EMERGENCE OF HEREDITARY HYPERPLASTIC GINGIVITIS IN NEWFOUNDLAND AND LABRADOR, CANADA: AN EXPLORATION INTO THE MOLECULAR AETIOLOGY AT BOTH THE GENE AND GENOME LEVELS

by

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Abstract

Hereditary hyperplastic gingivitis (HHG) is a benign fibrous enlargement of the gingival tissue progressing to dental encapsulation in ranched silver foxes (*Vulpes vulpes*). It is an autosomal recessive condition displaying male sex-biased penetrance HHG demonstrates a pleiotropic association with superior fur quality. In 2004, after the introduction of a Finnish fox line, HHG emerged in Newfoundland and Labrador, Canada. While the underlying HHG aetiology is unknown, an analogous condition called hereditary gingival fibromatosis (HGF) occurs in humans, providing a platform for investigation into the molecular mechanisms of HHG. A mutation in the *son of sevenless homolog 1* gene causes one form of HGF. Candidate gene sequencing of this and related genes including *epidermal growth factor receptor* (*EGFR*), *growth factor receptor bound protein 2*, and *mitogen-activated protein kinase kinase 6* did not uncover any putative coding sequence or splice-site mutations. HHG was also examined at the genomic level, integrating knowledge of the chromosomal loci associated with the analogous human condition, HGF. This exploration was divided into the known genetic causes of isolated HGF and HGF-associated syndromes characterized by both gingival and hair overgrowth. Global gene expression differences between affected and unaffected foxes pinpoint *SOS2* and *RASA1* as candidate genes for HHG; overall, the genomic expression patterns strongly indicate the involvement of the mitogen-activated protein kinase (MAPK) signalling pathway. Specifically, the error could occur prior to the Rat sarcoma protein in this pathway. Future exploration of this pathway could elucidate the genetic basis of HHG and more broadly the molecular aetiology of gingival

overgrowth in humans and canines. Additional information regarding the HHG phenotype was revealed, including the potential involvement of androgens in disease severity. The *steroid-5-alpha-reductase, alpha polypeptide 2* gene was found to be up-regulated in the HHG-affected foxes, and the link between androgens and gingival sensitivity in dogs might help explain the perceived sex biased penetrance originally noted in HHG. Overall the molecular mechanisms underpinning the HHG phenotype and future work revolve around the MAPK signalling pathway including influences that other gene products like androgens have on it.

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Abbreviations

°C	Degree Celsius
2ddCT	2 delta delta CT
A	Adenine
ABCA	ATP binding cassette transporter family A
ABCA5	ATP-binding cassette, sub-family A, member 5
ABCA6	ATP-binding cassette, sub-family A, member 6
ABCA10	ATP-binding cassette, sub-family A, member 10
ABCC9	ATP-binding cassette, sub-family C member 9
AD	Autosomal dominant
ADCY2	Adenylate cyclase 2
AGGF1	Angiogenic factor with G patch and FHA domains 1
ALK	Anaplastic lymphoma kinase
ALPL	Alkaline phosphatase
ANKRD31	Ankyrin repeat domain 31
ATG7	Autophagy related 7
ATP	Adenosine triphosphate
AZIN1	Antizyme inhibitor 1
BCR	Breakpoint cluster region
BIRC3	Baculoviral IAP repeat containing 3
bp	Base pair
BRAF	V-RAF murine sarcoma viral oncogene homolog B
C	Cytosine
C-terminal	Carboxy-terminal
C2orf18	Chromosome 2 open reading frame 18
CAAT	Upstream transcriptional binding box
CASP6	Caspase 6
CBL	Casitas B-lineage Lymphoma
CDC25	Cell division cycle 25 homolog
CDC37	Cell division cycle 37 homolog
cDNA	Complementary DNA
CH	Chapter
CHD1	Chromodomain helicase DNA binding protein 1
CHL	Congenital hypertrichosis lanuginose
cM	Centimorgan
CMA1	Chymase 1, mast cell
C-MYC	C-myelocytomatosis viral oncogene

CMYA5	Cardiomyopathy associated 5
COL14A1	Collagen, type XIV, alpha 1
CpG	Cytosine phosphodiester bond guanine
CPQ	Carboxypeptidase Q
CYP2C9	Cytochrome P450 enzyme family member 2C9
CYP3A4	Cytochrome P450 enzyme family member 3A4
ΔΔCT	Delta delta CT
dN	Nonsynonymous
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides
dS	Synonymous
DST	Dystonin
E2F4	E2F transcription factor 4
EGF	Epidermal growth factor
EGFR	Epidermal Growth Factor Receptor
EGFR1	Epidermal growth factor receptor 1
ELL2	Elongation factor, RNA polymerase II
EPO receptor	Erythropoietin receptor
ERK	Extracellular signal-regulated kinase
EST	Expressed sequence tag
et al	And others
F1	Filial generation
FAM174A	Family with sequence similarity 174, member A
FGF2	Fibroblast growth factor 5
FHL2	Four and a half LIM domains 2
FNTA	Farnesyltransferase
FOS	Finkel–Biskis–Jinkins murine osteogenic sarcoma virus
FU domain	Furin-like repeat domain
FZD7	Frizzled family receptor 7
G	Guanine
G protein	Guanine nucleotide binding protein
GAP	GTPase activating protein
GCRMA	Guanine cytosine robust multi-array average
GDP	Guanosine-5'-diphosphate
GEF	Guanine nucleotide exchange factor
GF	Gingival fibromatosis
GJA1	Gap junction protein, alpha 1
GNAI1	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1
GRB2	Growth factor receptor bound protein 2
GRP113	G-protein coupled receptor 110
GTF3C2	General transcription factor IIIC, polypeptide 2
GTP	Guanosine-5'-triphosphate
GTPase	Guanosine-5'-triphosphatase
GYG1	Glycogenin 1
h	Hour

HGF	Hereditary gingival fibromatosis
HGF1	Hereditary gingival fibromatosis locus 1
HGF2	Hereditary gingival fibromatosis locus 2
HGF3	Hereditary gingival fibromatosis locus 3
HHG	Hereditary hyperplastic gingivitis
HLA	Human leukocyte antigens
HLA A19	Human leukocyte antigen A19
HLA B27	Human leukocyte antigen B27
HLA DR1	Human leukocyte antigen DR1
HRAS	V-RAF murine sarcoma viral oncogene homolog H
HyPhy	Site-specific test of selection
ID	Indentifier
IL1B	Interleukin 1, beta
IL4	Interleukin 4
INS	Insulin
IRF3	Interferon regulatory factor 3
JNK	c-Jun N-terminal kinase
KCNJ11	Potassium inwardly-rectifying channel, subfamily J, member 11
KCNJ8	Potassium inwardly-rectifying channel, subfamily J, member 8
KIAA1429	KIAA1429 ortholog
KRAS	V-RAF murine sarcoma viral oncogene homolog K
KRT71	Keratin-71
KTN1	Kinectin 1
LAMP1	Lysosomal-associated membrane protein 1
Ltd	Limited
MAPK	Mitogen activated protein kinase
MAP2K	Mitogen-activated protein kinase kinase
MAP2K1	Mitogen-activated protein kinase kinase 1
MAP2K2	Mitogen-activated protein kinase kinase 2
MAP2K6	Mitogen-activated protein kinase kinase 6
MAP3K	Mitogen-activated protein kinase kinase kinase
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
MAP3K10	Mitogen-activated protein kinase kinase kinase 10
MAP4K	Mitogen-activated protein kinase kinase kinase kinase
MAP4K1	Mitogen-activated protein kinase kinase kinase kinase 1
MAPK3	Mitogen-activated protein kinase 3-like
MAPK4	Mitogen-activated protein kinase 4
Mb	Mega base
MCTP1	Multiple C2 domains, transmembrane 1
MED30	Mediator complex subunit 30
MEGA	Molecular evolutionary genetic analysis
MEK	Mitogen-activated protein kinase kinase
mg	Miligram
mL	Mililitre
µL	Microlitre
µM	Micromolar

mM	Milimolar
MMP	Matrix metallopeptidase 9
mRNA	Messenger ribonucleic acid
NA	Not available
NCALD	Neurocalcin delta
NCBI	National centre for biotechnology information
NF1	Neurofibromatosis 1
nM	Nanomolar
No.	Number
NRAS	V-RAF murine sarcoma viral oncogene homolog N
nt	Nucleotide
N-terminal	Amino terminal
NUDCD1	NudC domain containing 1
NUP214	Nucleoporin 214kDa
OXR1	Oxidation resistance 1
P120GAP	RAS p21 protein activator (GTPase activating protein) 1
p38	Protein 38
PAML	Phylogenetic analysis by maximum likelihood
PCR	Polymerase chain reaction
PEI	Prince Edward Island
PITPNC1	Phosphatidylinositol transfer protein, cytoplasmic 1
PRDM9	PR domain containing 9
PRKCζ	Protein kinase C, zeta
PRKD1	Protein kinase D1
PTK2	Protein tyrosine kinase 2
PTPN11	Tyrosine-protein phosphatase non-receptor type 11
RAC	RAS related C3 botulinum toxin
RAD21	RAD21 homolog
RAF	Rapidly accelerated fibrosarcoma
RAF1	Rapidly accelerated fibrosarcoma 1
RALBP1	RalA binding protein 1
RAS	Rat sarcoma protein family
RASA1	RAS p21 protein activator (GTPase activating protein) 1
RdgBβ	Class IIB PIP protein subfamily
RNA	Ribonucleic acid
RNF25	Ring finger protein 25
ROCK1	Rho-associated, coiled-coil containing protein kinase 1
ROR1	Receptor tyrosine kinase-like orphan receptor 1
RPL30	Ribosomal protein L30
RRAD	RAS-related associated with diabetes
RSPO2	R-spondin-2
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SELI	Ethanolaminephosphotransferase 1
SH2	Src homology 2
SH3	SRC Homology 3 Domain
SH3GL3	SH3-domain GRB2-like 3

SHC	Src homology 2 domain containing) transforming protein
SHOC3	Soc-2 suppressor of clear homolog
SHP2	Protein tyrosine phosphatase, non-receptor type 11
SLC30A3	Solute carrier family 30 (zinc transporter), member 3
SOS	Son of sevenless homolog
SOS1	Son of sevenless homolog 1
SOS2	Son of sevenless homolog 2
Sp1	Specificity protein 1
Spred1	Sprouty-related, EVH1 domain containing 1
SRD5A2	Steroid-5-alpha-reductase, alpha polypeptide 2
SRY	Sex determining region Y
T	Thymine
TAF2	TATA box binding protein associated factor
TATA	Goldberg-Hogness box
Temp	Temperature
TGFβ	Transforming growth factor beta
TGFβ3	Transforming growth factor beta , 3
TIMP	Tissue inhibitor of metalloproteins
Tm	Melting temperature
TMEM161B	Transmembrane protein 161B
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15
TNFα	Tumour necrosis factor alpha
TRIM36	Tripartite motif containing 36
tRNA	Transfer ribonucleic acid
U	Units
USP6NL	USP6 N-terminal like
v	Version
vs.	Versus
ω	Ratio of dN/dS
Wnt	Wingless/integrated
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4
χ^2	Chi square
γ	Rate variation among sites
ZNF512	Zinc finger protein 512

Chapter one:
Introduction and overview

Introduction

Hereditary hyperplastic gingivitis (HHG) is a benign, proliferative gingival overgrowth seen in foxes. It was first documented in the 1940s and to date the underlying molecular aetiology is still unknown (Dyrendahl and Henricson 1960). In 2004, HHG was documented for the first time in North America in farmed silver foxes after the introduction of a Finnish silver fox line (Clark *et al.* 2015). The emergence of this disease in Canada brought its aetiology to the forefront. Analogous conditions occur in other organisms, including humans and the domestic dog, which could provide clues to a common underlying aetiology to this disease.

Hereditary gingival fibromatosis (HGF) is a benign, proliferative gingival overgrowth affecting humans. Studies have demonstrated HGF to be a complex disease with numerous aetiologies. Broadly, these can be divided into three groups: isolated genetic inheritance, part of a genetic syndrome, or an acquired drug-induced phenotype (Shashi *et al*. 1999). Each of these categories can be further subdivided, further highlighting the complexity of this disease.

Gingival hyperplasia occurs in the domestic dog (Hale 2004). It has an association with certain breed dispositions, suggesting an underlying genetic aetiology; as well, there is an independent drug-induced phenotype. In either case, the underlying molecular mechanism is unknown.

From an evolutionary standpoint the fox and domestic dog are closely related (Wayne *et al*. 1997). The publication of the canine genome in 2005 (Lindblad-Toh *et al.* 2005) has enabled genomic research in other closely-related species such as the fox, thus providing a new platform of investigation. A co-operative strategy incorporating what is known about HGF along with genomic technologies based on the dog genome will aid in creating a comprehensive platform for the assessment of HHG genetics in silver foxes.

Fox farming and HHG

In Canada, fox farming originated in 1895 on Prince Edward Island (Rubtsov 1998). As part of this practice, various coat colour variants were selected for breeding. In the 1940s the first case of HHG was noted in a Norwegian male silver fox with superior fur quality that had been imported into Sweden from Norway. The disease quickly spread through fox farms in both Sweden and Norway. In 1960 Dyrendahl and Henricson published a seminal paper describing the genetic inheritance of HHG in the farmed silver fox population (Dyrendahl and Henricson 1960). They examined 1,080 parents and 7,238 silver fox pups over a six-year period. The manifestation of HHG occurred at approximately two to three years of age, with tumour proliferation continuing throughout life and resulting in dental encapsulation. Pathology showed large epithelial extensions in the keratinized collagen of the gingival tissue. Genetic analysis found HHG to be an autosomal recessive disease demonstrating incomplete penetrance, with an increased incidence in males relative to females. In addition, a pleiotropic relationship between HHG and superior fur quality

(typically length and density of the guard hairs) was implicated. Shortly after, the global price for silver fox furs decreased significantly resulting in the reduction of silver fox stocks in many countries until the early 1970s when the fox lines were re-established (R. Hudson, personal communication, 2012).

While the majority of HHG cases are found in farmed foxes there was a report in 2008 of a case of severe HHG in a wild European red fox (Schulze *et al*. 2008). A male red fox of at least six years of age was investigated as part of a national regulatory program for protection against rabies. Gross clinical presentation showed severe papillomatous growth on the both upper and lower jaws, encapsulating the teeth, similar to the HHG seen in the farmed silver fox population.

In Canada, Newfoundland and Labrador has established the largest collection of farmed foxes in North America, where approximately 2,200 foxes are housed each year (Bursey 2001). HHG was first documented in North America in 2004 in Newfoundland and Labrador in a farmed fox population (Clark *et al*. 2015). With the emergence in Canada questions about the molecular aetiology is once again being brought to the forefront.

Human hereditary gingival fibromatosis: clinical presentation

HGF is a rare, pernicious disease of the oral cavity for which the underlying cellular aetiology is unknown. It has a phenotypic frequency of 1:175 000, affecting males and females equally (Fletcher 1966). HGF is a genetically and phenotypically heterogeneous

disease characterized by progressive benign fibrous enlargements of the maxillary and mandibular keratinized gingival tissue (Hart *et al*. 2002). On average, manifestation occurs at permanent dentition but cases have been reported demonstrating involvement in primary dentition and even at birth (Breen *et al*. 2009; Coletta and Graner 2006). Manifestations typically worsens during adolescence (Coletta and Graner 2006).

Gross appearance

Clinically, HGF occurs asymptomatically with benign, non-hemorrhagic, non-exudative gingival overgrowths (Bittencourt *et al*. 2000; Hakkinen and Csiszar 2007). The gingival tissue is of normal colour, with a firm consistency and stippling on the adjoining gingiva (Singer *et al*. 1993). This fibrous tissue can appear nodular or have a pebbled surface (Brown *et al*. 1995). Involvement of the buccal and lingual tissue on both the mandible and maxilla can occur, but does not extend beyond the mucogingival junction (Katz *et al*. 2002; Ramer et al. 1996).

Two different forms of HGF have been classified, nodular and symmetric, that can occur together or in isolation (Bittencourt *et al*. 2000; Ramer *et al*. 1996). The nodular phenotype contains multiple focal lesions into the dental papillae. This form commonly presents in the maxillary tuberosity and the molar area, predominantly on the palatal area (Katz *et al*. 2002). The symmetric form is more generalized showing a more uniform gingival pattern. This form is more common and affects both the maxilla and the mandible equally (Bittencourt *et al*. 2000).

More severe presentations of HGF can involve the anterior mandible with tooth encapsulation (Bittencourt *et al*. 2000; Breen *et al*. 2009). While the alveolar bone is not involved in this disease there can be alteration of the contour of the palate and pseudo-pocketing can occur from the excess gingival tissue (DeAngelo *et al*. 2007; Hakkinen and Csiszar 2007). Numerous other dental problems can subsequently result, including: diastemas; malpositioning of the teeth; prolonged retention of primary dentition and delayed eruption (Bittencourt *et al*. 2000). While HGF disease originates as a result of gingival overgrowth it has profound effects on the entire oral cavity.

Microscopic structure

Ultrastructure studies conducted on gingival overgrowth lesions from human samples showed predominantly myoblast-like cells and fibroblast-like cells (Takagi *et al*. 1991). Another prominent feature included large amounts of collagen fibrils in the extracellular matrix. Electron microscopy demonstrated an abnormal variety in the collagen bundle diameter (Barros *et al*. 2001). In addition, there was an increased level of oxytalan fibres and minimal elastic fibres were present.

Histological examination of the human HGF gingival samples characterized changes in epithelial thickness, cellular structures such as collagen bundles and fibroblast, and general abnormalities. The squamous epithelium contained acanthosis and elongated rete pegs (Sengun *et al*. 2007; Takagi *et al*. 1991). Epithelial hyperplasia was seen as a result of the

acanthosis in cases where chronic inflammation was present (Hakkinen and Csiszar 2007). The collagen bundles appeared coarse, mature and acellular and were arranged mostly in parallel bundles (Hakkinen and Csiszar 2007, Takagi *et al*. 1991). Two populations of fibroblast cells were identified (Collan *et al*. 1982; Sakamoto *et al*. 2002). One population contained little perinuclear cytoplasm and had dense collagen bundles. The second contained inactive fibroblasts with large amounts of smooth and rough endoplastic reticulum, Golgi apparati and mitochondria in the cytoplasm. There were minimal signs of inflammatory cells unless there was a background of chronic inflammation or in cases with pseudo-pockets of excess gingival tissue (Hakkinen and Csiszar 2007; Takagi *et al*. 1991). Extensive cellular degradation was seen with few vascular structures present. There were a number of abnormal findings present in some but not all cases, including: mucosal ulceration; amyloid, calcified particles; epithelial metaplasia and islands of odontogenic epithelium (Gunhan *et al*. 1995). HGF demonstrated numerous histological findings that were non-specific and thus cannot be used in making a diagnosis; rather diagnosis is based on family history and clinical presentation.

Treatment

Treatment has evolved from more extreme measures including tooth removal and removing portion of the alveolar bone to more conservative measures including quadrant by quadrant internal or external bevel gingivectomy or carbon dioxide laser removal (Coletta and Graner 2006; Ramer *et al*. 1996). Ideal timing for gingival removal is often debated between pre- or post-arrival of permanent dentition. Post- operative surgical

patients are required to follow an oral hygiene regime and are followed routinely as reoccurrence rates can be high, especially in children and adolescents (Ramer *et al*. 1996). Removal of the gingival growth is not curative as the gingival tissue will continue to grow and require future resections.

Hereditary gingival fibromatosis: aetiology

Broadly, HGF can be classified as primary or secondary (Figure1.1) (Shashi *et al*. 1999). Primary HGF can be the result of an isolated disorder with a genetic cause, or a component of a larger syndrome. Secondary HGF is the result of an interaction with a drug that can induce the HGF phenotype. One approach to understanding the aetiology of HGF is to determine the genetic basis of isolated HGF and then examine the role that gene or genes play in syndromic form as well as in the genetic susceptibility for drug-induced HGF.

Primary HGF: isolated HGF genetics

HGF is a genetically heterogeneous disease with different patterns of inheritance including Mendelian autosomal dominant, Mendelian autosomal recessive, and maternal genome imprinting.

Mendelian HGF is predominantly autosomal dominant and displays incomplete penetrance and variable expression

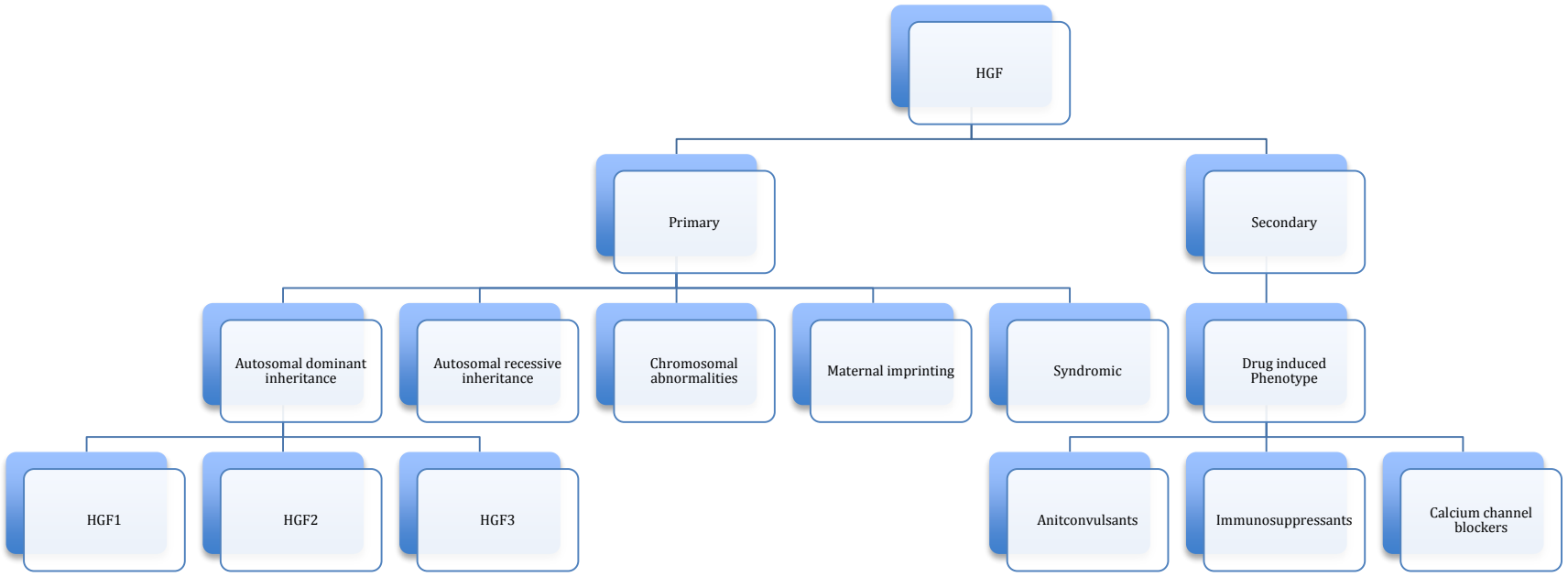

Figure 1.1: Schematic diagram depicting the known causes of human hereditary gingival fibromatosis (HGF).

(Goldblatt and Singer 1992; Hart *et al*. 1998; Singer *et al*. 1993). Autosomal recessive HGF is rare and often is the result of *de novo* mutations and is associated with other abnormalities (Goldblatt and Singer 1992; Hart *et al*. 1998; Singer *et al*. 1993). HGF inheritance via genome imprinting and maternal transmission (Zhu *et al*. 2007) has been linked to chromosome 11p15, which contains two imprinting clusters. The HGF phenotype has also been associated with chromosomal abnormalities involving chromosomal locations 7p, 8, and 14q22-q32 (Xiao *et al*. 2001).

While HGF has several modes of inheritance, the focus of research has been to elucidate the genes involved with the autosomal dominant forms of transmission. Autosomal dominant isolated HGF has been linked to three distinct chromosomal loci: HGF1 at 2p21-p22 (Hart *et al*. 1998; Xiao *et al*. 2001), HGF2 at 5q13-q22 (Xiao *et al*. 2001), and HGF3 at 2p22.3-p23.3 (Ye *et al*. 2005). The HGF1 locus was mapped through analysis of a Brazilian family and further refined to a 37cM genetic interval (Shashi *et al*. 1999). Later a single nucleotide insertion in codon 1,083 of the *Son of sevenless homolog 1* (*SOS1*) gene was found within the refined region and was proven to be the HGF1 causative mutation (Hart *et al*. 2002). HGF2 was mapped to the locus 5q13-q22 using samples from a four generation Chinese family (Xiao *et al*. 2001). This region contains 45 known genes including genes involved in calcium signalling mechanisms and pathways. These could be involved in one of the pharmacologic mechanisms causing the HGF phenotype (Xiao *et al*. 2001). Despite identifying the HGF2 locus, no genes have been identified as putative HGF2 genes. The third HGF locus at 2p22.3-p23.3 was identified in a five generation Chinese family, and two genes, GRP113 and SELI, were screened and eliminated as carrying HGF3 mutations (Ye *et al*. 2005). This locus was

further refined to a 6.56cM interval by Pampel *et al*. (2010). While no putative mutation has been determined, greater attention through sequence analysis has been placed on those genes in the region that are involved with the RAS/MAPK signalling pathway based on its involvement with SOS1 in HGF1 as well as its involvement with several syndromic forms of HGF.

Primary HGF: syndrome-associated HGF

HGF can occur as a part of a syndrome with high phenotypic variability (Haytac and Ozcelik 2007). For many syndromes the overall mode of inheritance is known but the specific mechanisms remain unknown (Table 1.1). Many of these syndromes have overlapping phenotypes and may represent a single common disorder with a spectrum of manifestations (Haytac and Ozcelik 2007). The most commonly associated presentation with HGF is hypertrichosis (Coletta and Graner 2006). This overgrowth of hair is reminiscent of the longer guard hairs present in the analogous silver fox version of this disease (Dyrendahl and Henricson 1960). In humans, this association can occur with or without mental retardation and has a dominant inheritance pattern (Coletta and Graner 2006). Haplotype analysis and linkage studies have demonstrated that when HGF with hypertrichosis occurs, it is not linked to HGF1 or HGF2, suggesting a distinct genetic form of HGF (Mangino *et al*. 2003) that needs to be characterized.

Hypertrichosis is defined as an abnormal excess of hair growth with either a generalized or localized distribution, that is neither primarily androgen-dependent

Table 1.1: General features, inheritance and age at onset of known syndromes where hereditary gingival fibromatosis can co-occur

Syndrome	Features (in addition to HGF)	Age of HGF onset	Mode of inheritance	Reference
Generalized gingival fibromatosis with hypertrichosis	Hypertrichosis with/without mental retardation	Within first year of life	Autosomal dominant	(Cuestas-Carnero and Bornancini 1988; Gorlin *et al*. 1990; Tay *et al*. 2001)
Epilepsy	Seizures with or without mental retardation	Age 5	Autosomal recessive	(Gorlin *et al*. 1990)
Murray-Puretic-Drescher syndrome	Multiple hyaline fibromas, white papules on the skin, flexion contractures, osteolytic lesions, recurrent infections and premature death	Within first year of life	Autosomal recessive	(Gorlin *et al*. 1990; Piattelli *et al*. 1996)
Rutherfurd syndrome	Corneal opacities and delayed tooth eruption	Not reported	Autosomal dominant	(Gorlin *et al*. 1990)
Zimmermann-Laband syndrome	Ear, nose abnormalities, hypoplastic changes in the terminal phalanges of the fingers and toes, hepatosplenomegaly	Birth	Autosomal dominant	(Gorlin *et al*. 1990; Holzhausen *et al*. 2003)
Cross syndrome	Microphthalmia, mental retardation, athetosis, hyperpigmentation	Not reported	Autosomal recessive	(Gorlin *et al*. 1990)
Ramon syndrome	Cherubic faces, delayed/ incomplete tooth eruption, narrow palate, mild retardation, epilepsy, stunted growth, hypertrichosis	Age 2	Autosomal recessive	(Gorlin *et al*. 1990)
GF and growth hormone deficiency	Growth retardation	Prior to age 8	Autosomal recessive	(Oikarinen *et al*. 1990)
Costello syndrome	Growth retardation, developmental delay, coarse face, decreased nasal bridge, full cheeks, thick lips, loose skin on hands and feet, deep palmer/plantar creases	Childhood	Autosomal dominant	(Hennekam 2003)
Jones syndrome	Progressive deafness	Not reported	Autosomal dominant	(Gorlin *et al*. 1990; Hartsfield *et al*. 1985)
Prune-belly syndrome	Facial dimorphism, abdominal defects	2 years	Autosomal recessive with sex influence	(Harrison *et al*. 1998)

nor influenced by race, sex or age (Beighton 1970). Estimates of the incidence of this rare condition in the early 1900s were judged to be "one in a million" but this is believed to be an underestimate, as it does not account for the incidence of hypertrichosis in association with numerous syndromes (Beighton 1970; Garcia-Cruz *et al*. 2002). There are three main kinds of hair: lanugo, vellus and terminal, each inserting into different places within the skin (Wendelin *et al*. 2003). Lanugo hair occurs in utero, disappearing shortly after birth consisting of a fine longer hair (Duggins and Trotter 1950). Vellus hair is an intermediate hair that is short, non-pigmented and covers the majority of the body after birth excluding the scalp and eyebrows. Terminal hair is wider, pigmented with a coarse quality (Stenn and Paus 2001). In normal development, vellus hair transforms into terminal hair as a part of an androgen surge during sexual maturity by mechanisms that are not well understood (Wendelin *et al*. 2003). One mechanism of hypertrichosis involves this hair transition but in places where it does not normally occur. Another mechanism involves a change in the hair follicle cycle, which consists of growth, apoptosis and shedding phases (Wendelin *et al*. 2003). Hypertrichosis results when the cycle has been altered and the hair spends more time in the growth phase (Paus and Cotsarelis 1999). A third mechanism involves an alteration in the density of the hair follicles (Wendelin *et al*. 2003). While these are the established mechanisms for hypertrichosis there may be other mechanisms and mechanisms may work concurrently in the same hypertrichosis presentation.

