. A few studies have shown that transgenic mice with moderate over-expression of MeCP2 have neurological impairments (Collins *et al.*, 2004; Luikenhuis *et al.*, 2004). This suggests that even mild over-expression of this protein is deleterious and any therapies directed at increasing the levels of MeCP2 in patients must be carefully considered to avoid any further neurological dysfunctions. Furthermore, a study by Samaco *et al.*, (2008) has shown that reducing MeCP2 levels by 50% results in a broad spectrum of phenotypic abnormalities such as learning and motor deficits, altered social behaviour and breathing irregularities. However, in contrast with *MeCP2*-null mice that die between 8 and 12 weeks of life, these mice have normal lifespan. Thus, even a 50% decrease in MeCP2 levels might still cause a disease. Collectively, these results suggest that the central nervous system is sensitive to MeCP2 expression levels and the protein`s levels and function must be critically maintained.

The finding that over-expression of MeCP2 is as deleterious as its loss of expression complicate the treatment of Rett syndrome. Using a therapy such as gene replacement therapy would not necessary be beneficial since in addition to adding function to the cells expressing the mutant *MECP2* allele, it would also increase the level of *MECP2* in cells expressing the functional *MECP2* allele. Thus, this procedure will introduce more MeCP2 protein than needed. Therefore, a therapeutic option would be to enhance endogenous MeCP2 levels selectively in cells expressing the mutant allele.
1.3 The molecular mechanism of premature stop mutations.

Protein translation occurs in the cytoplasm where the ribosomes are located. Eukaryotic ribosomes are made of a small subunit (40S) and a large subunit (60S) which form the 80S ribosomal complex. The small subunit (40S) also contains 18S subunit. In prokaryotes, the small ribosomal subunit is 30S which contains the 16S subunit, and the large subunit is 50S (Rodnina and Wintermeyer, 2009). In eukaryotes, the ribosomal complex has two sites: the A site (also called decoding site) within the 18S of rRNA which contains the bases A1492 and A1493 which are important in facilitating the pairing of the anticodon of aminoacyl tRNA with the complementary codon on mRNA, and a P site where the peptide bond is formed. Termination of the protein synthesis happens when a termination codon (UAA, UAG or UGA) on mRNA enters the A site of rRNA. At this stage, the translation stops since there is no tRNA with an anticodon complementary to any of the stop codons. This causes the release factors to bind to the stop codon (Kerem, 2004). In eukaryotes, the release factor eRF1 recognizes all three stop codons in the ribosomal A site and facilitates polypeptide chain release; another release factor eRF3 modulates the function of eRF1 (Zhouravleva et al., 1995). Nonsense mutations introduce a premature stop codon, which lead to premature binding of the eRF1 to this premature termination codon, and thus a truncated and non-functional protein is produced (Kerem, 2004).
1.4 Aminoglycosides

1.4.1 What are aminoglycosides?

Aminoglycosides are a class of structurally related antibiotics that are used in the treatment of bacterial infections (Wilhelm et al., 1978). They are hydrophilic sugars that contain several amino and hydroxyl groups. The amine groups are protonated in biological media (Botto and Coxon, 1983); thus, they have a high binding affinity for nucleic acids. Furthermore, due to their charged properties, they have poor permeability across the plasma membrane (Kondo and Hotta, 1999). Their antibacterial action results from an aminoglycoside binding to the decoding site (A site) on bacterial rRNA disrupting the translational process and thus inhibiting protein synthesis. Accumulation of erroneous proteins that are incorrectly folded and truncated accumulate in bacterial membrane leading to bacterial cell death (Magnet and Blanchard, 2005).

The majority of aminoglycosides contain a common non-sugar ring, called 2-deoxystreptamine (2-DOS) that carries sugar substituents at positions 4, 5, and 6. The 2-DOS ring is also called ring II, and represents the central ring. The sugar ring bound at position 4 of 2-DOS is ring I and the sugar ring bound either at position 5 or at position 6 of 2-DOS is ring III. The most important classes of aminoglycoside antibiotics are 4,5- and 4,6-disubstituted 2-DOS derivatives. Paromomycin is an example of 4,5 disubstituted 2-DOS and gentamicin, geneticin and amikacin are part of 4,6 disubstituted 2-DOS (Figure 4). In addition to having a distinct linkage between 2-DOS and ring III, the 4, 5- disubstituted class
Figure 4. Structures of aminoglycosides used in my study.

(All structures were copied from the following web-sites).

Amikacin:    http://www.rsc.org/ej/NP/2000/a902202c/a902202c-u6.gif
Paromomycin: http://upload.wikimedia.org/wikipedia/commons/thumb/l/l6/Paromomycin_structure.svg/522Px-Paromomycin_structure.svg.png

The structures of aminoglycosides could also be found in the review article by Hainrichson et al., 2008.
Figure 4

Gentamicin

Geneticin

Amikacin

Paromomycin
of aminoglycosides consist of four (or more) rings rather than the three rings found in 4, 6-disubstituted class members (Hermann, 2007).

1.4.2 Toxicity of aminoglycosides.

One of the major disadvantages in using aminoglycosides for long term is their toxicity through kidney (nephrotoxicity) and ear (ototoxicity) illnesses. A large amount of the intravenously administered dose of aminoglycosides is accumulated in the kidney (about 10% of dose) and in the inner ear, whereas little distribution is seen in other tissues (Nagai and Takano, 2004). The mechanism of aminoglycoside-induced toxicity involves a series of steps. Since aminoglycosides are positively charged in neutral environment, they are able to interact electrostatically with the negatively charged cell membranes. Upon entering the cells, aminoglycosides interact with acidic phospholipids in the lysosomal membranes (Nagai and Takano, 2004). This interaction generates free radical species which eventually leads to tissue damage (Keeling and Bedwell, 2005). Also, aminoglycosides inhibit phospholipases (enzymes that break down phospholipids) and this is another factor that contributes to toxicity of aminoglycosides in inner ear and kidneys (Forge and Schacht, 2000; Nagai and Takano, 2004).

There is a structure-toxicity relationship of aminoglycosides. A decrease in the number of amino groups results in diminished toxicity; however, a decrease in the number of hydroxyl groups results in elevated toxicity. The reduced toxicity due to a decrease in the number of charged amino groups could be explained by a decrease in nonspecific
interactions with many cell components and reduced formation of free radicals. The rank order of the binding affinity with cell components resulting in nephrotoxic and ear toxicity is: geneticin > gentamicin-paromomycin>amikacin (Humes et al., 1982; Williams et al., 1987). Currently, the aminoglycosides that are used in clinical use as antibiotics for administration in humans are amikacin, gentamicin and paromomycin amongst others (Figure 4). Due to its high toxicity, geneticin is not used for clinical practices (Hainrichson et al., 2008) (Figure 4).

1.4.3 Megalin receptor is important in the uptake of aminoglycosides in cells.

Megalin is an anionic, endocytic receptor (Moestrup et al., 1995). Since aminoglycosides are cationic at physiological pH, they can easily bind to megalin and are taken up in the cells via receptor-mediated endocytosis (Moestrup et al., 1995). Megalin is expressed on the membranes of most cells and organs of the body; however, it is most abundantly expressed in the renal proximal tubule of kidney and inner ear (Christensen et al., 1998). Thus, the nephrotoxicity and ototoxicity arise due to too much accumulation of aminoglycosides in kidney and inner ear. The role of megalin in aminoglycoside accumulation in kidney is supported by a study showing that a mouse model carrying a knockout of the megalin gene does not accumulate aminoglycosides in kidney (Schmitz et al., 2002). Furthermore, a study by Watanabe et al., (2004) has shown that administration of agonists that compete with aminoglycoside binding to megalin results in a decrease of aminoglycoside accumulation and toxicity. These studies indicate that one way aminoglycosides might get into cells is through megalin receptor.
1.4.4 Potential of aminoglycosides to treat genetic diseases with nonsense mutations

A large number of human genetic diseases arise from nonsense mutations, single point alterations in DNA that give rise to UAA, UAG, or UGA premature stop codons in mRNA coding regions, leading to premature termination of protein synthesis and eventually to truncated and non-functional proteins (Kerem, 2004). One approach to treat these diseases is to reduce the efficiency of translation termination, so production of some full-length and functional protein is restored. This mechanism is termed “termination suppression” or “read-through”. Through mechanisms not completely understood, in the past few years, several studies have shown that besides their use as antibiotics, aminoglycosides could have a therapeutic benefit in the treatment of genetic diseases caused by premature stop codons by inducing the ribosome to read-through these premature stop codons generating a full length protein (Howard et al., 1996; Bedwell et al., 1997; Barton-Davis et al., 1999; Rebibo-Sabbah et al., 2007; Lai et al., 2004; Pinotti et al., 2006).

The fact that aminoglycosides could suppress premature stop codons in mammalian cells was first demonstrated in 1985 by Burke and Mogg. They have shown that geneticin and paromomycin can suppress the TAG premature stop codon and restore the activity of a mutant gene to approximately 20% of wild type levels when it was transfected in COS-7 cells. Furthermore, they also mentioned the therapeutic potential of these drugs in the treatment of genetic diseases. Cystic Fibrosis (CF) was the first genetic disease studied and several experiments have shown that nonsense mutations in the CFTR gene (which encodes for cystic fibrosis transmembrane receptor protein) could be suppressed by geneticin and gentamicin as seen by the appearance of full length, functional CFTR protein in transfection
assays and in a bronchial epithelial cell line (Howard et al., 1996; Bedwell et al., 1997). Other genetic disorders where aminoglycosides were tested on are Duchenne Muscular Dystrophy (DMD) (Barton-Davis et al., 1999; Howard et al., 2004), Hurler Syndrome (Keeling et al., 2001), Usher Syndrome (Rebibo-Sabbah et al., 2007), Ataxia-Telangiectasia (Lai et al., 2004), Factor VII deficiency (Pinotti et al., 2006) and Nephropathic cystinosis (Helip-Wooley et al., 2002). The production of full length and functional proteins in these studies were demonstrated with efficiencies varying from 1% to 30%, depending on the sequence of the stop codon, the sequence context surrounding it and the aminoglycoside tested.

Several studies have shown that the premature stop codon TGA shows a greater translational read-through than TAG, and TAA stop codon is the most resistant to read-through. The nucleotide after the stop codon also plays an important role in determining the efficiency of aminoglycoside mediated read-through, but its effect is highly influenced by the stop codon present and sequence around it (Manuvakhova et al., 2000; Bidou et al., 2004; Keeling and Bedwell, 2002). Thus, it remains unclear whether the different nonsense mutations responsible for Rett syndrome would be responsive to aminoglycoside treatment.

1.4.5 Proposed mechanisms of aminoglycoside mediated read-through.

It is believed that the potential of aminoglycosides in the treatment of disorders with premature termination codons results from their ability to suppress nonsense mutations by inducing the ribosomes to “read-through” the premature stop codons generating full length
proteins by insertion of an amino acid by the near-cognate tRNA (Hainrichson et al., 2008). It has been suggested that in general tryptophan is inserted at TGA premature stop codon and glutamine is inserted at TAG and TAA premature stop codons (Nilsson and Ryden-Aulin, 2003). A proposed mechanism of how aminoglycosides may induce read-through is by their ability to bind to the decoding site (A site) of rRNA inducing conformational changes that stabilize the interaction between the stop codon of mRNA and the near-cognate aminoacyl tRNA (aminoacyl-tRNA that has an anticodon complementary to two of the three nucleotides of the stop codon). When this occurs, the release factor proteins do not bind, thus the elongation of the polypeptide chain in the correct reading frame continues and a full length protein is produced (Recht et al., 1996) (Figure 5).

