Project Number: DVG-LRNF

Effects of Silencing the Mental Retardation Gene Jarid1c on Neuronal Differentiation of Pluripotent P19 Cells

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By

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Abstract:

Jarid1c is a master regulator of transcription and represses expression of specific target genes. Mutations in the Jarid1c gene have been found to cause mental retardation, epilepsy, aggression, and autism, indicating that this gene plays an important role in brain development and function. It is not clear as to exactly how Jarid1c causes behavioral defects and it is thought that Jarid1c perhaps plays a role in neuronal differentiation in the embryonic brain. Jarid1c was silenced in embryonic carcinoma P19 cells using Lentiviral particles containing Jarid1c-shRNA constructs. Neuronal differentiation was then induced in these Jarid1c knockdown P19 cells as well as in control P19 cells, which were treated with non-targeting shRNAs. The effects of silencing the Jarid1c gene was assessed with SuperArray PCR panels analyzing 84 neurotrophin genes. Fus and Gfra3 were found to be up-regulated in the Jarid1c knockdown relative to control P19 cells on Day 0 before neuronal induction had begun. On Day 4, Ntrk1 was up-regulated while Il1r1 and Il6 were down-regulated in the Jarid1c knockdown cells. By Day 10, when P19 cells were fully differentiated, no detectable difference was found in the 84 genes' expression in the Jarid1c knockdown and control P19 cells. These results indicate that Jarid1c is involved in neuronal differentiation as well as in gene expression in pluripotent stem cells.

Introduction and Background:

This project studies the Jarid1c gene and its role in gene expression and neuronal differentiation. Jarid1c (Jumonji AT-rich interactive domain 1C), formally called 'SMCX,' is involved in transcriptional regulation and chromatin remodeling. Mutations in this gene can cause X-linked mental retardation (XLMR) in humans. XLMR is a heterogeneous disease and can involve genetic, stochastic, and/or environmental factors. The disease affects 2 out of 1000 males causing symptoms of mild to severe mental retardation (Ropers and Hamel, 2005).

In a study by Jensen et al. (2005), mutations in Jarid1c were found in several patients with XLMR, including frameshift mutations, nonsense mutations, and missense mutations. Frameshift mutations can lead to a premature stop codon. The mutations cosegregate with the phenotype. A northern blot hybridization of RNA on affected patients resulted in an almost undetectable Jarid1c transcript. This led the authors to conclude that the phenotype in these patients was due to a loss of the Jarid1c protein, likely due to the nonsense-mediated mRNA decay (NMD) mechanism (Holbrook et al., 2004). The Jarid1c RNA levels in patients with missense mutations were not notably different, due to extreme shortening of the protein or an abnormal protein (Jensen et al., 2005).

Missense changes in Jarid1c in different patients have been found in the conserved C5HC2 zinc-finger domain as well as outside of this area. Mutations outside of this domain involved changes such as the introduction of a proline which adds a kink to the backbone of a protein, or changing a glutamic acid to a lysine which changes the charge of a protein. The seven Jarid1c mutations found by Jensen et al. (2005) are thought to affect protein function and to be responsible for cognitive defects found in patients with XLMR. These mutations are found at a frequency of

2.8%, making Jarid1c one of the most prevalently mutated genes found in XLMR (Tzschach et al., 2006). They can cause sydromic and non-syndromic XLMR.

Jarid1c is a member of a subfamily of four proteins, Jarid1d (SMCY), Jarid1a (RBP2), and Jarid1b (PLU-1) (Klose et al., 2006), each with their own influence over biological processes and diseases. Jarid1d has been identified as a male-specific antigen. Jarid1a has been found to be a pRB binding protein and has a role monitoring cellular response when DNA has been damaged (Ahmed et al., 2004). Jarid1b is expressed in the ovary, testis, and transiently in mammary glands while pregnant and has been shown to be up-regulated in breast and testis cancer (Lu et al., 1999).

Jarid1c and Jarid1d are known to make up an X-Y homologous gene pair from the original mammalian sex chromosomes (Delbridge et al., 2004). This suggests the possibility that Jarid1c and Jarid1d are functionally equivalent. These two are expressed in sex-specific manners, with Jarid1c being expressed in a higher level in females during adulthood. The expression of Jarid1d cannot compensate for the female bias in X-gene expression (Xu et al., 2002). This led Jensen et al. (2005), to believe that they are not functionally equivalent after all, and that Jarid1c is essential for normal brain function.

Jarid1c as a Histone Demethylase

Jarid1c is also known to be a histone demethylase, meaning it regulates chromatin structure as well as gene transcription (Iwase et al., 2007). Five lysine residues on the histone tails of histones H3 and H4 as well as K79 within H3's core have been identified as methylation sites (Margueron et al., 2005). DNA-damage response as well as transcriptional activation and repression are affected by histone methylation (Sanders et al., 2004). Lysine methylation can be

done in three different ways: mono-, di-, and trimethylation. It has been found that histone H3 lysine 4 (H3K4) trimethylation regulates transcription in a positive manner by recruiting complexes that cause chromatin remodeling (Liang et al., 2004; Santos-Rosa et al, 2003).