Hypertrichosis can be broadly split into either congenital or acquired categories. Each category is further subdivided based on localized or general hair distribution. The acquired category also has a pharmaceutically induced hypertrichosis category. This is of particular

interest as phenytoin and cyclosporine are known to cause hypertrichosis as well as to induce gingival hyperplasia (Wendelin *et al*. 2003). There is a wide range of frequency of hypertrichosis with these medications but the hypertrichosis resolves with discontinuation of the medication. The mechanisms involved are unknown.

Within the congenital hypertrichosis category, the subtypes of interest are those cases occurring with gingival hyperplasia. They occur in syndromes where hypertrichosis is the primary symptom, including congenital hypertrichosis lanuginosa and gingival fibromatosis with hypertrichosis. In addition, osteochondrodysplasia hypertrichosis occurs within this congenital category but typically does not present with gingival fibromatosis (Czeschik *et al*. 2012). Its associated spectrum of disease, however, does have cases with gingival fibromatosis, making it another subtype of interest here.

Congenital hypertrichosis lanuginosa is often referred to as Ambras syndrome (Freire-Maia *et al*. 1976). The main features of this form of hypertrichosis are the excessive growth of lanugo hair and dental abnormalities (Garcia-Cruz *et al*. 2002). The lanugo hair generally covers the body but often recedes over the trunk and limbs later in childhood (Wendelin *et al*. 2003). Typically the associated dental anomalies involve the delayed presentation of primary and secondary teeth as well as the absence of some teeth (Tadin *et al*. 2001). However, one sporadic case of hypertrichosis lanuginosa with mild gingival hyperplasia has been reported (Lee *et al*. 1993). Lee *et al*. (1993) commented on the association of hypertrichosis with congenital ectodermal or mesodermal defects, suggesting a common origin for both the gingival hyperplasia and the hypertrichosis as phenotypic variants of

the same defect. The majority of congenital hypertrichosis lanuginosa cases are the result of autosomal dominant inheritance but sporadic cases occur (Tadin *et al*. 2001). The chromosomal region for the autosomal dominant form of this disease has been narrowed to 8q22-q24 (Balducci *et al*. 1998; Tadin *et al*. 2001) but a specific causative gene mutation has not been determined.

Gingival fibromatosis with hypertrichosis presents as an autosomal dominant condition with terminal hair covering the face, trunk and eyebrow, with progressive gingival hyperplasia starting in childhood (Wendelin *et al*. 2003). Additionally, individuals can present with coarse facial features including a wide flat nose, thick lips and large ears, and, in 50% of cases, mental retardation (Garcia-Cruz *et al*. 2002; Wendelin *et al*. 2003). To assess genetic causation, copy number variation was explored by characterizing cases of microdeletions associated with the congenital hypertrichosis with terminal hair, with and without gingival fibromatosis (Sun *et al*. 2009). A significant association was found with chromosomal region 17q24.2-q24.3. This region contained three ATP binding cassette transporter family proteins (ABCA) and the mitogen-activated protein kinase kinase 6 (MAP2K6) gene. Conflicting reports both implicate and eliminate MAP2K6 involvement in this syndrome (Blyth *et al*. 2008, Sun *et al*. 2009). This suggests complex involvement of this chromosomal region, supported by the fact that other conditions with hypertrichosis have been linked to the same chromosome (Sun *et al*. 2009). Again, no specific genetic mutation has been elucidated.

Osteochondrodysplasia with hypertrichosis is an autosomal recessive condition that presents with generalized hypertrichosis, macrosomia and cardiomegaly. While typically this syndrome is not associated with gingival fibromatosis, it is thought to be apart of a spectrum of acromegaloid phenotypes, with or without hypertrichosis and gingival fibromatosis (Czeschik *et al*. 2012). For these conditions the putative mutation is in the ABCC9 gene, which encodes another member of the ATP binding cassette family of proteins, and is located at 12p12.1. The high homology within this family of proteins suggests the potential for the involvement of related proteins, encoded within the 17q24.2-q24.3 chromosomal region in gingival fibromatosis with hypertrichosis.

Secondary HGF: drug-induced gingival overgrowth

Three main categories of drugs have been extensively documented for inducing gingival overgrowth phenotypes: anticonvulsants, immunosuppressants, and calcium channel blockers (Marshall and Bartold 1999). Pharmaceuticals that induce gingival overgrowth in the anticonvulsant category include phenytoin, sodium valproate, phenobarbitone and vigabatrin (Marshall and Bartold 1999). Notably, for phenytoin an average of 50% of patients report gingival hyperplasia (Hallmon and Rossmann 1999). The major immunosuppressant drugs that induce gingival overgrowth are cyclosporine and the tacrolimus medications (Ellis *et al*. 2004; Marshall and Bartold 1999). The prevalence of the cyclosporine-induced phenotype is approximately 25%, with some reports suggesting a higher prevalence in children than adults (Hassell and Hefti 1991; Nakib and Ashrafi 2011). Calcium channel blockers are used in the management of many cardiovascular diseases including hypertension, angina, some arrhythmias and myocardial infarctions. The main

drug in this class is nifedipine, for which 20-83% of patients report gingival hyperplasia (Barclay *et al*. 1992).

This diverse group of systemic medications does share commonalities in clinical presentation and potentially pathogenesis. Clinical manifestations of gum disease typically appear within the first three months of starting the medication (Hallmon and Rossmann 1999). Growth appears from the interdental papillae and extends to the facial and lingual portions of the gingival margin (Nakib and Ashrafi 2011). Similar to the other forms of HGF in severe cases the gingival tissue will encapsulate the teeth. Broadly, the histology shows a fibrotic change in the connective tissue as well as changes in the extracellular matrix, with variation in the number of immune cells (Nakib and Ashrafi 2011). Management involves excellent oral hygiene care to minimize plaque, discontinuation of the medication, and in severe cases gingival resection (Marshall and Bartold 1999). In many cases stopping or reducing the dosage of medication does diminish the phenotypic presentation.

The pathogenesis for each category of medication-induced overgrowth is controversial and poorly understood. The major areas of investigation include collagen production/degradation, fibroblasts, and inflammatory processes. There is an imbalance in the synthesis and degradation of collagen in the drug-induced gingival overgrowths. Despite decreases in collagen production, there is an accumulation of collagen, suggesting a decrease in collagen breakdown (Correa *et al*. 2011). Collagen is degraded both extracellularly and intracellularly (Ogino *et al*. 2005). Extracellular collagen is digested by collagenase and other matrix metalloproteins (MMP) (Correa *et al*. 2011). With respect to

the degradation by collagenase, all three classes of pharmaceuticals known to induce gingival overgrowth share a common feature: they induce a decrease in the influx of calcium into the cell through the sodium calcium exchanger (Brunet *et al*. 1996). This limits the amount of folic acid taken in by the cells, limiting the amount of collagenase produced. The other extracellular pathway involves MMPs that are controlled by tissue inhibitors (TIMPs) (Correa *et al*. 2011). In phenytoin-induced overgrowth TIMP mRNA is significantly increased, leading to increased inhibition of the MMPs (Kato *et al*. 2005). The levels of MMPs are also decreased, further reducing collagen degradation (Kato *et al*. 2005; Serra *et al*. 2010). Intracellular degradation of collagen is also reduced as the result of a decrease in collagen-binding α-2-β-1-integrin receptors (Kato *et al*. 2005). Through these numerous mechanisms the level of collagen degradation is decreased in drug-induced gingival overgrowth.

Like collagen, fibroblast cells are in greater numbers in gingival overgrowth tissue. There is some evidence to suggest that this is the result of altered apoptosis (Kantarci *et al*. 2007). A decrease in the death rate of fibroblast cells causes their accumulation in the extracellular matrix.

Anticonvulsants, calcium channel blockers and immuno-suppressants act to control the production of inflammatory factors. Inflammatory factors that are increased in gingival overgrowth tissue include interleukins 1 and 6, platelet derived growth factor, fibroblast growth factor-2, transforming growth factor β and connective tissue growth factor (Correa *et al*. 2011; Ellis *et al*. 2004; Wright *et al*. 2006). Transforming growth factor β regulates

collagen production, which, in combination with connective tissue growth factor, induces fibrosis (Correa *et al*. 2011).

These groups of medications are quite diverse each plays a role in the pathogenesis of the drug-induced HGF phenotype. Gaining a better understanding of the molecular aetiology of HGF might in turn aid in testing for genetic susceptibility to these broad classes of pharmaceuticals in the future.

The RAS/ Mitogen activated protein kinase signalling pathway (MAPK)

The RAS/MAPK signalling pathway is critical for normal mammalian development and has many roles including influencing cellular growth, differentiation and senescence, with cellular proliferation being of most importance (Rauen 2013, Bezniakow *et al*. 2014). The RAS superfamily is comprised of proteins that function as molecular switches through their GTPase activity (Wennerberg *et al.* 2005). Activation occurs when GTP is attached to the molecule. Its function can be altered through guanine nucleotide exchange factors (GEF) as well as GTPase activation proteins (GAP). A GAP increases the efficiency of GTP hydrolysis to GDP. GEFs work by exchanging the inactive GDP for GTP thus activating the protein. MAPK signaling is based upon activation through a phosphorylation event (Rauen 2013). Signalling in this pathway starts on the outside of the cell and finishes in the nucleus (Figure 1.2). Signalling is initiated by growth factors, hormones, cytokines, insulin or immune cells. For example epidermal growth factor activates a tyrosine kinase receptor resulting in receptor dimerization and autophosphorylation. The activated receptor interacts with growth factor receptor bound protein 2, resulting in son of sevenless (SOS)

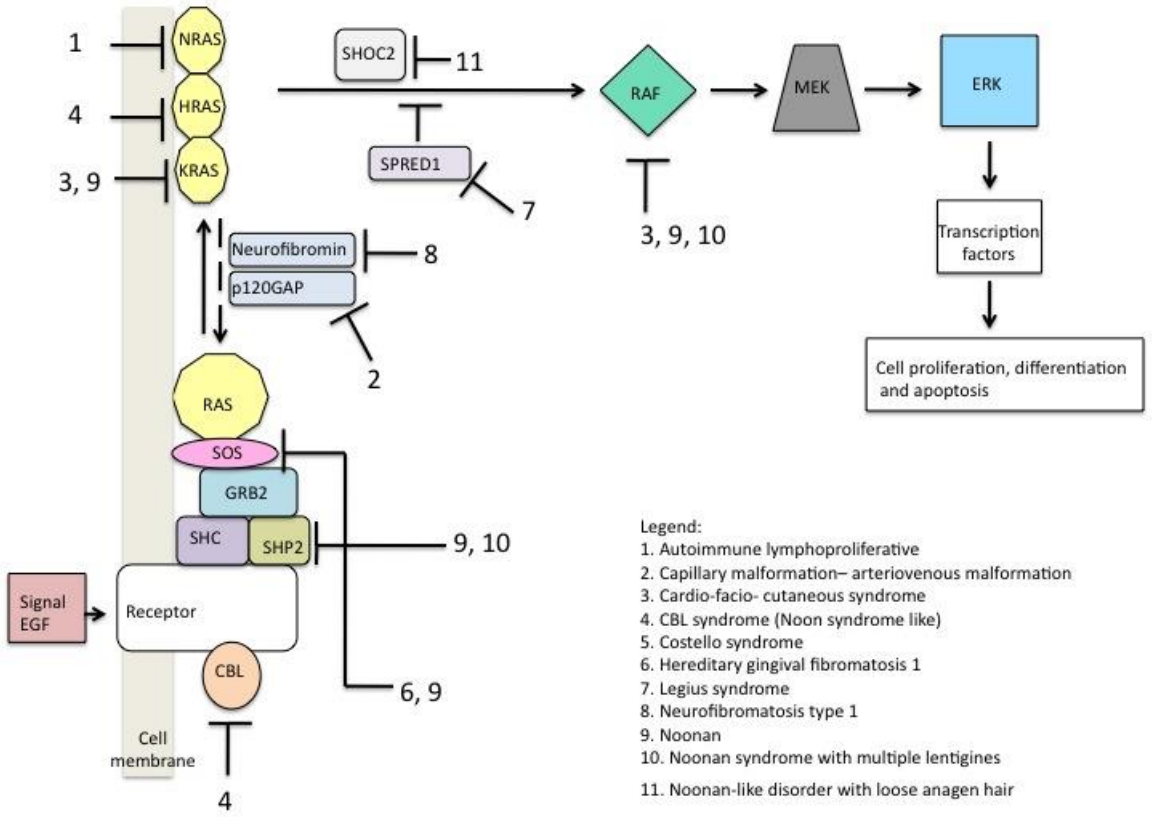

Figure 1.2: Diagram of several steps in the RAS/MAPK signalling pathway highlighting the proteins affected in various RASopthies. Adapted from Aoki and Matsubara 2013, Rauen 2013, Bezniakow *et al*. 2014, Tidyman and Rauen 2009

protein binding and recruitment to the membrane. SOS is a GEF and exchanges GDP with GTP bound to RAS. RAS proteins lead to a number of downstream pathways including the MAPK pathway. The MAPK signalling cascade follows a hierarchy of signalling from MAPK3K proteins which are also known as Raf family kinases to MAP2K, also known as the MEK kinases to MAPK, which are also known as the extracellular signal related kinases (ERK) (Bezniakow *et al*. 2014). Activated ERK proteins translocate into the nucleus and phosphorylate transcription factors, resulting in the change of gene expression.

Broadly, mutations can occur in either the germline or somatic cell lines (Tidyman and Rauen 2009). When a mutation occurs in a germline it occurs in cells that will be used in gamete formation and thus will be passed on to the subsequent generation (Rauen 2013). Conversely, when mutations are acquired in a somatic cell line they are not inherited by offspring (Rauen 2013). Twenty percent of malignancies are caused by somatic mutations in the RAS/MAPK signalling cascade (Tidyman and Rauen 2009). Germline mutations found in the RAS/MAPK signalling pathways result in developmental disorders and are classified as RASopathies (Rauen 2013) (Figure1.2). The specific disorders include: autoimmune lymphoproliferative, capillary malformation– arteriovenous malformation, cardio-facio- cutaneous syndrome, CBL syndrome (Noonan syndrome like), Costello syndrome, hereditary gingival fibromatosis 1, Legius syndrome, Neurofibromatosis type 1, Noonan, Noonan syndrome with multiple lentigines, and Noonan-like disorder with loose anagen hair (Table1.2) (Aoki and Matsubara 2013, Rauen 2013, Bezniakow *et al*.

Table 1.2: Syndrome, gene location, protein and inheritance pattern for RASopathies in humans. (Aoki and Matsubara 2013, Rauen 2013, Bezniakow *et al*. 2014, Tidyman and Rauen 2009)

Syndrome	**RAS/MAPK pathway gene**	**Protein**	**Chromosomal location**	**Inheritance**
Autoimmune lymphoproliferative	*NRAS*	NRAS	1p15.2	Autosomal dominant (AD)
Capillary malformation–arteriovenous malformation	*RASA1*	p120-RASGAP	5q14.3	AD
Cardio-facio-cutaneous syndrome	*BRAF* *MAP2K1* *MAP2K2* *KRAS*	BRAF MEK1 MEK2 KRAS	7q34 15q22.31 19p13.3 12p12.1	AD
CBL syndrome (Noon syndrome like)	*CBL*	CBL	11q23.3	AD
Costello syndrome	*HRAS*	HRAS	11p15.5	AD
Hereditary gingival fibromatosis 1	*SOS1*	SOS1	2p22.1	AD
Legius syndrome	*SPRED1*	SPRED1	15q14	AD
Neurofibromatosis type 1	*NF1*	Neurofibromin	17q11.2	AD
Noonan	*PTPN11* *SOS1* *RAF1* *KRAS* *NRAS*	SHP2 SOS1 CRAF KRAS NRAS	12q24.1 2p22.1 3p25.1 12p12.1 1p13.2	AD
Noonan syndrome with multiple lentigines	*PTPN11* *RAF1*	SHP2 RAF1/CRAF	12q24.1 3p25.1	AD
Noonan-like disorder with loose anagen hair	*SHOC2*	SHOC2	10q25	AD

2014, Tidyman and Rauen 2009). While each disorder has unique phenotypic features the group also has a spectrum of overlapping characteristics including: craniofacial dysmorphology, cardiac malformations and cutaneous, musculoskeletal and ocular abnormalities with varying degrees of neurocognitive impairment (Bezniakow *et al*. 2014). Collectively this group of RASopthies has a prevalence of one in 1000 individuals (Rauen 2013). Given the importance of the functions of this pathway modulation therapies including small molecule inhibition studies are currently under investigation (Rauen 2013).

Canine gingival disease

Canines can present with a spectrum of different gingival growths with infectious, inflammatory, benign and neoplastic aetiologies (Hale 2004). In addition, some breeds demonstrate a predisposition towards various kinds of growths. For example, the Collie, Great Dane, Dalmatian, Doberman and Boxer breeds show a higher propensity for generalized idiopathic gingival hyperplasia (Hale 2004). In particular, the Boxer breed is notorious for manifesting numerous types of tumourous growths including gingival hyperplasia and epulis. The presentation of canine gingival hyperplasia is comparable to that in foxes and humans.

Presentation

Broadly, gingival hyperplasia in the domestic dog (and also cat) is defined as an enlargement of the gingiva due to an increase in cell number that is not the result of an inflammatory process or the presence of a local irritation (Beckman 2010). In canines, the

gross appearance of gingival hyperplasia can be focal or generalized, manifesting as either a single, multiple or generalized growth (Beckman 2010; Hale 2004). The focal subtype appears as localized areas of redundant gingival tissue. There tends to be inflammation between these areas and the teeth. The histology is variable, ranging from islands of dystrophic calcifications to bony metaplasia (Hale 2004). The diagnosis is based on the histology of a biopsy sample.

Gingival hyperplasia has clinical implications for dogs. The growth itself is at risk for trauma from the opposing jaw. As the gingiva continues to grow the animal is no longer able to close its mouth and mastication is difficult and painful (Hale 2004). Given the force exerted by the tissue mass it can cause the underlying dentition to move. As the disease progresses, the gingiva expands both outwards and upwards along the teeth, engulfing them. This creates pseudo-pockets where bacteria can settle increasing the risk of infection, inflammation and periodontal disease. Periodontal disease then occurs secondary to the gingival hyperplasia, altering the clinical and histological appearance (Beckman 2010). Despite the extensive oral growth, there are no documented systemic symptoms associated with canine gingival hyperplasia.

Causation

Canine gingival hyperplasia can be divided into three categories: idiopathic, breed-predisposition, and drug-induced (Hale 2004). The aetiology for both the idiopathic and breed predisposition forms is not known. Similar to human HGF, three classes of drugs have been known to induce gingival hyperplasia: immuno-suppressants, calcium channel

blockers and anticonvulsant drugs. The commonality between these drugs is their alteration of calcium influx into the gingival cells.

Small dogs are prone to degenerative valvular heart disease as they age at which point calcium channel blockers like amlodipine are used to vasodilate blood vessels, decreasing peripheral resistance (Thomason *et al*. 2009). Thomason *et al*. (2009) demonstrated that 7/82 dogs developed gingival hyperplasia when placed on amlodipine. A minimum of five months on the drug was required before signs of growth were present. After discontinuation of amlodipine the gingival hyperplasia regressed partially within two to eight weeks with complete regression taking up to six months. This is not restricted to smaller canine breeds, as is evident by a case of gingival hyperplasia in a Great Dane on amlodipine (Pariser and Berdoulay 2011). The exact mechanism behind this drug-induced gingival hyperplasia is unclear. Some theories implicate testosterone, as calcium channel blockers result in the up-regulation of circulating androgens causing stimulation of the androgen sensitive gingiva (Pariser and Berdoulay 2011). This theory was further supported when castration of dogs with calcium channel blocker-induced gingival hyperplasia showed regression of the disease (Dayan *et al*. 1998). Other theories suggest that calcium channel blockers block other calcium-dependent enzymes involved in the apoptotic pathway, thus preventing cell death (Pariser and Berdoulay 2011). For this rare form of canine gingival hyperplasia, the underlying mechanism appears multifactorial and complex.

Immune-suppressing agents like cyclosporine are known to induce gingival hyperplasia in canines. The pathophysiology shows a link to calcium, similar to calcium channel blockers (Beckman 2010). A decrease in calcium levels induces a decrease in transglutinase, a protein that plays a role in regulating apoptosis. When transglutinase levels are reduced apoptosis is reduced, possibly contributing to the hyperplasia. A case was reported of cyclosporine-induced gingival hyperplasia in a long-haired Dachshund (Namikawa *et al*. 2012). Similar to other drug-induced forms of gingival hyperplasia, as the dose of the drug was reduced the hyperplasia regressed. The incidence of cyclosporine-induced gingival hyperplasia is anticipated to increase as the licensing of this compound for use in the pet population has increased.

Anticonvulsants such as phenytoin cause gingival hyperplasia in dogs, as well as cats (Beckman 2010). Little is known about these gingival growths as they have not been observed in rodent or other small-animals (Hassell and Hefti 1991). In felines, cells similar to fibroblasts are activated and thought to contribute to the gingival overgrowths (Beckman 2010). This mechanism may relate to the hypothesized role of TGFβ signalling in human HGF.

Treatment

Treatment is dependent on the cause of the disease. For the drug-induced phenotype stopping the medication that led to the induction of gingival overgrowth can reverse the phenotype. There is no curative treatment for idiopathic canine gingival hyperplasia. Severe disease can be managed with resection but the gingiva will continue to grow.

A canine disease model

Overall, the gingival disease in the canine is quite similar to human HGF. This allows the dog to be an excellent bridging point for research into HHG in foxes.

Publication of the domestic dog genome in 2005 greatly facilitates genomic research with phylogenetically related species such as the fox (Kohn *et al*. 2006; Lindblad-Toh *et al*. 2005). *Vulpes vulpes* and *Canis lupus familiaris* are both members of the mammalian family Canidae, and last shared a common ancestor 12 to 15 million years ago (Wayne *et al*. 1997). Karyotyping, cytogenic genome markers, and gene sequences have been examined in the red fox using the canine genome for comparison (Switonski *et al*. 2009). There is strong evidence supporting the close relationship between the two species. For instance, a 97.3% similarity between the red fox and dog coding sequences for the *SRY* (sex determining region Y) gene (Nowacka-Woszuk and Switonski 2009; Switonski *et al*. 2009). The dog genome has proven to be a useful model system for human disease as a potential bridge between human and mouse genetic studies as mice have a higher rate of evolution than humans creating more sequence divergence (Boyko 2011). For both foxes and humans, the dog has proven to be a suitable model for research.

The Canidae group consists of four groups: fox-like canids; wolf-like canids; South American canids; and island foxes (Lindblad-Toh *et al*. 2005). Canidae underwent rapid speciation resulting in the rearrangement of their chromosomes (Becker *et al*. 2011). The domestic dog (*Canis lupus familiaris*), a wolf-like canid, has 78 diploid chromosomes while

the red fox (*Vulpes vulpes*), a fox-like canid, has 34 (Becker *et al*. 2011). Mapping of the red fox genome began in the 1980s (Switonski *et al*. 2009). Early work established the chromosome assignment of numerous gene loci on most of the autosomes and fine mapping continued with canine microsatellite markers and aligned them with its canine counterpart (Kukekova *et al*. 2004). After microsatellite mapping was completed a linkage map of various fox pedigrees was constructed (Kukekova *et al*. 2011; Switonski *et al*. 2009). Once the framework for fox genomics was established, it opened the gates for many types of research including disease models, functional genetics, and behavioral studies.

Goals and outline of thesis (Figure 1.3)

Hereditary hyperplastic gingivitis is a progressive growth of gingival tissues in foxes resulting in dental encapsulation (Dyrendahl and Henricson 1960). Genetically, it is an autosomal recessive condition displaying a sex-biased penetrance, with an association with superior fur quality. This disease has been primarily described in European farmed foxes. **Chapter 2** documents the emergence of HHG in Canada. The goal was to perform a detailed examination of the history of HHG including what is known about its genetic aetiology and histology in Europe, and then extend this examination to the emergence of HHG in Newfoundland and Labrador again with respect to both the genetics and histology of the condition.

HHG and HGF are analogous conditions with very similar manifestations and disease progression. The known molecular basis of one form of HGF, the goal of **chapter 3** was to

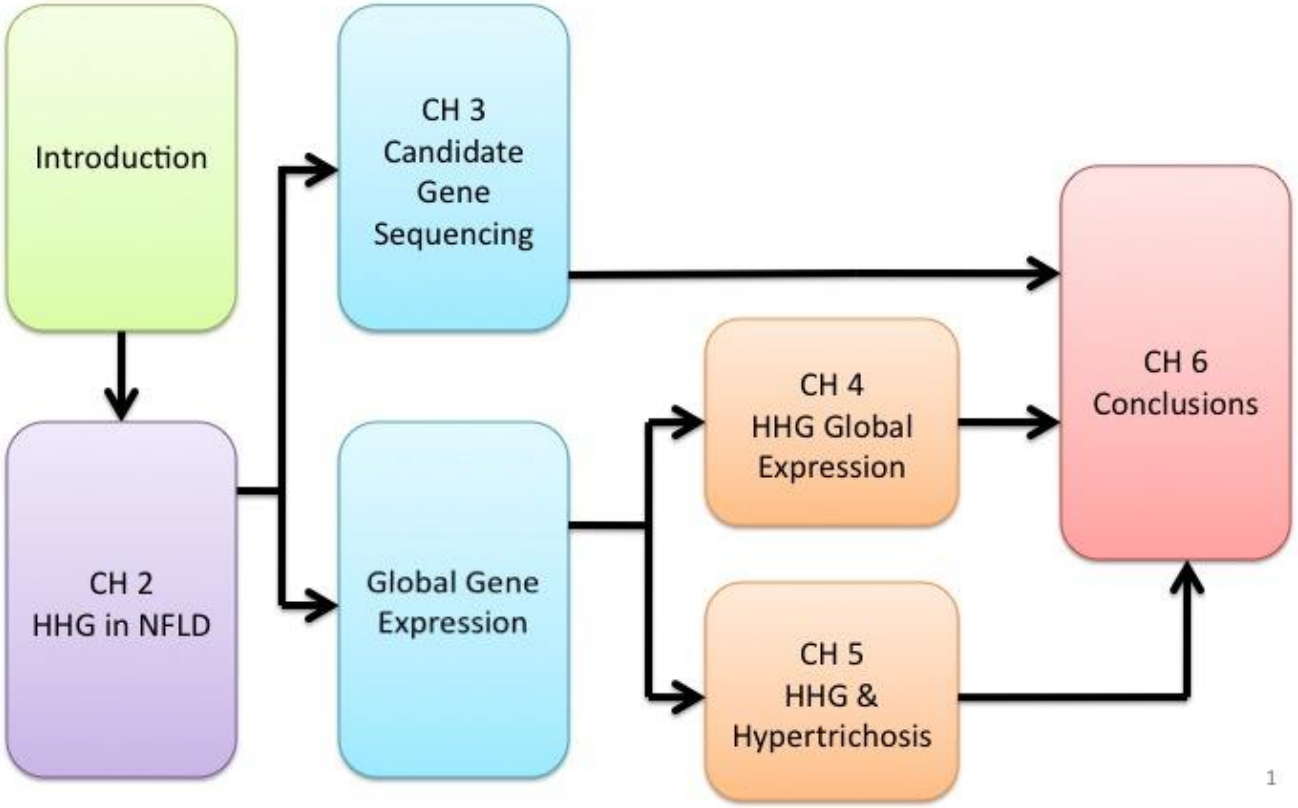

Figure 1.3: Schematic overview of thesis chapters

perform functional candidate gene analysis as a means to establish or eliminate particular gene mutations as causative of HHG in foxes. The premise of this functional candidate gene approach was the implication that a mutation causative of a disease in one organism may be the causative mutation of the analogous condition in another organism (Aguirre-Hernandez and Sargan 2005). The DNA sequences of the exonic coding regions, exon-intron boundaries and partial introns of *SOS1*, the known causative gene in HGF1 in humans, the closely-associated *GRB2,* and *EGFR* genes from both affected ranched silver foxes and unaffected wild red foxes were compared to determine if any fixed difference exist. Inferences were also made about the rates and patterns of evolution of the *SOS1, GRB2* and *EGFR* genes to assess their likelihood of association with disease.

A comprehensive examination of the genetic aetiology of HHG requires a multi-tier strategy. In addition to investigating specific candidate genes, examining global gene expression levels can provide additional insight. The goal of **chapter 4** of this thesis was to integrate the current knowledge of HGF with access to genomic technologies based on the dog genome to examine potential pathways involved in silver fox HHG. This was conducted using the Affymetrix GeneChip Canine Genome 2.0 Array to compare the global gene expression patterns of HHG-affected farmed silver foxes and HHG-unaffected silver foxes. This section of the thesis explored the many potential factors contributing to the HHG phenotype by reviewing the HGF literature and comparing it to the HHG genome expression, as well as looking for any new insights.

HHG can occur as an isolated genetic condition or as part of a syndrome, which most often presents with hypertrichosis. The HGF-associated syndrome with hypertrichosis is similar in presentation to the HHG-affected foxes with longer and denser fur. Exploring the connection between hypertrichosis and HGF could provide further insight into the molecular mechanisms of HHG. In **chapter 5** of this thesis the goal was to combine the current knowledge of HGF-associated syndromes with hypertrichosis with the genome-enabling capabilities of the dog genome to examine potential pathways involved in silver fox HHG.

HHG and its analogous condition, HGF, are heterogeneous conditions with potentially complex molecular mechanisms. To gain a better understanding of the underlying aetiology of each a cooperative, multi-faceted examination is required. Examining HHG in light of known HGF information at both the gene and genome level creates a platform to accomplish this goal.