It has been shown that there are several important structures that allow aminoglycosides to bind to the decoding site of rRNA. The bases G1408, A1492 and A1493 in the rRNA decoding site are necessary for high affinity binding to ring I of the aminoglycosides (Vicens and Westhof, 2003). A possible reason is that these nucleotides are unpaired and they create a suitable site for the aminoglycosides to bind and interact with nucleic acids and anionic phosphate groups. It has been shown that this cavity in the rRNA is necessary to allow binding of aminoglycosides (Figure 6) (Vicens and Westhof, 2003). Furthermore, it has been proposed that the central ring (2-DOS or ring II) of aminoglycosides is required for the precise anchoring of the aminoglycosides to the decoding site of rRNA (Vicens and Westhof, 2003; Hermann, 2005).
Figure 5. The mechanism of aminoglycoside interaction with ribosomal protein synthesis.

A) As an example, in the normal case, tRNA carrying the anticodon (CUU) of glutamic acid (E) matches the codon on mRNA (GAA). This match results in the conformational alignment of A1492 and A1493 in the decoding site of 18S rRNA leading to polypeptide chain elongation.

B) When a nonsense mutation occurs, in this example the codon for glutamic acid changes to a premature stop codon (UAA) in mRNA. This mutation prevents the codon-anticodon pairing since A1492 and A1493 in the ribosomal decoding site are not properly aligned and this causes translation to end and thus a truncated protein is produced.

C) Aminoglycosides bind to the decoding site of 18S rRNA and induce a conformational alignment of A1492 and A1493 in the ribosomal decoding site. When this occurs, the interaction between the premature stop codon of mRNA and the near-cognate aminoacyl tRNA is stabilized, leading to incorporation of an amino acid (in this example, glutamine or Q) and promoting chain elongation.

(Image modified from a review article by Zingman et al., 2007)
Figure 5
A study by Recht et al., (1999) has shown that aminoglycosides bind with a higher affinity to the rRNA of prokaryotes than to that of eukaryotes. Prokaryotic rRNA contains A1408 in the decoding site; however, eukaryotic rRNA contains G1408 base pair (Figure 6) (Recht et al., 1999). Crystal structure analysis of aminoglycoside complexes have shown that key hydrogen bonds occur at A1408 of the ribosomal decoding site of bacteria (Francois et al., 2005). Since eukaryotic rRNA decoding site has a G at this position, it is not capable of forming these critical hydrogen interactions with aminoglycosides. Thus, this single nucleotide change allows binding of aminoglycosides to bacterial rRNA decoding site with a much higher affinity than to eukaryotic rRNA. The antibacterial mode of these compounds is due to this single nucleotide change (Recht et al., 1999).

The precise mechanism(s) through which aminoglycoside mediated read-through is achieved remains unclear. During decoding, the aminoacyl-tRNA forms a minihelix between the codon of the mRNA and the anti-codon of aminoacyl-tRNA. During this process, the conformation of the A site is changed from an “off” state where the conserved adenines A1492 and A1493 are folded back within helix, to an “on” conformation, where the adenines are flipped out from the A-site, allowing the interaction between the cognate codon-anticodon to occur. This conformation is a molecular switch that determines the continuation of translation. It is believed that aminoglycosides facilitate read-through of nonsense mutations by binding to the A-site of rRNA and changing the conformation equilibrium of the two conserved adenines A1492 and A1493 to the “on” state (Figure 7). In the “on” state conformation the two adenines are able to create hydrogen bonds with the bases formed by near-cognate tRNA anticodon and the mRNA premature stop codon leading to continuation
Figure 6. The structures of ribosomal decoding sites of prokaryotes and eukaryotes. The A base (prokaryotes) and G base (eukaryotes) are indicated by arrows. The cavity formed by the unpaired nucleotides A1408 or G1408, A1492 and A1493 allows aminoglycosides to bind. (Abbreviations: A-adenine, G-guanine).

(Figure modified from a review article by Hainrichson et al., 2008)
Figure 6

Prokaryotic (16S) decoding site

Eukaryotic (18S) decoding site
of translation (Hainrichson et al., 2008; Keeling and Bedwell, 2005) (Figure 7). It has been shown that different aminoglycosides bind to decoding site of rRNA with different affinities. This might depend on the hydrogen bonds and electrostatic interactions between decoding site of rRNA and the rings of aminoglycosides (Vicens and Westhof, 2003; Carter et al., 2000; Yoshizawa et al., 1998; Vicens and Westhof, 2001).

Although it is not completely clear, several studies have proposed that another mechanism of how aminoglycosides might be able to induce read-through is by suppressing the nonsense mediated mRNA decay (NMD) pathway (Bedwell et al., 1997; Correa-Cerro et al., 2005; Buck et al., 2009). Approaches that inhibit NMD pathway increase the amount of mutated mRNA available for translation. This may greatly enhance the levels of protein produced by suppression therapy (Correa-Cero et al., 2005).

1.4.6 Do aminoglycosides facilitate read-through at normal stop codons?

Although not completely known, it is believed that aminoglycosides can only facilitate read through at premature stop codons, and not at normal stop codons. In a review article, Kerem (2004) has proposed that naturally occurring stop codons are found within a context that promotes efficient translation termination compared to premature stop codons. Furthermore, multiple stop codons are found at the end of an open reading frame of mRNA. The presence of many stop codons may reduce the ability of aminoglycosides to induce read-through at normal termination signals (Major et al., 2002). Also, the normal stop codons are located in proximity to the poly(A) tail and this may contribute to translational termination.
Figure 7. The molecular mechanism of the aminoglycoside mediated read-through.

At the ribosomal decoding site, A1492 and A1493 are in the “off” state conformation. When aminoglycosides bind to the decoding site, they change the conformation equilibrium of the two conserved adenines to the “on” state. In the “on” state conformation, A1492 and A1493 are able to create hydrogen bonds with the bases formed by near-cognate tRNA anticodon and the mRNA premature stop codon leading to continuation of translation.

(Figure modified from a review article by Hainrichson et al., 2008)
Figure 7
(Amrani et al., 2004). In addition, specific mRNA decay mechanisms are activated when translation extends into the 3′-untranslated region (UTR); thus, even if aminoglycosides would facilitate read through at normal stop codons, the proteins would not be produced (Hoof et al., 2002). Collectively, these factors have led to the hypothesis that aminoglycosides may be able to induce read through only at premature stop codons.

1.5 Aims of my thesis

Approximately 40% of mutations that cause Rett syndrome are of the nonsense mutation class (Percy et al., 2007). Several studies have shown that certain aminoglycosides facilitate premature termination stop codon read-through and allow the generation of a full-length and functional protein product (Bedwell et al., 1997; Howard et al., 1996). However, the aminoglycoside mediated read-through is highly dependent on the sequence of the stop codon, and nucleotides surrounding the stop codon. It remains unclear whether the different nonsense mutations of MECP2 that cause Rett syndrome will be responsive to aminoglycoside treatment.

Specifically, the aims of my thesis were:

- To generate epitope tagged cDNAs containing some nonsense mutations seen clinically in Rett syndrome girls.
- To transiently transfect HEK-293 cells with the mutant cDNAs in the presence and absence of different concentrations of aminoglycosides, and determine whether the
prevalence of full length MeCP2 protein is increased in the presence of aminoglycosides using western blot analysis.

- To determine whether the prevalence of full length MeCP2 protein is increased in lymphocyte cells derived from a Rett syndrome girl with R255X mutation when they were treated for four days with different concentrations of aminoglycosides.

- To determine whether the prevalence of full length MeCP2 protein is increased in the lymphocyte cells derived from a Rett Syndrome girl with R255X mutation when they were treated for 12 days with aminoglycosides at clinically relevant doses.

**MY OVERALL HYPOTHESIS** is that the premature terminating mutations of *MECP2* that cause Rett syndrome can be partially suppressed by aminoglycoside administration allowing a full length MeCP2 protein to be generated.
2 Methods

2.1 Molecular biological techniques

2.1.1 Construction of mutant forms of MECP2

Nonsense mutations corresponding to specific mutations seen clinically in Rett girls were generated by PCR-based site-directed mutagenesis using a myc or hemagglutinin-tagged (HA) full-length mouse MeCP2 cDNA (exon 2 form) as a template for generating the Y141X, Q170X, and E205X mutant forms, and a human MECP2 cDNA (exon 1 form) for generating the R294X mutant. The following primers were used for these reactions:

MeCP2-Y141X (C-G substitution):
Sense: 5' - GTAGAATTGATTGCATA\textsubscript{G}TTTGAAAAGGTGGGAGACACCTCC-3',
Antisense: 5' - GGAGGTGTCTTCCACCTTTTCTAAAACTATGCAATCAATTCTAC;

MeCP2-Q170X (C-T substitution):
Sense: 5' - CCCTCCAGGAGAGAG\textsubscript{T}AGAAACCACCTAAG-3',
Antisense 5' - CTTAGGTGTGTTCTACTCTCTCTGGAGGG-3';

MeCP2-E205X (G-T substitution):
Sense: 5' - GGCAGCAGCATCATAAGGTGGTGCAAGGT - `3,
Antisense: 5' - CACCTGAACACCTT\textsubscript{A}TGATGCTGCTGCC -`3;

MECP2-R294X (C-T substitution):
Sense: 5' - GTGAAGGAGTCTTCTATCT\textsubscript{T}GATCTGTGCAGGAGACC -3',
Antisense: 5' - GGTCTCCTGCACAGATCA\textsubscript{A}GATAGAAGACTCCTTCAC-3'.
The bolded and underlined nucleotide indicates the site targeted to generate the mutant form of MECP2.

In order to generate the mutant forms of MECP2, the site directed mutagenesis kit (STRATAGENE) was used. The PCR reaction mix was made by adding reaction buffer to 1X, 50 ng of wild type MECP2 cDNA (having HA or Myc epitope tags in a modified pTRACER-CMV2 expression vector in which cDNA encoding the green fluorescent protein had been excised), 125 ng of sense primer, 125 ng of antisense primer, 1 ul of dNTP mix and water to a final volume of 50 ul. After mixing the reaction, 1 ul of PfuUltra HF DNA polymerase (2.5 U/ul) was added. The reaction was added in a thermocycler with the conditions as illustrated in Table 2.

Table 2. Cycling Parameters for the Site-Directed Mutagenesis Method.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
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<td>30 seconds</td>
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<tr>
<td></td>
<td></td>
<td>55°C</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>1.5 minutes</td>
</tr>
</tbody>
</table>

Following the temperature cycling, the reaction was placed on ice for two minutes to cool to less than 37°C. Approximately 1 ul of Dpn I restriction enzyme (10 U/ul) was added directly to each amplification reaction, after which the reaction was mixed and incubated in 37°C water bath for two hours to digest the parental dsDNA (non-mutated DNA). The DNA was then transformed in SURE 2 Supercompetent E.coli cells (STRATAGENE), purified and
each mutant was verified by DNA sequencing of both strands (ACGT Inc, Toronto, Ontario). The generated truncated forms of MeCP2 are shown in Figure 8.

### 2.1.2 DNA transformation

All cDNA constructs were transformed in SURE 2 Supercompetent E. coli cells (STRATAGENE). The bacterial cells (100 ul cells) were allowed to thaw on ice. After thawing, 2 ul of Beta-mercaptoethanol was added on cells to increase transformation efficiency. The cells were then incubated on ice for 10 minutes, swirling gently every 2 minutes. Approximately 50 ng of each mutant cDNA was added to 100 ul bacterial cells, after which they were left on ice for 45 minutes. Then, the cells were heat-pulsed by placing them in a 42°C water bath for 30 seconds, and then incubated on ice for 2 minutes. Following this, 900 ul of preheated NZY+ (1% NZ amine, 0.5% yeast extract, 0.5% NaCl, pH 7.5) was added on bacterial cells, and the tubes were incubated at 37°C for 1-2 hours with shaking at 225-250 rpm in an incubator shaker (Series 25, New Brunswick Scientific CO., Inc).