In a study done by Iwase et al. (2007), it was shown that Jarid1c is a H3K4 trimethyl-demethylase and catalyzes the methylation of H3K4me3 to H3K4me1. When Jarid1c is overexpressed in cultured cells, there is a reduction of H3K4me3. A reduction was also found with the three other family members, Jarid1d, Jarid1a, and Jarid1b. One of the PHD fingers of Jarid1c bound to H3K9me3, which caused Iwase et al. to suggest that cross talk occurs with H3K4 and H3K9 methylation in repressing transcription. When there are mutations in Jarid1c, such as in XLMR, this compromises the demethylase activity and H3K9me3 binding ability (Iwase et al., 2007), which may be a common cause of the disease.

Iwase et al. (2007) used zebrafish as a model and inhibited Jarid1c. They found that there were defects in neuronal development as well as impaired dendritic morphogenesis in culture. An RNAi-resistant wild-type Jarid1c transgene was able to restore growth of dendrites after a Jarid1c knockdown. However, if the transgenes had point mutations, it caused the demethylation activity to be disrupted, and the dendrites' growth was unable to be restored. This suggests a link between Jarid1c's demethylase role and dendritic development.

P19 Embryonic Carcinoma Cells

Embryonal Carcinoma (EC) P19 cells can be used as a model system for neuronal differentiation. These cells are capable of differentiating into many types of neurons as well as glia (Bain et al., 1994). P19 stem cells can be cultured in the undifferentiated state and managed easily. They can be induced to differentiate all at once into neuron-like cells.

P19 cells were first isolated by Rogers and McBurney in 1982 (a) from a teratocarcinoma which was grafted onto C3H/He mice. The tumor that resulted was removed and dissociated so it could be grown in tissue culture. A clonal cell line was chosen and named P19 embryonal carcinoma cells. P19 cells and their clones are able to divide rapidly and can differentiate, even after many passages in culture (Bain et al., 1994). This allows both normal and mutated proteins to be overexpressed. They can also be blocked by anti-sense RNA.

When P19 cells are cultured, they must be induced to differentiate by a chemical inducer after formation of aggregates. To induce differentiation of cardiac- and skeletal muscle-like cells, dimethylsufoxide can be used (McBurney, 1982b). To induce neuron, fibroblast, and glia-like cell differentiation, retinoic acid (RA) can be used (Jones-Villeneuve et al., 1982).

The affects of RA on cells is mediated by two major families of receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Bain et al., 1994). Each family has α, β, and γ receptors, which are produced by separate genes with various isoforms that result from alternative splicing (Leid et al., 1992). Before cells are differentiated, there are very low levels of RARs. Once RA is introduced, the amounts of RARα and RARβ mRNA increase greatly, while encoding RARγ is greatly repressed. The RAR family has been found to play a key role in P19 neuronal differentiation (Bain et al., 1994). The RAR family members possess inducible *trans*-acting factors and have been shown to be regulators of gene expression (Leid et. al, 1992).

Neuron-like cells cultured from P19 cells are very similar to cultured brain cells (Bain et al., 1994). These cells have small cell bodies with long projections that resemble axons and dendrites. They are stable post-mitotic cells, as are normal neurons. Neurotransmitters, associated gene transcripts, and enzymes have been found to be expressed in these neuron-like

cells, most being characteristic of cells in the central nervous system. Functional synapses have also been discovered (McBurney, M.W. et al., 1988).

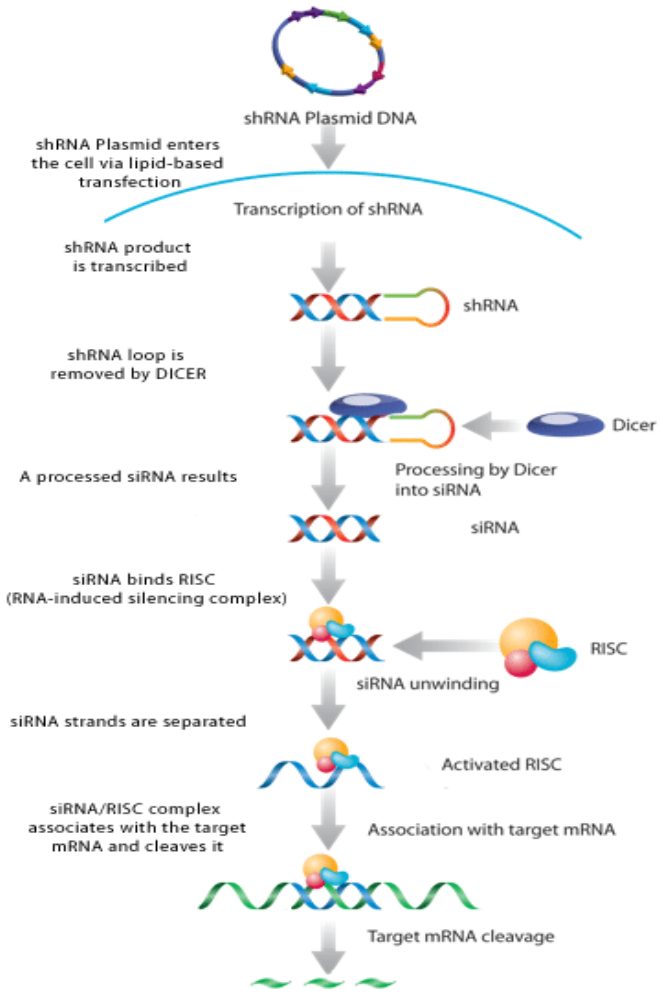
RNA Interference: Gene Silencing Methods