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Chapter two:
Index case report of hereditary hyperplastic gingivitis in North American farmed silver fox, *Vulpes vulpes*

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Co-authorship statement

The following is to provide clarification of the roles played by multiple co-authors in the manuscript published from chapter two. As per the guidelines set forth by the School of Graduate Studies my contributions as thesis author are divided into the following areas: *i) design and identification of the research proposal; ii) practical aspects of the research; iii) data analysis* and *iv) manuscript preparation*.

i) *Design and identification of the research proposal*

Given the lack of documentation of this fox gum disease in North America, Jo-Anna B.J. Clark conceived the idea of the case report developed in this chapter. All background research as well as reviewing all communications regarding this disease was completed by Jo-Anna B.J. Clark, as was the writing of the initial draft of the manuscript. Robert Hudson also initiated a staging system to describe the extent of gingival overgrowth.

ii) *Practical aspects of the research*

Jo-Anna B.J. Clark was present on numerous trips to the fox farm for documentation, staging, image collection and pedigree assessment.

iii) *Data analysis*

Not applicable to this chapter

iv) *Manuscript preparation*

Jo-Anna B.J. Clark prepared the initial draft of the report. H. Dawn Marshall and Robert Hudson provided critical review and editing.

Hereditary hyperplastic gingivitis (HHG) presents as a tumour-like growth of the gingival tissue on the mandible and maxilla in farmed silver foxes (Dyrendahl and Henricson 1960), a coat colour variant of the American subspecies (*Vulpes vulpes fulva*) of the red fox (*Vulpes vulpes*). It is an autosomal recessive inherited disease, with predominance in males, and is correlated with superior fur quality (Dyrendahl and Henricson 1960). Until 2008, HHG had only been described in the farmed fox population, at which time a case in a wild red fox was reported in Germany (Schulze et al. 2008). In Canada and the rest of North America, this condition has never previously been documented in the fox population. This chapter will review the origins of HHG and detail its discovery and manifestation in farmed silver foxes in Newfoundland, Canada.

Fox farming in Canada originated in the late 1800s in Prince Edward Island (PEI), with the successful raising of wild silver foxes in captivity and the pioneering of selective breeding in foxes, resulting in the first commercial production of silver fox pelts. The interest in this profitable new industry led to national distribution of breeding stock and exportation to the United States and Europe (Canada 1979; Colpitts 1997). The European fur farming industry began with the importation of two silver foxes from Canada into Norway in 1914. Norway went on to pioneer numerous silver fox colour mutations. The first documented case of HHG in Europe was in Sweden around 1940 in a young male fox with superior fur quality that had been imported from Norway (Dyrendahl and Henricson 1960).

Since the 1940s there have been two main aetiologies described for the silver fox gingival overgrowth. K.E. Kull, Swedish veterinarian, described it as a recessive sex-biased disease, more common in males than in females, where disease phenotype is induced by a viral infection (R. Hudson, personal communication, 2004). This theory was reiterated in the 1982 publication of Kangas' book entitled "Kettujen ja supikoirien sairaudet" (The Diseases of Foxes and Raccoon Dogs) (Kangas 1982). In 1960, Dyrendahl and Henricson published an extensive review of HHG (Dyrendahl and Henricson 1960). They stated that pronounced manifestation of HHG occurs at approximately two to three years of age, with gingival proliferation continuing throughout life, resulting in dental encapsulation. They concluded that HHG was an autosomal recessive disease demonstrating incomplete penetrance, with an increased incidence in males relative to females (Dyrendahl and Henricson 1960). In addition, there was a pleiotropic relationship between HHG and high fur quality (typically length and density of the guard hairs) (Dyrendahl and Henricson 1960). Pathology indicated large epithelial extensions in the keratinized collagen of the gingival tissue. Schulze et al. documented the first case of HHG in the wild fox population. In this case, both macroscopic and histopathological examination confirmed the diagnosis of HHG rather than a viral induced papillomatous growth (Schulze et al. 2008). Meanwhile, despite the rapid expansion using a limited gene pool, the Canadian stock had not shown any signs of HHG before 2004.

In Newfoundland and Labrador, twenty male and twenty female, ten-month-old silver foxes were imported from Finland in January 2003. By May 2004, five of the

male Finnish imported foxes were manifesting gingival proliferation. The extent of the gingival involvement varied with one fox displaying complete encapsulation of the incisors with symmetrical coverage extending back to the molars on both arcades of the maxilla and mandible. Neither the Finnish females nor any Canadian foxes housed in the same shed had any obvious signs of gingival proliferation. All foxes were fed a relatively soft diet of commercial fox pellets softened with water and mixed with ground chicken. All foxes appeared healthy and were in good body condition.

In June 2004, all imported Finnish foxes underwent thorough manual oral examinations performed by a provincial veterinarian. Six males were severely affected with gum tissue hyperplasia and encapsulated teeth; twelve had mild to moderate hyperplasia and two were unaffected. Seventeen of the twenty females examined showed mild to moderate gum hyperplasia that was more pronounced around the molars and premolars than the incisors. In addition, a one -year-old male from a mating of a Finnish male to a Canadian female in 2003 clearly showed early signs of the disease. Re-examination in December 2004 demonstrated rapid progression of the hyperplasia. Severe cases also showed gingivitis, plaque covered teeth, and irregular positioning of some teeth. Due to animal welfare concerns and the genetic implications of this disease, the severely affected animals were removed from breeding stock and pelted. In subsequent breeding seasons, the disease continued to be evident in offspring from crosses between remaining original foxes

and from crosses with Canadian foxes. It was not present in every generation nor did it progress as rapidly, or developed as severely, as in the original Finnish foxes.

Two additional trips were made to Norway to obtain fox breeding stock. In December 2004, thirty Norwegian males and thirty females were selected. Individual clinical examinations of prospective candidates, as well as familial examinations dating back two subsequent generations, were performed to ascertain whether the disease was present in their pedigree. These animals arrived in January 2005 and did not develop any signs of hyperplasia during the first year after importation but it became evident in a few, as they grew older. It was also evident in subsequent generations suggesting many of the original imports were non-symptomatic carriers. The final trip was to select thirty male Norwegian foxes with rare silver fox colour mutations. They were selected in December 2005 and arrived in January 2006. Again, meticulous examinations were performed on all possible candidates for acquisition but fewer familial connections were available for examination. Several of these males did show HHG within the first year after importation and many of their offspring mated with the previously imported Norwegian silver foxes did go on to manifest gingival hyperplasia.

Gross examination of the affected silver fox gingival tissue identified proliferative gingival tissue with a red granular surface (Figure 2.1). A grading system from zero (normal gingival tissue) to five (most severe gingival hyperplasia) was developed to

A.

B.

C.

Figure 2.1: A. An original Finnish silver fox gingival overgrowth upon full examination in 2004. B. An original Finnish silver fox post-mortem demonstrating gingival overgrowth resulting in dental rearrangement. C. An unaffected Canadian silver fox (Photographs provided by Robert Hudson)

quantify the degree of gingival hyperplasia (Figure 2.2). The tissue bled easily and profusely with minimal trauma to which the animals appeared insensitive. Surgical biopsy samples contained a rich vascular fibrous tissue that was tough and resilient when cut below the mucosa. In all affected gingiva, the hyperplastic tissue occurred outward along the lingual and buccal aspects, and then extended up or down, covering a variable amount of the crowns of the teeth. The gingival hyperplasia was initially bilaterally symmetrical, but often progressed to surround some teeth more than others. In moderate to advanced cases, the incisors were completely buried in the convergence of hyperplasic tissue from both the lingual and buccal sides. In advanced cases, the proliferative gingiva on the buccal side formed large multilobular, cauliflower-like masses that often completely obscured the teeth. Post mortem examination of the severely affected fox heads obtained after pelting in 2004 showed the hyperplastic gum tissue to be pale, lacking firm consistency with occasional areas of necrosis in the flattened multilobulated masses. Deep pockets containing food and debris were present between large gingival tissue flaps and the teeth on both the buccal and lingual sides. This provided the environment for premature periodontal disease and may explain irregular teeth placement and asymmetrical dental arcades noticed in the advanced cases.

Histological sections were taken from a variety of fox gingival samples, representing a range of gingival proliferation from normal to severe. In all affected samples, thick collagen bundles, a few fibroblast cells and small blood vessels expanded the submucosa (Figure 2.3). The degree of submucosal expansion ranged from mild to

A

B

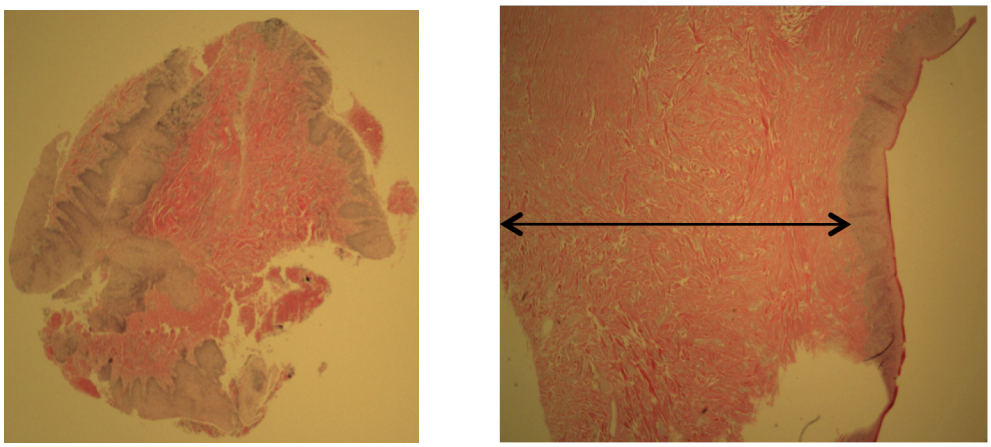
C

D

E

F

Figure 2.2: Silver fox gingival hyperplasia grading system
A. Grade 0 (normal): The gingiva is pale pink, smooth and relatively flat.
B. Grade 1: A thin layer of gingival tissue at its margin with the crown of the tooth is slightly raised, red and granular. There may be enlargement of the interdental papillae.
C. Grade 2: A somewhat thicker layer of the gingiva is moderately rounded, mildly thickened, red and covers up to approximately a third of the crown of at least some teeth. Most times it is bilaterally symmetrical at this stage, affects both upper and lower jaws equally, and is clearly evident on both the buccal and lingual gingival as raised red tissue. At this stage all teeth are still clearly visible.
D. Grade 3: The gingiva is diffusely raised, thickened, red and rounded and covers a half to almost all of the crowns of most teeth. The tips of the crown of the teeth are still visible in most cases, but the development of large flaps and nodules are now clearly visible and there may be total encapsulation of some teeth, but the majority of the tips of the crowns are still visible.
E. Grade 4: The dental arcade is almost completely covered by diffusely thickened, red and proliferative gingival tissue, forming confluent multi-nodular masses with large lateral flaps resulting in numerous teeth becoming encapsulated. The tips of the crowns of a few teeth are visible. By this stage the canine teeth are at least 50% encapsulated
F. Grade 5: The dental arcade is completely covered by large cauliflower like masses of red, friable granular tissue. Only a few teeth are visible and at least three quarters of the canine teeth are buried in the masses. Massive gum proliferation is evident everywhere.
(Photos provided by Robert Hudson)



A. B.

Figure 2.3: Histology slides viewed at 25X magnification of hereditary hyperplastic gingivitis (HHG) silver fox gingival samples. A. Histological presentation of mild HHG. B. Histological presentation of severe HHG. Arrow demonstrating the thickness of the submucosa. (Photographs provided by Laura Rogers)

marked, corresponding to the degree of gingival proliferation. In all affected histology sections, the mucosa was undermined by moderate numbers of lymphocytes and plasma cells whereas the normal gingival sections contained minimal numbers of lymphocytes and plasma cells. Based on histology the diagnosis was fibrous gingival hyperplasia ranging in severity from mild to marked.

The Newfoundland and Labrador fox farming industry underwent rapid expansion during the 1980s with the addition of many new farms and increases in the size of existing farms. Despite the increased inbreeding that resulted from the demand for more breeding stock, there was no evidence of HHG in the Newfoundland and Labrador farmed fox population until after the introduction of Scandinavian foxes in 2003. Similarly in the maritime provinces and the rest of Canada, a century of breeding silver foxes from the original PEI stock, as well as cross breeding with animals imported from Scandinavia, including colour mutations, had not resulted in any documented cases of HHG prior to 2003. Histologically, the presence of expanded submucosa due to increased collagen in the 2004-2006 cases in Newfoundland were congruent with both the original HHG description and the HHG case in a wild fox, supporting the HHG diagnosis (Dyrendahl and Henricson 1960; Schulze et al. 2008). Original HHG descriptions suggest an autosomal recessive mode of inheritance. Prior to the Scandinavian importation of foxes to Newfoundland and Labrador in 2003, this disease was not documented in Canada. In addition, since 2004 the disease has not been present in every subsequent

generation from imported foxes, suggesting a complex process behind the development of HHG.

This disease impacts both economics and animal welfare. As this disease appears more often when selecting for superior fur quality in affected populations, it has the potential for high propagation and wide dissemination. As the aetiology is genetic, the only prevention of further propagation of the disease is cessation of breeding of the affected animals and potential carriers of the genes for this disease. Also, affected animals often have to be culled prematurely before living out a normal productive life and this can have an enormous economic impact on the fox farming industry. This disease may come with a high cost as animal welfare concerns are being heightened globally. Hereditary hyperplastic gingivitis also has the potential to affect wild populations creating a potential ecosystem health concern, if affected or carrier animals inadvertently escape from captivity and breed with indigenous populations. The industry would benefit from a thorough understanding of the genetics of this disease as well as a commercial genetic test that could be used to help farmers select stock that are free of the undesirable gene.

The disease can still be found today in the offspring of the original imported foxes. The next step in understanding this disease is to investigate its underlying genetic mechanisms. This work is currently ongoing, utilizing co-operative strategies such as candidate gene sequencing and whole genome microarray expression techniques based on canine genome resources.

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We gratefully acknowledge the late Dr. Bruce Hunter, Ontario Veterinary College, University of Guelph, for his careful original histological examinations and consultations regarding the presentation of this disease in Newfoundland and for making us aware of the Dyrendahl and Henricson study. We also thank Dr. Laura Rogers, Animal Health Division, Department of Natural Resources, Government of Newfoundland and Labrador, for pathological examination of the additional histological slides of both the HHG affected and HHG unaffected samples, and Dr. Hugh G. Whitney, also with the Animal Health Division for helpful discussion. We acknowledge Merv Wiseman for bringing this issue to our attention and providing us samples of affected fox and for the many opportunities he gave us to observe and examine foxes on the farm as well as review his breeding records.

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Schulze C, Bensch M, Winterhoff N, Ansorge H, Teifke JP (2008) [Gingival fibromatosis (hereditary hyperplastic gingivitis) in a wild European red fox (*Vulpes vulpes*)]. Dtsch Tierarztl Wochenschr 115, 471-474

Chapter three:
Sequence analysis of the RAS-MAPK pathway genes *SOS1; EGFR* & *GRB2* in silver foxes (*Vulpes vulpes*): candidate genes for hereditary hyperplastic gingivitis

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Co-authorship statement

The following is to provide clarification of the roles played by multiple co-authors in the manuscript published from chapter three. As per the guidelines set forth by the School of Graduate Studies my contributions as thesis author are divided into the following areas: *i) design and identification of the research proposal; ii) practical aspects of the research; iii) data analysis;* and *iv) manuscript preparation*.

i) *Design and identification of the research proposal*

The fox candidate gene sequencing study commenced looking at a single gene (*SOS1*) with an initial draft sequence obtained by Honours student, Sara J. Tully, under the supervision of H. Dawn Marshall. Jo-Anna B.J. Clark extended the candidate gene sequencing concept to include additional functional candidate genes which she selected from the literature.

ii) *Practical aspects of the research*

Honours student Sara J. Tully obtained an initial draft *SOS1* gene sequence for a sample of affected and wild foxes. Jo-Anna B.J. Clark designed new primers and re-sequenced *SOS1* and included a larger cohort of samples. Primer design, PCR amplification, and DNA sequencing of additional candidate genes was completed by Jo-Anna B.J. Clark.

iii) *Data analysis*

Sequence analysis, including sequence editing, alignment, and detection of mutations and polymorphisms, of all candidate genes was performed by Jo-Anna B.J. Clark. Molecular tests of evolution were conducted in consultation with H. Dawn Marshall.

iv) *Manuscript preparation*

Jo-Anna B.J. Clark prepared the manuscript with critical review and editing from H. Dawn Marshall.

Introduction

Hereditary hyperplastic gingivitis (HHG) is a rare disease of the oral cavity that occurs primarily in captive silver foxes (a coat colour variant of the red fox, *Vulpes vulpes*), and is characterized by progressive proliferation of the gingival tissues starting at two to three years of age. The fibrous overgrowths eventually lead to encapsulation of the teeth and inhibition of normal function. HHG is inherited in an autosomal recessive pattern, with sex-biased penetrance favouring males over females (Dyrendahl and Henricson 1960). HHG frequently co-occurs with superior fur quality (length and thickness of guard hairs) suggesting the possibility that a pleiotropic gene is responsible for both phenotypes. Its pathology indicates large epithelial extensions in the keratinized collagen of the gingival tissue. In 2004 HHG was first documented in Newfoundland and Labrador, Canada, in a farmed fox population after importation of European silver fox lines (Clark *et al*. 2015). Gross examination demonstrated proliferative gingival tissue containing a red granular surface. The histology demonstrated thick collagen bundles. These results were in keeping with both the original Dyrendahl and Henricson report as well as with those reported by Schulze *et al*. (2008), who documented the first case of HHG in a wild fox (Clark *et al*. 2015; Dyrendahl and Henricson 1960; Schulze *et al*. 2008). However, despite initial documentation, the underlying genetics and cellular mechanisms causing HHG remain unknown.

An analogous condition that affects humans is hereditary gingival fibromatosis (HGF). HGF typically manifests with the onset of permanent dentition and results in

slow, progressive, benign fibrous enlargements of the maxillary and mandibular keratinized gingival tissue (Hart *et al*. 2002; Ye *et al*. 2005). HGF is both functionally and aesthetically problematic. Treatment involves quadrant-by-quadrant gingivectomy, but there is a common recurrence of the overgrowth (Ramer *et al*. 1996). The majority of HGF cases show autosomal dominant inheritance, with autosomal recessive cases occurring rarely (Goldblatt and Singer 1992; Shashi *et al*. 1999). HGF is a genetically heterogeneous disease that can occur as an isolated condition, part of an underlying syndrome or chromosomal abnormality, or in a non-hereditary form where it occurs in association with certain pharmacological agents (Shashi *et al*. 1999). Linkage analysis of isolated cases has mapped HGF to three different chromosomal locations: 2p21-p22, 5q13-q22 and 2p22.3-p23.3 (Hart *et al*. 2002; Ye *et al*. 2005). At 2p21-p22, the causative mutation is a single nucleotide insertion in codon 1083 of the *Son of sevenless homolog 1* (*SOS1*) gene (Hart *et al*. 2002). This causes a frameshift mutation, leading to the premature truncation of the C-terminal domain and resulting in constitutive activation of its downstream products. Within the 5q13-q22 loci the RAS pathway genes *G protein coupled receptor 113 (GPR113)* and *ethanolaminephosphotransferase 1* (*SELI*) have been screened with no reported causative mutations (Ye *et al*. 2005). The 2p22.3-p23.3 locus has recently been refined to a 6.56cM region (Pampel *et al*. 2010), within which no causative mutations have been reported.

HHG and HGF are analogous conditions with very similar manifestations and disease progression. A family has been reported that displays hypertrichosis

(excessive hair growth) in addition to the gingival overgrowth (Mangino *et al*. 2003), which may be reminiscent of the dense fur of HHG-affected silver foxes. Given the strong similarity between the human and fox diseases and the known molecular basis of one form of HGF, our goal was to perform functional candidate gene analysis as a means to establish or eliminate particular gene mutations as causative of HHG in foxes. The premise of this functional candidate gene approach is the implication that a mutation or gene associated with a disease in one organism may be associated with the analogous condition in another (Aguirre-Hernandez and Sargan 2005). Additionally, candidate genes can be identified on the basis that disruption of their predicted gene function may be expected to result in the disease phenotype.

Here we report and compare the DNA sequences of three candidate genes for HHG in silver foxes: *SOS1* (KP099615), where the single known causative mutation of HGF is located, and the closely-associated *Growth factor receptor bound 2 protein* (*GRB2)* (KP099613) and *Epidermal growth factor receptor* (*EGFR*) (KP099614) genes. These three genes encode components of the RAS cell signalling pathway. The pathway is initiated by ligand binding to the epidermal growth factor receptor (EGFR) which results in activation of its tyrosine receptor kinase, which then activates Growth factor receptor bound 2 protein (GRB2) (Qian *et al*. 2000). GRB2 sequesters SOS1 to the cell membrane, which binds to form a GRB2-SOS1 complex. Then SOS1 activates RAS via its guanine nucleotide exchange factor function. Once initiated, RAS activates numerous pathways involved in the cell cycle, cell migration

and cell proliferation. To perform candidate gene analysis, exonic coding regions, exon-intron boundaries, and partial introns of these three genes were sequenced from both affected ranched silver foxes and unaffected wild and farmed red foxes and compared. To further interpret any candidate mutations identified, we also examine rates and patterns of molecular evolution in *SOS1, GRB2* and *EGFR* genes among mammals.

Materials and Methods

Sample collection and DNA extraction

In 2004, provincial government veterinarian, Robert Hudson, on a fox farm in Newfoundland and Labrador, Canada, first documented the presence of HHG (Figure 3.1). It was determined that the affected silver foxes were either original Finnish silver foxes imported for their superior fur quality or first generation descendants of a cross between the new Finnish silver fox males with the Canadian silver fox females (Clark *et al*. 2015).

HHG-affected and unaffected silver foxes were obtained from the same Newfoundland fox farm. Provincial government veterinarians examined these animals and after gross manual examination determined the presence or absence of HHG. HHG showed phenotypic variation in severity, with diagnosis being made in the early stages of the disease when there was the presence of a thin layer of gingival tissue at the dental margin with the crown of the tooth that was slightly raised, red and granular (Hudson 2014). Provincial government veterinarians

A B

Figure 3.1: A. Silver fox with no gingival overgrowth in 2004. B. Original Finnish Silver fox with gingival overgrowth documented in 2004 (Photos provided by Robert Hudson)

anesthetized these animals and small gingival sections were collected. Additionally, gingival and skeletal muscle samples were obtained from both HHG-affected and unaffected foxes post-mortem during pelting season. The Newfoundland and Labrador Department of Environment and Conservation Wildlife Division and Department of Natural Resources Animal Health Division provided wild unaffected red fox samples. The wild unaffected foxes from the Newfoundland and Labrador Department of Environment and Conservation Wildlife Division were captured from the area surrounding the St. John's International Airport and skeletal muscle was sampled. Animal Health Division unaffected foxes were obtained during a rabies eradication program where salivary glands were sampled. All sampling was compliant with Canadian Council on Animal Care regulations, and approved by the Memorial University Institutional Animal Care Committee.

DNA extractions were performed with the QiaAmp DNA Mini kit (Qiagen Inc., Mississauga, Canada) according to the manufacturer's tissue protocol.

Primer design

Primers were designed using *Canis lupus familiaris* sequences with GenBank Accession Numbers NC_006600.3, NC_006591.3, and NC_006599.3, as references for *EGFR* found on chromosome 18, *GRB2* found on chromosome 9 and *SOS1* found on chromosome 17, respectively, according to their reported sequences from 2008-2011. Primer pairs were selected from the intronic regions flanking each target region using either Primer3 (Rozen and Skaletsky 2000) or Oligo 4.1 (Molecular

Biology Insights, Inc., Cascade, USA). The following criteria were used to choose primer pairs in Primer3: 23-29 bp length; 20-80% GC content; primer Tm range 57-63°C; and expected amplicon size 700-800 bp. The following criteria were used to choose primer pairs using Oligo 4.1: 20-22 bp length; 40-60% GC content; Tm difference for the pair less than 12°C; expected amplicon size 128-1500 bp; optimal annealing temperature 50-57°C. Some primers were tailed with standard M13 forward or reverse sequences for subsequent sequencing. Primers were manufactured by Operon (Huntsville, USA).

Polymerase Chain Reaction (PCR) and DNA sequencing

Each PCR contained 2.5 μL 10X buffer, 0.5 μL dNTPs (New England Biolabs Ltd., Whitby, Canada), 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 0.2 μL Hot Star Taq Plus (Qiagen Inc., Mississauga, Canada), 19 μL distilled water and 1 μL template DNA. The thermal profile used was 95°C for 15 minutes, followed by 40 cycles of 93°C for 30 seconds, target-specific annealing temperature for 30 seconds, and 72°C for 2 minutes, followed by 72°C for 6 minutes. Amplified PCR products were purified using either Pall Life Sciences Multi-Well Plate Manifolds (Pall Corporation, Port Washington, USA) or the QIAquick PCR purification kit (Qiagen Inc., Mississauga, Canada). Target-specific annealing temperatures were as recommended by the primer selection software. For detailed primer pair sequences, annealing temperatures and amplicon lengths refer to Appendix 1.

DNA sequencing reactions were performed with both forward and reverse primers for each PCR amplicon using BigDye Terminator v3.0 chemistry (Applied Biosystems Inc., Foster City, USA) utilizing the following thermal profile: 96°C for 6 minutes, then 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes. Sequencing reaction purification was carried out using either ethanol precipitation or an Agencourt CleanSeq method (Beckman Coulter Inc., Danvers, USA). Purified DNA sequencing reactions were electrophoresed on the Applied Biosystems Inc. 3730 DNA Analyzer, in the GaP Facility of the CREAIT Network at Memorial University of Newfoundland.

Data analysis

Raw data was collected using Sequence Analysis v5.2 (Applied Biosystems Inc., Foster City, USA) and imported into Sequencher v4.8 (Gene Codes Corp., Ann Arbor, USA). Contigs were created by assembling reads to the reference sequence using an 85% minimum gap percentage and a 20% minimum overlap, followed by manual trimming and editing of sequence each read. For each gene individual foxes with maximum sequence data were used for comparisons. Consensus sequences for each individual animal for each gene were constructed and exported.

For each of the three genes examined, HHG-affected farm foxes and wild HHG unaffected foxes were sequenced. Initial sequence comparisons were performed using the HHG-affected and wild HHG-unaffected foxes. For any DNA sequence that demonstrated a trend segregating the HHG-affected farm foxes and HHG unaffected

wild foxes, additional samples including HHG-unaffected farm foxes with no direct familial relation to the HHG-affected farm foxes were sequenced to explore any true segregating sequence differences between affected and unaffected groups of foxes.

Analyses of molecular evolution were conducted within MEGA 5.0 (Tamura *et al*. 2011). These included rates of synonymous and non-synonymous sequence variation (Nei-Gojobori algorithm), Z-tests of selection (using Nei-Gojobori proportions), and HyPhy tests of site-specific selection. Three levels of taxonomic hierarchy were considered for each gene: within *Vulpes vulpes*; between *V. vulpes* and *Canis lupus familiaris* (domestic dog); and among mammals using sequences accessed from GenBank (*Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *Pan troglodytes*, *Homo sapiens*). For intraspecies comparisons maximum sequence data available for each individual was used. For interspecies comparisons consensus sequence data was used. For the *EGFR* gene, a McDonald-Kreitman test of selection (MacDonald and Kreitman 1991) was performed and a test of lineage-specific selection was conducted by comparing a model with a fixed dN/dS (ω) ratio parameter to a model in which this parameter was free to vary, using the log-likelihood ratio test implemented in PAML v4.5 (Yang 2007).

Results

Mutational differences between affected and unaffected foxes

The *EGFR* gene was sequenced for 19 unaffected foxes and 13 HHG-affected foxes; the total coverage for each individual ranged from 2,731 to 9,418 bp. The *SOS1* gene

was sequenced for 15 unaffected foxes and 14 HHG-affected foxes. The total coverage for each individual for this gene ranged from 194 bp to 12,454 bp. The *GRB2* gene was sequenced for 12 unaffected foxes and 10 HHG-affected foxes, and the total coverage for this gene for each individual ranged from 318 bp to 2,139 bp. For each gene, there was 100% coverage of the protein-coding region and the intron/exon boundaries for all HHG-affected and wild unaffected foxes. There were no fixed mutations segregating the HHG-affected from the unaffected sets of samples in any coding portions or intron/exon boundaries of the *EGFR*, *SOS1* or *GRB2* genes. There were no heterozygous sites conserved across affected foxes in any of these gene regions, as may be expected for a dominant mutation.

The 5' regulatory region of the *EGFR* gene was examined. In humans, this region is still not fully characterised but it contains several features within the 500 bp upstream of the 5' start codon. This is a GC rich section (GC content of 88%) that contains multiple transcriptional start sites and several CCGCCC repeats (Ishii *et al*. 1985; Liu *et al*. 2005). Here, 1,775 bp upstream of the 5'start codon of the EGFR gene using the 19 unaffected foxes and 13 HHG-affected fox sequences were examined and no CCGCCC repeat was present. In the 500 bp immediately upstream of the EGFR start codon in foxes the GC content was 52.4% (158 Gs and 104 Cs). This region did not contain any fixed differences between the HHG-affected and unaffected foxes. In humans, a polymorphism at position -216 G/T is associated with increased promoter activity (Liu *et al*. 2005). This site was an invariant G in foxes.