Approximately 50-100 ul of the transformation mixture was plated on Luria-Bertaini (LB) agar plates (1% NaCl, 1% tryptone, 0.5% yeast extract, 1.5% agar, 1% ampicillin) and incubated at 37°C for 12-16 hours. Next day, a single colony from a plate was inoculated in approximately 3 mL LB medium (1% NaCl, 1% tryptone, and 0.5% yeast extract) and incubated for 12-16 hours at 37°C with shaking at 300 rpm.
Figure 8. The truncated forms of MeCP2 that I used in my study. On the right side of the figure, the induced premature stop codon, the nucleotide downstream of stop codon, and their expected migration sizes in kilodaltons (kDa) are shown.
Figure 8

Y141X  TAC T > TAG T (15 kd)
Q170X  CAG A > TAG A (20 kd)
E205X  GAA G > TAA G (25 kd)
R294X  CGA T > TGA T (35 kd)
2.1.3 DNA purification

For DNA purification, the QIAprep Spin Miniprep Kit (QIAGEN) was used. Approximately 3 mL of bacteria were pelleted at 13,200 rpm (16,300 x g; 851 IEC MicroMax) in a tabletop microfuge for 1 minute, after which the pellet was re-suspended in 250 ul buffer P1 (50 mM glucose, 25 mM Tris.Cl pH 8, 10 mM EDTA pH 8, RNAse). The bacteria were re-suspended in this buffer until no cell clumps were visible. Then, 250 ul of buffer P2 (0.2 M NaOH, 1% SDS) was added to lyse the cells, and the tube was inverted slowly six times until the solution became slightly clear. The lysis reaction was allowed to proceed for 5 minutes. To neutralize the reaction, 350 ul buffer N3 (3 M potassium acetate, 11.5 % glacial acetic acid) was further added and the solution was mixed several times until it became cloudy. The reaction was then centrifuged for 10 minutes at 13,000 rpm. The supernatant was applied to the QIAprep spin column and centrifuged for 1 minute. The flow-through was discarded and the DNA in the QIAprep spin column was washed with 500 ul buffer PB (guanidinium hydrochloride, isopropanol) to remove trace nuclease activity. The column was centrifuged again for 1 minute, the flow-through was discarded, and the DNA was washed by adding 750 ul buffer PE (70% ethanol). The QIAprep spin column was centrifuged for 1 minute to remove the wash buffer. The column with the DNA was placed in a clean 1.5 microcentrifuge tube and 50 ul buffer EB (10 mM Tris-Cl pH 8.5) was added to the center of spin column. The tube was left on the bench for 1 minute and then centrifuged again for 1 minute at 13,000 rpm to elute the DNA. DNA concentration was measured using Nanodrop.
2.1.4 Preparation of cell lysates

Treated and non-treated transfected HEK-293 cells plated in 6-well plates were washed once with cold PBS and lysed on ice with 100 ul Mammalian Protein Extraction Reagent (M-PER-PIERCE) lysis buffer supplemented with proteinase inhibitors per well for 5 minutes. Lysates were then centrifuged at 13,200 rpm (16,300 x g) for 10 minutes in a tabletop microfuge (851 IEC MicroMax). The supernatant fractions were collected, aliquoted and stored at -20°C until use.

2.1.5 Nuclear extraction

Treated and untreated lymphocytes were washed 3X with PBS at 4°C by sequential centrifugation at 2000 rpm (688 x g) (model #3840, Omnifuge). The washed cell pellet was re-suspended in 4X the pellet volume (roughly 800 ul) of hypotonic solution (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, protease inhibitors) and incubated on ice for 10 minutes to allow the cells to swell. The swollen cells were then centrifuged for 15 minutes at 2000 rpm (688 x g) at 4°C. The pellet was re-suspended in 2X the pellet volume (roughly 400 ul) in hypotonic solution, and disrupted by manual homogenization (40 strokes) in a round bottom Dounce tube fitted with a pestle. The nuclei were then collected by centrifugation at 2000 rpm (688 x g) for 15 minutes at 4°C as above. The pellet was collected, re-suspended in 300 ul ice cold lysis buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitors), and homogenized 40X as above in the Dounce homogenization tube. Nuclear proteins were extracted from this homogenate by incubation on a rotating platform for 30 minutes at 4°C,
and then cleared by centrifugation for 30 minutes at 14,500 rpm (26,000 x g) (SW-41T1 Beckman rotor). The nuclear extract was aliquoted into cryovials, and snap-frozen by submerging in liquid nitrogen. The extracts were stored at -80°C until use.

### 2.1.6 Western blot analysis

The protein concentrations of individual samples were determined using the Bradford protein assay (Invitrogen, Carlsbad CA) at 595 wavelength using the spectrophotometer (Beckman model DU640). For nuclear extractions 3 ug proteins were loaded on each well. For total extractions, 15 ug proteins were loaded on each well. Samples were prepared for gel electrophoresis by addition of loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% Beta-mercaptoethanol, 12.5 mM EDTA and 0.02% bromophenol blue) and heating to 95°C for five minutes to denature the proteins. The samples were then resolved by electrophoresis on a 5% acrylamide stacking gel and 12.5% resolving acrylamide gel in Tris-glycine Laemmli running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS) at a constant voltage of 100 V for 2 hours. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane overnight in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at a constant voltage of 23V at 4°C. The membranes were then incubated for two hours at room temperature with a blocking solution containing TBST washing buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween 20) and 5% non-fat dry skim milk to diminish non-specific binding, and then incubated overnight with specific primary antibodies diluted in blocking solution at 4°C. The primary antibodies used were a chicken anti-human MeCP2 C-terminus antibody at a dilution of 1:15,000 (a gift from Dr. Janine LaSalle, University of
California, Davis) and monoclonal antibodies raised in mouse towards the HA (COVANCE, dilution 1:1000) and Myc (Cell Signaling Technology, dilution 1:1000) epitope tags. Following primary incubation, the blots were washed extensively in TBST washing buffer, and then incubated for two hours with secondary horseradish peroxidase-conjugated species-specific antibodies diluted in blocking solution. The antibodies were used at concentrations recommended by the manufacturer (anti-chicken 1:10,000 and anti-mouse 1:5,000). Reaction products were visualized using enhanced chemiluminescence (GE Healthcare, Amersham ECL Western Blotting Detection Reagents). Molecular weights were determined with pre-stained markers. To control for the amount of loaded protein in lymphocytes, the membranes were stripped by incubation at 55°C for 30 minutes in stripping buffer (10% SDS, 1 M Tris pH 6.7, Beta-mercaptoethanol) followed by washing in TBST (3 X 10 minutes per wash) and blocking for 2 hours at room temperature in blocking solution. The blots were re-probed with an antibody against MBD2 (Sigma) at a dilution of 1:10,000.

2.1.7 Immunocytochemistry

Transfected HEK-293 cells grown on glass coverslips were washed twice with PBS and fixed in cold PBS containing 4% paraformaldehyde for 20 minutes. The cells were washed with PBS (5 minutes 3X), and permeabilized with 0.25% Triton X-100 (Sigma), followed by washing with PBS (5 minutes 3X). The cells were incubated overnight at 4°C with blocking solution containing 4% BSA, 4% goat serum and 0.5% non-fat dry skim milk to eliminate nonspecific antibody binding. The following day, the cells were incubated overnight at 4°C with primary antibodies diluted in the blocking solution (Anti HA 1:500; Anti Myc 1:500).
The cells were washed with PBS (5 minutes 3X) after which they were incubated with secondary antibody conjugated with fluorochromes for 1 hour at room temperature. The cells were washed again with PBS (5 minutes 3X) and stained with DAPI, which fluorescently labels nuclei for 20 minutes. Cells were viewed using a Zeiss Axioplan with Deconvolution Imaging microscope.

2.2 Statistical analysis

The read-through in transfected HEK-293 cells was determined by dividing the full length MeCP2 protein by the total amount of MeCP2 protein (full length + truncated form) after subtracting film background, to determine the percent recovery using densitometry (MCID Elite 6.0 Program). In lymphocytes, the read through was determined by dividing the full length MeCP2 protein by the control load (MBD2) and then divide the ratio of treated cells by the ratio of untreated cells using densitometry. Data are expressed as means +/- standard error of the mean (SEM). Statistical analysis was performed by one way analysis of variance and student t-test. Statistical significance was accepted at p<0.05 following a post hoc Bonferroni correction for multiple comparisons.

2.3 Aminoglycosides used in my study

All aminoglycosides used (amikacin, paromomycin, gentamicin and geneticin) (Figure 4) were purchased from SIGMA in powder form. A stock solution of 50 mg/mL in distilled water was made and stored at 4°C.
2.4 Cell culture

2.4.1 HEK-293 cell culture and transfection

HEK-293 cells were grown as monolayer cultures in Dulbecco’s Modified Eagle Medium (DMEM) with 1,000 mg/L of D-glucose, L-glutamine, pyridoxine hydrochloride, and 110 mg/L of sodium pyruvate supplemented with 10% fetal bovine serum (FBS) and 50 units/ml penicillin, and 50 ug/mL streptomycin in 10-cm dishes. The cells were incubated at 37°C at an atmosphere of 5% CO₂. Twenty four hours before transfection, the cells were split in 6-well plates. Next day, at approximately 80% confluency, the cells were transiently transfected with the purified plasmids using Lipofectamine 2000 (Invitrogen). Cells were transfected with a total amount of 4 ug of DNA/well and 10 ul of Lipofectamine/well (for immunoblotting) and 0.8 ug of DNA/well and 2 ul of Lipofectamine/well (for immunocytochemistry). The purpose of immunocytochemistry was to determine the transfection efficiency in HEK-293 cells. The transfection efficiency was determined by dividing the number of transfected cells by the total cell number (DAPI staining). Four hours later, the transfection medium was removed and replaced with fresh medium containing aminoglycosides diluted at the indicated concentrations (without streptomycin and penicillin). The cells were treated for 48 hours. Fresh aminoglycoside-containing media was replaced every 24 hours.
2.4.2 Lymphocyte culture and drug treatment

Lymphocyte cells derived from a Rett girl expressing an R255X nonsense mutation of MECP2 (Corriell Cell Repository, Stock Number 16497) were grown in RPMI medium (SIGMA) with 15% FBS, 1% glutamine and 1% penicillin/streptomycin at 37°C and 5% CO₂. The cells were treated with aminoglycosides at the indicated doses for four days (acute treatment) and twelve days (long term treatment). Fresh media containing aminoglycosides was replaced every 48 hours. Nuclear extractions were done at the fifth day (acute treatment) and thirteenth day (long term treatment) after the aminoglycosides were added.
3 Results

3.1 In vitro

3.1.1 Nonsense mutations generating truncated forms of MeCP2 are expressed in transiently transfected HEK-293 cells.

Although nonsense mediated decay is primarily seen in *in vivo* settings, I first confirmed that each of the nonsense mutant forms of MeCP2 to be used in my study were successfully expressed in human embryonic kidney cells. As shown in Figure 9, expression cassettes containing the Y141X, Q170X, E205X and R294X mutations each generated a truncated MeCP2 protein in transient transfection assays. I chose these mutations because they generate all three premature stop codons in different surrounding contexts. While this does not rule out the possibility of nonsense mediated mRNA decay affecting any or all of these nonsense mutations *in vivo*, these results do establish that the mutant forms are generated from their respective transcripts in the assay conditions used for this study.

Using immunocytochemistry, the transfection efficiency in HEK-293 cells was determined to be approximately 10% (32 transfected cells out of 300 cells) (Figure 10).
Figure 9. The mutant forms of MeCP2 are expressed in transiently transfected HEK-293 cells. Western blot analysis of protein samples extracted from HEK-293 cells transfected with epitope tagged wild type MECP2 or tagged Y141X, Q170X, E205X and R294X mutant forms of MECP2. Immunoreactivity was detected using monoclonal antibodies: anti-Myc for R294X and anti-HA for Y141X, Q170X and E205X, which detect the N-terminal part of MeCP2 protein. Because of the basic nature of MeCP2, its electrophoretic mobility is slower than its predicted mass. The 60 kDa product is a non-specific product seen in all of the transfected HEK-293 cells with the Myc antibody.
Figure 9

[Image of a gel electrophoresis with bands labeled by different antibodies and molecular weights.]