Double-stranded RNA (dsRNA), for example, short-hairpin RNAs (shRNAs), can be utilized to silence target gene expression (Applied Biosystems, 2009). Gene silencing by dsRNAs has been termed RNA interference (Howard Hughes Medical Institute, 2005). RNAi occurs in many mammals including mice and humans. It is able to protect the genome from certain viruses as well as 'jumping genes.' It plays an important role in gene expression regulation by down-regulating certain genes at specific times. RNAi can be triggered by dsRNAs which are homologous to portions of target genes.

RNA interference begins when a long dsRNA molecule is present, which could have been introduced or the product of a virus or a jumping gene (HHMI, 2005). Viruses can be used to target a specific gene. In the case of Jarid1c, the Lentivirus is used. The Lentivirus is used for cells that do not divide or are hard to transfect such as blood or stem cells (System Biosciences, 2009). The constructs integrate into the genomic DNA and do not require cell replication.

The process of RNAi using the Lentivirus starts with a short-hairpin Plasmid DNA (System Biosciences, 2009). This plasmid enters the cell by lipid-based transfection. The shRNA is transcribed once inside the cell. The loop in the shRNA is removed by the RNase III-like enzyme, Dicer, and processed into small, 20-25 nucleotide long small interference RNAs (siRNAs). The siRNA then binds to several proteins which form an RNA-induced silencing complex (RISC). The siRNA are then separated utilizing energy from adenosine triphosphate (ATP), which causes the RISC to be activated. The siRNA/RISC complex associates with target

RNA and cleaves it. Other proteins further degrade the mRNA, which prevents them from producing proteins. This process can affectively and permanently silence any gene using Lentiviral transduction particles with shRNA constructs (See Figure 1).



(System Biosciences, 2009)

Figure 1: RNA Interference Process Using shRNA Plasmid DNA

The XLMR gene, Jarid1c, causes many neurological disorders including mental retardation, epilepsy, aggression, and autism. It is unclear as to exactly how Jarid1c causes these behavioral defects. A possibility is that Jarid1c plays a role in neuronal differentiation in the embryonic brain. To model what occurs during embryonic development, embryonic carcinoma P19 cells

were used because they can be induced to differentiate into neurons. To explore Jarid1c's role in neuronal differentiation, it was necessary to knockout or knockdown Jarid1c, which was done by Lentivirus and RNAi. The effect of this silencing of Jarid1c was assessed with SuperArray PCR panels on a real-time PCR machine.

Materials and Methods:

P19 Cell Culture:

EC P19 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). This medium contained 10% fetal bovine serum (FBS) and 1% P/S antibiotic. The cultures were grown in a 37˚C incubator supplemented with 5% CO_2. The cells were cultured in NUNC^{TM} adhesive Petri dishes from frozen stock kept in an -80˚C freezer. The medium was refreshed every other day.

Lentiviral Transduction:

The Lentiviral transduction (See Figure 3) was initiated on Day 1 by inoculating dispersed P19 cells into a 12 well plate with 1mL DMEM. On Day 2, the medium was refreshed and Lentiviral particles containing Jarid1c shRNA constructs were added, while non-targeting shRNAs were added to form a negative line (Figure 2).