Molecular evolution of the EGFR, GRB2, and SOS genes in foxes and other mammals

Across all measures of sequence diversity at all hierarchical levels *EGFR* demonstrated the highest level of variation among the three genes (Table 3.1). *SOS1* was more variable than *GRB2* at non-synonymous sites, but less so at synonymous sites (Table 3.1) both between canids and among mammals. Variability of all genes was consistent with purifying selection (Z-test dN/dS << 1; P=0) with the highest functional constraints associated with *GRB2* and the lowest with *EGFR*. As the Z-test is a very conservative test of positive selection requiring dN/dS (ω) > 1 to reject neutrality, we used site-specific test of selection (HyPhy) for all genes, and a McDonald-Kreitman test of selection for the EGFR gene, where the most variability was observed. HyPhy tests did not reveal evidence of site-specific selection in any gene (P>0.15 for all sites in *EGFR*; P>0.29 for all sites in *SOS1*; P>0.67 for all sites in *GRB2*). The McDonald-Kreitman ratio of non-synonymous-to-synonymous changes was higher between *Vulpes vulpes* and *Canis lupus familiaris* (20/20) than within *Vulpes vulpes* (4/7), suggestive of positive selection, but the difference was not significant (Fisher exact test P=0.298).

A lineage-specific test of selection was performed using phylogenetic analysis by maximum likelihood (PAML) with the branch-site model for the multispecies phylogeny of the *EGFR* gene (Yang 2007). The PAML model allowing ω (dN/dS) ratios to vary was significantly more likely than the model in which they were fixed (χ^2=32.7; P=0), to suggest the possibility of positive selection affecting amino acid sequence evolution in some of the mammalian lineages. The branch model indicated

Table 3.1: Measures of sequence diversity in the coding portions of the *EGFR*, *GRB2*, and *SOS1* genes, within *Vulpes vulpes*, between *Vulpes vulpes* and *Canis familiaris*, and among mammals (pairwise averages)

Comparison	Gene (nt)		
	EGFR (3543)	GRB2 (654)	SOS1 (4032)
Within *V. vulpes*			
No. variable sites (%)	12 (0.339)	2 (0.306)	4 (0.099)
Nonsynonymous	4	0	0
Synonymous	8	2	4
V. vulpes vs. *C. familiaris*			
No. variable sites (%)	41 (1.157)	5 (0.760)	19 (0.470)
Nonsynonymous (dN)	20 (0.008)	0 (0)	9 (0.003)
Synonymous (dS)	21 (0.031)	5 (0.036)	10 (0.011)
Among Mammals			
p-distance	0.129	0.062	0.069
dN	0.047	0.001	0.014
dS	0.401	0.288	0.257
dN/dS	0.118	0.003	0.054
Z-test P-value	0 (purifying)	0(purifying)	0 (purifying)

a range of lineage-specific ω ratios from 0.0453 for *Homo sapiens* to 0.2627 for *Canis familiaris* (Table 3.2). The highest ω ratios were seen in the *Canis* (0.2627) and *Vulpes* (0.2580) lineages, suggesting the possible presence of positive selection in these lineages.

Discussion

HHG is an autosomal recessive gum disease occurring almost exclusively in ranched silver foxes (Dyrendahl and Henricson 1960). While nothing is known about the underlying genetic mutation(s) leading to its prolific gingival overgrowth phenotype, an analogous condition occurring in humans, HGF, has a known causative genetic mutation (Hart *et al*. 2002). In the present study, we explore the gene containing this mutation, *SOS1*, and two genes with functions in the same cell signalling pathway, *EGFR* and *GRB2*, to determine if these genes carry any potential HHG causative mutations, in addition, to elicit the molecular evolutionary patterns of these genes in foxes and compare to other canids and mammals to assess the likelihood that mutations in these genes could drastically change the phenotype.

Mutational differences between HHG-affected and unaffected foxes

Since research into HGF suggests involvement of the RAS signalling pathway (Xiao *et al*. 2001), our aim was to start with the known HGF-associated gene *SOS1*, then examine the closely-associated *GRB2* gene and the adjacent upstream pathway gene *EGFR*, to determine whether any mutational differences exist between HHG-affected

Table 3.2: Lineage-specific rates of nonsynonymous (dN) and synonymous (dS) substitution in the *EGFR* gene of mammals, and their ratio (ω), determined using phylogenetic analysis by maximum likelihood (PAML) branch-site model

Species	dN	dS	ω
Rattus norvegicus	0.0088	0.1353	0.0652
Mus musculus	0.0097	0.0987	0.0980
Homo sapiens	0.0075	0.0165	0.0453
Pan troglodytes	0.0019	0.0122	0.0603
Vulpes vulpes	0.0062	0.0241	0.2580
Canis familiaris	0.0019	0.0074	0.2627
Bos taurus	0.0370	0.2754	0.1343

and unaffected foxes that could affect protein function and potentially be causative of HHG.

The *SOS1* gene in humans spans 138,915 bp, contains 23 exons and encodes a seven domain protein consisting of a histone folding domain, a Dbl homology domain, a Pleckstrin homology domain cassette, a helical linker, a RAS exchange motif, a Cdc25 domain and a proline rich C-terminal which binds to GRB2 (Findlay and Pawson 2008). The human *GRB2* gene is located at chromosomal position 17q24-q25, spans 87,634 bp, and consists of five exons. The ubiquitously expressed GRB2 protein contains three domains: a Src homology 2 (SH2) domain flanked on either side by SH3 domains (Dharmawardana *et al*. 2006). The SH2 domain binds to tyrosine phosphorylated regions while the SH3 domains bind to other proline rich regions on other proteins such as to the C-terminal domain of the SOS1 protein. Duplication of the *GRB2* gene has been associated with leukemia's and tumours (Dharmawardana *et al*. 2006).

The results of sequence analyses of both the *SOS1* and *GRB2* genes in HHG-affected and unaffected fox demonstrated similar findings. Neither gene showed any fixed differences between these two groups that could represent putative causative mutations of HHG in either the coding regions or at the exon-intron boundaries. There were very few polymorphic sites in these genes among foxes, and no non-synonymous changes. These findings do not rule out the possibility that a mutation could exist in the upstream promoter/enhancer regions for either of these genes, or

potentially elsewhere in the introns. Nonetheless, they do suggest that amino acid differences in the protein products of these genes, frameshift mutations leading to truncation or extension of the protein product, or failure to correctly excise introns, which would radically alter the protein product, are not the cause of the gum disease phenotype in foxes. We can confirm that the known HGF causative mutation in *SOS1* is not found in HHG-affected foxes.

The 188,307 bp human *EGFR* gene, located on chromosome 7 position p12, consists of 34 exons that are expressed as four protein variants (Lurje and Lenz 2009). All EGFR protein variants contain three domains: a variable extracellular receptor domain; a short, hydrophobic, membrane-spanning domain; and a tyrosine kinase intracellular carboxy-terminal domain (Scaltriti and Baselga 2006). Ligand binding results in a conformational change in the extracellular domain leading to receptor dimerization and subsequent autophosphorylation of the intracellular tyrosine residues, enabling the "docking" and activation of the GRB2 protein. Constitutive activity of this gene has been linked to cancer and cancer progression, and EGFR has been researched as a target for drug therapies (Scaltriti and Baselga 2006). Here, no mutational differences separated HHG-affected foxes from unaffected ones, indicating that similar to GRB2 and SOS1, structural differences in the encoded protein are not associated with HHG.

In humans, the 5' regulatory region of the EGFR gene, unlike most eukaryotic 5' regulatory regions, does not contain TATA or CAAT boxes. Instead this region has a GC content of ~88%, and contains several CCGCCC repeats and multiple RNA

initiation sites. A polymorphism at position -216G/T acts independently of other promoter region sequences to bind specificity protein 1 (Sp1), a transcription factor required for promoter activity (Liu *et al*. 2005); the thymine causes an increase in promoter activity. We found no fixed differences in the region immediately upstream of the *EGFR* gene in the two groups of foxes, including at position -216, hence have no evidence to suggest a promoter difference as causative of HHG. But, the GC content in foxes, similar to *Canis familiaris,* was not as high as would be expected if this region functions the way it does in humans, so further exploration of the promoter region will be required before this gene's involvement in HHG can be eliminated. However there are fewer CpG islands in promoter regions in canines than in humans (Auton *et al*. 2013), which could account for the lower CG content seen in the upstream region of the *EGFR* gene in both *Canis* and *Vulpes*.

Molecular evolution of the SOS1, GRB2, and EGFR genes in mammals

The *SOS1* and *GRB2* genes showed similar molecular evolutionary patterns to each other, in that both genes were highly conserved at the intraspecies, intra-family, and among-general levels in mammals. Less than 1% of sites were variable in either gene between the domestic dog and the fox, separated by ~ 15 million years of evolution, while among mammals ~6% of sites varied in pairwise comparisons (Wayne *et al*. 1997). Additionally, non-synonymous substitutions and ω ratios were low for both these genes, especially *GRB2*, consistent with strong functional constraints. The GRB2 protein functions as an adapter protein between a receptor and the cytoplasmic kinases (Kraskouskaya *et al*. 2013), while SOS1 is a nucleotide exchange factor activating both the RAS and RAC cascade pathways (Pierre *et al*.

2011). Consistent with high conservation, disruptions in both these genes have known disease consequences; the absence of GRB2 protein in mice, for example, is incompatible with life (Cheng *et al*. 1998), and in humans, mutations in *SOS1* lead to diseases like HGF1 and Noonan syndrome (Pierre *et al*. 2011).

Unlike *SOS1* and *GRB2*, *EGFR* showed high levels of sequence variation at both synonymous and non-synonymous sites, and *EGFR*'s ω ratio was about 40 times greater than *GRB2*. Elevated synonymous substitution rates may indicate a high mutation rate, relaxed selection related to alternative codon usage, or a recombination hotspot (Auton *et al*. 2013; Comeron and Aguade 1998). Elevated dN rates and ω values suggest either reduced functional constraints or positive Darwinian selection. To determine which, we considered four tests of selection. The Z-test could not be rejected, but this test is known to be conservative, requiring ω ratios greater than unity. Nonetheless, site-specific tests also did not indicate positive selection at any codon sites ($P > 0.15$) nor could the McDonald-Kreitman test reject the equality of the non-synonymous-to-synonymous ratios of polymorphisms within *Vulpes* to fixed differences between *Vulpes* and *Canis*. However, the latter was higher, consistent with adaptive evolution; furthermore, of the 20 fixed non-synonymous differences between *Canis* and *Vulpes*, eight (40%) were located in the third FU (Furin-like repeat) domain while only seven were between domains. The PAML lineage-specific test of selection supported a model allowing the ω ratio to vary among lineages, pointing to the possibility of positive

selection in certain lineages (Yang 2007). Notably the *Canis*, *Vulpes, and Bos* ω values are the highest among mammals.

Why might there be positive selection in *EGFR* in the *Vulpes*, *Canis*, or *Bos* lineages? One possibility is that selection pressure is diversifying this receptor's ability to respond to different stimuli or participate in multiple pathways. The *EGFR* ω ratios pattern supports the deep evolutionary divergence between the clade of mammals containing the primates and rodents relative to the one containing the Cetartiodactyla 64-104 million years ago (Murphy *et al*. 2001), so the molecular evolutionary pattern of either relaxed functional constraints or positive selection we observe in *EGFR* may date to this divergence. Nonetheless this pattern suggests that mutations in EGFR may be more readily tolerated and less likely to lead to disease than those in *GRB2* or *SOS1*.

Conclusions

We have determined that HHG-affected silver foxes do not carry the same *SOS1* mutation that causes HGF in humans. We ruled out the involvement of coding region or exon-intron boundary mutations in two adjacent RAS pathway genes, *EGFR* and *GRB2*, although we cannot eliminate the possibility that promoter, upstream binding, or other regulatory mutations might be changing gene expression patterns of these genes in affected versus unaffected foxes. It is possible that the mutation still lies in a gene somewhere within the RAS pathway. We have also demonstrated high evolutionary conservation of the *SOS1* and *GRB2* genes among mammals. The EGFR gene is more highly variable than the other two genes, in a pattern consistent with relaxed functional constraints or possibly even positive Darwinian selection especially in the lineage containing *Canis* and *Vulpes*.

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Chapter four:
Genome-wide expression analysis of hereditary hyperplastic gingivitis in silver foxes (*Vulpes vulpes*) using canine microarrays

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Co-authorship statement

The following is to provide clarification of the roles played by multiple co-authors in the manuscript published from chapter four. As per the guidelines set forth by the School of Graduate Studies my contributions as thesis author is divided into the following areas: *i) design and identification of the research proposal; ii) practical aspects of the research; iii) data analysis;* and *iv) manuscript preparation*.

i) *Design and identification of the research proposal*

All experiments in this chapter by Jo-Anna B.J. Clark with interest in the fox gum disease initiated by Robert Hudson and the concept of a transpecies microarray platform for identification of functional candidate genes by H. Dawn Marshall as part of an NSERC Discovery Grant. Jo-Anna B.J. Clark carried out gene selection and RT-qPCR study design.

ii) *Practical aspects of the research*

Jo-Anna B.J. Clark identified all pairs of foxes that were used in this study, and obtained the necessary tissue samples, working with a veterinarian at the fox farm. RNA preparation and quantification for the microarrays was conducted by Jo-Anna B.J. Clark and sent to The Center for Applied Genomics in Toronto where the microarray experiments were performed. For the first batch of RT-qPCR reactions, cDNA was prepared by Jo-Anna B.J. Clark while RT-qPCR was conducted by a student research assistant under Dr. Clark's supervision. When additional RT-qPCR

trials became necessary the cDNA was prepared by Jo-Anna B.J. Clark and sent to The Center for Applied Genomics for the RT-qPCR.

iii) *Data analysis*

Jo-Anna B.J. Clark from raw data files completed all microarray and RT-qPCR data analysis and interpretation with guidance provided by Marije Booman.

iv) *Manuscript preparation*

Jo-Anna B.J. Clark prepared the manuscripts with critical review and editing provided by H. Dawn Marshall and Marije Booman.

Introduction

Hereditary hyperplastic gingivitis (HHG) presents as tumourous growths of the gingival tissue on the mandible in the farmed silver fox, a coat colour variant of the red or coloured fox (*Vulpes vulpes*). Its pathology includes large epithelial extensions in the keratinized collagen of the gingival tissue (Dyrendahl and Henricson 1960). Pronounced manifestation of HHG occurs at approximately two to three years of age, with tumour proliferation continuing throughout life, resulting in dental encapsulation. HHG is an autosomal recessive disease demonstrating incomplete penetrance, with an increased incidence in males relative to females. In addition, a pleiotropic relationship between HHG and high fur quality, typically length and density of the guard hairs, has been implicated. While most cases of HHG are found in fox farms, there was a report in 2008 of a case of severe HHG in a wild European red fox (Schulze *et al*. 2008). Although HHG was first discovered in Sweden in the 1940s, and has been described in different parts of Europe since then, it was only documented in Newfoundland and Labrador, Canada, in 2004 (Figure 4.1), and its molecular aetiology remains unknown (Clark *et al*. 2015).

Hereditary gingival fibromatosis (HGF) in humans is an analogous condition to HHG. HGF is characterized as a genetically heterogeneous disease with progressive benign fibrous enlargements of the maxillary and mandibular keratinized gingival tissues (Hart *et al*. 2002). HGF is a rare disease with a phenotypic frequency of 1:175 000 individuals, affecting males and females equally (Fletcher 1966). Clinically, HGF presents with benign, non-haemorrhagic, non-exudative gingival overgrowths

A. B. C.

Figure 4.1: A, B, C: Hereditary hyperplastic gingivitis (HHG) in several original Finish foxes in 2004. Each panel demonstrates a different case of HHG (Photographs by Robert Hudson)

(Bittencourt *et al*. 2000; Hakkinen and Csiszar 2007). Manifestation occurs approximately at the advent of permanent dentition with progressive worsening during adolescence (Coletta and Graner 2006). Treatment involves quadrant by quadrant gingivectomy (Coletta and Graner 2006; Ramer *et al*. 1996), and despite resection, the gingival tissue continues to proliferate. HGF has a heterogeneous aetiology, occurring either because of an underlying isolated genetic cause, in association with other symptoms as a syndrome, or as the result of a drug interaction (Shashi *et al*. 1999). Inheritance patterns of the genetic forms include Mendelian autosomal inheritance and maternal genome imprinting. Mendelian autosomal inheritance is primarily dominant with rare case reports of autosomal recessive transmission (Goldblatt and Singer 1992; Hart *et al*. 1998; Singer *et al*. 1993). Maternal imprinting has been linked to two imprinting clusters on chromosome 11p15 (Zhu *et al*. 2007). To date, the focus has been on elucidating the mechanisms of autosomal dominant transmission.

Autosomal dominant HGF displays incomplete penetrance and variable expression (Hart *et al*. 1998) and has been linked to three chromosomal loci: HGF1 at 2p21-p22 (Hart *et al*. 1998; Xiao *et al*. 2000); HGF2 at 5q13-q22 (Xiao *et al*. 2001); and HGF3 at 2p22.3-p23.3 (Ye *et al*. 2005). Only HGF1 has a known causative mutation, which affects codon 1083 of the *Son of sevenless homolog 1* (*SOS1*) gene, the guanine nucleotide exchange factor involved in signal transmission from the receptor tyrosine kinase to the rat sarcoma viral (RAS) protein in the mitogen-activated protein kinase (MAPK) signalling cascade (Hart *et al*. 2002). Given the strong

similarity between human HGF and fox HHG, the known molecular basis of one form of HGF establishes a basis for the study of the analogous gum disease in the fox.

The publication of the domestic dog (*Canis lupus familiaris*) genome (Lindblad-Toh *et al*. 2005) greatly facilitates genomic research with closely related species such as the red fox (Kohn *et al*. 2006; Lindblad-Toh *et al*. 2005). *Vulpes vulpes* and *Canis lupus familiaris* are both members of the mammalian family Canidae and last shared a common ancestor 12 to 15 million years ago (Kukekova *et al*. 2004; Wayne *et al*. 1997). There is strong molecular evidence supporting the close relationship between the two species, such as a 97.3% similarity between their *sex determining region Y* (*SRY)* gene coding sequences (Nowacka-Woszuk and Switonski 2009; Switonski *et al*. 2009). Mouse genetic studies are hampered by the high evolutionary rate of the rodent lineage (Boyko 2011). The dog genome has proven to be a useful model system for human diseases and as a potential bridge between human and mouse studies.

Interpreting genome-wide HHG expression patterns in the silver fox in the context of the genetics of HGF should provide insights into many facets of this disease; specifically it can be used as a gene discovery tool to identify putative genes involved. The goal of this study was to integrate genetic knowledge of HGF with genomic technologies accessible from the dog genome to examine molecular pathways involved in silver fox HHG. We used the Affymetrix GeneChip Canine Genome 2.0 Array, which contains over 20 000 non-redundant predicted genes as

well as 18 000 *C. familiaris* mRNA/EST-based transcripts, to compare the global gene expression patterns within gum tissue from farmed HHG-affected and unaffected silver foxes. We evaluated our findings with a focus on the genes within the known HGF chromosomal regions and, additionally, the RAS/ MAPK pathway thought to be involved in HHG, demonstrating differential expression between HHG-affected and HHG-unaffected silver foxes.

Materials and Methods

Sample collection

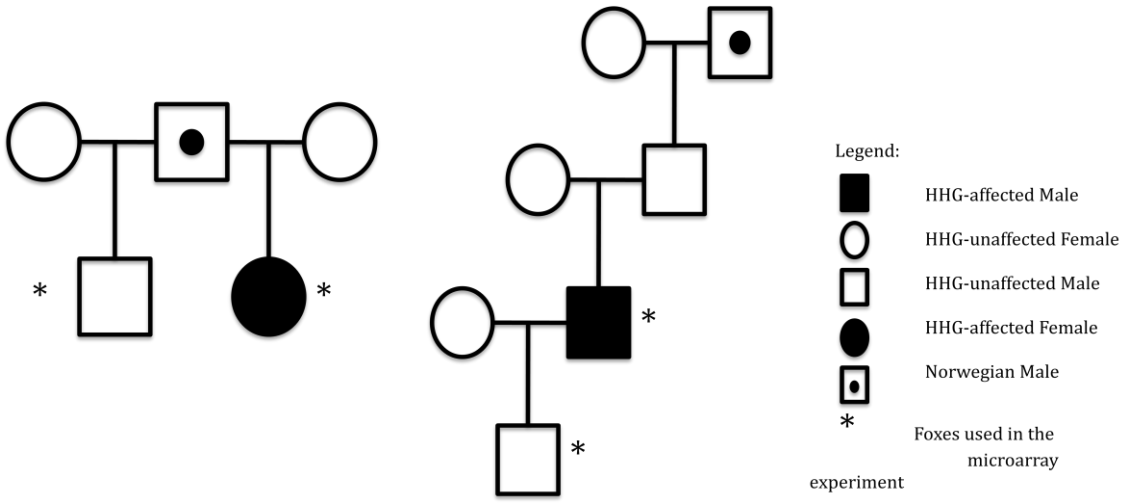
Diagnosis of HHG was made based on early markers of the disease where red, raised, granular gingival tissue was present at the dental margins on the crowns of the teeth. Provincial government veterinarians, based on gross manual examination of each fox's gingival tissue, made this diagnosis (Hudson 2014, Personal communication). A phenotypic severity scale was created ranging from no signs of disease at zero to severe disease at five to document severity of initial presentation and for monitoring disease progression. Samples of HHG-affected and HHG-unaffected gum tissues were taken from a total of thirty-two foxes from a Newfoundland and Labrador fox farm. All HHG-unaffected samples displayed normal pale pink, smooth, flat gingival tissue (a zero on the phenotypic severity scale). The HHG-affected samples ranged from a two (a moderately thick, rounded red layer of gingiva, that is bilateral, with no complete dental encapsulation) to a four (the dental arcade is mostly covered by diffusely thickened, red and proliferative gingival tissue, forming confluent multi-nodular masses with large

lateral flaps resulting in numerous encapsulated teeth) on the phenotypic severity scale. Eight of the samples (five HHG-affected and three HHG-unaffected) were used for the microarray experiment and the remainder for reverse quantitative transcriptase polymerase chain reaction (RT-qPCR) experiments (eleven HHG-affected and thirteen HHG-unaffected samples). Within the microarray samples, there were two pairs of related foxes (Figure 4.2) and four individuals sharing no direct familial connections.

Gingival samples were collected either during pelting season or in the late spring after whelping season. During pelting season, samples were collected immediately after the animal was euthanized. Samples collected after whelping season were taken from live anesthetised foxes. Provincial government veterinarians performed all sampling and the Institutional Animal Care Committee at Memorial University of Newfoundland approved use of samples for this research, in accordance with Canadian Council of Animal Care guidelines. Gingival sections (<500 mg) were immediately placed into 5 mL of RNAlater (Qiagen Inc., Mississauga, Canada) for four hours followed by storage at -20°C.

RNA preparation

RNA was extracted using an RNeasy Fibrous Tissue Mini kit, according to protocol specifications (Qiagen Inc., Mississauga, Canada). An additional off-column DNase procedure was performed to ensure the quality of the RNA (Qiagen Inc., Mississauga, Canada). RNA quality was assessed using 1.5% agarose gel



A. B.

Figure 4.2: Pedigrees of two separate silver fox families (A and B) demonstrating the relationship between a hereditary hyperplastic gingivitis (HHG)-affected members and HHG-unaffected members

electrophoresis and spectrophotometry using the NanoDrop ND1000 (NanoDrop Technologies, Inc., Wilmington, USA).

Microarray experiments

RNA samples were sent to the Microarray Facility at the Toronto Centre for Applied Genomics, Hospital for Sick Children, in Toronto, Canada, where RNA integrity was assessed using the Agilent BioAnalyzer (Affymetrix Inc. and Agilent Technologies Inc., Santa Clara, USA). RNA was labeled using the Affymetrix 3'IVT Express kit (Affymetrix Inc., Santa Clara, USA) according to the manufacturer's protocol. Each sample was hybridized to an Affymetrix GeneChip Canine Genome 2.0 Array and scanned using an Affymetrix GeneChip Scanner 3000 (Affymetrix Inc., Santa Clara, USA).

Data analysis

Microarray '.CEL' files were analyzed using a Bioconductor 'gcrma' package (Gentleman *et al*. 2004, Wu and Gentry 2014). Raw data was converted to expression measures using a GCRMA normalization and summarization algorithm. Non-parametric Mann-Whitney U-tests were used to test for statistical significance of differential expression values. Data was submitted to NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). Gender was initially assessed but did not create a bias. Multiple comparisons of the means were completed however given the diversity of the near-wild animal a reliable list was not obtained.

Lists of probesets showing statistically significant differential expression between affected and unaffected animals were compiled, annotated with gene names and functions using NetAffx Analysis Centre (Affymetrix Inc., Santa Clara, USA), and placed in Wikipathways to search for involvement in known canine signalling pathways (Kelder *et al*. 2011). Using the NCBI human genome build 36.3 (http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9606&query=human&SITE=HumanGuide&SUBMIT=y&submit=Go) lists of the genes found within the chromosomal loci known to be associated with HGF2 and HGF3 were created and then cross-referenced to the microarray expression gene lists.

Reverse transcription quantitative PCR (RT-qPCR)

cDNA was transcribed from total RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems Inc., Foster City, USA). Initial RT-qPCR assays were conducted in house or at the Microarray Facility at the Toronto Centre for Applied Genomics, Hospital for Sick Children in Toronto, Canada (see Appendix 2). For each assay, cDNA was combined with 10 µL Taqman Master Mix (2X) and 1 µL Taqman Gene Expression Assay (20X; 900 nM primer and 250 nM probe) (Applied Biosystems Inc., Foster City, USA.). Reaction mixtures were placed into MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems Inc., Foster City, USA) and subjected to thermal cycling in the StepOnePlus Real Time PCR System (Applied Biosystems Inc., Foster City, USA) according to the following profile: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. Six genes that are involved in MAPK signalling were selected for RT-qPCR: *Son of sevenless homolog 1*

(*SOS1*) (Cf02704868_m1), *Son of sevenless homolog 2* (*SOS2)* (Cf02699545_m1), *RAS p21 protein activator* 1 (*RASA1*) (Cf02676354_m1), *protein kinase C, ζ* (*PRKCζ*) (Cf02655062_m1), *mitogen-activated kinase kinase kinase 1* (*MAP3K1*) (Cf02635276_m1) and *Finkel–Biskis–Jinkins murine osteogenic sarcoma virus* (*FOS*) (Cf02741684_m1). As the endogenous control gene, *elongation factor, RNA polymerase II* (*ELL2*) (Cf02707156_m1) was selected for the in house RT-qPCR, and the *breakpoint cluster region* (*BCR)* (Cf02664178_m1) for the remaining RT-qPCR samples (see Appendix 2). Genes were selected either due to relation to the MAPK pathway or from literature reviews or based out of interest from the microarray data.

Relative quantification of each gene was calculated using the ΔΔCT method (Livak and Schmittgen 2001). For the in house RT-qPCR the unaffected sample with the lowest standard error was selected as the reference sample while for the remaining RT-qPCR tests the unaffected sample with the lowest expression was selected as the reference sample. Fold changes were calculated using Log2RQ method and the relative fold changes were tested for significance with two-tailed t-tests. All samples were tested in triplicate and samples with a standard deviation greater than 0.5 were removed from the dataset. Any samples that were identified as outliers within biological groups according to Data Assist v3.0 (Life Technologies, Grand Island, USA) were also discarded. Validation experiments were conducted to standardize amplification efficiency.

Results

Differential gene expression in HHG-affected versus HHG-unaffected silver foxes

The analysis generated a list of 1,154 probesets demonstrating a statistically significant differential gene expression when comparing the HHG-affected to HHG-unaffected samples (Mann-Whitney U-test $P < 0.05$) (see Appendix 3). Of these 1,154 differentially expressed probesets, 563 were significantly down-regulated in the HHG-affected versus HHG-unaffected samples and 591 were significantly up-regulated. Probesets representing genes with no known function as identified through the NetAffx Analysis Centre were removed from the list, and the remaining probesets represent 671 unique genes. Wikipathways analysis showed that of the 671 genes, over 135 were associated with known *Canis lupus familiaris* pathways, several of which were related to cell differentiation and proliferation and thus could be related to HHG aetiology (Table 4.1).

RT-qPCR

RT-qPCR assays were performed for 6 genes, with fold changes that generally support the trend observed with the microarray data (Table 4.2). Of the 6 genes, *FOS* demonstrated a statistically significant differential expression at a relaxed significance criterion of $P<0.10$, which was used to account for non-model organism biological diversity (Table 4.2). Figure 4.3 demonstrates the general expression trend comparison between the HHG-affected and unaffected samples for the genes involved in the MAPK signalling cascade.