HA antibody | MYC antibody
---|---
Y141X | Q170X | E205X | WT-MeCP2 | R294X | WT-MeCP2

Molecular weights: 72 kd, 55 kd, 43 kd, 34 kd, 26 kd
Figure 10. Transfection efficiency in HEK-293 cells is about 10%. I have transiently transfected HEK-293 cells with MECP2-R294X mutation. Forty eight hours after transfection, immunocytochemistry was done to determine the transfection efficiency. Panel A shows the total cell number (DAPI staining). Panel B shows the transfected cells (using Texas Red anti-Myc antibody) and Panel C shows the emerged cells.
Figure 10

A

B

C
3.1.2 The aminoglycosides gentamicin and geneticin facilitate read-through of the R294X Rett syndrome causing nonsense mutation.

The first part of my study was to test whether the administration of different doses of specific aminoglycosides would promote read-through of specific nonsense mutant forms of MECP2. I have transiently transfected HEK-293 cells with the mutant forms of MECP2 in the presence and absence of different concentrations of aminoglycosides for 48 hours. In the absence of aminoglycosides, no full length MeCP2 protein was detected for any of the mutant forms tested.

However, administration of the aminoglycosides geneticin, or gentamicin, partially suppressed the R294X nonsense mutation (TGA T) and facilitated the generation of a full length MeCP2 protein in a dose-dependent manner. A statistically significant effect for these aminoglycosides started to be observed at a concentration of 0.6 mg/mL. Analysis of densitometric levels revealed that at this concentration gentamicin induced the full length MeCP2 protein by 8 +/- 1.5% while geneticin induced the full length MeCP2 protein by 11 +/- 1.8% (Figure 11 C). The maximal levels of stop codon read-through for these aminoglycosides were detected at 2 mg/ml. The relative efficiency of read-through for geneticin at 2 mg/ml was 30 +/- 1.7%, while the efficiency for gentamicin was 22% +/- 1.8% (Figure 11 C).
**Figure 11. Gentamicin and geneticin induce read-through of the R294X mutation in a dose response manner.**

Western blot analysis of protein samples extracted from treated and non-treated HEK cells transfected with R294X mutation (TGA T) (A and B). The negative control represents protein samples extracted from non-transfected HEK-293 cells and the positive control represents protein samples extracted from HEK-293 cells transfected with wild type Myc-MECP2 cDNA. Immunoreactivity was detected with anti-Myc antibody which detects the N-terminal part of MeCP2 protein. The 60 kDa protein is a non-specific product detected by the Myc antibody in all of the transfected HEK-293 cells used in this study. Panel C shows the mean and standard errors from 4 independent experiments for each aminoglycoside, each performed in duplicate. Percent read-through was determined by dividing the full length MeCP2 by the total amount of protein (full length + truncated form). Astericks * and # represent statistical significance compared to non-treated cells for geneticin and gentamicin, respectively, at p<0.05, following a post-hoc Bonferroni correction for multiple comparisons.
Figure 11

A

Gentamicin (mg/mL)

Full length MeCP2 protein
Truncated MeCP2 protein (R294X)

B

Geneticin (mg/mL)

Full length MeCP2 protein
Truncated MeCP2 protein (R294X)

C

% Readthrough

Gentamicin
Geneticin

Concentration (mg/mL)
3.1.3 Amikacin and paromomycin are not effective in inducing read-through of R294X mutation.

I then tested the read through potential of two additional aminoglycosides on the R294X mutation that are used clinically with less toxicity than gentamicin: namely amikacin and paromomycin. I have transiently transfected HEK-293 cells with R294X mutation in the presence and absence of different concentrations of amikacin or paromomycin for 48 hours. Amikacin produced a small increase in the full length MeCP2 protein (in 2 out of 3 assays) only at the highest concentrations used, however this did not reach statistical significance (Figure 12 A, C). Paromomycin did not induce full length MeCP2 protein at any of the concentrations tested (Figure 12 B, C).
**Figure 12. Amikacin and paromomycin do not facilitate read-through of the R294X mutation.**

Western blot analysis of protein samples extracted from non-treated, amikacin-treated (A) and paromomycin-treated (B) HEK-293 cells transfected with R294X mutation. The negative control represents protein samples extracted from non-transfected HEK-293 cells and the positive control represents protein samples extracted from HEK-293 cells transfected with wild type *MECP2* cDNA. Immunoreactivity was detected with an anti-Myc antibody which detects the epitope tag at the N-terminal part of MeCP2 protein. The 60 kDa protein is a non-specific product detected by the Myc antibody in all of the transfected HEK-293 cells used in this study. Panel C shows the mean and SEM from 3 independent experiments with each aminoglycoside, each performed in duplicate. The percent read-through was determined by dividing the full length MeCP2 by the total amount of protein (full length + truncated form). Statistical significance was accepted at p<0.05 following a post-hoc Bonferroni correction for multiple comparisons. Amikacin and paromomycin did not significantly induce full length MeCP2 protein.
Figure 12

A  Amikacin (mg/mL)

72 kd  55 kd

0  0.3  0.6  1  2  3  4  5  -  +

Full length MeCP2 protein
Truncated MeCP2 protein (R294X)

B  Paromomycin (mg/mL)

72 kd  55 kd

0  0.3  0.6  1  2  3  4  5  -  +

Full length MeCP2 protein
Truncated MeCP2 protein (R294X)

C

% Readthrough

0  5  10  15  20  25  30  35  40

0  0.3  0.6  1  2  3  4  5

Concentration (mg/mL)

- Amikacin
- Paromomycin
3.1.4 Aminoglycoside treatment induces read-through of Q170X mutation.

In addition to the nonsense mutations involving arginine codons, another mutation seen in Rett syndrome girls is glutamine (Q170X). This mutation induces the stop sequence TAG A. I have transiently transfected HEK-293 cells with Q170X mutation in the presence and absence of different concentrations of gentamicin or geneticin for 48 hours. Gentamicin induced the read-through of Q170X mutation at a concentration of 2 mg/mL, where it increased the prevalence of full length MeCP2 protein by approximately 9 +/- 2%. Lower concentrations were not effective in restoring the full length MeCP2 protein (Figure 13 A, C). Furthermore, geneticin suppressed this tetranucleotide termination signal in a dose response manner (Figure 13 B, C). A statistical significant effect started to be detected at a concentration of 0.6 mg/mL where geneticin induced full length MeCP2 protein from this mutation by 7 +/- 1.2%. The highest read-through occurred at a dose of 2 mg/mL where geneticin suppressed this premature stop codon by 11 +/- 1.7% (Figure 13 C).
**Figure 13. Aminoglycoside treatment induces read-through of Q170X mutation.**

Western blot analysis of protein samples extracted from non-treated, gentamicin-treated (A) and geneticin-treated (B) HEK-293 cells transfected with Q170X (TAG A) mutation. The negative control represents protein samples extracted from non-transfected HEK-293 cells and the positive control represents protein samples extracted from HEK-293 cells transfected with wild type *MeCP2* cDNA. An anti-HA antibody was used to detect the epitope tag located at the N-terminal part of the recombinant MeCP2 proteins. Panel C shows the cumulative densitometric results (mean and SEM) from 3 independent experiments for each aminoglycoside, each performed in duplicate. Percent read-through was determined by dividing the full length MeCP2 by the total amount of protein (full length + truncated form). * and # denote statistical significance compared to non-treated cells for geneticin and gentamicin, respectively, at p<0.05 following a post-hoc Bonferroni correction for multiple comparisons.
Figure 13

A

Gentamicin (mg/mL)

Full length MeCP2 protein

Truncated MeCP2 protein (Q170X)

B

Geneticin (mg/mL)

Full length MeCP2 protein

Truncated MeCP2 protein (Q170X)

C

% Readthrough

Concentration (mg/mL)

\[
\begin{array}{c}
\text{Gentamicin} \\
\text{Geneticin}
\end{array}
\]

*  

#
3.1.5 Aminoglycosides induce read-through of Y141X mutation with different efficiencies.

The nonsense mutation Y141X induces the premature termination codon TAG T. I have transiently transfected HEK-293 cells with this mutation in the presence and absence of different concentrations of gentamicin or geneticin for 48 hours. Western blot analysis shows that gentamicin did not induce the prevalence of full length MeCP2 protein from this mutation at any concentrations tested (Figure 14 A). However, geneticin suppressed this mutation only at a concentration of 2 mg/mL where it induced the full length MeCP2 protein by approximately 10 +/- 0.67% (Figure 14 B, C). Thus, at the concentrations tested, geneticin is more efficient in suppressing this type of mutation than gentamicin.
Figure 14. Gentamicin and genetin induce read-through of Y141X mutation with different efficiencies.

Western blot analysis of protein samples extracted from non-treated, gentamicin-treated (A) and genetin-treated (B) HEK-293 cells transfected with Y141X (TAG T) mutation. The negative control represents protein samples extracted from non-transfected HEK-293 cells and the positive control represents protein samples extracted from HEK-293 cells transfected with wild type MeCP2 cDNA. An anti-HA antibody was used to detect the epitope tag located at the N-terminal part of the recombinant MeCP2 proteins. Panel C shows the cumulative densitometric results (mean and SEM) from 3 independent experiments for each aminoglycoside, each performed in duplicate. Percent read-through was determined by dividing the full length MeCP2 by the total amount of protein (full length + truncated form). * denotes statistical significance compared to non-treated cells for genetin at p<0.05 following a post-hoc Bonferroni correction for multiple comparisons.
Figure 14

A

B

C

Full length MeCP2 protein

Truncated MeCP2 (Y141X)

Full length MeCP2 protein

Truncated MeCP2 (Y141X)

% Readthrough

Gentamicin

Geneticin

Concentration (mg/mL)

0 0.3 0.6 1 2

0 5 10 15 20 25 30 35 40

*
3.1.6 Aminoglycosides are not effective in inducing read-through of E205X mutation.

Another nonsense mutation seen in Rett girls is E205X. This mutation introduces the premature stop codon TAA G. I have transiently transfected HEK-293 cells with E205X mutation in the presence and absence of different concentrations of gentamicin or geneticin for 48 hours. Western blot analysis shows that the aminoglycosides gentamicin and geneticin had no effect on this type of mutation, as full length MeCP2 protein was not detected at any concentrations tested (Figure 15). These results illustrate that the identity of the stop codon plays an important role in determining the efficiency of aminoglycoside-mediated read-through.
Figure 15. Aminoglycosides fail to increase the prevalence of full length MeCP2 from E205X mutation.

Western blot analysis of protein samples extracted from non-treated, gentamicin-treated (A) and geneticin-treated (B) HEK-293 cells transfected with E205X (TAA G) mutation. The negative control represents protein samples extracted from non-transfected HEK-293 cells and the positive control represents protein samples extracted from HEK-293 cells transfected with wild type Mecp2 cDNA. An anti-HA antibody was used to detect the epitope tag located at the N-terminal part of the recombinant MeCP2 proteins. Panel C shows the cumulative mean data from 3 independent experiments for each aminoglycoside, each performed in duplicate. Gentamicin and geneticin did not increase full length MeCP2 protein from E205X mutation at any concentrations tested.
Figure 15

A

Gentamicin (mg/mL)

Full length MeCP2 protein

Truncated MeCP2 protein (E205X)

B

Geneticin (mg/mL)

Full length MeCP2 protein

Truncated MeCP2 protein (E205X)

C

% Readthrough

- Geneticin
- Gentamicin

Concentration (mg/mL)
3.2 In vivo

3.2.1 Acute aminoglycoside treatment increases the prevalence of full length MeCP2 protein in a lymphocyte cell line with R255X mutation.

Next, I tested whether aminoglycoside treatment would be effective at increasing full-length MeCP2 levels in a lymphocyte cell line derived from a Rett girl with an R255X nonsense mutation. Although this nonsense mutation is an R-X conversion, in this case the nucleotide after stop codon differs from the R294X mutation tested above in the transient transfection assays. The mutation R255X induces the premature stop codon TGA A while the mutation R294X induces the premature stop mutation TGA T. Cultured lymphocyte cells were treated for four days with different concentrations of gentamicin, geneticin or amikacin, and then harvested for nuclear protein extraction. Since MeCP2 is a nuclear protein, nuclear extractions were done to minimize the unspecific binding of the antibody to the proteins from the cytoplasm.