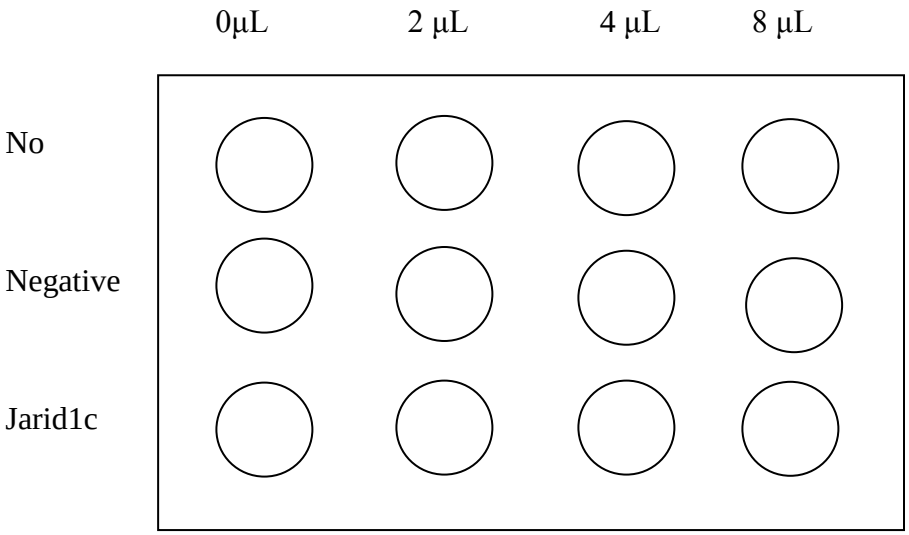


Figure 2: Lentiviral transduction 12-well plate. Row 1, no particles were added. Row 2, non-targeting shRNAs were added to create a negative control cell line. Row 3, Lentiviral particles containing Jarid1c shRNA constructs were added.

On Day 3, the viruses were removed by replacing the medium. On Day 4, DMEM containing puromycin was added to the wells to select for cells that had been successfully transduced with Lentivirus. On Day 5 and on, the medium was replaced every other day, until a culture of stable puromycin-resistant cells was established.

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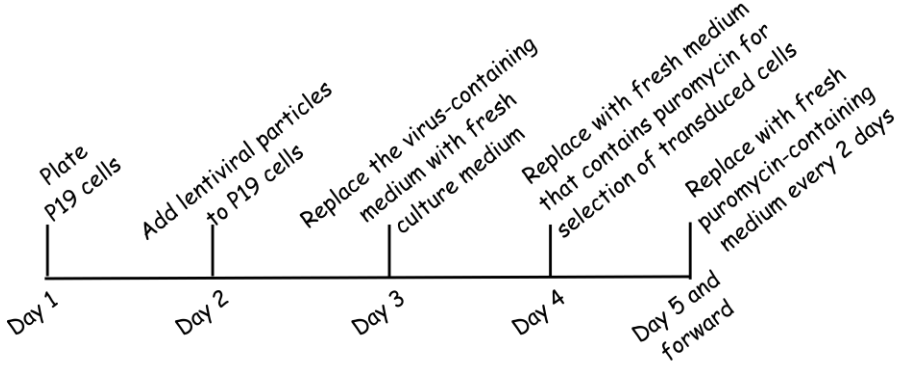

Figure 3: Timeline of Lentiviral transduction.

Neuronal Induction:

Neuronal differentiation (Figure 4) was initiated by plating the Jarid1c Lentivirus and negative cell lines created in the Lentiviral induction in DMEM containing 0.3μM retinoic acid (RA). The cells were transferred to non-adhesive Falcon^{TM} Petri dishes, which persuades the cells to form aggregates by Day 4. On Day 4, the cells were dispersed using trypsin, a serine protease, and then replated to adhesive NUNC plates.

On Day 5, DMEM containing cytosine arabinoside (Ara-C) was used to refresh the cells. Ara-C inhibits cell division, so only neuron-like cells will be selected. Neurons were differentiated by Day 6 and the medium was replaced every other day. On Day 10, cells were collected for gene analysis.

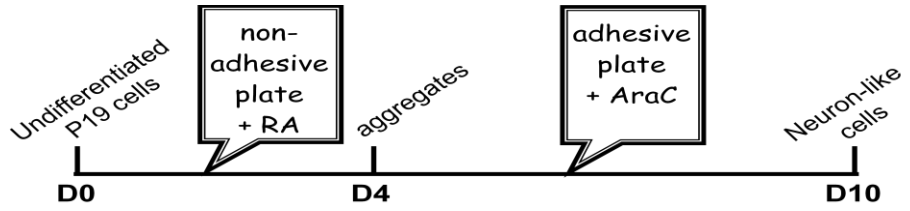

Figure 4: Timeline of Neuronal differentiation.

Expression of Neurotrophin Genes:

RNA was extracted from samples collected at each of the three time points: D0, D4, and D10 using the RNeasy kit by Qiagen. Any DNA left in the samples was removed using the DNA-free Kit by Applied Biosystems. cDNA synthesis was done using RT^2 Real Strand Kit. The cDNA expression quantification was performed following SABiosciences instructions on an ABI 7500 real-time PCR system. The relative expression of each gene was calculated using the following formula:

$Expression_{GOI} = 2^{-\Delta Ct}$

$\Delta Ct = Ct_{GOI} - Ct_{AVG(HKG)}$

Ct_{GOI}: Ct value of gene of interest;

$Ct_{AVG(HKG)}$: average Ct value of five housekeeping genes.

Mouse neurotrophin plates were used for real-time PCR and were laid out as shown in Figure 5.