Table 4.1: Microarray-identified genes significantly differentially expressed in hereditary hyperplastic gingivitis (HHG) affected foxes relative to unaffected foxes that have roles in signalling pathways involved in cell differentiation and proliferation

Gene symbol	Gene title	Affymetrix probe ID	Fold change	Mann Whitney U-test P value	Pathways
ADCY2	Adenylate cyclase 2	Cfa.19117.1.S1_s_at	-1.143	0.036	Guanine nucleotide binding protein (G protein)
ALPL	Alkaline phosphatase	Cfa.13804.1.A1_at	1.251	0.036	Tumour necrosis factor α (TNFα)
ATG7	Autophagy related 7	Cfa.14549.1.A1_at	1.257	0.036	Senescence and autophagy
BIRC3	Baculoviral IAP repeat containing 3	CfaAffx.23263.1.S1_at	1.194	0.036	TNFα, Apoptosis
CASP6	Caspase 6	CfaAffx.17770.1.S1_at	-1.213	0.036	Mitogen activated protein kinase (MAPK), Apoptosis
CDC37	Cell division cycle 37 homolog	CfaAffx.27245.1.S1_s_at	1.282	0.036	TNFα
CMA1	Chymase 1, mast cell	CfaAffx.19295.1.S1_s_at	1.372	0.036	TNFα
E2F4	E2F transcription factor 4	CfaAffx.31199.1.S1_at	-1.234	0.036	Transforming growth factor β (TGFβ)
EGF	Epidermal growth factor	Cfa.3524.1.S2_at	1.216	0.036	Epidermal growth factor receptor 1 (EGFR1), MAPK, TGFβ
FNTA	Farnesyltransferase	Cfa.18192.1.S1_at	-1.293	0.036	TGFβ
FHL2	Four and a half LIM domains 2	Cfa.1493.1.A1_at	-1.269	0.036	Wingless/integrated (Wnt)
FZD7	Frizzled family receptor 7	Cfa.15623.1.A1_at	1.251	0.036	Wnt and pluripotnecy

GJA1	Gap junction protein, alpha 1	Cfa.119.1.S1_at	-5.484	0.036	EGFR1
GYG1	Glycogenin 1	Cfa.4710.1.A1_at	2.078	0.036	Insulin
GNAI1	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	CfaAffx.25135.1.S1_s_at	-1.870	0.036	G protein
INS	Insulin	Cfa.18796.1.S1_s_at	-1.053	0.036	Insulin, senescence and autophagy
IRF3	Interferon regulatory factor 3	Cfa.18322.1.S1_at	1.234	0.036	Apoptosis
IL1β	Interleukin 1, β	CfaAffx.11741.1.S1_s_at	1.189	0.036	MAPK, senescence and autophagy
KTN1	Kinectin 1	CfaAffx.23345.1.S1_s_at	-1.114	0.036	TNFα
LAMP1	Lysosomal-associated membrane protein 1	CfaAffx.10473.1.S1_s_at	-2.425	0.036	Senescence and autophagy
MAP3K10	Mitogen-activated protein kinase kinase kinase 10	CfaAffx.9015.1.S1_at	-1.168	0.036	Insulin
MAP4K1	Mitogen-activated protein kinase kinase kinase kinase 1	CfaAffx.9778.1.S1_s_at	-1.267	0.036	MAPK, Insulin
NUP214	Nucleoporin 214kDa	CfaAffx.30488.1.S1_s_at	-1.376	0.036	TGFβ
PRKCζ	Protein kinase C, ζ	Cfa.19185.2.A1_at	-1.204	0.036	EGFR1, MAPK. TNFα, Wnt and pluripotency, Insulin, G protein
PRKD1	Protein kinase D1	Cfa.12558.1.A1_s_at	-1.612	0.036	EGFR1, Wnt and pluripotnecy
RALBP1	RalA binding protein 1	Cfa.14547.1.A1_at	2.421	0.036	EGFR1

RNF25	Ring finger protein 25	Cfa.11589.1.A1_at	1.192	0.036	TNFα
ROCK1	Rho-associated, coiled-coil containing protein kinase 1	CfaAffx.27952.1.S1_at	1.240	0.036	TGFβ
RRAD	RAS-related associated with diabetes	Cfa.17583.1.S1_at	1.874	0.036	Insulin
SH3GL3	SH3-domain GRB2-like 3	CfaAffx.20540.1.S1_at	1.160	0.036	EGFR1
TNFSF15	Tumour necrosis factor (ligand) superfamily, member 15	CfaAffx.5977.1.S1_at	1.220	0.036	Senescence and autophagy
USP6NL	USP6 N-terminal like	Cfa.19504.1.S1_at	1.392	0.036	EGFR1

Table 4.2: Differential expression in hereditary hyperplastic gingivitis (HHG) affected foxes compared to unaffected foxes of genes from RT-qPCR validation assays, compared with fold-change found by microarray analysis

Gene symbol	Gene title	RT-qPCR Fold change	t-test P Value	Microarray Affymetrix probe ID	Fold change	Mann Whitney student U-test P value
FOS*	Finkel–Biskis–Jinkins murine osteogenic sarcoma virus	3.385	0.077	CfaAffx.26065.1.S1_at	2.863	0.393
MAP3K1	Mitogen-activated protein kinase kinase kinase	-2.313	0.112	Cfa.19524.1.S1_at	-1.959	0.071
PRKCζ	Protein kinase C, ζ	1.299	0.382	Cfa.19185.2.A1_at	-1.204	0.036
RASA1	RAS p21 protein activator 1	3.727	0.116	Cfa.350.1.S1_at	1.344	0.071
SOS1*	Son of sevenless homolog 1	-1.225	0.406	CfaAffx.10496.1.S1_s_at	-1.400	0.250
SOS2	Son of sevenless homolog 2	1.214	0.182	CfaAffx.22062.1.S1_s_at	1.000	1.000

*RT-qPCR gene assays conducted in house

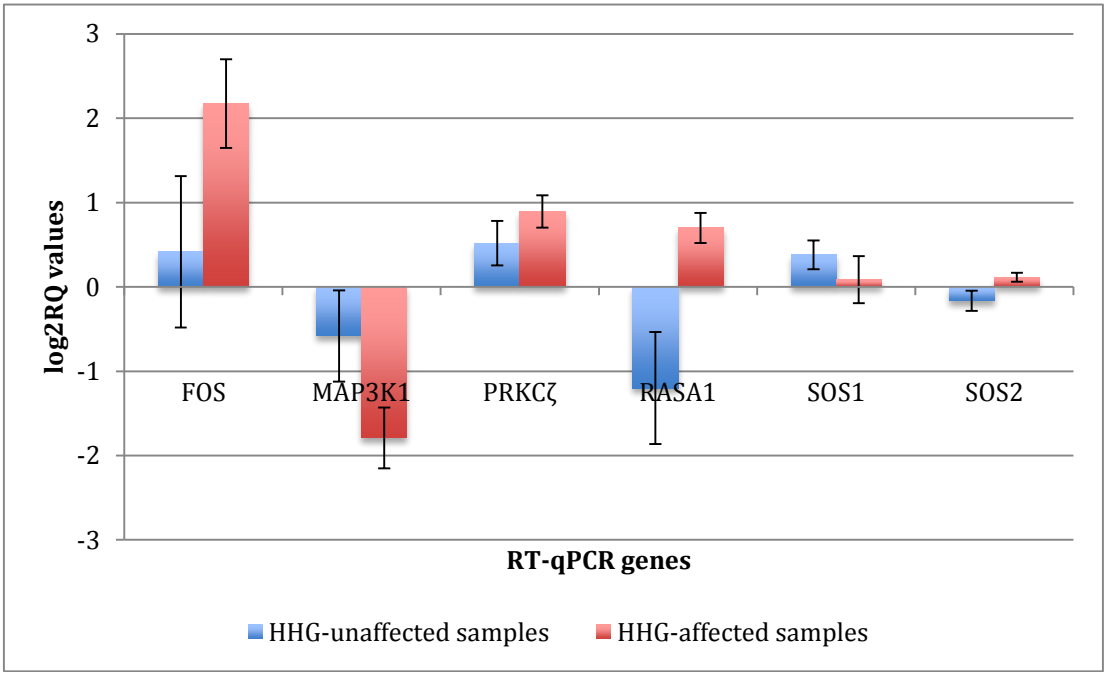


Figure 4.3: Mean $\log_2$ relative expression ($\log_2$RQ) values for RT-qPCR genes in the hereditary hyperplastic gingivitis (HHG)-affected and HHG-unaffected fox samples. Standard errors are indicated by the bars.

Comparison of HHG microarray gene lists with known HGF genes and chromosomal loci

Human HGF has been linked to three loci: HGF1(Hart *et al*. 1998; Xiao *et al*. 2000), HGF2 (Xiao *et al*. 2001), and HGF3 (Ye *et al*. 2005). Genes located within all three loci were compared to the HHG microarray gene lists. A mutation in the *SOS1* gene has been identified as the causative mutation in HGF1 (Hart *et al.* 2002). This gene was represented on the canine microarray by three probes, all of which showed down-regulation (1.14, 1.25 and 1.40-fold) of the gene in the HHG-affected foxes compared to the HHG-unaffected foxes, although this down-regulation was not statistically significant (Mann-Whitney U-test $P>0.10$). This result is opposite from what would be expected if *SOS1* were involved in HHG similarly to HGF, as in HGF mutation in the *SOS1* gene leads to a constitutively active gene (Hart *et al*. 2002).

HGF2 was mapped to the locus 5q13-q22 using a four-generation Chinese family (Xiao *et al*. 2001). This chromosomal region spans approximately 500 genes according to NCBI human genome build 36.3 (accessed July 2013). After removing those with unknown function or redundancy the remaining genes were cross-referenced to the canine genome and their Affymetrix gene IDs determined. The entire microarray gene list was queried with these IDs, generating a list of 273 Affymetrix probe set IDs, where some genes were represented more than once. Of these, six showed a statistically significant differential expression (Mann-Whitney U-test $P<0.05$) when comparing the HHG-affected to the HHG-unaffected samples (Table 4.3); two up-regulated and four down-regulated. Relaxing the required

Table 4.3: Microarray analysis of differential expression in hereditary hyperplastic gingivitis affected foxes compared to unaffected foxes for genes found in the human chromosome loci associated with hereditary gingival fibromatosis (HGF)

HGF subtype	Gene symbol	Gene title	Fold change	Mann Whitney U-test P value
HGF2	ANKRD31	Ankyrin repeat domain 31	1.100	0.036
HGF2	CHD1	Chromodomain helicase DNA binding protein 1	1.347	0.036
HGF2	CMYA5	Cardiomyopathy associated 5	-1.210	0.036
HGF2	MCTP1	Multiple C2 domains, transmembrane 1	-1.233	0.036
HGF2	TMEM161B	Transmembrane protein 161B	-1.523	0.036
HGF2	XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	-1.538	0.036
HGF2	AGGF1	Angiogenic factor with G patch and FHA domains 1	-1.190	0.071
HGF2	FAM174A	Family with sequence similarity 174, member A	-1.560	0.071
HGF2	RASA1	RAS p21 protein activator (GTPase activating protein) 1	1.344	0.071
HGF2	TRIM36	Tripartite motif containing 36	1.239	0.071
HGF3	GTF3C2	General transcription factor IIIC, polypeptide 2	-1.182	0.036
HGF3	SLC30A3	Solute carrier family 30 (zinc transporter), member 3	1.234	0.036
HGF3	SRD5A2	Steroid-5-alpha-reductase, alpha polypeptide 2	1.162	0.036
HGF3	ZNF512	Zinc finger protein 512	-1.172	0.071

significance level to P=0.10 to accommodate high biological variability among the samples generated an additional four differentially-expressed genes (Table 4.3), two up-regulated and two down-regulated in affected versus unaffected animals.

The third HGF locus was originally mapped to 2p22.3-p23.3 (Ye *et al*. 2005); this was subsequently refined to a 6.56cM interval within which 112 genes were found (Pampel *et al*. 2010). According to NCBI human genome build 36.3 (accessed July 2013), the 2p22.3-p23.3 chromosome region contains 158 genes of which 84 have canine equivalents. Three of these genes showed a statistically significant differential expression when comparing the HHG-affected to the HHG-unaffected microarray samples using a P<0.05 criterion (Table 4.3). Two were up-regulated in the HHG-affected samples relative to the unaffected samples, and one was down-regulated. One additional differentially expressed gene was identified with a relaxed P<0.10 criterion (Table 4.3). The additional gene was down-regulated in the HHG-affected samples. None of these genes were found to be associated with known signalling pathways.

Comparison of HHG microarray genes lists to MAPK pathway genes

Involvement of genes in the MAPK signalling pathway in the HGF phenotype has been suggested (Hart *et al*. 2002; Xiao *et al*. 2001). The canine MAPK signalling pathway was examined using Wikipathways (Kelder *et al*. 2011) to determine if any significantly differentially expressed genes within the fox HHG-affected vs. HHG-unaffected microarray comparison could be identified. Three genes showed

statistically significant differential expression (Mann-Whitney U-test P< 0.05) and six in total with a relaxed Mann Whitney criterion (P< 0.10) (Table 4.4). Of these six genes, three showed up-regulation in the HHG-affected samples and three showed down-regulation in the HHG-affected samples compared to the HHG-unaffected samples.

Discussion

Microarray analysis revealed a substantial list of genes expressed differentially in gum tissue from HHG-affected silver foxes compared with unaffected foxes. This gene list implicated numerous cell signalling pathways including epidermal growth factor receptor 1 (EGFR1), mitogen-activated protein kinase (MAPK), tumour necrosis factor alpha (TNFα), wingless/integrated (Wnt), insulin signalling, senescence and cell autophagy (Table 4.1). We first interpreted these gene lists and pathways in the context of genes or loci associated with human HGF, and then discuss new insights into the molecular and cellular mechanisms underlying expression of the HHG phenotype.

HGF genetics includes cases of maternal imprinting, chromosomal abnormalities, and autosomal recessive and autosomal dominant aberrations. With our microarray experimental design, chromosomal aberrations cannot be distinguished nor can maternal imprinting clusters be identified. Autosomal recessive cases of HGF appear sporadically throughout the literature (Goldblatt and Singer 1992; Hart *et al*. 1998; Singer *et al*. 1993). Given the rareness of the autosomal recessive mode of HGF

Table 4.4: Microarray analysis of differential expression in hereditary hyperplastic gingivitis (HHG)-affected foxes compared to unaffected foxes for genes found in the extracellular signal-regulated kinases portion of the mitogen-activated protein kinase (MAPK) signalling cascade

Gene symbol	Gene title	Fold change	Mann Whitney U-test P value
EGF	Epidermal growth factor	1.216	0.036
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	-1.959	0.071
MAPK3	Mitogen-activated protein kinase 3-like	1.541	0.036
MAPK4	Mitogen-activated protein kinase 4	-1.285	0.071
PRKCζ	Protein kinase C, ζ	-1.204	0.036
RASA1	RAS p21 protein activator (GTPase activating protein) 1	1.344	0.071

inheritance, there have been no definitive causative genes isolated and the penetrance and expression patterns are unknown, although novel mutations are thought to be responsible. The lack of understanding of the recessive HGF aetiology makes it difficult to explicitly compare to the HHG microarray data, but an autosomal recessive mode of inheritance of HHG in the farmed fox population cannot be ruled out and indeed was the conclusion drawn by Dyrendahl and Henricson (1960). Autosomal dominant isolated HGF is associated with three known chromosomal loci and we discuss each of these below.

Comparison of HHG microarray gene lists with known HGF genes and chromosomal loci

HGF1 at 2p21-p22 (Hart *et al*. 1998; Xiao *et al*. 2000) is the only locus with a known aetiology. A single nucleotide insertion in codon 1,083 of the *Son of sevenless homolog 1* (*SOS1*) gene was found within the refined chromosomal region and proven to be the HGF1 causative mutation (Hart *et al*. 2002). This insertion results in a protein with constitutive membrane recruitment, which allows constitutive activation of the MAPK pathway leading to cell proliferation (Pierre *et al*. 2011). In both the microarray and RT-qPCR experiments, *SOS1* is expressed at a lower level in the HHG-affected foxes when compared to the unaffected foxes, although neither result was statistically significant. Given that the mutation causing HGF1 results in constitutive membrane recruitment of the SOS1 protein, it is possible that the mutation does not result in increased gene expression and thus would not appear up-regulated in the HHG-affected foxes compared to the unaffected foxes. Further

investigation of fox *SOS1*, (Clark *et al*. 2014a) has shown that there are no mutations within the coding region or within the intron-exon boundaries of this gene in the HHG-affected silver fox population. This conclusively rules out the same nucleotide insertion in *SOS1* that causes HGF1 as causative of HHG or any other structural change in the SOS1 protein.

We also consider the possibility of a putative mutation in the *SOS2* gene. While located on different chromosomes, the SOS1 and SOS2 proteins share a high amino acid similarity (overall 65%), especially at their amino termini, but not at their carboxy termini (Yang *et al*. 1995). The carboxy-terminal end of SOS2 contains several proline rich areas whereas SOS1 only contains one such region. These proline rich regions confer a stronger affinity for the SH3 domain of the GRB2 protein, which is an activator of SOS2. While both SOS1 and SOS2 are present in most human cells, they have different signalling capabilities (Qian *et al*. 2000). Both interact with the MAPK pathway through the extracellular signal-regulated kinase path, but SOS1 has the capability to elicit both long- and short-term signalling, while SOS2 can only participate in short-term signalling. The RT-qPCR data shows a small up-regulation in the *SOS2* gene in the HHG-affected foxes compared with the unaffected foxes (fold change 1.214, P=0.182). This minor increase in fold change is likely insufficient to implicate the *SOS2* gene as a candidate gene; however, it is possible that a nucleotide alteration exists which would change the structure of the expressed protein without resulting in increased gene expression. Given the high similarity and similar function of the two *SOS* homologs, it is possible that the

causative mutation of HHG occurs in the *SOS2* gene rather than *SOS1* but does not cause up-regulation of the gene.

HGF2 was mapped to 5q13-q22 (Xiao *et al*. 2001). Of particular interest in this region are the genes involved in the cell cycle and calcium signalling. Of the ten genes in Table 4.3 two are involved in known canine pathways, *XRCC4* (X-ray repair cross-complementing protein 4) and *RASA1* (rat sarcoma viral p21 protein activator 1). *RASA1* was highlighted by Xiao *et al*. (2001) as a potential candidate gene. The known pathways involving *RASA1* include MAPK signalling pathway, EGFR1 signalling pathway, EPO receptor signalling, signalling of hepatocyte growth factor receptor, IL-4 signalling pathway, Kit receptor signalling pathway, T cell receptor signalling pathway, and B cell receptor signalling pathway (Kelder *et al*. 2011). Given its involvement in MAPK signalling and its up-regulation in HHG-affected foxes, *RASA1* is an excellent candidate for involvement, either directly or through a subsequent pathway, in the HHG phenotype.

A third HGF locus was determined at 2p22.3-p23.3 and two genes within this region, *GRP113* and *SELI*, were screened and ruled out as not containing putative HGF3 causative mutations (Ye *et al*. 2005). This locus was further refined to a 6.56cM interval (Pampel *et al*. 2010). Based on the canine microarray data, none of the genes showed a statistically significant expression difference in this region, including *SLC30A3*, *GTF3C2* and *ZNF512*. Of interest is the *Steroid-5-alpha-reductase, alpha polypeptide 2* (*SRD5A2*) which encodes an enzyme that converts testosterone

into a more potent androgen (Ye *et al*. 2011). The *SRD5A2* gene was significantly up-regulated in HHG-affected foxes. In dogs, a similar gingival hyperplasia occurs as a result of pharmaceutical interaction (Pariser and Berdoulay 2011). The proposed mechanism for this involves blocking of calcium channels resulting in testosterone sensitive gingival tissue. Although the fox gum condition is not drug-induced, the underlying mechanism may be similar as SRD5A2 results in a higher potency androgen, resulting in androgen sensitive gingival tissue.

Comparison of HHG microarray gene lists to MAPK pathway genes

Particular emphasis has been placed on the MAPK signalling pathway as it has a direct link to HGF1 (Hart *et al*. 1998) and a suggested link to HGF2 (Hart *et al*. 1998; Xiao *et al*. 2001). MAPK signalling incorporates a multi-tier cascade of kinase phosphorylations whereby a mitogen-activated protein kinase kinase kinase (MAP3K) phosphorylates a mitogen-activated protein kinase kinase (MAP2K), which phosphorylates the MAPK (Yang *et al*. 2013). In mammals, MAPK signalling broadly consists of four major pathways: the extracellular signal-regulated kinase (ERK1/2), extracellular signal-regulated kinase 5 (ERK5), c-Jun N-terminal kinase (JNK), and p38 pathway (Krishna and Narang 2008; Wang and Tournier 2006). The HGF1 and HGF2 studies place emphasis on the ERK1/2 pathway and its activation by growth factors.

The general mechanism of the ERK pathway in both humans and canines starts at the tyrosine kinase receptor EGFR. Once activated by a ligand, the receptor undergoes autophosphorylation (Pierre *et al*. 2011). GRB2 acts as an adaptor between the EGFR and SOS1 or SOS2 proteins. It binds to the intracellular component of the transmembrane EGFR, resulting in relocation of the SOS1 or SOS2 protein to the membrane where it also binds to GRB2. The SOS protein in turn interacts with the rat sarcoma viral (RAS) protein through two domains: one that is the active site for nucleotide exchange and the other that is responsible for autoinhibition. The phosphorylation cascade continues on to rapidly accelerated fibrosarcoma 1 (Raf1), followed by MAP2K then MAPK (Roskoski 2012). The MAPK then interacts with transcription factors c-Myc and SAP-1 (Whitmarsh and Davis 1996). These transcription factors interact with one of the three transcriptional control elements of the Finkel–Biskis–Jinkins murine oestrogenic sarcoma virus (FOS) promoter to enhance transcription of FOS protein which forms transcription factor complexes which alters cellular growth and differentiation (Figure 4.4).

Within the ERK1/2 pathway several genes showed statistically significant expression changes between the HHG-affected and HHG-unaffected foxes with the microarray or RT-qPCR data. The *protein kinase C zeta* (*PRKCζ*) and *RAS p21 protein activator (GTPase activating protein) 1* (*RASA1*) genes were of particular interest. *PRKCζ* is a member of the AGC Ser/Thr protein kinase family, belonging to the atypical protein kinase C subfamily that works independently of calcium

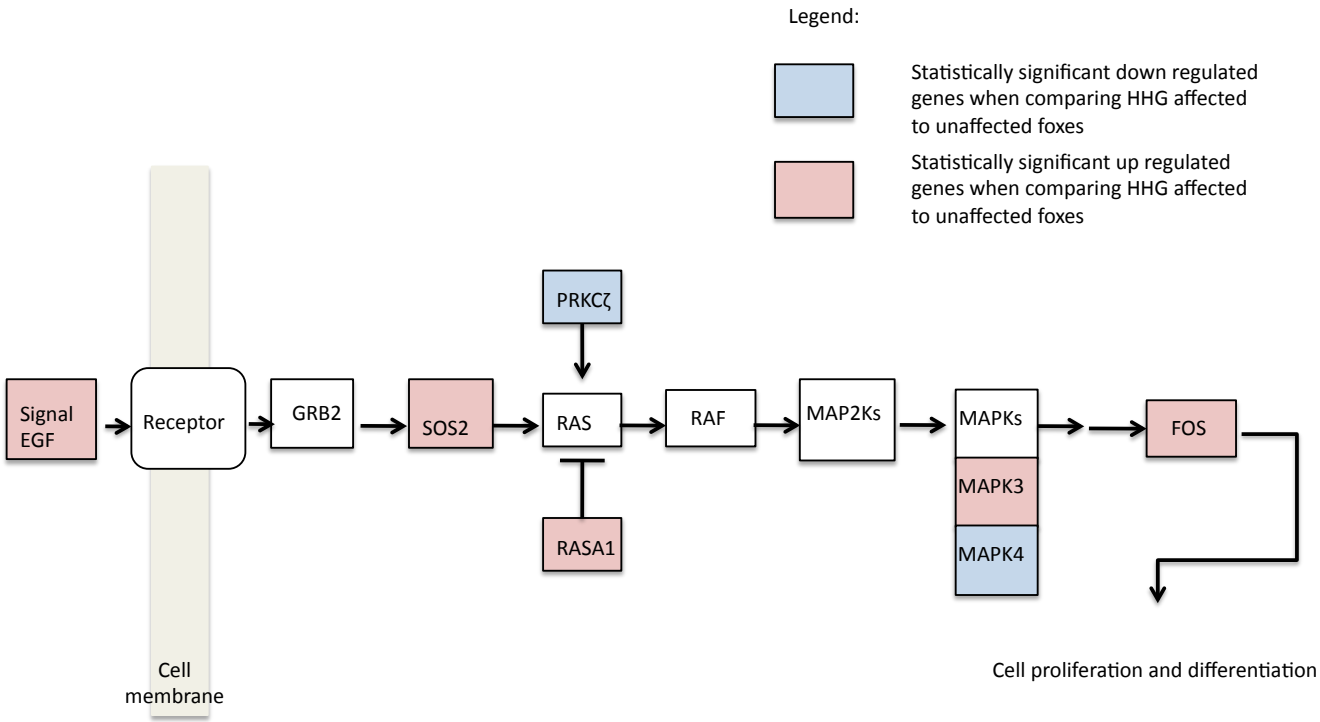


Figure 4.4: Diagram of several steps in the extracellular signal-regulated kinase (ERK) sub-pathway of the mitogen-activated protein kinase (MAPK) pathway in canines, highlighting genes differentially expressed on the microarray or in the RT-qPCR results in foxes affected with hereditary hyperplastic gingivitis (HHG). Adapted from Kelder, van Iersel, Hanspers, Kutmon, Conklin, Evelo and Pico 2011.

(Hirai and Chida 2003). *RASA1* is an inhibitor of *RAS* by accelerating the GTPase hydrolysis reaction converting guanosine-5'-triphosphate (GTP) to guanosine-5'-diphosphate (GDP) thus inactivating *RAS* (Rudack *et al*. 2012). In the ERK pathway both gene products act upon *RAS*: *PRKCζ* activates it while *RASA1* inhibits it (Figure 4.4). According to the microarray data, PRKC*ζ* is significantly down-regulated in HHG-affected foxes, while RASA1 is significantly up-regulated. The combined effect of the differential expression of these two genes results in the decreased activity of *RAS*.

One hypothesis for the differential expression of the ERK pathway in HHG is that the putative mutation occurs before the *RAS* gene. Upstream of the *RAS* gene in this signalling cascade, two other genes demonstrated an up-regulation in the HHG-affected compare to the HHG-unaffected foxes in the microarray analysis, the *epidermal growth factor* (*EGF*) and *son of sevenless homolog 2* (*SOS2*) genes. The hypothesis is that if the EGF signal is mutated, then the downstream *SOS2* gene product would in turn be up-regulated in the HHG-affected samples. Alternatively, given the similarity between *SOS1* and *SOS2*, and the fact that *SOS1* contains the putative mutation for HGF1 in humans, perhaps *SOS2* carries the HHG causative mutation. The differential expression of *RASA1* and *PRKCζ* could then represent a compensatory mechanism to offset increases in *RAS* expression. Nonetheless the up-regulation of FOS downstream in the pathway (statistically significant with the RT-qPCR assay) indicates that this strategy is only partially successful, and further implicates this pathway in HHG.

qPCR assay) indicates that this strategy is only partially successful, and further implicates this pathway in HHG.

Interpreting global expression data is a complex process whereby it can be difficult to determine how the normal expression pattern changes in response to the disease process. In the case of HHG, the disease process can be altering several pathways simultaneously, some directly due to the underlying disease mutation and others in response to the disease process. In addition HHG is associated with a superior fur quality meaning that the complex process might change depending on the tissue type that is being examined. To help eliminate some of this complexity our study design only examined gene expression in the gingival tissue. In addition, we utilized known and speculated genetic information about the analogous disease HGF as a starting point when exploring potential pathways, to help determine what pathways were more likely involved in pathogenesis as opposed to in reaction to the disease.

The canine genome has enabled genetic investigation in *Vulpes* (Kukekova *et al*. 2004). While conducting a microarray experiment based upon genomic similarities between these species enables new avenues for research in non-traditional model organisms, it creates new challenges. This study dealt with two major challenges. The first was the cross-species microarray hybridization. While the dog and fox are evolutionarily closely-related, a non-exact match to a probe set could result in the loss of signal and thus loss of the ability to detect potential differential expression (Kukekova *et al*. 2004; Wayne *et al*. 1997). The second major obstacle was utilizing

near-wild animal samples in a controlled experimental design. While the fox samples collected were from fox farms, the animals are still biologically diverse when compared to laboratory-bred model organisms. These obstacles might account for some of the discrepancies seen between the microarray and RT-qPCR data. This however does not discount the general trends that we discuss.

Conclusions

Thorough examination of the genetic loci involved in HGF in comparison to the fox HHG microarray experiment has led to several significant findings. First, the *SOS1* mutation that causes HGF1 is not causative of HHG, a result confirmed by sequence analysis (Clark *et al*. 2014a). Second, we find evidence that androgens play a role in the HHG phenotype, as indicated by the increased expression of *SRD5A2* in the HHG-affected foxes. Third, microarray and RT-qPCR analysis support the involvement of the MAPK pathway in HHG, as has been suggested for HGF1 and HGF2. We recommend further investigation of this pathway, specifically with respect to pathway members upstream of *RASA1,* including *EGF* and *SOS2,* to determine the HHG-causative mutation, both with single gene sequencing, and whole exome sequencing.

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Chapter Five:
Examination of hereditary hyperplastic gingivitis in association with superior fur quality in farmed silver foxes using canine microarrays and candidate gene sequencing

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Co-authorship statement

The following is to provide clarification of the roles played by multiple co-authors in the manuscript published from chapter five. As per the guidelines set forth by the School of Graduate Studies my contributions as thesis author is divided into the following areas: *i) design and identification of the research proposal; ii) practical aspects of the research; iii) data analysis* and *iv) manuscript preparation*.

i) *Design and identification of the research proposal*

The concept for this chapter and all experimental design were developed by Jo-Anna B.J. Clark. Jo-Anna B.J. Clark identified MAP2K6 as a candidate gene for hypertrichosis-related gum disease.

ii) *Practical aspects of the research*

Jo-Anna B.J. Clark identified all pairs of foxes that were used in this study, and obtained the necessary tissue samples, working with a veterinarian at the fox farm. RNA preparation and quantification for the microarrays was conducted by Jo-Anna B.J. Clark and sent to The Center for Applied Genomics in Toronto where the microarray experiments were performed. For the first batch of RT-qPCR reactions, cDNA was prepared by Jo-Anna B.J. Clark while RT-qPCR was conducted by a student research assistant under Dr. Clark's supervision. When additional RT-qPCR trials became necessary the cDNA was prepared by Jo-Anna B.J. Clark and sent to The Center for Applied Genomics for the RT-qPCR itself. MAP2K6 sequencing was

conducted by Desmond Whelan, as part of a BSc Honours project supervised by H. Dawn Marshall for which Jo-Anna B.J. Clark provided input and guidance.

iii) *Data analysis*

All microarray and RT-qPCR data analysis and interpretation was completed by Jo-Anna B.J. Clark from raw data files with guidance provided by Marije Booman. *MAP2K6* sequence analysis was completed by Desmond Whelan, under the guidance of Jo-Anna B.J. Clark and supervision of H. Dawn Marshall.

iv) *Manuscript preparation*

Jo-Anna B.J. Clark prepared the manuscripts with critical review and editing from H. Dawn Marshall.