Western blot analysis of these nuclear extracts revealed that all three aminoglycosides induced full length MeCP2 protein in a dose-dependent manner. These cells are heterozygous for MeCP2: it is expected that half of the cells express the full length copy of MeCP2 and half of the cells express the truncated form. For geneticin, the highest read-through occurred at 0.1 mg/mL, where a 35 +/- 8% increase in the prevalence of full length MeCP2 protein was observed (Figure 16). Higher doses than 0.1 mg/mL were associated with poor cell growth and some cell death. Gentamicin increased the prevalence of full length MeCP2 protein in a dose response manner starting at a concentration of 0.05 mg/mL. The highest read-through for this aminoglycoside was observed at a concentration of 0.3
mg/mL, where full-length MeCP2 levels were increased by 30 +/- 2.6% (Figure 17). Similarly, amikacin increased the levels of full-length MeCP2 by 32 +/- 2.6% at a concentration of 0.3 mg/ml. Lower concentrations were not effective in inducing read-through (Figure 18).
Figure 16. Geneticin induces the prevalence of full length MeCP2 protein in a dose response manner.

Representative western blot of nuclear proteins extracted from treated and non-treated lymphocyte cell line having R255X mutation. The antibody used is an anti-human MeCP2 raised in chicken that detects the C-terminal part of MeCP2 protein. The cells were treated for 4 days with the indicated concentrations of geneticin. Positive control represents nuclear extracts from MBD2-null mouse brain. Following initial hybridization, the blots were stripped and re-probed with an antibody against MBD2 to serve as a loading control. The histogram shows the densitometric data (mean and SEM) from 5 independent experiments, each performed in duplicate, normalized to MBD2. * denotes statistical significance compared to non-treated cells at p<0.05 following a post-hoc Bonferroni correction for multiple comparisons.
Figure 16

Rett lymphocytes treated with geneticin

Geneticin (mg/mL)

<table>
<thead>
<tr>
<th>0</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>+</th>
</tr>
</thead>
</table>

72 kd

[Image: Full length MeCP2]

34 kd

[Image: MBD2 (control load)]

OD ratio (normalized to MBD2)

Concentration (mg/mL)
Figure 17. Gentamicin induces the prevalence of full length MeCP2 protein in a dose response manner.

Representative western blot of nuclear proteins extracted from treated and non-treated lymphocyte cell line having R255X mutation. The antibody used is an anti-human MeCP2 raised in chicken that detects the C-terminal part of MeCP2 protein. The cells were treated for 4 days with the indicated concentrations of gentamicin. Positive control represents nuclear extracts from MBD2-null mouse brain. Following initial hybridization, the blots were stripped and re-probed with an antibody against MBD2 to serve as a loading control. The histogram shows the densitometric data (mean and SEM) from 5 independent experiments, each performed in duplicate, normalized to MBD2. * denotes statistical significance compared to non-treated cells at p<0.05 following a post-hoc Bonferroni correction for multiple comparisons.
Figure 17

Gentamicin (mg/mL)

<table>
<thead>
<tr>
<th>Concentration (mg/mL)</th>
<th>0</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD ratio (normalized to MBD2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

72 kd

Full length MeCP2

34 kd

MBD2 (control load)

Rett lymphocytes treated with gentamicin

Concentration (mg/mL)

OD ratio (normalized to MBD2)
Figure 18. Amikacin is effective in restoring the full length MeCP2 protein at a high concentration.

Representative western blot of nuclear proteins extracted from treated and non-treated lymphocyte cell line having R255X mutation. The antibody used is an anti-human MeCP2 raised in chicken that detects the C-terminal part of MeCP2 protein. The cells were treated for 4 days with the indicated concentrations of amikacin. Positive control represents nuclear extracts from MBD2-null mouse brain. Following initial hybridization, the blots were stripped and re-probed with an antibody against MBD2 to serve as a loading control. The histogram shows the densitometric data (mean and SEM) from 6 independent experiments, each performed in duplicate, normalized to MBD2. * denotes statistical significance compared to non-treated cells at p<0.05 following a post-hoc Bonferroni correction for multiple comparisons.
Figure 18

Amikacin (mg/mL)

0 0.05 0.1 0.2 0.3 +

72 kd Full length MeCP2 protein

34 kd MBD2 (control load)

Rett lymphocytes treated with amikacin

OD ratio (normalized to MBD2)

Concentration (mg/mL)

0 0.05 0.1 0.2 0.3

0.5 0.7 0.9 1.1 1.3 1.5 1.7 1.9
3.2.2 Long-term treatment of R255X lymphocyte cells at clinically-relevant concentrations of aminoglycosides fails to increase the prevalence of full length MeCP2.

The doses of aminoglycosides required to significantly elevate full-length MeCP2 protein in lymphocytes treated for four days exceeded clinically-tolerable levels. This led me to test whether culturing the lymphocyte cells for twelve days in concentrations of amikacin or gentamicin that are more appropriate for clinical use would be sufficient to increase full length MeCP2 levels. The maximal clinical accepted dose for amikacin is approximately 100 ug/mL and for gentamicin is approximately 30 ug/mL (Du et al., 2006). Western blot analysis shows that at these concentrations aminoglycosides did not significantly induce full length MeCP2 protein (Figure 19).
Figure 19. Long-term culture of R255X lymphocytes at clinically-relevant concentrations of aminoglycosides does not induce a significant increase in full-length MeCP2 protein.

Western Blot analysis of nuclear proteins extracted from lymphocyte cell line having R255X mutation treated with aminoglycosides for 12 days using the same anti-human C-terminus antibody as above. The cells were treated with either gentamicin (0.03 mg/mL), or amikacin (0.1 mg/mL) throughout the incubation period. The positive control represents nuclear proteins extracted from MBD2 null mouse brain. Following initial hybridization, the blots were stripped and re-probed with an antibody against MBD2 to serve as a loading control. The histogram represents the cumulative data normalized to MBD2 (mean and SEM) from 3 independent experiments, each performed in duplicate. No significant increases in MeCP2 protein were detected under these conditions.
Figure 19

![Image of gel electrophoresis with protein bands at 72 kd and 34 kd, labeled as Full length MeCP2 and MBD2 (control load) respectively. The gel shows the effect of different concentrations of G+ and A+ on the protein bands. Below the gel, a bar graph displays the OD ratio (normalized to MBD2) for Untreated, Gentamicin, and Amikacin treatments.]
### 3.3 Summary of results

**Table 3: Effect of 48 hours treatment of aminoglycosides on HEK-293 cells transfected with the mutant forms of MeCP2**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Stop codon</th>
<th>Aminoglycoside</th>
<th>Concentration</th>
<th>Readthrough</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>R294X</td>
<td>TGA T</td>
<td>Gentamicin</td>
<td>2 mg/mL</td>
<td>22% *</td>
<td>+/- 1.8%</td>
</tr>
<tr>
<td>R294X</td>
<td>TGA T</td>
<td>Geneticin</td>
<td>2 mg/mL</td>
<td>30% *</td>
<td>+/- 1.7%</td>
</tr>
<tr>
<td>R294X</td>
<td>TGA T</td>
<td>Amikacin</td>
<td>5 mg/mL</td>
<td>6%</td>
<td>+/- 2.3%</td>
</tr>
<tr>
<td>R294X</td>
<td>TGA T</td>
<td>Paromomycin</td>
<td>4 mg/mL</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Q170X</td>
<td>TAG A</td>
<td>Gentamicin</td>
<td>2 mg/mL</td>
<td>9% *</td>
<td>+/- 2.4%</td>
</tr>
<tr>
<td>Q170X</td>
<td>TAG A</td>
<td>Geneticin</td>
<td>2 mg/mL</td>
<td>11% *</td>
<td>+/- 1.7%</td>
</tr>
<tr>
<td>Y141X</td>
<td>TAG T</td>
<td>Gentamicin</td>
<td>2 mg/mL</td>
<td>1%</td>
<td>+/- 1%</td>
</tr>
<tr>
<td>Y141X</td>
<td>TAG T</td>
<td>Geneticin</td>
<td>2 mg/mL</td>
<td>10% *</td>
<td>+/- 0.7%</td>
</tr>
<tr>
<td>E205X</td>
<td>TAA G</td>
<td>Gentamicin</td>
<td>2 mg/mL</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E205X</td>
<td>TAA G</td>
<td>Geneticin</td>
<td>2 mg/mL</td>
<td>1%</td>
<td>+/- 0.9%</td>
</tr>
</tbody>
</table>

*denotes statistical significance compared to non-treated cells at p<0.05 following a post-hoc Bonferroni correction for multiple comparisons.
**Table 4: Effect of 4 days aminoglycoside treatment on a lymphocyte cell line**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Stop codon</th>
<th>Aminoglycoside</th>
<th>Concentration</th>
<th>Readthrough</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>R255X</td>
<td>TGA A</td>
<td>Geneticin</td>
<td>0.1 mg/mL</td>
<td>35% *</td>
<td>+/-8%</td>
</tr>
<tr>
<td>R255X</td>
<td>TGA A</td>
<td>Gentamicin</td>
<td>0.3 mg/mL</td>
<td>30% *</td>
<td>+/-2.6%</td>
</tr>
<tr>
<td>R255X</td>
<td>TGA A</td>
<td>Amikacin</td>
<td>0.3 mg/mL</td>
<td>32% *</td>
<td>+/-2.6%</td>
</tr>
</tbody>
</table>

*denotes statistical significance compared to non-treated cells at p<0.05 following a post-hoc Bonferroni correction for multiple comparisons.

**Table 5: Effect of 12 days aminoglycoside treatment on a lymphocyte cell line**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Stop codon</th>
<th>Aminoglycoside</th>
<th>Concentration</th>
<th>Readthrough</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>R255X</td>
<td>TGA A</td>
<td>Gentamicin</td>
<td>0.03 mg/mL</td>
<td>3%</td>
<td>+/-3.2%</td>
</tr>
<tr>
<td>R255X</td>
<td>TGA A</td>
<td>Amikacin</td>
<td>0.1 mg/mL</td>
<td>8%</td>
<td>+/-6.2%</td>
</tr>
</tbody>
</table>
4 Discussion

4.1 Principal findings of my study

In this study, I tested the hypothesis that the premature terminating mutations of \textit{MECP2} that cause Rett syndrome can be partially suppressed by aminoglycoside administration, allowing a full length MeCP2 protein to be generated. My results partially support this hypothesis, as aminoglycoside treatment facilitated full length MeCP2 protein in both HEK-293 cells transiently expressing the R294X mutation, and in a lymphocyte cell line expressing the R255X nonsense mutation of \textit{MECP2}. Thus, the results of my study show that nonsense mutations that generate a TGA premature stop codon are responsive to this treatment. However, other nonsense mutations of \textit{MECP2} seen clinically in Rett girls that have different premature stop codon sequences responded to aminoglycoside treatment less efficiently. No full length MeCP2 protein was detected in transient transfection assays with the induced TAA stop sequence, and only marginal increases were observed in assays testing the induced TAG stop sequence. Furthermore, the ability of the aminoglycosides to suppress premature stop mutations also depended on the sequence context surrounding the stop codon and on the aminoglycoside tested.
4.2 Wild type and mutant forms of MeCP2 migrate at higher sizes than expected in SDS-PAGE.

Wild type MeCP2 protein is 52 kDa, however it migrates at about 72 kDa in SDS-PAGE. Furthermore, the truncated mutant forms of MeCP2 also migrate higher than expected (compare Figure 8 for expected sizes with Figure 9 for actual sizes). Since MeCP2 is a highly basic protein (approximately one-fifth of all residues in MeCP2 are basic) (Kumar et al., 2008) it does not bind SDS in a uniform manner. Thus, MeCP2 does not follow the expected charge-to-mass ratio and migrates slower than expected.