Adcyapr	Artn	Bax	Bcl2	Bdnf	Cbln1	Cckar	Cntfr	Crh	Crhbp	Crhr1	Crhr2
A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
Cx3cr1	Cxcr4	Fas	Fgf2	Fgf9	Fgfr1	Fos	Frs2	Frs3	Fus	Galr1	Galr2
B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
Gdnf	Gfra1	Gfra2	Gfra3	Gmfb	Gmfg	Npffr2	Grpr	Hcrt	Hcrtr1	Hcrtr2	Hspb1
C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
Il10	Il10ra	Il1b	Il1r1	Il6	Il6ra	Il6st	Lif	Lifr	Maged1	Mc2r	Mef2c
D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
Mt3	Myc	Nf1	Ngf	Ngfr	Ngfrap1	Nmbr	Npff	Npy	Npy1r	Npy2r	Nr1i2
E1	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
Nrg1	Nrg4	Ntf3	Ntf5	Ntrk1	Ntrk2	Ntsr1	Ppyr1	Pspn	Ptger2	Stat1	Stat2
F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
Stat3	Stat4	Tfg	Tgfa	Tgfb1	Tgfb1i1	Cd40	Tro	Trp53	Ucn	Zfp110	Zfp91
G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
Gusb	Hprt1	Hsp90a	Gapdh	Actb	MGDC	RTC	RTC	RTC	PPC	PPC	PPC
H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

Figure 5: Layout for Neurotrophin receptor real-time PCR plates

Appendix I displays the detailed gene descriptions for the 84 genes above.

Results:

It was found by PCR analysis that Jarid1c expression was indeed diminished in P19 Jarid1c knockdown cells when compared with negative control cells that had been transfected with non-targeting shRNAs. Expression of Jarid1d, a member of the Jarid1c family, was not affected, which argues against an off-target effect.

P19 Jarid1c knockdown cells were induced to differentiate into neuron-like cells. This is a clear indication that Jarid1c is not indispensable for neuronal induction to occur. Figure 6 shows the process of neuronal induction. It shows the P19 cells growing on NUNC plates on Day 0. On Day 4 the cells have formed aggregates in Falcon plates while being treated with retinoic acid. By Day 10 the cells had fully differentiated after being treated with Ara-C and replated on adhesive NUNC plates. The neuronal cell body and neurite can be clearly seen in the photograph.

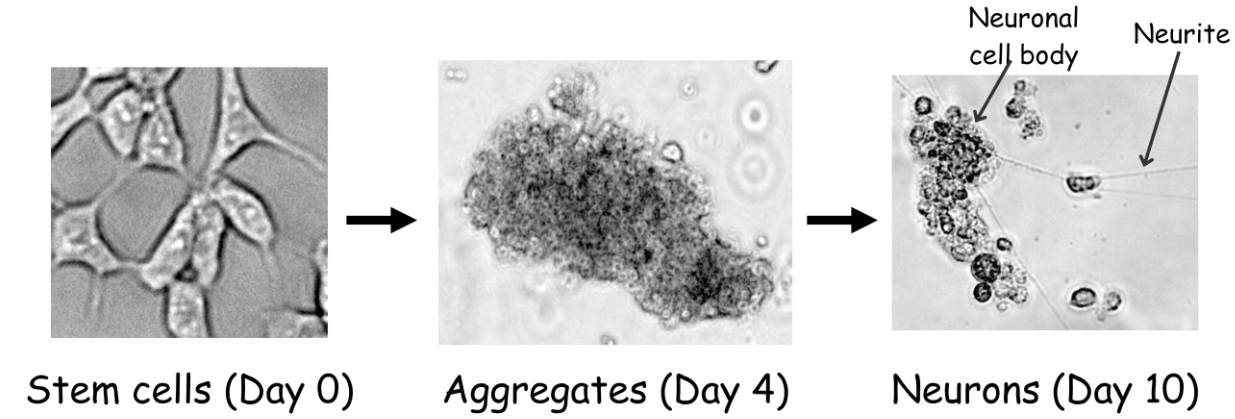

Figure 6: Process of Neuronal Differentiation

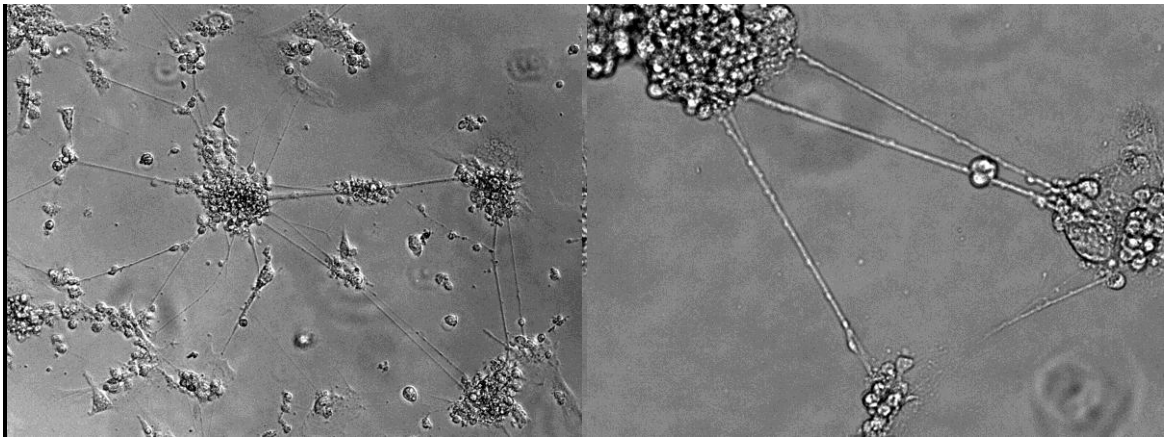

Figure 7: Neurons, Day 10

In Figure 7, different views of neurons can be seen at 10x and 20x magnification. These photos show the complex connections between neuronal cell bodies that occur.