Introduction

Hereditary hyperplastic gingivitis (HHG) presents as tumour-like growths of the gingival tissue on the mandible in farmed silver foxes, a coat colour variant of the red fox (*Vulpes vulpes*) (Dyrendahl and Henricson 1960). HHG is characterized by progressive proliferation of the gingival tissues starting at approximately two to three years of age leading to the encapsulation of the teeth and inhibition of normal function. The HHG inheritance pattern is autosomal recessive with sex-biased penetrance displaying an increased occurrence in males over females (Dyrendahl and Henricson 1960). HHG frequently co-occurs with superior fur quality, described as length and thickness of guard hairs, suggesting that a pleiotropic relationship is responsible for both phenotypes. Despite its discovery in Sweden in the 1940s (Dyrendahl and Henricson 1960) this disease was only documented in Newfoundland and Labrador, Canada, in 2004, when Finnish foxes selected for their denser, longer fur quality were introduced into the breeding population of farmed Canadian silver fox lines (Figure 5.1) (Clark *et al*. 2015). The molecular aetiology of HHG and its association with fur quality remain unknown.

Human hereditary gingival fibromatosis (HGF), an analogous condition to HHG, is a rare disease of the oral cavity with progressive benign fibrous enlargements of the maxillary and mandibular keratinized gingival tissue (Hart *et al*. 2002). HGF affects both sexes equally and average disease onset occurs during permanent dentition, with progression worsening through adolescence (Breen *et al*. 2009; Coletta and Graner 2006; Fletcher 1966). While its underlying origin and cellular mechanisms

Figure 5.1: Initial hereditary hyperplastic gingivitis presentation in original Finnish silver foxes in 2004 (Photos provided by Robert Hudson)

are still mostly unknown, HGF can be broadly classified as primary or secondary HGF (Shashi *et al*. 1999). Primary HGF can be further divided into genetic aetiologies and HGF-associated syndromes, while secondary HGF involves an acquired drug-induced HGF phenotype. The focus to date has been on the understanding the genetic aetiologies of HGF with little focus on the HGF-associated syndromes.

Syndrome-associated HGF presents with high phenotypic variability (Haytac and Ozcelik 2007). The most commonly associated presentation with HGF is hypertrichosis (Coletta and Graner 2006). This association can occur with or without mental retardation and displays a dominant inheritance pattern (Coletta and Graner 2006). Haplotype analysis and linkage studies have demonstrated that when HGF with hypertrichosis occurs, it is not linked to two other known, genetic forms of HGF (HGF1 and HGF2) suggesting a distinct genetic form and aetiology of HGF (Mangino *et al*. 2003).

Hypertrichosis is defined as an abnormal excess of hair growth that is not primarily androgen dependent nor influenced by race, sex or age (Beighton 1970). The incidence estimates of this rare condition have been about one in a billion but as these do not account for hypertrichosis in association with numerous syndromes they are probably under-estimates (Beighton 1970; Garcia-Cruz *et al*. 2002). Hypertrichosis can be broadly split into congenital or acquired categories, and the congenital category is divided based on a general or localized hair distribution. The generalized distribution is considered a syndrome and is further subdivided

according to whether the hypertrichosis is the primary or secondary syndrome characteristic. The subtypes with gingival hyperplasia are classified as syndromes with hypertrichosis as the primary symptom, and include congenital hypertrichosis lanuginose (CHL) and gingival fibromatosis with hypertrichosis. In addition, there is a group of syndromes with hypertrichosis and acromegaloid facial features with or without gingival fibromatosis, for example Cantu syndrome, that are thought to be phenotypic variations of the same disease (Czeschik *et al*. 2012).

HHG and HGF are diseases with similar manifestations and presentations; in particular the HGF-associated syndrome with hypertrichosis is similar in presentation to HHG-affected foxes with longer denser fur qualities. The goal of this study was to combine the current knowledge of HGF-associated syndromes with hypertrichosis with the genome-enabling capacity of the dog genome to examine potential molecular and cellular pathways involved in silver fox HHG. Utilization of a cross-species platform is supported by karyotyping, cytogenetic genome markers and gene sequence comparisons between *Vulpes vulpes* and *Canis lupus familiaris* that provide strong evidence supporting the close relationship between the two species (Switonski *et al*. 2009). Specifically, the Affymetrix GeneChip Canine Genome 2.0 Array was employed to compare the global gene expression patterns of HHG-affected farmed silver foxes and HHG-unaffected farmed silver foxes. We conducted an extensive review of the congenital forms of hypertrichosis with gingival fibromatosis in humans and examined their known chromosomal regions and in the context of the HHG microarray expression patterns. In addition we report candidate

gene sequencing based mutational analysis of the *mitogen activated protein kinase kinase 6* (*MAP2K6*) gene in the silver fox, for which a role in hypertrichosis has been debated (Sun *et al*. 2009).

Materials and Methods

Please see chapter four (Pages 87-92) for description of sample collection, RNA preparation and microarray experiment and analysis.

Microarray analysis

Using the NCBI human genome build 36.3 gene lists were created based on the genes found within the chromosomal loci 17q24.2-q24.3 and 8q22, which are known to be associated with hypertrichosis with gingival fibromatosis (http://www.ncbi.nlm.nih.gov/projects/mapview/map_search.cgi?taxid=9606&query=human&SITE=HumanGuide&SUBMIT=y&submit=Go). These gene lists were cross-referenced to the microarray expression results and annotated with gene names and functions using NetAffx Analysis Centre (Affymetrix Inc., Santa Clara, USA). Additionally, genes were placed in Wikipathways to investigate involvement in known canine signalling pathways (Kelder *et al*. 2011).

Reverse transcription quantitative PCR (RT-qPCR)

cDNA was transcribed from total RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems Inc., Foster City, USA). Initial RT-qPCR assays were conducted in house or at the Microarray Facility at the Toronto Centre for Applied Genomics,

Hospital for Sick Children in Toronto, Canada (see Appendix 2). For each assay, cDNA was combined with 10 µL Taqman Master Mix (2X) and 1 µL Taqman Gene Expression Assay (20X; 900 nM primer and 250 nM probe) (Applied Biosystems Inc., Foster City, USA.). Reaction mixtures were placed into MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems Inc., Foster City, USA.) and subjected to thermal cycling in the StepOnePlus Real Time PCR System (Applied Biosystems Inc., Foster City, USA.) according to the following profile: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. A selection of MAPK signalling pathway genes (Assay ID) were selected for RT-qPCR testing including: *transforming growth factor β 3* (*TGFβ3*) (Cf02625275_m1), *mitogen-activated protein kinase kinase kinase 6* (*MAP2K6*) (Cf02683089_m1) and *mitogen-activated kinase kinase kinase 1* (*MAP3K1*) (Cf02635276_m1). As the endogenous control gene, *elongation factor, RNA polymerase II* (*ELL2*) (Cf02707156_m1) was selected for the in house RT-qPCR, and the *breakpoint cluster region* (*BCR)* (Cf02664178_m1) for the remaining RT-qPCR samples.

Relative quantification of each gene was calculated using the ΔΔCT method (Livak and Schmittgen 2001). For the in house RT-qPCR the unaffected sample with the lowest standard error was selected as the reference sample while for the remaining RT-qPCR samples the unaffected sample with the lowest expression was selected as the reference sample. Fold changes were calculated using Log2RQ method and the relative fold changes were tested for significance with two-tailed t-tests. All samples were tested in triplicate and samples with a standard deviation greater than 0.5

were removed from the dataset. Any samples that were identified as outliers within biological groups according to Data Assist v3.0 (Life Technologies, Grand Island, USA) were also discarded. Validation experiments were conducted to standardize amplification efficiency.

Primer design for candidate gene sequencing

Primers were designed using the *Canis lupus familiaris* sequences with GenBank Accession Number NC_006591.3, found on chromosome 9, for the *MAP2K6* gene (http://www.ncbi.nlm.nih.gov/). Primer pairs were selected from the intronic regions flanking each target region using NCBI Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All parameters were set to default except for the "Primer Melting Temperatures" which were as follows: maximum melting temperature: 65^0C; minimum melting temperature: 50^0C; and optimum melting temperature: 57^0C. Primers were manufactured by Operon Inc. as PCReady primers (Huntsville, USA). Refer to Appendix 1 for primer sequences.

DNA preparation and polymerase chain reaction (PCR)

DNA extractions were performed with the QiaAmp DNA Mini kit (Qiagen Inc., Mississauga, Canada) according to the manufacturer's tissue protocol for six HHG-affected and six HHG-unaffected samples. Each PCR contained 2.5 μL of 10X buffer, 0.5 μL dNTPs (New England Biolabs Ltd., Whitby, Canada), 1 μL of 10 μM forward primer, 1 μL of 10μM reverse primer, 0.2 μL of 5 U/μL Qiagen Hot Star Taq Plus (Qiagen Inc., Mississauga, Canada), 15 μL distilled nuclease free water and 1 μL

template DNA. The thermal profile used was 95°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, target-specific annealing temperature for 30 seconds, and 72°C for 1 minute, followed by 72°C for 10 minutes. Amplified PCR products were purified using either Pall Life Sciences Multi-Well Plate Manifolds (Pall Corporation, Port Washington, USA) or the QIAquick PCR purification kit (Qiagen Inc., Mississauga, Canada). Target-specific annealing temperatures were as recommended by the primer selection software. PCR products were tested using 1.5% agarose gel electrophoresis

DNA sequencing and analysis

DNA sequencing reactions were performed with both forward and reverse primers for each PCR amplicon using BigDye Terminator v3.0 chemistry (Applied Biosystems Inc., Foster City, USA) utilizing the following thermal profile: 96°C for 6 minutes, then 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes. Sequencing reaction purification was carried out using either ethanol precipitation or an Agencourt CleanSeq method (Beckman Coulter Inc., Danvers, USA). Purified DNA sequencing reactions were electrophoresed on the Applied Biosystems Inc. 3730 DNA Analyzer, in the GaP Facility of the CREAIT Network at Memorial University of Newfoundland and at Genome Quebec located at McGill University. For detailed primer pair sequences, annealing temperatures and amplicon lengths refer to Appendix 1.

Raw data was collected using Sequence Analysis v5.2 (Applied Biosystems Inc., Foster City, USA) and imported into Sequencher v4.8 (Gene Codes Corp., Ann Arbor, USA). Contigs were created by assembling reads to the reference sequence, Accession Number NC_006591.3 (chromosome 9) using an 85% minimum gap percentage and a 20% minimum overlap, followed by manual trimming and editing of each sequence read. Sequence was submitted to NCBI.

Results

Differential gene expression in HHG-affected compared to HHG-unaffected silver foxes based on the Affymetrix GeneChip Canine Genome 2.0 Array, 43,035 gene ID probes in both the HHG-affected and HHG-unaffected groups were analyzed. Of these, 1,154 probesets demonstrated a statistically significant differential gene ID probe expression when comparing the HHG-affected to HHG-unaffected samples as determined by a Mann-Whitney *t*-test ($P < 0.05$). 508 probesets demonstrated at least a two-fold differential up-regulation when comparing HHG-affected foxes to HHG-unaffected foxes and 446 Probe IDs demonstrated a least a two-fold differential down-regulation when making the same comparison.

Figure 5.2 demonstrates the general expression trend comparison between the HHG-affected and unaffected samples for a selection of RT-qPCR genes involved in the MAPK signalling cascade. The *TGFβ3* gene showed a statistically significant fold change ($P<0.05$) (Table 5.1).

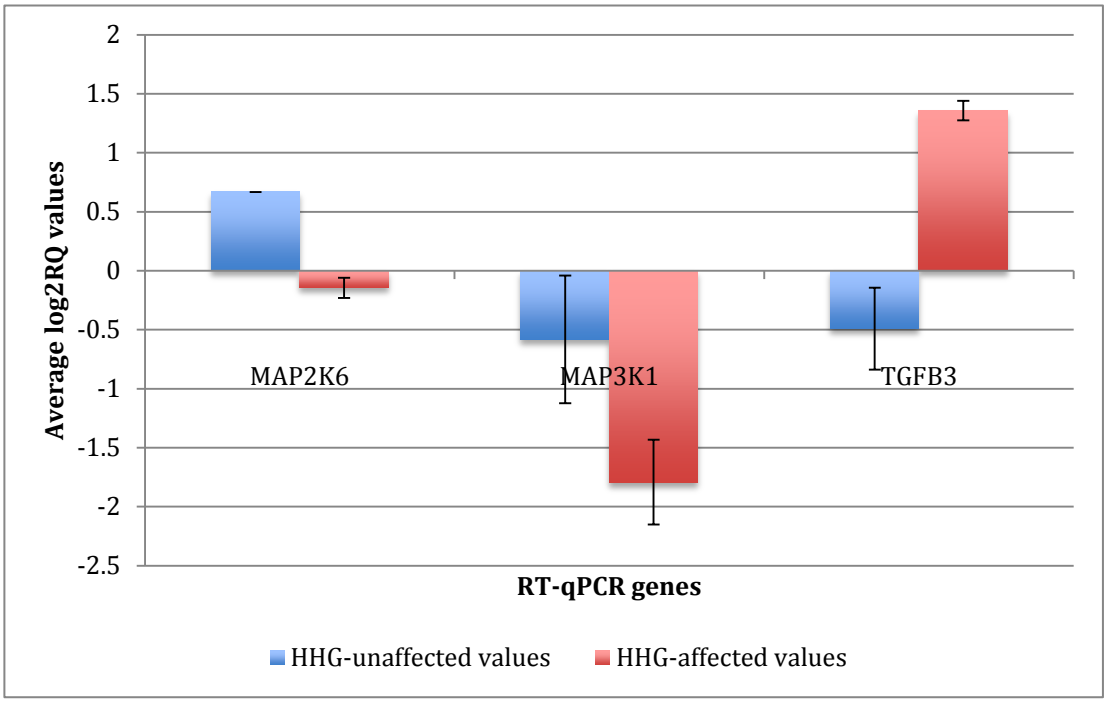

Figure 5.2: Mean $\log_2$ relative expression ($\log_2$RQ) values for RT-qPCR genes in the hereditary hyperplastic gingivitis (HHG)-affected and HHG-unaffected fox samples. Standard errors are indicated by the bars.

Table 5.1: Differential expression of genes in hereditary hyperplastic gingivitis-affected foxes compared to unaffected foxes from quantitative polymerase chain reaction (RT-qPCR) validation assays, compared with fold-change found by microarray analysis

Gene symbol	**Gene name**	**RT-qPCR**		**Microarray**		
		Fold change	**P value**	**Affymetrix probe ID**	**Fold change**	**P value**
MAP2K6	Mitogen-activated protein kinase kinase 6	-1.106	NA*	CfaAffx.16824.1.S1_at	-1.203	0.250
MAP3K1	Mitogen-activated protein kinase kinase kinase	-2.313	0.112	Cfa.19524.1.S1_at	-1.959	0.071
TGFβ3	Transforming growth factor beta 3	3.603	0.026	CfaAffx.26185.1.S1_at	1.084	0.788

*NA Insufficient samples present in triplicate to perform a *t*-test

Comparison of gene expression patterns from microarrays to chromosomal loci associated with hypertrichosis with gingival fibromatosis

There are a number of congenital hypertrichosis syndromes that occur with gingival overgrowth, including CHL, Cantu syndrome and hypertrichosis with gingival fibromatosis (Garcia-Cruz *et al*. 2002). Genes within the loci for each of these syndromes were examined and compared with the differential expression HHG-affected versus HHG-unaffected microarray data in the silver fox samples.

In humans, CHL is associated with chromosomal region 8q22-24 (Tadin *et al*. 2001). NCBI human genome build 36.3 contained 215 genes in this chromosomal region (accessed July 2013). Eighty-two of those genes correspond to equivalent known canine genes. On the Affymetrix GeneChip Canine Genome 2.0 Array those 82 genes were represented by 170 probesets. Five of those probe IDs demonstrated statistically significant expression differences (Mann Whitney $P<0.05$) and an additional nine when the requirement for statistical significance was relaxed to $P<0.10$ to account for the high biological diversity of the samples (Table 5.2). These 14 probe IDs represented 11 different genes with three that were differentially up-regulated in the HHG-affected versus HHG-unaffected fox samples and eight that were differentially down-regulated in the same comparison.

Cantu syndrome in humans has a putative causative mutation in the *ATP-binding cassette, sub-family C member 9* (*ABCC9*) gene (Czeschik *et al*. 2012). There is a

Table 5.2: Microarray-identified differential expression in hereditary hyperplasticgingivitis (HHG)-affected foxes to unaffected foxes for genes found in the human chromosome loci associated with hypertrichosis occurring with gingival overgrowth

Gene symbol	Gene name	Human chromosome region	Fold change	P value
ABCA5	ATP-binding cassette, sub-family A, member 5	17q24.2-q24.3	1.022	0.393
ABCA6	ATP-binding cassette, sub-family A, member 6	17q24.2-q24.3	1.014	0.571
MAP2K6	Mitogen-activated protein kinase kinase 6	17q24.2-q24.3	-1.203	0.250
PITPNC1	Phosphatidylinositol transfer protein, cytoplasmic 1	17q24.2-q24.3	1.358	0.036
OXR1	Oxidation resistance 1	8q22	-1.288	0.036
NCALD	Neurocalcin delta	8q22	-1.232	0.036
MED30	Mediator complex subunit 30	8q22	-1.789	0.036
COL14A1	Collagen, type XIV, alpha 1	8q22	1.242	0.036
AZIN1	Antizyme inhibitor 1	8q22	-1.228	0.036
TAF2	TATA box binding protein associated factor	8q22	-1.258	0.071
RPL30	Ribosomal protein L30	8q22	-1.303	0.071
RAD21	RAD21 homolog	8q22	-1.431	0.071
NUDCD1	NudC domain containing 1	8q22	-1.551	0.071
KIAA1429	KIAA1429 ortholog	8q22	1.124	0.071
CPQ	Carboxypeptidase Q	8q22	1.122	0.071

homologous gene in the canine microarray represented by two probe IDs. Both probe IDs demonstrated an up-regulation in the HHG-affected compared to the HHG-unaffected microarray fox samples but not significantly so (P>0.10). The ABCC9 protein is part of an ATP potassium-sensitive channel composed of four pore-forming subunits of either KCNJ8 or KCNJ11 and four regulatory subunits of the ABCC9 protein (Harakalova *et al*. 2012). KCNJ8 and KCNJ11 microarray gene expression levels were examined and neither showed any statistically significant differential expression (P>0.10). Hypertrichosis with gingival fibromatosis is associated with the chromosome region 17q24.2-q24.3 in humans (Sun *et al*. 2009) and this region contains the following genes: *ABCA5*, *ABCA6*, *ABCA10* and *MAP2K6*. ABCA10 was not on the Affymetrix GeneChip Canine Genome 2.0 Array, nor was a description of the gene found in NCBI for canines. The remaining genes were compared to the HHG microarray data but none yielded a statistically significant differential expression with a Mann Whitney *t*-test at P<0.05 (Table 5.2). The *MAP2K6* gene did show statistically significant (P<0.05) differential expression in the RT-qPCR assay with a down-regulation of the gene in the HHG-affected foxes compared to the unaffected foxes (Table 5.1). NCBI human genome build 36.3 listed 36 genes within that chromosome region (accessed July 2013). Of these there were 20 canine equivalents that were represented on the Affymetrix GeneChip Canine Genome 2.0 Array by 46 probe IDs. Of those probe IDs, the *Phosphatidylinositol Transfer Protein, Cytoplasmic 1* (*PITPNC1*) gene showed was significantly (P<0.05) differentially up-regulated in the HHG-affected versus HHG-unaffected microarray fox samples.

Mutational differences between affected and unaffected foxes in the MAP2K6 gene

The *MAP2K6* gene was sequenced for six HHG-unaffected foxes and six HHG-affected foxes. There was 100% coverage of the protein-coding region and the intron/exon boundaries for several HHG-affected and several HHG-unaffected animals. There were no fixed mutations segregating the HHG-affected from the HHG-unaffected sets of samples in any of coding portions or intron/exon boundaries of the *MAP2K6* gene, nor were there any apparent fixed heterozygous sites.

Comparison of HHG microarray gene lists to MAPK pathway genes

The canine c-Jun N-terminal kinase (JNK), and p38; portions of mitogen-activated protein kinase (MAPK) signalling cascade; were examined using Wikipathways (Kelder *et al*. 2011) to determine if any significantly differentially expressed genes within the fox HHG-affected vs. HHG-unaffected microarray comparison could be identified. Three genes showed statistically significant differential expression (Mann-Whitney *t*-test $P < 0.05$) and four in total with a relaxed Mann Whitney criterion ($P < 0.10$) (Table 5.3). Of these four genes, one showed up-regulation in the HHG-affected samples and three showed down-regulation in the HHG-affected samples compared to the HHG-unaffected samples.

Discussion

HHG is a condition that occurs predominantly in the farmed silver fox population, where HHG appears to have been selected when choosing to breed animals with

Table 5.3: Microarray analysis of differential expression of hereditary hyperplastic gingivitis (HHG)-affected foxes to unaffected foxes for genes found in the c-Jun N-terminal kinase (JNK) and p38 portions of the mitogen-activated protein kinase (MAPK) signalling cascade

Gene symbol	Gene title	Fold change	Mann Whitney P value
CASP6	Caspase 6, apoptosis-related cysteine peptidase	-1.213	0.036
IL1B	Interleukin 1, beta	1.189	0.036
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	-1.959	0.071
MAP4K1	Mitogen-activated protein kinase kinase kinase kinase 1	-1.270	0.036

longer, denser fur (Dyrendahl and Henricson 1960). HGF is an analogous condition in humans with a complex aetiology. The genetic basis of syndromic HGF associated with hypertrichosis is distinct from the isolated genetic forms of HGF (Coletta and Graner 2006; Mangino *et al*. 2003). While often viewed as separate but co-occurring, fibromatosis and hypertrichosis may be different phenotypic expressions of a common ectodermal or mesodermal congenital abnormality (Lee *et al*. 1993). Here we use a canine microarray platform to investigate the aetiology of hypertrichosis with HHG in the context of what is known about the genetic basis of the human syndrome.

Comparison of HHG microarray gene lists to chromosomal loci of gingival fibromatosis associated with hypertrichosis

CHL is often referred to as Ambras syndrome after paintings that were discovered in the Ambras castle in Austria depicting the child Peter Gonzales, who had this form of hypertrichosis (Freire-Maia *et al*. 1976). The main features of CHL are the excessive growth of lanugo hair and dental abnormalities (Garcia-Cruz *et al*. 2002). Typically the latter involve the delayed presentation of primary and secondary teeth as well as the absence of some teeth (Tadin *et al*. 2001). However, there has been a report of a sporadic case of CHL with mild gingival hyperplasia (Lee *et al*. 1993). CHL has autosomal dominant inheritance with the occurrence of sporadic cases (Tadin *et al*. 2001). The CHL mutation has been mapped to the 8q22-q24 chromosomal region (Tadin *et al*. 2001). The HHG microarray demonstrated 11 genes with a statistically significant ($P<0.10$) differential expression between the HHG-affected and HHG-

unaffected fox samples. While none of these genes is thought to contain the causative mutation, their expression levels and possible contributions to disease phenotype are of note. Of particular interest was the *COL14A1* gene. The *COL14A1* gene was up-regulated in the HHG-affected foxes compared to the HHG-unaffected foxes. This gene encodes collagen 14 protein produced by fibroblasts, myofibroblasts and hepatic stellate cells during late embryogenesis (Ruehl *et al*. 2005). It plays a role in cellular quiescence and differentiation, such as during wound healing, and has been known to induce fibroblast differentiation (Ruehl *et al*. 2005). As HGF is associated with a more friable tissue (Clark *et al*. 2015) it is plausible that the increased need for wound healing has resulted in the up-regulation of this gene.

Cantu syndrome is classified primarily with hypertrichosis, cardiomegaly and bone abnormalities and can result from autosomal dominant or recessive inheritance (Kurban *et al*. 2011). While typically not associated with gingival fibromatosis it is thought that Cantu syndrome along with the phenotypes of acromegaloid with or without hypertrichosis and gingival fibromatosis are variations along a spectrum of the same disease (Czeschik *et al*. 2012). The putative causative mutation of this condition is in the ABCC9 gene, located at 12p12.1. In the silver fox microarray study the gene is up-regulated in HHG-affected compared to the HHG-unaffected fox samples but not significantly so. Although the HHG-affected foxes have both gingival and hair overgrowth they do not show the bone or cardiac abnormalities associated

with Cantu syndrome. Hence it is unlikely that ABCC9 is involved in HHG, although RT-qPCR validation of the up-regulation and mutational analysis may challenge this.

Gingival fibromatosis with hypertrichosis is an autosomal dominant condition with terminal hair covering the face, trunk and eyebrows, with progressive gingival hyperplasia starting in childhood (Wendelin *et al*. 2003). It is associated with chromosomal region 17q24.2-24.3 (Sun *et al*. 2009). Of interest, this region contains several members of the ATP binding cassette transporter family A (ABCA) family of genes, *MAP2K6,* and the *PITPNC1* gene. The ABCA is a large family of proteins (Albrecht and Viturro 2007). ABCA5 has been found in lysosomes and endosomes and is thought to be involved in intracellular trafficking (Kubo *et al*. 2005). In rat mRNA expression the *ABCA5* transcript has been identified in Leydig cells where testosterone production occurs (Petry *et al*. 2006). ABCA5 was down-regulated in both male and female HHG-affected fox samples when compared to the unaffected samples so the gingival fibromatosis phenotype is unlikely to be associated with Leydig cell biological function. However, in dogs, the gingival tissue is androgen sensitive, such that testosterone exacerbates the phenotypic presentation of gingival hyperplasia (Pariser and Berdoulay 2011). If the fox gingival tissues are similar to the canine counterpart then the down-regulation of the *ABCA5* gene may be in response to the already hypertrophied androgen-sensitive gingival tissue. ABCA6 is responsive to cholesterol concentrations and is up-regulated during macrophage differentiation (Kaminski *et al*. 2001). The up-regulation of ABCA6 in the HHG-affected foxes could be related to disease aetiology, although it is plausible that it

may be up-regulated as part of an inflammatory response in the gingival tissue leading to an increase in macrophage differentiation.

In the human genome version 36.3 the *PITPNC1* gene is identified in the 17q24.2-24.3 chromosomal region and this gene was up-regulated in the HHG-affected foxes when compared to the HHG-unaffected foxes. The *PITPNC1* gene encodes the RdgBβ protein, a Class IIB PIP protein subfamily (Cockcroft 2012). RdgBβ expression is tightly regulated, with a substantial level of expression in cardiac and renal tissue (Garner *et al*. 2011). This protein has been implicated in a variety of processes and the full scope of it biological roles is unknown (Trivedi and Padinjat 2007). One of its roles is during wound healing (Gu and Iyer 2006). As previously mentioned HGF is a delicate tissue prone to injury (Clark *et al*. 2015) contributing to an increased need for wound healing, potentially explaining the up-regulation of this gene.

Candidate gene, microarray, and RT-qPCR analysis of the MAP2K6 gene in HHG-affected foxes

The original interest in the 17q24.2-24.3 chromosomal region was in the *MAP2K6* gene (Sun *et al*. 2009) . While other MAPK pathway proteins have been implicated in both hair growth and gingival overgrowth conditions there was some debate about the *MAP2K6* gene involvement in the combined phenotype (Hart *et al*. 2002; Sun *et al*. 2009). In 2008, a human case was reported with a 2.3Mb deletion encompassing this gene that did not result in either hypertrichosis or gingival overgrowth (Blyth *et al*. 2008). The microarray analysis of this gene showed a differential down-

regulation in the HHG-affected foxes compared to the HHG-unaffected foxes but this difference was not statistically significant. RT-qPCR however confirmed the differential down-regulation in the HHG-affected fox samples again without statistical significance. Depending on the nature of the mutation however and due to the inherent biological variability this does not definitely rule it out as a putative HHG gene.

Mutational analysis of the coding regions and exon-intron splice sites of the *MAP2K6* gene did not show any putative candidate mutations within HHG-affected foxes in this gene. The *MAP2K6* gene did demonstrate variability between the fox sequences and the *Canis* reference sequence. In particular, exon 3 carried a nonsynonymous sequence difference between the dog and the foxes, which was variable within foxes. In dogs, the sequence is CGT (codon for histidine) while in the fox samples the variant form of this same allele was CAT (arginine). Although some foxes were heterozygous for these two alleles, there were no fixed differences between affected and unaffected individuals. While *MAP2K6* does not appear to be the causative gene for HHG at least with respect to coding portion of the gene, it is possible that allelic variations contribute to phenotype severity; for example this amino acid change could potentially result in a greater propensity for foxes to develop HGF. The absence of a putative mutation does not completely exclude the possibility of the *MAP2K6* gene involvement in the phenotype of HHG with hypertrichosis as it is still possible that there is a mutation in the promoter region or within the introns that

leads an enhanced expression of the gene. The expression pattern we observe supports the potential involvement of this pathway in the HHG presentation.