4.3 Different Rett syndrome causing mutations responded differently to aminoglycoside treatment.

I have transiently transfected HEK-293 cells with some mutant forms of MECP2 in the presence and absence of different concentrations of aminoglycosides. I tested four different types of nonsense mutations: arginine which produces TGA T premature stop codon; glutamine which produces TAG A stop codon; tyrosine which produces TAG T stop codon; and glutamate which produces TAA G premature stop codon. The mutations I chose generate all three premature stop codons (TGA, TAG, and TAA) in different surrounding contexts, thus, I was able to determine how aminoglycosides differ in their ability to induce read-through based on the sequence of the stop codon and surrounding context. These nonsense mutations are seen clinically in Rett girls. I chose to treat these cells for 48 hours with aminoglycosides since several studies have shown that treatment with aminoglycosides
for 48 hours leads to higher levels of full length protein than treatment for a lower period of time in transiently transfected cells (Azimov et al., 2008; Sangkuhl et al., 2004).

My results suggest that the aminoglycoside mediated read-through was highly dependent on the sequence context of the stop codon. The pattern of suppression observed as a function of stop codon was TGA > TAG > TAA with gentamicin and geneticin. These data are consistent with other studies that have demonstrated that the sequence of the stop codon plays an important role in determining the efficiency of aminoglycoside mediated read-through (Howard et al., 2000; Bedwell and Keeling, 2002; Manuvakhova et al., 2000; Bidou et al., 2004).

I have also shown that the context surrounding a stop codon can have a strong influence on the aminoglycoside mediated read-through. My results show that gentamicin induced full length MeCP2 protein from Q170X mutation (TAG A premature stop codon) by 9%; however, gentamicin had no effect on Y141X mutation (TAG T premature stop codon). Thus, the order of read-through as a function of 3' nucleotide that I observed was TAG A > TAG T with gentamicin. This pattern of suppression differs from a study by Keeling and Bedwell (2002) who have shown that the order of susceptibility of gentamicin as a function of 3' nucleotide after stop codon is TAG T > TAG G > TAG C > TAG A. Thus, in MECP2 the TAG A is more responsive to gentamicin treatment, distinguishing it from the series of read-through reporter constructs that Keeling and Bedwell have used. It is likely that the sequence context around the tetranucleotide termination signal accounts for these differences. In agreement with this, Manuvakhova et al., (2000) have shown that the sequence context beyond the tetranucleotide termination signal influences the level of read-
through induced by aminoglycosides. This study used an in vitro translation system to test the ability of aminoglycosides to suppress termination in the presence of different contexts of the tetranucleotide termination signal (Manuvakhova et al., 2000). Also, a study by Bidou et al., (2004) using an in vitro translation system has demonstrated that the impact of the nucleotide downstream of the stop codon on gentamicin mediated read-through is largely dependent on the surrounding context. My study also showed that geneticin suppressed these mutations with the same efficiency. Taken together, these findings suggest that each of these aminoglycosides can suppress premature stop codons in a context-dependent manner in MECP2.

4.4 Different aminoglycosides suppress nonsense mutations with different efficiencies in transfected HEK-293 cells.

Different read-through efficiencies were obtained depending on the aminoglycoside tested. Efficiency is determined as the amount of full length MeCP2 protein produced at concentrations of aminoglycosides that will not kill the cells. At a concentration of 2 mg/mL gentamicin and geneticin induced full length MeCP2 protein in HEK-293 cells transfected with R294X mutation by 22% and 30%, respectively. Higher doses than 2 mg/mL geneticin and 3 mg/mL gentamicin were associated with poor cell growth and toxicity in these cells. Furthermore, amikacin at a concentration of 5 mg/mL induced very little read-through, and paromomycin at 4 mg/mL had no effect on R294X mutation under these acute conditions (48 hours treatment) (Figure 12). First, I started treating HEK-293 cells transfected with R294X mutation with lower concentrations of amikacin and paromomycin (up to 2 mg/mL);
however, since I saw no full length MeCP2 protein, I increased the concentrations. Higher concentrations than 4 mg/mL paromomycin and 5 mg/mL amikacin were associated with poor cell growth and signs of toxicity in HEK-293 cells. Since amikacin and paromomycin were not effective in inducing read-through on the nonsense codon TGA, which is the most susceptible mutation to read-through, I did not test the effect of these aminoglycosides on HEK-293 cells transfected with the mutations that cause TAG and TAA premature stop codons. Collectively, these results suggest that in these transfection assays, gentamicin and geneticin suppress nonsense mutations of MECP2 with a higher efficiency than amikacin or paromomycin.

My results in the transfection assays are consistent with other studies, which have also demonstrated that another factor that can affect the response to read-through is the chemical composition of aminoglycosides. A study by Manuvakhova et al., (2000) has demonstrated that amikacin was not able to induce a significant level of read-through in a reporter system using all three stop codons surrounded by different sequences using a rabbit reticulocyte translation system. They also showed that paromomycin induced read-through less efficiently than gentamicin, and geneticin showed the highest effect of read-through in all the constructs tested (Manuvakhova et al., 2000). Also, a study by Sangkuhl et al., (2004) has shown that geneticin was approximately 2-fold more efficient than paromomycin in restoring the full length and function of AVPR2 protein containing TAG C stop codon in transfected COS-7 cells. However, amikacin had no effect on this type of mutation (Sangkuhl et al., 2004). Taken together, these results may suggest that in contrast to gentamicin and geneticin, amikacin or paromomycin are not able to induce efficient conformational changes on these type of mutations that would allow the premature stop
codon on mRNA to interact with a near cognate tRNA molecule. Thus, the ability of aminoglycosides to suppress termination at premature stop codons is also largely affected by the structure of aminoglycosides.

4.5 Possible reasons of the context dependence effects of aminoglycosides.

It is not yet clear how the composition of the nonsense mutation and its surrounding sequence influence the efficiency of an aminoglycoside to suppress a premature stop codon. A proposed mechanism of how aminoglycosides may induce read-through is through their ability to bind to the decoding site of rRNA (A site) inducing conformational changes that allow the near-cognate tRNA-mRNA complexes to occur (Figure 5 C) (Recht et al., 1996). Although not completely understood, it was suggested that the mRNA context surrounding the stop codon may affect the ability of aminoglycosides to bind to the A site directly, or it is possible that mRNA context may limit the conformational change induced in the decoding site by aminoglycosides (VanLoock et al., 1999). Furthermore, it was proposed that aminoglycosides form hydrogen bonds with the mRNA molecule directly in the decoding site (VanLoock et al., 1999). Thus, it is possible that the complexity of the context-dependence observed in aminoglycoside mediated read-through may be due to the formation of different hydrogen bonding between the different aminoglycosides, the mRNA and the decoding site of rRNA, thus influencing conformational changes within the decoding site of rRNA (VanLoock et al., 1999; Keeling and Bedwell, 2002).

When a termination codon enters the decoding site of rRNA, it is recognized by release factors which cause the release of the polypeptide chain (Zhouravleva et al., 1995).
Interestingly, several studies have shown that the identity of the stop codon and the sequence context around it influence termination efficiency. Termination is most efficient at TAA stop codons, followed by the TAG, and TGA is the least efficient (McCaughan et al., 1995; Poole et al., 1998). The release factor eRF1 and near-cognate aminoacyl-tRNAs normally compete for A site binding when a termination stop codon within the mRNA reaches this site (Zhouravleva et al., 1995). It was proposed that the aminoglycosides bound to the decoding site of rRNA may reduce the efficiency of release factor recognition of the stop codon (Keeling and Bedwell, 2005). Thus, the complexity of the context dependence seen in aminoglycoside mediated read-through may also be due to differences in the ability of stop codon to recognize the release factors.

### 4.6 NMD pathway and aminoglycoside mediated read-through.

In addition, it is possible that aminoglycosides induce full length MeCP2 protein by suppressing the NMD pathway. Approaches that suppress NMD pathway increase amount of mutated mRNA available for translation. This, in turn, may greatly enhance the levels of protein produced by suppression therapy. A study by Bedwell et al., (1997) has demonstrated that in a human bronchial respiratory epithelial cell line from a Cystic Fibrosis patient having TGA A premature stop codon, the mutated mRNA levels were increased after incubation with geneticin for 24 hours. Also, a 2 fold increase was observed in mRNA levels derived from fibroblasts from a patient with Smith-Lemli-Opitz syndrome having a premature termination codon in DHCR7 gene after treatment with geneticin for 48 hours (Correa-Cerro et al., 2005). These results suggest that aminoglycosides might be able to
inhibit the NMD pathway. However, the mechanism(s) by which aminoglycosides may suppress the NMD pathway is currently unknown.

In order to determine whether NMD is inhibited by aminoglycosides in my study, mRNA levels could be assessed before and after treatment by quantitative RT-PCR. If mRNA levels were increased after treatment with aminoglycosides, this would suggest that aminoglycosides may be able to suppress the NMD pathway.

Furthermore, it was proposed that not all transcripts containing premature termination codons (PTCs) are targeted by NMD (Holbrook et al., 2004), and this has been shown to have a benefit in the response of aminoglycosides to read-through (Linde et al., 2007). Some transcripts containing PTCs are markedly reduced by NMD, while others are not affected as much (Linde et al., 2007; Kerr et al., 2001; Azimov et al., 2008). This pathway may vary depending where on the gene the mutation is found. It has been proposed that the more upstream a nonsense mutation is found in mRNA, the more likely it is to be degraded by NMD pathway (Holbrook et al., 2004). Consistent with this idea, it has been shown that mutations near the 3` end of the open reading frame usually escape nonsense-mediated mRNA decay (Kerr et al., 2001). Another explanation why in my study the R294X mutation is more susceptible to suppression by aminoglycosides might be because it is located further downstream in the gene and it is not degraded by NMD as much as the other mutations. If this is the case, then the level of R294X nonsense transcripts available for read-through would be higher and read-through would be more effective. In order to determine whether NMD acts differently on the different nonsense mutations of MECP2 used in my study,
mRNA levels of each mutant in transfection assays could be compared by quantitative RT-PCR.

A possible role for NMD in regulating the response to aminoglycoside mediated read-through came from a study by Linde et al., (2007) who has shown that there is no increase in full length CFTR protein and no correction of the CFTR function with gentamicin in patients with low levels of nonsense transcripts. However, full length CFTR protein and a significant improvement in CFTR function with gentamicin treatment was achieved by increasing the level of CFTR nonsense transcripts. In this study, Linde et al., (2007) have shown that CFTR mRNA nonsense transcript levels with the same mutation (W1282X generating TGA A premature stop codon) vary between patients. Following treatment of epithelial cell lines from these patients with 50-200 ug/mL gentamicin for 18-24 hours, a dose-dependent function of CFTR was detected in cells with higher levels of transcripts compared to lower levels. Also, this study showed that inhibition of NMD pathway using the cycloheximide (CHX) inhibitor, and downregulation of UPF proteins (proteins involved in regulating NMD pathway) using siRNA oligonucleotides, in cells carrying low levels of CFTR transcripts led to an increase in CFTR nonsense transcripts and enhanced CFTR function in response to gentamicin treatment. Taken together, these results suggest that aminoglycosides may be more effective on nonsense mutations that escape or are not degraded as much by NMD pathway.
4.7 Aminoglycoside treatment in a lymphocyte cell line with R255X mutation (CGA A>TGA A).

To test whether the suppression of premature stop mutations in Rett syndrome can be extended to a more physiological, clinical setting, we turned to a lymphocyte cell line derived from a Rett syndrome girl with R255X mutation. The mutation R255X (resulting in TGA A premature stop codon) is one of the most common nonsense mutations seen in Rett syndrome girls (Figure 3) and has the premature stop codon most susceptible to suppression. These cells are heterozygous: it is expected that half of the cells express full length and functional copy of MeCP2 and half of the cells express the truncated, non-functional form of MeCP2. First, I have treated these cells for four days with aminoglycosides in different concentrations, since a study by Lai et al., (2004) has shown that treatment for four days with aminoglycosides induced the most read-through in lymphocytes from Ataxia Telangiectasia (AT) patients at a concentration of 125 ug/mL. At the fifth day, nuclear extractions were done to determine whether the prevalence of full length MeCP2 protein was elevated with increasing concentrations of aminoglycosides. Since MeCP2 is a nuclear protein, nuclear extractions were done to minimize the unspecific binding of the antibody to the proteins from the cytoplasm.