Mouse neurotrophin receptor PCR panels were used to analyze the expression of 84 different neurotrophin signaling-related genes (See Figure 5) in Jarid1c knockdown and control cells at Day 0, Day 4, and Day 10. Most genes were expressed at relatively the same rate between cell types. However, on Day 0, prior to initiation of neuronal differentiation, Fus and Gfra3 were found to be up-regulated in the Jarid1c knockdown when compared to the negative control cells. This was important to note as a baseline when comparing Day 4 and Day 10.

On Day 4, the neurotrophin receptor gene, Ntrk1 (Neurotrophic tyrosine kinase receptor, type 1) was upregulated in Jarid1c knockdown cells. Il6 (Interleukin 6) and Il1r1 (Interleukin receptor 1, type1) were found to be down-regulated at Day 4 in Jarid1c knockdown cells. Figure 8 shows a quantification of the relative expression of each of the three genes.

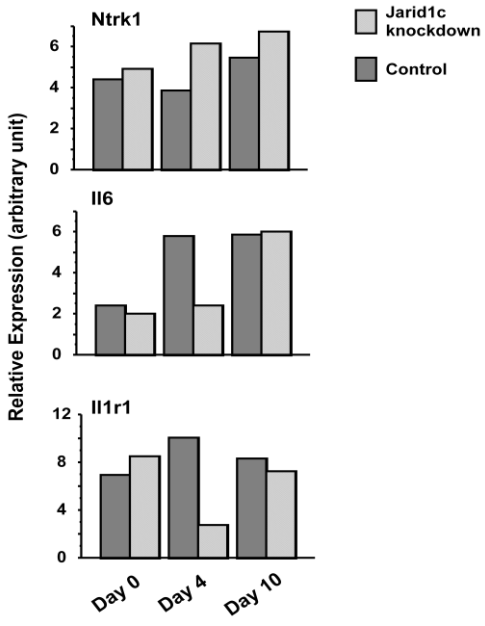

Figure 8: Relative Expression of three neurotrophin receptor genes in Jarid1c knockdown cells vs. negative control cells.

On Day 10, when P19 cells were fully differentiated there was no difference detected by PCR between the expression of the 84 genes in Jarid1c knockdown and negative control.

Discussion:

Jarid1c is a histone demethylase master regulator gene which suppresses transcription of certain target genes. Mutations in this gene can cause neurological disorders such as mental retardation, aggression, epilepsy, and autism. It is unclear as to how exactly Jarid1c causes these behavioral defects. One hypothesis was that Jarid1c plays a role in neuronal differentiation in the embryonic brain. This study used P19 embryonic carcinoma stem cells as an in vitro model, because they can easily be induced to differentiate into neurons. Lentiviral transduction particles were used to permanently silence Jarid1c expression using shRNA constructs. The affects of this silencing at three time points, Day 0, Day 4, and Day 10 were analyzed with SuperArray PCR panels on a real-time PCR machine.

Jarid1c was stably silenced using the Lentivirus transduction method. These Jarid1c knockdown cells were able to fully differentiate into neurons, indicating that Jarid1c is not essential for neuronal differentiation.

On Day 4 it was found that the expression of three genes was different between Jarid1c and negative control cell lines. Ntrk1 was found to be up-regulated in Jarid1c knockdown cells. This gene encodes for a neurotrophic tyrosine kinase receptor (Weizmann Institute of Science, 2009). It is capable of self phosphorylation. It is thought to lead to cell differentiation and play a role in specifying the subtype of neuron. The up-regulation of Ntrk1 may be compensating for the lack of Jarid1c in the Jarid1c knockdown cells and taking over some of its roles.

Il6 and Il1r1 were found to be down-regulated in the Jarid1c knockdown cells. Il6 is a cytokine with many different functions, such as playing a role in differentiating B-cells and inducing myeloma and plasmacytoma growth (Weizmann Institute of Science, 2009). It also induces nerve cell differentiation. A down regulation of this gene suggests that Jarid1c normally would induce this gene to help in neuronal differentiation. A mutation in Jarid1c may cause a similar down-regulation in Il6, which could be tied to certain XLMR phenotypes.

Il1r1 was also down-regulated. It belongs to a family of interleukin 1 receptors (Weizmann Institute of Science, 2009). It is a receptor for interleukin alpha, beta, and interleukin 1 receptor type 1. It is also a mediator in cytokine induced inflammatory responses. Il1r1 is a member of a cytokine receptor gene cluster on chromosome 2q12. A study conducted by Wainwright et al. (2005) suggests that genes on chromosome 2 may play an important role in human intelligence. A down-regulation in this receptor gene may also occur in Jarid1c mutations and could also be linked to XLMR phenotypes, such as lowered intelligence.