Mitogen activated protein kinase pathway

Emphasis has been placed on the MAPK signalling pathway with a direct link to several of the known isolated genetic forms of HGF and again its role in hypertrichosis with gingival fibromatosis, warranting a further examination in the MAPK pathway (Hart *et al*. 1998; Sun *et al*. 2009; Xiao *et al*. 2001). MAPK signalling starts with a signal that is transmitted through a receptor that in turn activates a multi-tier cascade of mitogen-activate protein kinase phosphorylations ultimately resulting in a cellular event at the nucleus (Yang *et al*. 2013) (Figure 5.3). MAP2K6 signalling occurs in the c-Jun N-terminal kinase (JNK), and p38 sub-pathways in the MAPK signalling schematic (Krishna and Narang 2008; Wang and Tournier 2006). The p38 and JNK pathways are activated by signals such as interleukin B (ILB), transforming growth factor beta (TGFβ) and stress and end within a common pathway of cell proliferation, differentiation or cellular apoptosis (Kim and Choi 2010). Based on both the microarray and RT-qPCR data there are several genes for both the JNK and p38 pathways demonstrating a differential expression between the HHG-affected and unaffected samples.

At the start of the JNK pathway is the cellular signal *interleukin 1, beta* (*IL1B*) which is up regulated in the HHG-affected foxes. After the receptor the *caspase 6, apoptosis-*

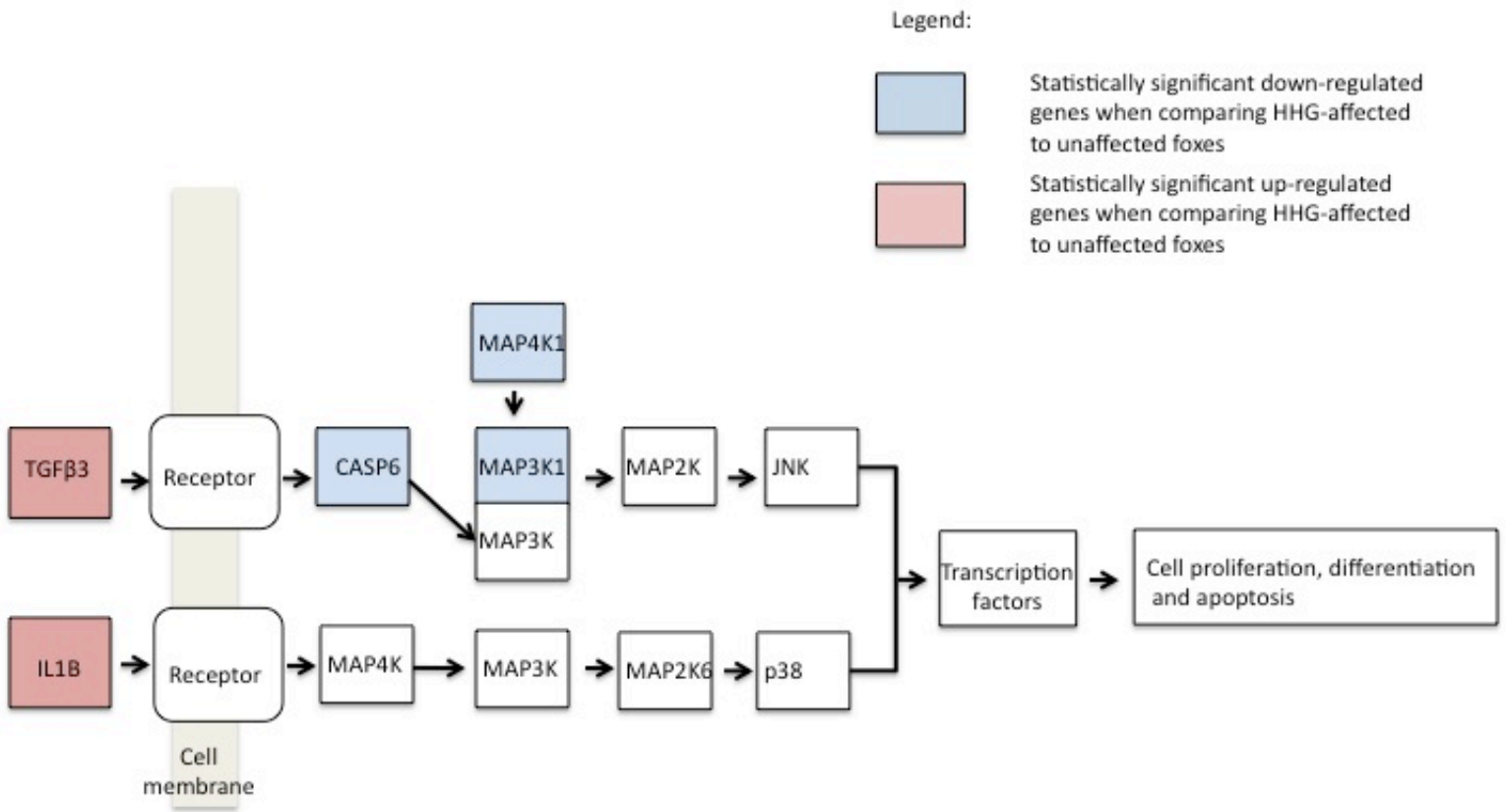

Figure 5.3: Diagram of several steps in the c-Jun N-terminal kinase (JNK), and p38 sub-pathway of the mitogen-activated protein kinase (MAPK) pathway in canines, highlighting genes differentially expressed on the microarray or in the RT-qPCR results in foxes affected with hereditary hyperplastic gingivitis (HHG). Cross talk between the pathways not illustrated. Adapted from Kelder, van Iersel, Hanspers, Kutmon, Conklin, Evelo and Pico 2011.

related cysteine peptidase (*CASP6*) adapter protein is down regulated in the HHG-affected foxes. Subsequent MAPK proteins *mitogen-activated protein kinase kinase kinase kinase 1* (*MAP4K1*) and *mitogen-activated protein kinase kinase kinase 1*(*MAP3K1*) also display down regulation in the HHG-affected foxes all with statistical significance (P<0.100). While the downstream portion of the pathway did not display any statistically significant differential expression the general trend appears to be a down regulation of this part of the MAPK pathway. The p38 pathway shows the inverse trend. This pathway had two up-regulated cellular signals including *TGFβ3* and the previously mentioned *IL1B*which acts as a signal for both pathways (Kelder *et al*. 2011). The *MAP2K6* gene, which was down-regulated in the HHG-affected foxes. As the end of this and the JNK pathway is the same we cannot comment on the overall affect of the up-regulation of the upstream members of this pathway. One hypothesis for the combined trend is that the JNK portion of the pathway is down-regulated in the HHG-affected foxes as a compensatory mechanism for the up-regulation of the p38 portion of the pathway in the HHG-affected foxes when compared to the HHG-unaffected foxes. Another hypothesis is that the portions of the pathway that are associated with apoptosis, like CASP6, are down-regulated to prevent death adding to the lifespan of the hypertrophied cells. While DNA sequencing has shown no mutation in the coding region of the *MAP2K6* gene the overall pattern of differential gene expression in the MAPK pathway still calls its involvement into question.

Hypertrichosis and gingival hyperplasia have overlapping phenotypic presentations that could support the notion of a shared underlying aetiology. Using the HHG-affected versus HHG-unaffected microarray platform the expression of genes in known hypertrichosis regions were explored. While a putative gene was not determined the expression of various genes helps to uncover potential contributing mechanisms such as the MAPK pathway involvement. While there was no putative mutation found in the MAP2K6 gene in the coding region, the overall differential gene expression trend still suggests involvement of the MAPK pathway.

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Chapter six:
Conclusions on the hereditary hyperplastic gingivitis in the silver fox (*Vulpes vulpes*)

Hereditary hyperplastic gingivitis (HHG) is a rare disease that is characterized by the progressive proliferation of the gingival tissues with fibrous overgrowths that inhibit normal functions like mastication and is found predominantly in ranched silver foxes (*Vulpes vulpes*) (Dyrendahl and Henricson 1960). Despite the existence of documentation as early as the 1940s, little is known about the molecular mechanisms underpinning its prolific phenotype. Analogous conditions occur in both humans and dogs that can aid in providing clues into the silver fox variant using both candidate gene and genome expression microarray approaches (Hale 2004; Hart *et al*. 1998). The first goal of this thesis was to document its emergence in Canada and perform a detailed examination on both the genetics and histological features of the disease. The second goal was to explore the molecular mechanisms underpinning the HHG phenotype, using a combination of candidate gene studies and examination of the HHG phenotype at the level of genome wide expression.

The functional candidate gene analysis was used to examine the DNA sequences of the exonic coding regions, exon-intron boundaries and partial introns of *SOS1*, the known causative gene of HGF1 in humans (Hart *et al*. 2002), as well as the closely-associated *GRB2,* and *EGFR* genes from both affected ranched silver foxes, a coat colour variant of the red fox, and unaffected wild red foxes were compared. Inferences were also made about the rates and patterns of evolution of the *SOS1, GRB2* and *EGFR* genes.

Examining HHG from the genomic standpoint allowed for the integration of the current knowledge of the analogous condition in human, HGF. This exploration was divided into examining both the known isolated genetic causes of HGF and the main HGF-associated syndromes. For the isolated genetic causes, I explored the many potential facets contributing to the HHG phenotype by reviewing the literature and the main cell signalling pathway highlighted therein, as well as looking for new insights by close examination of each mapped HGF locus. The HGF-associated syndrome portion focused on known genetic associations of syndromes with both gingival and hair overgrowth. The isolated and syndromic facets were both explored by comparison to HHG genome-wide differential gene expression data.

Examining HHG in light of known HGF information at both the gene and genome levels created a platform to gain a better understanding of the silver fox HHG phenotype.

HHG index case for North America

Prior to 2004, there were no documented cases of HHG in the North American silver fox, *Vulpes vulpes*. After the introduction of a Finnish fox line, the disease became evident not only in the original Finnish foxes but also in some of the offspring. Subsequent to its first appearance in Newfoundland and Labrador, HHG was extensively documented. Histological studies of affected fox gum tissue showed a submucosal expansion with thick collagen bundles. In response to the increase in HHG prevalence, a scoring system was devised to rank the severity of the disease

based on gross appearance. This system will help with future cases and it will aid with assessing disease progression. Utilizing co-operative strategies like candidate gene sequencing and whole genome microarray expression techniques based on canine genome resources with help to gain a better understanding of the molecular mechanisms underlying HHG.

Candidate gene sequencing

HHG and HGF are analogous conditions with very similar manifestations and disease progression. One major research goal was to use the known molecular basis of one form of HGF to perform a functional candidate gene analysis as a means to establish or eliminate particular gene mutations as causative of HHG in foxes. The DNA sequences of the *SOS1*, *GRB2*, and *EGFR* genes were obtained for candidate gene analysis of the exonic coding regions, exon-intron boundaries, and partial introns of these three genes in both affected ranched silver foxes and unaffected wild and farmed red foxes. In addition to mutational analysis, the sequences were used in multispecies comparisons for examination of patterns of molecular evolution.

No mutational differences separated HHG-affected foxes from unaffected ones for the *SOS1*, *GRB2* or *EGFR* genes. While these findings do not rule out the possibility that a mutation could exist in the upstream promoter/enhancer regions for either of these genes, or potentially elsewhere in the introns, it does conclude that there are no amino acid differences in the protein products of these genes that could alter the

protein function. The upstream region of the EGFR gene showed a GC content similar to *Canis familiaris*, which is different from that of humans. While canines demonstrate fewer CpG islands in the promoter regions than humans (Auton *et al*. 2013), further characterization of the promoter region is required before its involvement in the HHG phenotype can be eliminated.

The *SOS1* and *GRB2* genes showed similar molecular evolutionary patterns to each other, in that both genes were highly conserved at the intra-species, intra-family, and inter-genus levels in mammals. The non-synonymous substitutions and ω ratios were low for both genes, especially *GRB2*, consistent with strong functional constraints. The involvement of protein products of both genes in the activation of multiple signalling pathways and RAS pathway cellular functions such as proliferation, transformation and survival (Pierre *et al*. 2011), is consistent with the requirement of a high level of functional constraints, which was demonstrated. Across all measures of sequence diversity at all hierarchical levels *EGFR* demonstrated the highest level of variation among the three genes, suggesting that the *EGFR* gene has been subject to different molecular processes than the other two genes. At this time the reason for the increased variability seen in the EGFR gene cannot solely be attributed to either a reduction in functional constrains or positive Darwinian selection and may involve elements of both.

The HHG-affected silver foxes do not carry the same *SOS1* mutation that causes HGF in humans. At this time the involvement of coding region or exon-intron boundary

mutations in *SOS1* and two adjacent RAS pathway genes, *EGFR* and *GRB2* can also be ruled out as causative of the HHG phenotype in silver foxes.

Global HHG expression

Human HGF and silver fox HHG are analogous conditions. The aetiology of HGF is complex, as it presents in a number of ways including as an isolated genetic condition, as part of a syndrome or as a pharmaceutically induced phenotype (Shashi *et al*. 1999). The silver fox presentation of HHG was not the result of pharmaceuticals as the animals were not taking any medications, leaving an isolated genetic condition or as part of a syndrome as viable aetiological possibilities. Genome-wide HHG expression patterns in the silver fox were interpreted in the context of both the genetics of HGF and the most commonly associated syndrome characteristic of hypertrichosis. Each evaluation was conducted in the context of global differential gene expression patterns within gum tissue from farmed HHG-affected and unaffected silver foxes. In both comparisons portions of the MAPK signalling pathway were implicated. Additionally each comparison shed light on genes expressed differentially that while they may not contain causative mutations for HHG, could potentially be contributing to the overall molecular phenotype.

MAPK pathway is a cascading kinase pathway that is involved in a diverse array of cellular processes including cellular proliferation, differentiation and cellular death (Kim and Choi 2010). Given its diversity, malfunctions within the pathway are known to cause numerous disease conditions. Mutations in the *EGFR* gene are

associated with lung and colorectal cancer (Kim and Choi 2010). Noonan syndrome is associated with mutations in any of eight different MAPK genes (Roberts *et al*. 2013). A mutation in the *SOS1* gene is responsible for one of the genetic subtypes of HGF (Hart *et al*. 2002). The sequencing of this gene in the silver fox did not reveal any putative mutations making it an unlikely candidate as a causative gene for HHG.

The HHG comparison to the known HGF genetics revealed several interesting points. First it showed that overall the ERK portion of the MAPK pathway was differentially up-regulated in the HHG-affected foxes. Next it showed that activators of the RAS protein were down-regulated while inhibitors of the same protein were up-regulated in the HHG-affected animals leading to the hypothesis that the mutation could be occurring at or before the RAS protein in the signalling cascade. Finally, while the SOS1 gene was considered an unlikely candidate gene the SOS2 gene did show up-regulation in the HHG-affected foxes. SOS1 is required for early development such that large errors are lethal while an error in the SOS2 protein is not be lethal (Qian *et al*. 2000). Given the high level of sequence homology it is possible that an error in the *SOS2* gene would cause the overall up-regulation of the MAPK pathway leading the HHG phenotype, making *SOS2* a candidate gene for HHG.

The HHG comparison to the syndromes with both hypertichosis and gingival hyperplasia also implicated the MAPK pathway, more specifically the JNK and p38 portions of the pathway. Initial emphasis was placed on the *MAP2K6* gene, however DNA sequencing identified no coding or splice site mutations within this gene,

rendering it unlikely as a candidate gene for HHG. While the end of this portion of the MAPK pathway did not show statistically significant differential expression of any genes, the overall trend showed that the JNK pathway was down-regulated while the p38 portion was up-regulated in the HHG-affected foxes. One hypothesis is that the down-regulation of the JNK portion of the pathway is a compensatory mechanism for the up-regulation of the p38 portion of the MAPK pathway. Further studies are required to determine if this compensation is sufficient thus ruling out this portion of the MAPK pathway or if despite the compensation the pathway end products are still up-regulated and thus contributing to the HHG disease phenotype. While the differential expression pattern does not implicate a singular gene, overall MAPK pathway involvement is likely.

In both the isolated HHG genetic and hypertrichosis with gingival hyperplasia comparisons there were genes that could be contributing to the overall HHG phenotype. In the HHG to known HGF genetic comparison the *SRD5A2* gene was significantly up-regulated in HHG-affected foxes and it plays a role in testosterone conversion (Ye *et al*. 2011). In an analogous canine condition of gingival hyperplasia, one mechanism for the pharmaceutically-induced phenotype proposes that blocking of calcium channels interrupts the intracellular calcium dynamic, rendering the gingival tissues sensitive to testosterone (Pariser and Berdoulay 2011). The potential involvement of androgens opens up a novel avenue for future research. If HHG demonstrates a variable penetrance then the higher levels of testosterone in the male foxes could explain the sex bias observed originally in HHG (Dyrendahl and

Henricson 1960). Androgen receptors also participate in non-androgen signalling (Heinlein and Chang 2002). They have been linked to the activation of the MAPK pathway and the alteration of intracellular calcium levels. Further evaluation of the MAPK pathway could possibly help explain the HHG phenotype and provide the groundwork for a unifying mechanism underpinning all aetiologies.

Conclusions

HHG is a complex disease with analogous conditions seen in other animals. In both the canine and human variants, the aetiology of the disease is broad. Candidate gene sequencing eliminates both the *SOS1* and *MAP2K6* genes as likely disease-causing genes. Overall, the genomic expression data points to the involvement of the MAPK signalling pathway and opens the door for further research into the MAPK pathway and its potential to provide an all-encompassing mechanism underpinning the various aetiologies in different species.

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Appendix 1:
Additional sequencing data

Table A1.1: Primers for PCR and sequencing of the *epidermal growth factor receptor* gene in foxes, based on the Canis familiaris gene sequence (NC_006600.3 on chromosome 18)

Exon/ Intron	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temp (°C)	Product length (bp)
1	ACGTAGGACGTTCATGCAGA	TCTGTGCTGCAGGAACTGGA	57	670
2	CTAGAAAGCACAGGGTCATG	GGGTCATACAGGGTCCGTCC	56	240
3	TTGCTTCCTCTTCCCACCTG	AATGGCTTATGGCTTCCTTC	57	482
4	TTCCTGGCCTCCAGTATCTTC	AACAAGGGAGCAATCACTAAG	57	346
5	CTCTTACAGCTGTACAGGTGC	TTGTTTGGCCACTTCCTCATT	51	217
6/7	TCTCTGTACATGGAGGAAGG	AGAGTCAAGACGCAGGTACT	55	770
8	TTCTTGTCGCATTCATCCTAG	CCAGGACACATAACTGCTGTA	51	422
9	TAGAGGCAGAAGTACCTGAG	TCTCCATCACCTACTGTCTT	52	554
10	AATGTGAAGAAGTATTGCATA	ACATGAACTCTGTTCTCTGTC	50	315
11	GGCAGGTTGTCATCAGTTCCT	AGGATGTCATTCACGCAACTG	54	257
12	CCAGTGACCAAAATCATCTG	CTTACCAGGCAGTCGCTCTC	55	128
13	TCCACAGTTACCACAGTAGT	AGTGAGGCAAATATATGGAC	50	555
14	GAACATGAGACAATGGTGTGG	GGCTTCTAATAAGGAGGAGTC	55	340
15/16	AGAGTTACCAGTACCCAGCA	GCATCTCCCGGCTCACTCTC	52	541
17	ATTCTAGAGGAGAGGCAGGT	TGCGTAGGTGAGATGTTAAC	52	466
18	GACTTCCTGCAATATGACTTA	TGATCCATGTGAACCATCTCC	51	386
19	CCACTTACAGGAGCGGTCTCA	TTTGTGATTAACAGTTGTCAA	50	368
20	AAGGTGACTTGTGTCTGATTA	GGCTTGGTGGCTACAGAGTGT	50	296
21	AGTCACATCAAGCAACAGCAG	CCTTGGGATGTGCAGAAATGA	54	478
22	CGTCATGCTCTCTCTTTCAAG	CCAGGAGAACCGGTCAAGATA	51	274
23	AGAATCCACATGGCATCTAC	TGCCAAGACTTGGCAGGAGA	55	356
24	AAGCTCTGAGAAGGTGTGCAT	CCGAACTGTGAGCAACACAGA	57	392
25	TTGGAGTGCACAGCTCTGCTC	TGCTGCTGCCAGACACCACAA	57	317
26	AATCACAGTTGTGCTTTGCTG	CTCTCCACTTACCTTTGCAAT	57	308
27	GTTGCAGGGCATGAACTACC	CTTCTTACTTTGCCTCCTTC	57	171

28	CCTCAGCTGAGACCTTCATG	CCTGGTGCACTGCAGGATGC	55	653
29	CAGGCGTCACCGTGTGGGAG	TTGACCATGATCATGTAGAC	57	148
30	GGCGAGATGTCTAGATGTCTA	TTCACCAAGGCTCGCTCTAAC	55	396
31	GGAAGGAAGCAGCTGTACCTC	TTATGCTAAGTTCCACTAGGT	53	410
32	TTAGCTCCACTCTGTGAGACC	CGGAAGGAACATCAAGGCTAG	54	250
33	GGCTTGCTGGAAACGCAGTGT	TATTTCTCCGCCTCTTACTCC	52	394
34	TGCCCTCAGCTGCCTCCTGGA	CCAAGAGTTAATGTGGGCATG	57	578

Table A1.2: Primers deigned for sequencing *growth factor receptor bound protein 2* gene in foxes based on the Canis familiaris gene sequence (NC_006591.3 on chromosome 9)

Exon	Forward primer (5' -3')	Reverse primer (5'-3')	Annealing temp (°C)	Product length (bp)
1	Never obtained			
2	GTGCTGGCCACTTCTCTCAC	CTATCCAGAGCCCGCCAGAC	57	484
3	ATCCATGTAACTGACCATACTTCAA	TTGGAATAAGCAGATGGGTCTGACT	52	239
4	CAAGGAGTGTTCTGTTACTCACATT	TCAGATGAGGAGGGAGAGGACTCCA	56	264
5	TCAGAAGATGCCTCCTTGATGTGAG	GAGAGGGTACCTGCCTATCTGAGGA	56	336
6	CCACGTAGAGTAGACTTAGGACCTG	CACTCCAACTACAACAGACATTTTG	54	715

Table A1.3: Primers deigned for sequencing *SOS1* gene in foxes based on the Canis familiaris gene sequence (NC_006599.3 on chromosome 17)

Exon/ Intron	Forward primer (5' -3')	Reverse primer (5'-3')	Annealing temp (°C)	Product length (bp)
1	CCTGATGAGTTGGAAGAGGA	GGATAGGCATGTGATAAAGC	50	475
2	GAATAAGCCTAGATGGTATATGTAGCTGGG	GGACTTGAAGAGCAAACTCTTCCGCTGAC	53	380
3	GATTTTCCCCTGAGATAATATAAGGG	GAATTTCACCCTTCAACTCACTATCATCC	51	470
4	GCATTGTTTCTTTTTAATCGGTGTGTC	GCTATTGGGTTACTGGAAATGTATCCTAAC	51	364
5	GGTATTAATGGACATGTTTCATCAAGATG	GAGATCATAGTCACTATGAATTAAATCCCAC	51	353
6	CAATGCCGTTTGCTTCATTCACTCAACTG	CAAGGAACAGACAATACGGCTAAGTAGTC	53	564
7/8	CTGTATTTCTGGTGGTAGGTTATTTATG	GCAATTAGATATAGACTAATGTTCATGGGTGC	53	480
9	GCCTGTCACAAGGCATGACATCTTTTAACAC	CACTTCCTCTGAAAGACCAAGCTTGTCAC	53	511
10	GTGAATCTGCATGTCGGTTTTATAGTCAGC	GGCACAATGACCTGTGCAGCAAAGAAAATC	51	735
11	CCAAAGCATTCTATGTGGTCAGAACATTTTGGG	GAGAAACATGTAGGATCTTTTAGCTCAATCT	51	286
12	GATCCCAATTTTGTTCGGACATTTC	CCTCTCACTGTTCCAATAAATTCTTCCATTCG	53	1348
13	GAGGGAAGCAAAAGTGTAAGCCACTG	CAACTTTTTCAAGTGCTTATGCTGCCAC	53	387
14	GGTAAAGCAATGAAAAAATGGGTTGAATCC	CTTCTCAAACCACAGAGTGAGATTAGTGG	54	1458
15	GTTTTCATAAACCTTTCTGTTGGTGTTC	GGACATGAGGCATACAAAAGCAGAACTGC	53	341
16	GATTCTCTCTATAACTATAACCATCTGCCCC	CCAAAGAAAGGCACACATGGTGGATTAATAG	53	919
17	GGCTTTATTTTTAGCCCAGCATTTTGGG	GTCCTAATAGAGTAAAATTCTATGGTGATTCAC	52	357
18	GGAAATCTGTGCTTTAAATCTGTGGTTATGG	CTTTGATATCTGATTCTACTCGTAAAC	52	305
19	CCAGGAATTTATCTAACTAATATCTTG	GACCAAGCCTTTGTGCCTGCGGGAC CAAATTTAAAGAGGGCTATTCAAACTGG	50	2213

20	CGAAGATATTAGTTGAGTTTTACCAGGC	CAAATTTAAAGAGGGCTATTCAAACTGG	53	567
21	GACTGCTGCACCATTCTTTACAACTAG	CAAGCAATGTACCAATGCCGCCAGACCC	53	287
22	GGATCTGCTTCAGTATCATCTATAAGTTTAACC	CCCTGAAGTTTCAGCCTAACCTGGTGGG	52	207
23	CCCCAACTTAATTCATAATATGTTTG	GCATATTTTGAATTACAAAAACTGTC	52	954

Table A1.4: Primers deigned for sequencing *MAP2K6* gene in foxes based on the Canis familiaris gene sequence (NC_006591.3 on chromosome 9)

Exon	Forward primer (5' -3')	Reverse primer (5'-3')	Annealing temp (°C)	Product length (bp)
1	CCACGTTTCCTTAAATCCGAG	ATGCATGCACAACCCTTTG	53	458
2	GGCTAGCCCTTTCTCCTTCA	GGCATTGCTCATCAGCCCAA	58	257
3	GACTAAATATACCTCATGTTTTTCT	TGCATGAGAAATGGTCAAGA	52	147
4	TAGAACGTTGGGAGAAGCAA	AACGGATACACCACAGACAG	53	248
5	AGAGCAGTAACCATGCATTT	AGAGCAGTAACCATGCATTT	52	202
6	TCCTTTTCTACCCTCTCTAACA	AGACGTCTCATATTCTCCTTGT	53	208
7	TTCCAGTGATGGAGAAAAATCT	TGGATTTCATAAAAGGAACAGCC	53	140
8	GCCTGGGACCATGGGCTTTT	GCTTTGCTGGCAGCTGCATTT	58	219
9	AGCTAAGAACATATCTCATTTGTC	GCCCAGAACCTCACAGTC	53	162
10	CACAGCAGATTCCCTAAAGTA	GCAGATGAGGCTCTCTTACC	53	238
11	AGATGGTTCTTAGATTTTAACGCA	TACTACCTGTCGGTTTGGAC	53	138
12	AGTGGTGAATATTAATACCATGTG	ACAACAGCTGGAACAGGT	53	740

Appendix 2:
Additional RT-qPCR gene data

Table A2.1: Mean log_2 relative expression (log_2RQ) values for qPCR genes in the hereditary hyperplastic gingivitis (HHG)-affected and HHG-unaffected fox samples and corresponding micraoarray fold change values.

		qPCR					Microarray
Gene symbol	**Gene Name**	**Fold change**	**Test location**	**T-test**	**Probe ID**	**Fold change**	**Mann Whittney P value**
COL14A1	*Collagen, type XIV, alpha 1*	NA	Toronto	NA	Cfa.1972.1.A1_at	1.242	0.036
DST	*Dystonin*	1.028	Toronto	0.947	CfaAffx.4544.1.S1_at	1.216	0.036
E2F4	*E2F transcription factor 4*	1.662	Toronto	0.354	CfaAffx.31199.1.S1_at	-1.234	0.036
FOS	*Finkel–Biskis–Jinkins murine osteogenic sarcoma virus*	3.385	St. John's	0.077	CfaAffx.26065.1.S1_at	2.863	0.393
GPR110	*G-protein coupled receptor 110*	4.247	St. John's	0.261	CfaAffx.4026.1.S1_at	2.072	0.393
MAP2K6	*Mitogen-activated kinase kinase 6*	-1.106	Toronto	NA	CfaAffx.16824.1.S1_at	-1.203	0.250
MAP3K1	*Mitogen-activated kinase kinase kinase 1*	-2.313	St. John's	0.112	Cfa.19524.1.S1_at	-1.959	0.071
MMP9	*Matrix metallopeptidase 9*	1.245	St. John's	0.811	Cfa.3470.1.S1_s_at	2.873	0.143
NCALD	*Neurocalcin delta*	1.776	Toronto	NA	Cfa.16838.1.S1_at	-1.231	0.036
OXR1	*Oxidation resistance 1*	1.210	Toronto	0.531	CfaAffx.1950.1.S1_s_at	-1.288	0.036
PITPNC1	*Phosphatidylinositol transfer protein, cytoplasmic 1*	1.060	Toronto	0.907	CfaAffx.17853.1.S1_s_at	1.358	0.036
PRKCZ	*Protein kinase C, ζ*	1.299	Toronto	0.382	Cfa.19185.2.A1_at	-1.204	0.036
PTK2	*Protein tyrosine kinase 2*	1.477	Toronto	0.433	Cfa.5377.1.A1_at	1.207	0.036
RASA1	*RAS p21 protein activator 1*	2.058	Toronto	0.116	Cfa.350.1.S1_at	1.344	0.071
ROCK1	*Rho-associated, coiled-coil containing protein kinase 1*	1.054	Toronto	0.837	CfaAffx.27952.1.S1_at	1.240	0.036
ROR1	*Receptor tyrosine kinase-like orphan receptor 1*	-2.394	St. John's	0.237	CfaAffx.28472.1.S1_at	-1.577	0.393

SLC30A3	*Solute carrier family 30 (zinc transporter), member 3*	NA	Toronto	NA	Cfa.6122.1.A1_at	1.234	0.036
SOS1	*Son of sevenless homolog 1*	-1.225	St. John's	0.406	CfaAffx.10496.1.S1_s_at	-1.400	0.250
SOS2	*Son of sevenless homolog 2*	1.214	Toronto	0.182	CfaAffx.22062.1.S1_s_at	1.000	1.000
TGFβ3	*Transforming growth factor beta , 3*	3.603	Toronto	0.026	CfaAffx.26185.1.S1_at	1.084	0.786

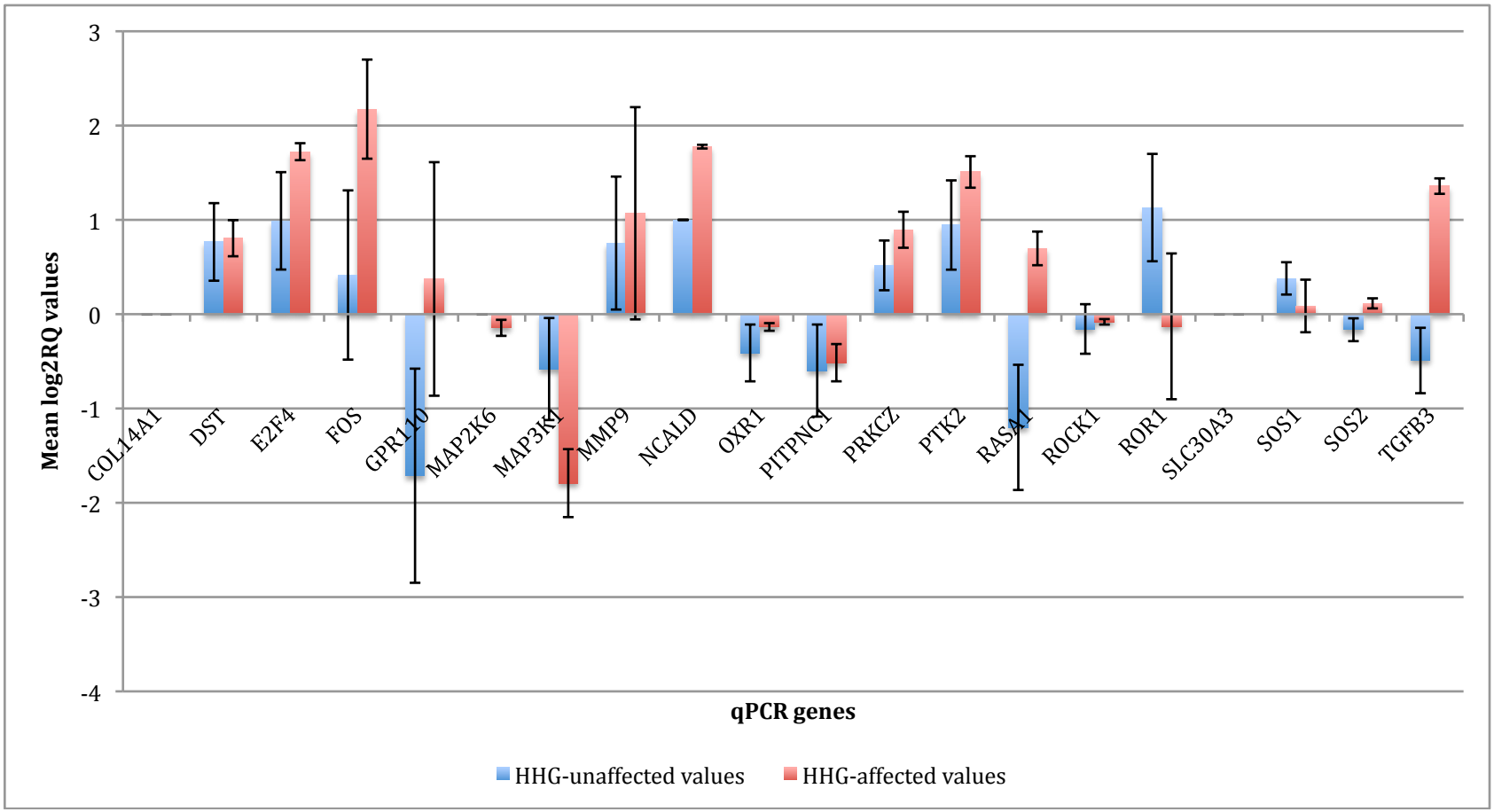

Figure A2.1: Mean $\log_2$ relative expression ($\log_2$RQ) values for qPCR genes in the hereditary hyperplastic gingivitis (HHG)-affected and HHG-unaffected fox samples. Standard errors are indicated by the bars.