The aminoglycosides (gentamicin, geneticin and amikacin) each elevated the relative prevalence of full length MeCP2 protein in the lymphocyte cell line with the same efficiency. However, they differed in the doses where they induced the most read through. For example, geneticin induced the highest amount of full length MeCP2 protein at a concentration of 100 ug/mL, while gentamicin and amikacin induced approximately the same amount of full
length MeCP2 protein at a concentration of 300 ug/mL. These data are consistent with previous studies that have shown that geneticin induces read-through at lower doses than gentamicin (Yang et al., 2007) and amikacin (Heier and DiDonato, 2009). In lymphocytes treated with geneticin for 4 days, I started noticing poor cell growth and obvious cell death starting at a concentration of 200 ug/mL; illustrating the toxicity of this drug in more long-term cultures. Consistent with my results, a study by Lai et al., (2004) has shown that geneticin increased the full length ATM protein having a premature stop codon in lymphocytes from Ataxia Telangiectasia (AT) patients treated for four days in a dose response manner. This study demonstrated that the highest read through occurred at 125 ug/mL geneticin; higher concentrations were associated with poor cell growth and toxicity (Lai et al., 2004). Consistent with my transfection assays and my in vivo results, it has been shown that geneticin starts to be toxic at concentrations that are lower than gentamicin and amikacin (Chernikov et al., 2003). Thus it is possible that it needs lower doses of geneticin to bind effectively to the decoding site of rRNA and change its conformation in order to allow read-through to occur. This may explain why geneticin induces read-through levels at lower doses than gentamicin and amikacin.

The concentration of aminoglycosides required to significantly elevate full-length MeCP2 protein in lymphocytes treated for four days exceeded clinically-tolerable levels. This led me to test whether culturing the lymphocyte cells for twelve days in concentrations of amikacin or gentamicin that are more appropriate for clinical use would be sufficient to increase full length MeCP2 levels. The maximal clinical accepted dose for gentamicin is approximately 30 ug/mL and for amikacin is approximately 100 ug/mL (Du et al., 2006). However, the results of these long-term culture experiments did not reveal any significant
increase in full length MeCP2 protein. It is possible that these low doses of aminoglycosides are not enough to bind effectively to the decoding site of rRNA and change its conformation in order to allow the suppression of premature stop codon to occur. These results are consistent with the transient assays where I have shown that treatment with gentamicin for 48 hours did not induce read through of R294X mutation (TGA T) or Q170X (TAG A) at low concentrations. Furthermore, treatment of lymphocytes for 4 days with 100 ug/mL amikacin did not induce a significant amount of full length MeCP2 protein; by treating these cells for 4 days with 50 ug/mL gentamicin, a 14% increase in the prevalence of full length MeCP2 protein was detected. Since I saw only a 14% increase in full length MeCP2 protein at a concentration of 50 ug/mL gentamicin, I have not tested lower concentrations of this drug. Thus, it can be concluded that while nonsense mutation read-through of the R255X MECP2 mutation is feasible, the concentration of aminoglycoside required to elicit an effect exceeds what could be tolerated clinically for prolonged use.

4.8 Possible reasons for the difference in aminoglycoside mediated read-through in lymphocytes vs. transfected HEK-293 cells.

Amikacin did not significantly induce full length MeCP2 protein in the transfected HEK-293 cells with MECP2-R294X mutation; however in Rett lymphocyte cell line carrying MECP2-R255X mutation at a concentration of 300 ug/mL, amikacin induced full length MeCP2 protein by approximately 32 +/- 2.6%. A possible reason for this difference might be because the sequence context around the stop codon is different. The mutation R294X has ATC TGA TCT sequence while R255X mutation has GGC TGA AAG sequence. Consistent
with this idea, a study by Keeling and Bedwell (2002) has shown that amikacin produced higher levels of read-through than gentamicin at certain contexts, demonstrating a unique pattern of context dependence. Furthermore, it is possible that lymphocytes are more sensitive to aminoglycoside treatment than HEK-293 cells. Consistent with my results, a study by Heier and DiDonato, (2009) has shown that amikacin was only capable of inducing read-through of the SMN premature stop codon (TAG A) in primary fibroblasts; not in transfected HEK-293 cells. This suggests that there might be differences in translational machinery between different types of cells. Another possible explanation is that the transfected HEK-293 cells were treated with amikacin only for 48 hours; however, the lymphocytes were treated for 4 days. Thus, duration might be an important factor determining how amikacin influences the read-through of these mutations. It is possible that longer than 48 hours treatment may be needed to be able to see a significant read-through effect in R294X mutation treated with amikacin. However, it was not possible to treat the cells for a longer period of time since the plasmid in transiently transfected HEK-293 cells after 48 hours might lose its expression. For this, we would need stable cell lines where the plasmid is incorporated in the cell’s genome. These are all possibilities that may explain why amikacin was able to induce read through in lymphocytes, but not in transfected HEK-293 cells.

Furthermore, both gentamicin and geneticin induced higher levels of read through at lower concentrations in lymphocytes carrying R255X mutation than in HEK-293 cells transfected with R294X, even though they produce the same premature stop codon: TGA. A possible reason for this is that the context sequence of the gene surrounding the stop codon is different. Consistent with this, the study by Keeling and Bedwell (2002) has demonstrated
that the absolute levels of aminoglycoside-induced suppression at the same tetranucleotide termination signals can differ among different contexts. Also, the duration of treatment (4 days in lymphocytes and 48 hours in transfected HEK-293 cells), and different cell types might account for these differences. Geneticin started to be toxic as seen by the poor cell growth and death in lymphocytes, at a concentration of 200 ug/mL; however, in transfected HEK-293 cells, geneticin started to be toxic at concentrations higher than 2 mg/mL at 48 hours. This suggests that lymphocytes might be more sensitive than HEK cells to aminoglycoside treatment, or it is possible that treatment for longer time at a lower dose causes toxicity. Collectively, these are possible explanations which may account for the difference in the doses where gentamicin and geneticin had the highest read-through effect in lymphocytes vs. transfected HEK-293 cells.

4.9 Related study

While my study was in progress, Brendel et al., (2009) reported that gentamicin effectively induced read-through of different TGA mutations associated with the most common R-X nonsense mutations seen clinically in Rett girls. The efficiencies in their report ranged between 10% and 22%, with the highest effect being observed for the R294X mutation. My results are consistent with this study, as I also showed a read-through efficiency of 22% for gentamicin in transfected HEK-293 cells employing this same R294X mutation. However, the study by Brendel et al., (2009) tested only one type of mutation - arginine which produces TGA stop codon. Furthermore, in their study Brendel et al., only discussed one aminoglycoside - gentamicin. My study is different because in addition to
gentamicin I tested three other aminoglycosides: geneticin, amikacin and paromomycin. Furthermore, I have tested more mutations that are known to cause Rett syndrome: tyrosine, glutamine, glutamic acid and arginine. In addition, my study extends from the study of Brendel et al., (2009) by testing whether acute and chronic aminoglycoside treatment affects full-length MeCP2 levels in a lymphocyte cell line derived from a girl with Rett syndrome who has an R255X nonsense mutation.

4.10 Aminoglycosides may be able to facilitate read-through at premature stop codons and not at normal stop codons.

Although not completely known, it is believed that aminoglycosides can only facilitate read-through at premature stop codons, and not at normal stop codons. A few reasons have been suggested for the apparent lack of read-through at normal stop codons. In a review article, Kerem (2004) has proposed that naturally occurring stop codons are found within a context that promotes efficient translation termination compared to premature stop codons. Consistent with this, a study by McCaughan et al., (1995) has shown that in mammalian genes certain signals such as UAAG are overrepresented and some are not used as much (such as UGAC, UGAT). Furthermore, multiple stop codons are frequently found at the end of an open reading frame. The presence of many stop codons may reduce the ability of aminoglycosides to induce read-through at normal termination signals (Major et al., 2002). Furthermore, the termination complex formed at premature stop codons appears to differ from the complex at normal stop codons (Amrani et al., 2004). This suggests that the ribosome might terminate translation at the normal stop codon more efficiently than at
premature stop codons. Also, it has been shown that when the normal stop codons are located in proximity to the poly(A) tail, termination is more efficient and this might contribute to translational termination (Amrani, et al., 2004). All these factors have led to the hypothesis that aminoglycosides may be able to facilitate read-through only at premature stop codons.

My results also suggest that aminoglycosides only induce read-through at premature stop mutations in MECP2. There are four in frame normal stop codons in MECP2 sequence: TGA, TGA, TGA and TAA. The first normal stop codon is followed by 78 bases before the next one; thus, if aminoglycosides would read-through this normal stop codon, in addition to MeCP2 which is 72 kDa, an extra product of approximately 75 kDa with aminoglycoside treatment should be detected in SDS-PAGE. However, only one clear product of approximately 72 kDa in HEK-293 cells transfected with the mutant forms of MeCP2 in the presence of aminoglycosides is detected, which is the same size as the wild-type MeCP2. As a control, I have treated HEK-293 cells transfected with wild type MECP2 cDNA with aminoglycosides; however, SDS-PAGE showed no difference in migration of protein lysates from aminoglycoside-treated and non-treated transfected HEK-293 cells (data not shown). If aminoglycosides would read-through the normal stop codon of MECP2, then we would expect the protein lysates from aminoglycoside treated transfected cells to have an extra product with a higher migration size than the protein lysates from non-treated wild-type transfected cells; however, this was not the case. Consistently, the protein extracts from aminoglycoside treated lymphocytes were not different than the positive control or non-treated cells, as they did not show an extra product at a higher mass. Collectively, these results suggest that aminoglycosides are only able to facilitate read-through at premature stop codons in MECP2.
4.11 Future directions and potential clinical implications

There are currently no effective treatments for Rett syndrome. However, transgenic studies in mouse models of Rett syndrome have revealed that re-introducing or re-activating functional MeCP2 into specific regions of the brain in pre-symptomatic mice, or throughout the brain in mice displaying Rett-like behaviour, improves at least some of their behavioral deficits (Luikenhuis et al., 2004; Collins et al., 2004; Guy et al., 2007; Jugloff et al., 2008). The fact that the loss of MeCP2 function during embryonic and prenatal brain development does not cause an irremediable condition is encouraging for prospective treatments. Furthermore, the finding that over-expression of MeCP2 is also deleterious (Collins et al., 2004; Luikenhuis et al., 2004) complicate the treatment of Rett syndrome. Using a therapy such as gene replacement therapy would not be beneficial since in addition to adding function to the cells expressing the mutant MECP2 allele, it would also increase the level of MECP2 in cells expressing the functional MECP2 allele. Thus, this procedure will introduce more MeCP2 protein than needed. Therefore, a therapeutic option would be to enhance endogenous MeCP2 levels selectively in cells expressing the mutant allele. The pharmacological approach is attractive for Rett syndrome since it circumvents these problems.