In future studies of Jarid1c and neuronal differentiation, it would be interesting to see how mutated forms of Jarid1c, instead of simply knocking down Jarid1c, affect neuronal differentiation. It is possible that the same genes would be up- and down-regulated, but it is possible that additional genes may be affected. This study could also be linked with a mouse model so that the affects of the Jarid1c silencing could be seen. Behavioral studies could be conducted to see if lack of Jarid1c causes changes in behavior. Detailed studies could be done on the differences in neuron structure between the Jarid1c knockdown cells and negative control cells. This would shed more light on Jarid1c's role in neuronal differentiation.

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Appendix I: Mouse Neurotrophin and Receptor PCR plate Gene Descriptions

Gene Table

Position	Unigene	GeneBank	Symbol	Description	C
A01	Mm.44245	NM_007407	Adcyap1r1	Adenylate cyclase activating polypeptide 1 receptor 1	290002
A02	Mm.56897	NM_009711	Artn	Artemin	
A03	Mm.19904	NM_007527	Bax	Bcl2-associated X protein	
A04	Mm.257460	NM_009741	Bcl2	B-cell leukemia/lymphoma 2	AW
A05	Mm.1442	NM_007540	Bdnf	Brain derived neurotrophic factor	
A06	Mm.4880	NM_019626	Cbln1	Cerebellin 1 precursor protein	
A07	Mm.3521	NM_009827	Cckar	Cholecystokinin A receptor	
A08	Mm.425178	NM_016673	Cntfr	Ciliary neurotrophic factor receptor	
A09	Mm.290689	NM_205769	Crh	Corticotropin releasing hormone	C
A10	Mm.316614	NM_198408	Crhbp	Corticotropin releasing hormone binding protein	
A11	Mm.1892	NM_007762	Crhr1	Corticotropin releasing hormone receptor 1	
A12	Mm.236081	NM_009953	Crhr2	Corticotropin releasing hormone receptor 2	CRF-
B01	Mm.44065	NM_009987	Cx3cr1	Chemokine (C-X3-C) receptor 1	
B02	Mm.1401	NM_009911	Cxcr4	Chemokine (C-X-C motif) receptor 4	CD
B03	Mm.1626	NM_007987	Fas	Fas (TNF receptor superfamily member 6)	AI1
B04	Mm.457975	NM_008006	Fgf2	Fibroblast growth factor 2	
B05	Mm.8846	NM_013518	Fgf9	Fibroblast growth factor 9	
B06	Mm.265716	NM_010206	Fgfr1	Fibroblast growth factor receptor 1	AW
B07	Mm.246513	NM_010234	Fos	FBJ osteosarcoma oncogene	D
B08	Mm.135965	NM_177798	Frs2	Fibroblast growth factor receptor substrate 2	4732458
B09	Mm.89912	NM_144939	Frs3	Fibroblast growth factor receptor substrate 3	493041
B10	Mm.277680	NM_139149	Fus	Fusion, derived from t(12;16) malignant liposarcoma (human)	D430004D1
B11	Mm.6219	NM_008082	Galr1	Galanin receptor 1	

B12	Mm.57149	NM_010254	Galr2	Galanin receptor 2	
C01	Mm.4679	NM_010275	Gdnf	Glial cell line derived neurotrophic factor	
C02	Mm.88367	NM_010279	Gfra1	Glial cell line derived neurotrophic factor family receptor alpha 1	
C03	Mm.32619	NM_008115	Gfra2	Glial cell line derived neurotrophic factor family receptor alpha 2	
C04	Mm.16520	NM_010280	Gfra3	Glial cell line derived neurotrophic factor family receptor alpha 3	
C05	Mm.87312	NM_022023	Gmfb	Glia maturation factor, beta	3110001H2
C06	Mm.194536	NM_022024	Gmfg	Glia maturation factor, gamma	0610039G1
C07	Mm.447881	NM_133192	Npffr2	Neuropeptide FF receptor 2	G
C08	Mm.4687	NM_008177	Grpr	Gastrin releasing peptide receptor	
C09	Mm.10096	NM_010410	Hcrt	Hypocretin	
C10	Mm.246595	NM_198959	Hcrtr1	Hypocretin (orexin) receptor 1	
C11	Mm.335300	NM_198962	Hcrtr2	Hypocretin (orexin) receptor 2	OX
C12	Mm.465216	NM_013560	Hspb1	Heat shock protein 1	2
D01	Mm.874	NM_010548	Il10	Interleukin 10	
D02	Mm.26658	NM_008348	Il10ra	Interleukin 10 receptor, alpha	AW5
D03	Mm.222830	NM_008361	Il1b	Interleukin 1 beta	IL
D04	Mm.896	NM_008362	Il1r1	Interleukin 1 receptor, type I	CD
D05	Mm.1019	NM_031168	Il6	Interleukin 6	
D06	Mm.2856	NM_010559	Il6ra	Interleukin 6 receptor, alpha	C
D07	Mm.4364	NM_010560	Il6st	Interleukin 6 signal transducer	513340
D08	Mm.4964	NM_008501	Lif	Leukemia inhibitory factor	
D09	Mm.149720	NM_013584	Lifr	Leukemia inhibitory factor receptor	A230075
D10	Mm.27578	NM_019791	Maged1	Melanoma antigen, family D, 1	2810433C1
D11	Mm.426053	NM_008560	Mc2r	Melanocortin 2 receptor	
D12	Mm.24001	NM_025282	Mef2c	Myocyte enhancer factor 2C	5430401D1
E01	Mm.2064	NM_013603	Mt3	Metallothionein 3	
E02	Mm.2444	NM_010849	Myc	Myelocytomatosis oncogene	AU