Roughly 40% of the mutations that cause Rett syndrome are nonsense mutations (Percy et al., 2007) and of these the most common involve R-X transformations which produce TGA premature stop codons (Dragich et al., 2000; IRSF database). As my results show, aminoglycosides are effective in partially suppressing some of these nonsense mutations of MECP2.
However, aminoglycosides are able to suppress nonsense mutations by inducing the ribosomes to “read-through” the premature stop codons generating full length proteins by insertion of an amino acid by the near-cognate tRNA in place of the premature stop codon. It has been proposed that tryptophan is usually inserted at TGA stop codon and glutamine is inserted at TAG and TAA premature stop codons (Nilsson and Ryden-Aulin, 2003). Following this hypothesis, it is possible that the read-through of MECP2 TGA premature stop codon would lead to the replacement of the normal arginine by a tryptophan. MeCP2 is a highly disordered protein (Adams et al., 2007) and this amino acid might impair the proper folding of the MeCP2 protein. It is possible that read-through might also insert other amino acids to generate a range of missense-mutated proteins. Thus, the random nature of amino acid incorporation at the premature stop codon leads to production of full length MeCP2 protein that may or may not be functional. However, database analysis shows no arginine missense mutations involving the key R sites associated with nonsense mutations (IRSF database). While not conclusive, the lack of any Rett girls with R-missense mutations suggests that some tolerance may exist for these R sites. First, it is important to determine the exact amino acid which is inserted in the full length MeCP2 protein by mass spectrometry.

If the amino acid that is introduced is not the wild-type, then functional studies in cells would be required to determine whether the functional capacity of MeCP2 is restored. Most nonsense mutations are located in transcriptional repressor domain (TRD) and interdomain (Figure 3), so the TRD and the Carboxy-terminus in these mutants are disrupted. Since the TRD domain of MeCP2 recruits Sin3A and histone deacetylases to repress transcription (Nan et al., 1998; Jones et al., 1998), it is important to determine whether these
mutant forms of MeCP2 in the presence of aminoglycosides can bind Sin3A and histone deacetylases in transfected HEK-293 cells (since they do not express endogenous MeCP2) using western blot analysis and immunoprecipitation. If successful, this would provide proof that aminoglycosides may be able to restore the function of MeCP2 protein.

If the above functional studies show an improvement in MeCP2 function with aminoglycoside treatment, the next step would be to determine whether the aminoglycosides ameliorate the symptoms of Rett syndrome in transgenic mice containing nonsense mutations. If successful, this would provide more evidence that aminoglycosides restore MeCP2 function and thus this would suggest that pharmacological treatment might be a therapeutic approach for a subset of Rett syndrome patients with nonsense mutations. A mouse model is useful to test the efficiency of this pharmacological approach before more expensive clinical trials are undertaken. A mouse model containing R168X nonsense mutation exists (Lawson-Yuen et al., 2007). This mutation introduces TGA premature stop codon, which is the most susceptible to suppression. Furthermore, this is one of the most severe (Neul et al., 2008) and common nonsense mutation seen in Rett syndrome girls (Figure 3). The mice containing this mutation show features similar to Rett syndrome, including breathing irregularities, hypoactivity, forelimb stereotypies, and social impairment (Lawson-Yuen et al., 2007). These mice could be treated with aminoglycosides in order to determine whether these Rett-like features are reduced. However, toxicity remains an issue, and it is not known whether aminoglycosides can effectively cross the blood brain barrier. If the aminoglycosides do not get into the brain, then they will not be effective in reducing some symptoms associated with Rett syndrome with the exception of bone deficits. A mouse model is useful to test some of these possibilities.
It is also possible that the truncated proteins generated by nonsense mutations might have a dominant negative effect and compete for the binding with the wild-type or the full length MeCP2 protein generated by aminoglycoside mediated read-through. If aminoglycosides suppress the NMD pathway and if there is a dominant negative effect of the truncated proteins, then this may suggest that aminoglycosides would not be beneficial to Rett syndrome. Furthermore, it may also be possible that the full length proteins generated by aminoglycoside mediated read-through may (if the amino acid introduced is not the wild type) have a dominant negative effect. These are possibilities that we do not yet know.

Shahbazian et al., (2002) have developed a mouse model where they replaced the wild-type MeCP2 allele with one encoding a truncated protein after amino acid 308 (MeCP2 308/y). These mice appeared normal for first 6 weeks, but then developed a neurological disease that includes many features of Rett syndrome: abnormal motor function, abnormal social interaction, seizures, tremors and stereotypic forelimb motions (Shahbazian et al., 2002). Alvarez-Saavedra et al., (2007) have expressed a transgene of functional MeCP2 in mice with a background of the endogenous truncated MeCP2 and have shown that the expression of transgenic MeCP2 did not result in the prevention of the development of some symptoms associated with Rett syndrome. This could be due to a dominant negative effect of MeCP2 308 allele which competes with the functional MeCP2 transgene for DNA binding, since MeCP2 308/y mice make a truncated protein that also binds DNA (Shahbazian et al., 2002). However, in order to test this possibility, Alvarez-Saavedra and his group, (2008) also introduced the functional MeCP2 transgene in MeCP2-null mice background, in which no MeCP2 mRNA or protein was observed (Guy et al., 2001). They again have shown that with the exception of locomotion, the Rett-like behaviour was not improved and there was no increase in lifespan.
These results contrast with other studies that have shown an increase in lifespan upon re-activation or re-introduction of functional MeCP2 in MeCP2-null mice (Giacometti et al., 2007; Luikenhuis et al., 2004). Although not completely clear, these results do not support the possibility of a dominant negative effect of truncated MeCP2 proteins.

However, it is also possible that truncated proteins might have residual function, and if aminoglycosides suppress the NMD pathway, they may partially restore the function of MeCP2 by just stabilizing the truncated form. The fact that individuals with R294X mutations and late C-terminal truncating mutations are less severely affected suggests that these mutants may still have some partial function of MeCP2. Most nonsense mutations occur on TRD and interdomain of MeCP2, thus they still have the MBD intact. Thus, it is possible that aminoglycosides by stabilizing truncated proteins might have a benefit since most of these proteins have the MBD intact and are able to bind methylated DNA.

It is not exactly known how much functional MeCP2 protein is required to confer a therapeutic improvement in Rett syndrome patients. Samaco et al., (2008) generated a mouse model that contains a conditional hypomorphic allele of MeCP2 which expresses 50% of the wild-type level of MeCP2 (they called this mouse MeCP2^{Flox-y}). In this study, they have shown that a 50% reduction of MeCP2 levels results in a variety of abnormalities such as altered social behaviour, learning and motor deficits, movement abnormalities, and breathing irregularities (Samaco et al., 2008). However, in contrast with MeCP2-null mice that die between 8 and 12 weeks of life (Chen et al., 2001; Guy et al., 2001), these mice have a normal lifespan. Furthermore, MeCP2^{Flox-y} mice do not show some overt abnormalities observed in MeCP2-null mice such as body tremor or hindlimb clasping. They also show
decreased anxiety (Samaco et al., 2008). These results suggest that a 50% decrease in MeCP2 levels might still cause a disease, however, it is not as severe as Rett syndrome. Taken together, these data suggest that it is possible that restoring low levels of MeCP2 may ameliorate some symptoms associated with Rett syndrome.

Although some nonsense mutations that cause Rett syndrome can be suppressed by aminoglycoside administration allowing a full length MeCP2 protein to be produced, the dose of aminoglycosides required to see a significant effect exceed the clinical accepted range. Thus, my data suggests that aminoglycosides may not be effective for treating Rett syndrome patients. However, this study is important because it establishes the “proof of principle” that a subset of nonsense mutations that cause Rett syndrome can be suppressed by drug treatment. My results suggest that if aminoglycosides also restore the function of MeCP2 protein (as determined by functional studies and transgenic mice containing nonsense mutations), then screening for other drugs with improved termination suppression activity and lower toxicity may have a great potential for reducing the symptoms in a subset of Rett syndrome patients with TGA mutations.

Drugs such as PTC124 and NB54 have been recently indentified (Figure 20). NB54 is a newly-derived aminoglycoside specifically tailored for nonsense mutation suppression which exhibits several fold greater suppression activity than gentamicin. The superior read-through efficiency compared to gentamicin was demonstrated in vitro in PCDH15, CFTR, Dystrophin, and IDUA genes carrying nonsense mutations and representing the genetic causes for Usher Syndrome (USH1), Cystic Fibrosis (CF), Duchenne Muscular Dystrophy (DMD) and Hurler Syndrome (HS), respectively and in transfected COS-7 culture cell line.
Importantly, NB54 also displays far less toxicity than either gentamicin or amikacin (Nudelman et al., 2009), increasing its potential for chronic therapeutic use. Furthermore, like aminoglycosides, the read-through of this compound is highly dependent on the context of the gene and the composition of nonsense mutation, with TGA having higher read-through than TAG and TAA (Nudelman et al., 2009). PTC124 is a new orally, bio-available drug, developed from screening as one potential lead to treat genetic diseases with nonsense mutations. It has read-through ability without the side effects associated with aminoglycosides. This compound has no structural similarities with aminoglycosides (Figure 20). Furthermore, it displays significantly higher read-through ability at TGA nonsense codons than aminoglycosides, and is effective at concentrations that are clinically tolerated (Welch et al., 2007). Encouraging results have been obtained in a clinical trial in which PTC124 was administered to Cystic Fibrosis patients (Kerem et al., 2008), but to date its effects on central nervous system disorders have not been investigated and we do not know if it crosses the blood brain barrier. It will clearly be of interest to determine whether either of these new drugs will facilitate effective read-through of nonsense mutations of MECP2.
Figure 20. The chemical structures of PTC124 and NB54

NB54: structure modified from the research article by Nudelman et al., 2009.

PTC124: structure copied from:

The structure of PTC124 can also be found in the research article by Auld et al., 2009.
Figure 20

NB54

PTC124
5 Summary

To summarize, my project focused on investigating whether the nonsense mutations of *MECP2* that cause Rett syndrome can be suppressed by aminoglycosides. With the help of my lab members, I have generated four mutant forms of MeCP2 seen clinically in Rett patients and using these constructs, I first tested the efficiency of read-through in transfection assays. I have shown that aminoglycoside treatment facilitated full length MeCP2 protein in a dose response manner from the premature stop codon TGA. However, other mutations that cause the TAG termination codon were less efficiently suppressed, and no full length MeCP2 protein was detected when the premature stop codon was TAA. Furthermore, the ability of aminoglycosides to suppress nonsense mutations also depended on the sequence context surrounding the stop codon and the aminoglycoside tested (Figure 21). Since full length MeCP2 protein was detected in transiently transfected cells, my final aim was to test whether and with what efficiency the restoration of full length MeCP2 protein can be achieved in a lymphocyte cell line from a Rett girl having TGA premature stop codon. Exposure of these lymphocyte cells acutely (4 days) to high concentrations of aminoglycosides increased the overall prevalence of full-length MeCP2 protein, indicating that the read-through effects observed in the transfection assays are recapitulated in cells with stable genomic nonsense mutations. Taken together, my results help to establish the “proof of principle” that a subset of nonsense mutations that cause Rett syndrome can be suppressed by drug treatment.
Figure 21. Model of aminoglycoside mediated read-through.

A) My data suggest that the R294X Rett syndrome mutation is the most amenable to read-through. Gentamicin and geneticin are the most effective of the drugs tested. These aminoglycosides bind to the decoding site of rRNA through hydrogen (H) bonds and electrostatic interaction (red dashes). This stabilizes the interaction of the third base of stop codon (Adenine or A) to pair with the cytosine (C) of a tryptophan (W) t-RNA anticodon. My results indicate that this context allows approximately 30% of the total MeCP2 protein generated in cells to be full length rather than truncated.

B) In contrast, my data show that amikacin and paromomycin are not as effective in inducing read-through at this same premature termination codon. This could be due to weaker binding of these specific aminoglycosides to ribosomal decoding site (red dashes), thereby not allowing the codon-anticodon stabilization to occur as efficiently as with geneticin and gentamicin.

C and D) In the TAG or TAA context, gentamicin and geneticin still facilitate some read-through, but with lower efficiencies. This may relate to the first base of the codon-anticodon pairing being mis-matched with TAG and TAA stop codons compared to the third base being mis-matched with TGA.
Figure 21

A

R294X

H bonding, Electrostatic interaction

Gentamicin

Geneticin

22%

30%

B

R294X

H bonding, Electrostatic interaction

Amikacin

Paromomycin

C

Q170X

H bonding, Electrostatic interaction

Gentamicin

Geneticin

9%

D

E205X

H bonding, Electrostatic interaction

Gentamicin

Geneticin
References