E03	Mm.255596	NM_010897	Nf1	Neurofibromatosis 1	AW4942
E04	Mm.1259	NM_013609	Ngf	Nerve growth factor	
E05	Mm.283893	NM_033217	Ngfr	Nerve growth factor receptor (TNFR superfamily, member 16)	LN
E06	Mm.90787	NM_009750	Ngfrap1	Nerve growth factor receptor (TNFRSF16) associated protein 1	AL
E07	Mm.425622	NM_008703	Nmbr	Neuromedin B receptor	
E08	Mm.208714	NM_018787	Npff	Neuropeptide FF-amide peptide precursor	
E09	Mm.154796	NM_023456	Npy	Neuropeptide Y	07
E10	Mm.5112	NM_010934	Npy1r	Neuropeptide Y receptor Y1	
E11	Mm.1433	NM_008731	Npy2r	Neuropeptide Y receptor Y2	
E12	Mm.8509	NM_010936	Nr1i2	Nuclear receptor subfamily 1, group I, member 2	
F01	Mm.153432	NM_178591	Nrg1	Neuregulin 1	6030
F02	Mm.443874	NM_032002	Nrg4	Neuregulin 4	
F03	Mm.267570	NM_008742	Ntf3	Neurotrophin 3	AI31
F04	Mm.20344	NM_198190	Ntf5	Neurotrophin 5	290004
F05	Mm.80682	XM_283871	Ntrk1	Neurotrophic tyrosine kinase, receptor, type 1	
F06	Mm.130054	NM_008745	Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	AI8483
F07	Mm.301712	NM_018766	Ntsr1	Neurotensin receptor 1	N
F08	Mm.57059	NM_008919	Ppyr1	Pancreatic polypeptide receptor 1	N
F09	Mm.86487	NM_008954	Pspn	Persephin	
F10	Mm.4630	NM_008964	Ptger2	Prostaglandin E receptor 2 (subtype EP2)	E
F11	Mm.277406	NM_009283	Stat1	Signal transducer and activator of transcription 1	201000
F12	Mm.293120	NM_019963	Stat2	Signal transducer and activator of transcription 2	160001
G01	Mm.249934	NM_011486	Stat3	Signal transducer and activator of transcription 3	111003
G02	Mm.1550	NM_011487	Stat4	Signal transducer and activator of transcription 4	
G03	Mm.425970	NM_019678	Tfg	Trk-fused gene	
G04	Mm.137222	NM_031199	Tgfa	Transforming growth factor alpha	
G05	Mm.248380	NM_011577	Tgfb1	Transforming growth factor, beta 1	TGF-

G06	Mm.3248	NM_009365	Tgfb1i1	Transforming growth factor beta 1 induced transcript 1	A
G07	Mm.271833	NM_011611	Cd40	CD40 antigen	AI
G08	Mm.3597	NM_019548	Tro	Trophinin	AA4
G09	Mm.222	NM_011640	Trp53	Transformation related protein 53	
G10	Mm.377116	NM_021290	Ucn	Urocortin	
G11	Mm.292297	NM_022981	Zfp110	Zinc finger protein 110	29000
G12	Mm.290924	NM_053009	Zfp91	Zinc finger protein 91	9130014I0
H01	Mm.3317	NM_010368	Gusb	Glucuronidase, beta	A
H02	Mm.299381	NM_013556	Hprt1	Hypoxanthine guanine phosphoribosyl transferase 1	C8
H03	Mm.2180	NM_008302	Hsp90ab1	Heat shock protein 90kDa alpha (cytosolic), class B member 1	90k
H04	Mm.343110	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	
H05	Mm.328431	NM_007393	Actb	Actin, beta, cytoplasmic	Actx/
H06	N/A	SA_00106	MGDC	Mouse Genomic DNA Contamination	
H07	N/A	SA_00104	RTC	Reverse Transcription Control	
H08	N/A	SA_00104	RTC	Reverse Transcription Control	
H09	N/A	SA_00104	RTC	Reverse Transcription Control	
H10	N/A	SA_00103	PPC	Positive PCR Control	
H11	N/A	SA_00103	PPC	Positive PCR Control	
H12	N/A	SA_00103	PPC	Positive PCR Control	