Appendix 3:
Comprehensive list of statistically significant fold changes form the microarray experiment

Table A3.1: Microarray-identified genes significantly differentially expressed in hereditary hyperplastic gingivitis (HHG)-affected foxes relative to unaffected foxes

Gene title	Affymetrix probe ID	Fold change	Mann Whittney P value
ATP-binding cassette, sub-family F (GCN20), member 2	CfaAffx.8212.1.S1_s_at	-1.180	0.036
v-abl Abelson murine leukemia viral oncogene homolog 2	Cfa.7403.1.A1_s_at	1.301	0.036
Actin binding LIM protein family, member 2	CfaAffx.22406.1.S1_s_at	1.403	0.036
Ankyrin repeat and BTB (POZ) domain containing 1	CfaAffx.7032.1.S1_at	1.986	0.036
Acetyl-CoA acyltransferase 1	Cfa.6351.2.A1_s_at	1.090	0.036
Acetyl-CoA carboxylase alpha	CfaAffx.27754.1.S1_s_at	1.247	0.036
Acyl-CoA synthetase long-chain family member 5	CfaAffx.17119.1.S1_s_at	-1.681	0.036
ADAM metallopeptidase domain 20	CfaAffx.12696.1.S1_at	-1.137	0.036
ADAM metallopeptidase domain 22	CfaAffx.3686.1.S1_at	1.148	0.036
ADAM metallopeptidase with thrombospondin type 1 motif, 20	CfaAffx.15306.1.S1_s_at	1.231	0.036
ADAMTS-like 1	CfaAffx.3277.1.S1_s_at	1.120	0.036
ADAMTS-like 3	Cfa.2566.1.S1_s_at	-1.177	0.036
Adenylate cyclase 2 (brain)	Cfa.19117.1.S1_s_at	-1.143	0.036
Alcohol dehydrogenase 4 (class II), pi polypeptide	Cfa.2072.1.S1_at	1.293	0.036
Adiponectin receptor 2	CfaAffx.24419.1.S1_at	1.450	0.036
Activity-dependent neuroprotector homeobox	CfaAffx.17978.1.S1_at	-1.494	0.036
AF4/FMR2 family, member 3	CfaAffx.4242.1.S1_at	-1.121	0.036
AFG3 ATPase family gene 3-like 2 (S. cerevisiae)	Cfa.7454.1.A1_at	1.154	0.036
ATP/GTP binding protein-like 3	Cfa.7400.1.A1_s_at	1.296	0.036
Acylglycerol kinase	CfaAffx.6782.1.S1_at	-2.058	0.036
AT hook containing transcription factor 1	CfaAffx.24433.1.S1_at	1.230	0.036
Androgen-induced 1	Cfa.13228.1.A1_at	1.385	0.036
Akirin 2	Cfa.9601.1.A1_at	-1.524	0.036

Alkaline phosphatase, liver/bone/kidney	Cfa.13804.1.A1_at	1.251	0.036
Adenosine monophosphate deaminase 2	CfaAffx.30325.1.S1_at	-1.154	0.036
Angiopoietin-like 6	CfaAffx.27426.1.S1_at	-1.243	0.036
Ankyrin repeat and IBR domain containing 1	CfaAffx.3876.1.S1_at	-1.235	0.036
Ankyrin repeat domain 13A	CfaAffx.16669.1.S1_at	1.138	0.036
Ankyrin repeat domain 13 family, member D	CfaAffx.18188.1.S1_s_at	-1.195	0.036
Ankyrin repeat domain 31	CfaAffx.14986.1.S1_at	1.100	0.036
AN1, ubiquitin-like, homolog (Xenopus laevis)	Cfa.19921.1.S1_s_at	1.116	0.036
Annexin A1	CfaAffx.3571.1.S1_s_at	1.805	0.036
Annexin A2	Cfa.138.1.A1_at	1.104	0.036
Adaptor-related protein complex 4, mu 1 subunit	Cfa.18599.1.S1_at	1.141	0.036
Amyloid beta (A4) precursor protein-binding, family B, member 3	Cfa.8054.1.S1_s_at	1.212	0.036
ADP-ribosylation factor 3	Cfa.6419.1.S1_at	1.900	0.036
ADP-ribosylation factor guanine nucleotide-exchange factor 1(brefeldin A-inhibited)	CfaAffx.12196.1.S1_s_at	-1.215	0.036
Armadillo repeat containing, X-linked 3	CfaAffx.27095.1.S1_s_at	-1.316	0.036
Actin related protein 2/3 complex, subunit 5, 16kDa	CfaAffx.20654.1.S1_at	-1.100	0.036
ADP-ribosyltransferase 1	Cfa.13089.1.A1_at	1.275	0.036
Ankyrin repeat and SOCS box-containing 18	CfaAffx.18888.1.S1_at	-1.215	0.036
Activating signal cointegrator 1 complex subunit 3	CfaAffx.6271.1.S1_s_at	-1.771	0.036
ATPase family, AAA domain containing 5	CfaAffx.6271.1.S1_s_at	-1.771	0.036
Ataxia, cerebellar, Cayman type	CfaAffx.29229.1.S1_s_at	1.097	0.036
ATG7 autophagy related 7 homolog (S. cerevisiae)	Cfa.14549.1.A1_at	1.257	0.036
Atonal homolog 7 (Drosophila)	CfaAffx.20942.1.S1_at	1.174	0.036
ATPase type 13A5	CfaAffx.21810.1.S1_s_at	-1.220	0.036
ATP synthase, H+ transporting, mitochondrial Fo complex, subunit B1	CfaAffx.30193.1.S1_s_at	-1.053	0.036
ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1	CfaAffx.31171.1.S1_s_at	1.270	0.036
ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D	Cfa.1081.2.S1_at	1.256	0.036
ataxin 7-like 1	CfaAffx.6925.1.S1_s_at	-1.267	0.036

AVL9 homolog (S. cerevisiase)	CfaAffx.5672.1.S1_s_at	1.306	0.036
Alpha-2-glycoprotein 1, zinc-binding	Cfa.11319.1.A1_at	-1.133	0.036
Antizyme inhibitor 1	Cfa.4399.1.S1_at	-1.228	0.036
BCL2-associated athanogene 4	CfaAffx.10159.1.S1_at	-1.259	0.036
BarH-like homeobox 1	CfaAffx.30442.1.S1_at	1.069	0.036
HLA-B associated transcript 2-like 2	Cfa.3163.1.A1_s_at	-1.179	0.036
Bromodomain adjacent to zinc finger domain, 2B	CfaAffx.14872.1.S1_at	-1.503	0.036
Brevican	CfaAffx.25582.1.S1_s_at	1.108	0.036
Breast carcinoma amplified sequence 3	Cfa.8596.2.A1_a_at	1.343	0.036
BRCA2 and CDKN1A interacting protein	CfaAffx.19936.1.S1_s_at	-1.201	0.036
Branched chain ketoacid dehydrogenase kinase	CfaAffx.25727.1.S1_at	1.238	0.036
BCL2-associated transcription factor 1	CfaAffx.1327.1.S1_s_at	-1.445	0.036
Baculoviral IAP repeat-containing 3	CfaAffx.23263.1.S1_at	1.194	0.036
Biorientation of chromosomes in cell division 1	Cfa.8007.1.A1_at	-1.139	0.036
Biorientation of chromosomes in cell division 1-like	Cfa.10683.1.S1_s_at	-1.382	0.036
Bromodomain containing 2	CfaAffx.2253.1.S1_s_at	-1.336	0.036
Bromodomain, testis-specific	CfaAffx.30888.1.S1_at	1.115	0.036
Bone marrow stromal cell antigen 1	CfaAffx.23551.1.S1_at	1.314	0.036
Butyrophilin, subfamily 1, member A1	CfaAffx.16956.1.S1_s_at	1.295	0.036
Complement component 5	CfaAffx.6326.1.S1_s_at	-1.300	0.036
Complement component 9	Cfa.13405.1.A1_at	-1.171	0.036
Carbonic anhydrase XI	CfaAffx.6852.1.S1_s_at	-2.193	0.036
Calcium channel, voltage-dependent, L type, alpha 1S subunit	CfaAffx.17088.1.S1_at	1.234	0.036
Calcyclin binding protein	CfaAffx.22188.1.S1_s_at	-1.159	0.036
Ca++-dependent secretion activator 2	Cfa.10726.1.A1_s_at	1.541	0.036
Calcitonin/calcitonin-related polypeptide, alpha	Cfa.3806.1.S1_at	-1.201	0.036
Calcium binding and coiled-coil domain 1	CfaAffx.10971.1.S1_s_at	1.213	0.036
Caspase recruitment domain family, member 11	CfaAffx.24989.1.S1_at	1.217	0.036
CAS1 domain containing 1	CfaAffx.4056.1.S1_s_at	-1.793	0.036

Caspase 6, apoptosis-related cysteine peptidase	CfaAffx.17770.1.S1_at	-1.213	0.036
Cation channel, sperm associated 2	CfaAffx.20427.1.S1_at	-1.351	0.036
Cation channel, sperm-associated, gamma	CfaAffx.9881.1.S1_at	1.277	0.036
Cystathionine-beta-synthase	CfaAffx.16501.1.S1_s_at	-1.609	0.036
Coiled-coil domain containing 110	CfaAffx.12192.1.S1_at	1.248	0.036
Coiled-coil domain containing 81	CfaAffx.7617.1.S1_s_at	-1.051	0.036
Coiled-coil domain containing 88C	CfaAffx.26864.1.S1_s_at	-1.239	0.036
Chemokine (C-C motif) ligand 3	Cfa.14352.1.A1_at	1.965	0.036
Chemokine (C-C motif) ligand 3	CfaAffx.27805.1.S1_s_at	1.105	0.036
Cell cycle progression 1	CfaAffx.24405.1.S1_at	-1.580	0.036
CD300a molecule	CfaAffx.7773.1.S1_at	-1.338	0.036
Cell division cycle 37 homolog (S. cerevisiae)	CfaAffx.27245.1.S1_s_at	1.282	0.036
Chymotrypsin-like elastase family, member 1	Cfa.12.1.S1_s_at	8.778	0.036
CUGBP, Elav-like family member 3	CfaAffx.19849.1.S1_s_at	1.181	0.036
Centromere protein J	CfaAffx.11960.1.S1_at	-1.353	0.036
Centromere protein M	CfaAffx.2400.1.S1_at	1.161	0.036
Ceramide kinase-like	CfaAffx.21887.1.S1_at	1.390	0.036
ChaC, cation transport regulator homolog 1 (E. coli)	CfaAffx.14841.1.S1_s_at	1.866	0.036
Chondroadherin-like	CfaAffx.2541.1.S1_at	1.148	0.036
Choline O-acetyltransferase	CfaAffx.10949.1.S1_s_at	-1.110	0.036
Chromodomain helicase DNA binding protein 1	Cfa.2790.1.A1_at	1.347	0.036
Chromodomain helicase DNA binding protein 2	CfaAffx.17145.1.S1_at	-1.222	0.036
Chromodomain helicase DNA binding protein 6	CfaAffx.14589.1.S1_at	-1.520	0.036
Choline phosphotransferase 1	Cfa.9478.1.A1_at	1.196	0.036
Cholinergic receptor, muscarinic 5	CfaAffx.229.1.S1_at	1.137	0.036
Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6	Cfa.17231.1.S1_s_at	-1.127	0.036
Corepressor interacting with RBPJ, 1	CfaAffx.20461.1.S1_at	1.178	0.036
C-type lectin domain family 17, member A	CfaAffx.24920.1.S1_at	-1.292	0.036
Clathrin, light chain A	CfaAffx.4312.1.S1_s_at	1.236	0.036

Chymase 1, mast cell	CfaAffx.19295.1.S1_s_at	1.372	0.036
Cardiomyopathy associated 5	Cfa.2645.1.S1_at	-1.210	0.036
CCR4-NOT transcription complex, subunit 4	CfaAffx.5770.1.S1_s_at	1.132	0.036
CCR4-NOT transcription complex, subunit 7	Cfa.18438.1.S1_s_at	-1.197	0.036
Coilin	Cfa.20787.1.S1_at	-1.306	0.036
Collagen, type XIV, alpha 1	Cfa.1972.1.A1_at	1.242	0.036
Collagen, type XVI, alpha 1	CfaAffx.17108.1.S1_at	1.155	0.036
Collagen, type XIX, alpha 1	CfaAffx.4714.1.S1_s_at	-1.138	0.036
Collagen, type XXII, alpha 1	CfaAffx.2659.1.S1_s_at	1.137	0.036
Collagen, type VI, alpha 3	Cfa.99.1.S1_s_at	-1.144	0.036
Coenzyme Q3 homolog, methyltransferase (S. cerevisiae)	CfaAffx.6146.1.S1_s_at	-1.410	0.036
cOR8B15 olfactory receptor family 8 subfamily B-like	CfaAffx.548.1.S1_x_at	-1.184	0.036
cOR9K3 olfactory receptor family 9 subfamily K-like	CfaAffx.14945.1.S1_x_at	-1.078	0.036
Carboxypeptidase A5	CfaAffx.3118.1.S1_at	1.423	0.036
Carboxypeptidase O	CfaAffx.20812.1.S1_at	2.225	0.036
Cleavage and polyadenylation specific factor 4, 30kDa	CfaAffx.23159.1.S1_s_at	1.389	0.036
Carboxypeptidase Z	CfaAffx.22474.1.S1_s_at	-1.156	0.036
Crumbs homolog 1 (Drosophila)	CfaAffx.17703.1.S1_at	-1.152	0.036
Cysteine-rich secretory protein LCCL domain containing 1	CfaAffx.13121.1.S1_s_at	-1.374	0.036
CSE1 chromosome segregation 1-like (yeast)	Cfa.21178.2.S1_at	-1.263	0.036
Casein kinase 1, gamma 3	Cfa.18620.1.S1_s_at	-1.427	0.036
Cysteine and glycine-rich protein 1	Cfa.15285.3.S1_at	-1.079	0.036
Catenin (cadherin-associated protein), alpha 1, 102kDa	Cfa.9512.1.A1_at	1.152	0.036
chymotrypsinogen B1	CfaAffx.30742.1.S1_s_at	1.137	0.036
Cathepsin D	Cfa.17547.1.S1_at	1.350	0.036
Chemokine (C-X-C motif) receptor 3	CfaAffx.26233.1.S1_s_at	-1.114	0.036
Aspartyl-tRNA synthetase	CfaAffx.8689.1.S1_s_at	-1.357	0.036
Diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein)	CfaAffx.8277.1.S1_at	1.146	0.036

dCMP deaminase	Cfa.13873.1.S1_at	-1.328	0.036
DEAD (Asp-Glu-Ala-Asp) box polypeptide 18	CfaAffx.8348.1.S1_at	-1.964	0.036
DEAD (Asp-Glu-Ala-Asp) box polypeptide 20	Cfa.19991.1.S1_at	-1.285	0.036
DEAD (Asp-Glu-Ala-Asp) box polypeptide 46	CfaAffx.2470.1.S1_s_at	-1.288	0.036
DEAD (Asp-Glu-Ala-Asp) box polypeptide 56	CfaAffx.5520.1.S1_at	1.220	0.036
Death effector domain containing	CfaAffx.19733.1.S1_at	1.170	0.036
DENN/MADD domain containing 1A	Cfa.12113.1.A1_at	-1.336	0.036
DENN/MADD domain containing 4B	CfaAffx.26653.1.S1_s_at	-1.256	0.036
Diacylglycerol O-acyltransferase 2	Cfa.21170.1.S1_s_at	1.165	0.036
Dehydrogenase/reductase (SDR family) member 7C	Cfa.2916.1.A1_at	-1.230	0.036
DEAH (Asp-Glu-Ala-His) box polypeptide 16	CfaAffx.1606.1.S1_s_at	-1.388	0.036
DEAH (Asp-Glu-Ala-His) box polypeptide 9	CfaAffx.20455.1.S1_s_at	-1.157	0.036
Dihydrolipoamide dehydrogenase	Cfa.862.1.S1_s_at	-1.157	0.036
Dystrophia myotonica, WD repeat containing	CfaAffx.7514.1.S1_s_at	-1.346	0.036
DNA replication helicase 2 homolog (yeast)	CfaAffx.21183.1.S1_at	-1.137	0.036
Dynein, axonemal, heavy chain 10	CfaAffx.11481.1.S1_s_at	1.119	0.036
Dynein, axonemal, heavy chain 14	Cfa.7202.1.S1_a_at	-1.375	0.036
DnaJ (Hsp40) homolog, subfamily B, member 12	Cfa.17078.1.S1_s_at	1.088	0.036
DnaJ (Hsp40) homolog, subfamily C, member 15	Cfa.7675.1.A1_s_at	1.145	0.036
DnaJ (Hsp40) homolog, subfamily C, member 16	Cfa.18042.1.S1_s_at	-1.218	0.036
DnaJ (Hsp40) homolog, subfamily C, member 19	CfaAffx.18083.1.S1_s_at	-1.173	0.036
Deoxyribonuclease I	CfaAffx.29403.1.S1_s_at	1.146	0.036
aspartyl aminopeptidase	CfaAffx.23735.1.S1_x_at	1.102	0.036
Dipeptidase 1 (renal)	CfaAffx.30336.1.S1_at	-1.194	0.036
Dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit	Cfa.1802.3.S1_s_at	-1.297	0.036
Dihydropyrimidinase	CfaAffx.1938.1.S1_at	1.247	0.036
Drosha, ribonuclease type III	CfaAffx.28945.1.S1_s_at	-1.433	0.036
Dystonin	CfaAffx.4544.1.S1_at	1.216	0.036

Dihydrouridine synthase 4-like (S. cerevisiae)	Cfa.2748.1.A1_at	-1.317	0.036
E2F transcription factor 4, p107/p130-binding	CfaAffx.31199.1.S1_at	-1.234	0.036
Early B-cell factor 1	CfaAffx.26525.1.S1_at	-1.323	0.036
Extracellular matrix protein 1	CfaAffx.18675.1.S1_s_at	1.622	0.036
Endothelin 2	Cfa.2.1.S1_s_at	1.119	0.036
Epidermal growth factor	Cfa.3524.1.S2_at	1.216	0.036
Eukaryotic translation initiation factor 2B, subunit 3 gamma, 58kDa	CfaAffx.7951.1.S1_s_at	-1.232	0.036
Eukaryotic translation initiation factor 5A	Cfa.8657.1.A1_at	1.213	0.036
Eukaryotic translation initiation factor 5B	CfaAffx.4292.1.S1_at	-1.272	0.036
ElaC homolog 2 (E. coli)	Cfa.12195.10.A1_at	-1.274	0.036
Elongation factor RNA polymerase II	CfaAffx.22762.1.S1_s_at	1.515	0.036
Elongation protein 2 homolog (S. cerevisiae)	Cfa.17346.1.S1_s_at	-1.645	0.036
Echinoderm microtubule associated protein like 4	CfaAffx.10731.1.S1_s_at	-1.583	0.036
Echinoderm microtubule associated protein like 5	CfaAffx.26723.1.S1_s_at	-1.084	0.036
Enolase family member 4	CfaAffx.18482.1.S1_at	1.199	0.036
Erythrocyte membrane protein band 4.1-like 2	CfaAffx.2592.1.S1_s_at	-1.070	0.036
Erythrocyte membrane protein band 4.1-like 3	Cfa.2671.2.S1_at	1.130	0.036
Epithelial cell adhesion molecule	Cfa.12746.1.S1_at	-1.245	0.036
EPH receptor B2	CfaAffx.22516.1.S1_s_at	1.177	0.036
Erythropoietin receptor	CfaAffx.26647.1.S1_at	-1.200	0.036
Epithelial stromal interaction 1 (breast)	CfaAffx.18139.1.S1_s_at	-2.300	0.036
Excision repair cross-complementing rodent repair deficiency, complementation group 6	Cfa.1909.1.A1_s_at	-1.264	0.036
ERI1 exoribonuclease family member 3	CfaAffx.8156.1.S1_s_at	-1.509	0.036
ESF1, nucleolar pre-rRNA processing protein, homolog (S. cerevisiae)	CfaAffx.9329.1.S1_s_at	-1.778	0.036
Ewing tumor-associated antigen 1	CfaAffx.5785.1.S1_at	-1.363	0.036
Ethanolamine kinase 2	Cfa.6777.1.S1_s_at	-1.194	0.036
Exocyst complex component 6B	CfaAffx.14182.1.S1_s_at	1.398	0.036
Exosome component 8	CfaAffx.10167.1.S1_at	-1.232	0.036

Exostoses (multiple)-like 2	Cfa.19919.1.S1_s_at	-1.216	0.036
Fumarylacetoacetate hydrolase domain containing 2A	Cfa.15241.1.S1_s_at	1.289	0.036
Fibulin 2	CfaAffx.7550.1.S1_s_at	-1.128	0.036
F-box and WD repeat domain containing 10	Cfa.11549.1.A1_s_at	1.145	0.036
Free fatty acid receptor 2	CfaAffx.11413.1.S1_at	3.257	0.036
Fibroblast growth factor 19	CfaAffx.16710.1.S1_at	1.188	0.036
Four and a half LIM domains 2	Cfa.1493.1.A1_at	-1.269	0.036
FK506 binding protein 15, 133kDa	CfaAffx.5753.1.S1_at	1.212	0.036
Farnesyltransferase, CAAX box, alpha	Cfa.18192.1.S1_at	-1.292	0.036
Folate receptor 1 (adult)	CfaAffx.9619.1.S1_s_at	-1.186	0.036
Forkhead box I3	CfaAffx.11968.1.S1_at	-1.078	0.036
Forkhead box K2	Cfa.8775.1.A1_at	-1.419	0.036
FERM and PDZ domain containing 2	CfaAffx.10768.1.S1_at	1.287	0.036
Fibroblast growth factor receptor substrate 2	Cfa.1958.1.S1_at	-1.490	0.036
FUN14 domain containing 1	Cfa.17008.1.S1_at	-1.329	0.036
Fuzzy homolog (Drosophila)	CfaAffx.6191.1.S1_at	-1.223	0.036
Frataxin	Cfa.9049.1.A1_at	-1.125	0.036
FYVE and coiled-coil domain containing 1	Cfa.21347.1.S1_s_at	1.528	0.036
FRizzled homolog 7 (Drosophila)	Cfa.15623.1.A1_at	1.251	0.036
Glucose-6-phosphatase, catalytic subunit	Cfa.204.1.S1_s_at	1.223	0.036
Gamma-aminobutyric acid (GABA) A receptor, delta	CfaAffx.29604.1.S1_at	-1.262	0.036
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 6 (GalNAc-T6)	CfaAffx.12475.1.S1_s_at	1.691	0.036
gGyceraldehyde-3-phosphate dehydrogenase	AFFX-Cfa-gapdh-M_x_at	1.316	0.036
GAR1 ribonucleoprotein homolog (yeast)	Cfa.21398.1.S1_s_at	-1.280	0.036
GATA zinc finger domain containing 1	CfaAffx.3883.1.S1_at	-1.272	0.036
Group-specific component (vitamin D binding protein)	CfaAffx.5352.1.S1_at	-1.173	0.036
Golgi-associated, gamma adaptin ear containing, ARF binding protein 1	Cfa.17715.1.S1_s_at	1.148	0.036

Gypsy retrotransposon integrase 1	CfaAffx.12178.1.S1_s_at	-1.403	0.036
GINS complex subunit 2 (Psf2 homolog)	CfaAffx.30492.1.S1_at	-1.265	0.036
Gap junction protein, alpha 1, 43kDa	Cfa.119.1.S1_at	-5.484	0.036
GLI family zinc finger 3	CfaAffx.6242.1.S1_s_at	-1.035	0.036
Glucagon-like peptide 2 receptor	CfaAffx.26693.1.S1_at	1.190	0.036
Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	CfaAffx.25135.1.S1_s_at	-1.870	0.036
N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits	CfaAffx.11738.1.S1_s_at	-1.692	0.036
Golgin A2	Cfa.19862.1.S1_s_at	-1.315	0.036
Golgin A3	Cfa.4969.1.A1_at	1.342	0.036
Golgin A4	Cfa.2021.2.S1_at	-1.434	0.036
Golgi integral membrane protein 4	Cfa.15237.2.S1_x_at	-1.171	0.036
Glucose-6-phosphate isomerase	Cfa.11243.2.S1_s_at	1.303	0.036
G protein-coupled receptor 107	CfaAffx.30541.1.S1_s_at	1.387	0.036
G protein-coupled receptor 124	CfaAffx.10264.1.S1_at	-1.146	0.036
G protein-coupled receptor 153	CfaAffx.29925.1.S1_at	-1.182	0.036
Glutathione peroxidase 2 (gastrointestinal)	Cfa.12202.1.A1_at	1.442	0.036
Glutathione peroxidase 7	CfaAffx.6540.1.S1_at	-1.313	0.036
GRB2-related adaptor protein 2	CfaAffx.2836.1.S1_s_at	1.242	0.036
Glutamate receptor, ionotropic, kainate 2	CfaAffx.6317.1.S1_at	-1.158	0.036
Glutamate receptor, ionotropic, N-methyl D-aspartate 2D	CfaAffx.6928.1.S1_at	-1.134	0.036
Germ cell associated 1	CfaAffx.20415.1.S1_s_at	1.771	0.036
G1 to S phase transition 2	Cfa.16646.1.S1_at	-1.319	0.036
Glutathione transferase zeta 1	CfaAffx.26294.1.S1_at	1.252	0.036
General transcription factor IIIC, polypeptide 2, beta 110kDa	Cfa.13466.1.A1_at	-1.182	0.036
General transcription factor IIIC, polypeptide 4, 90kDa	CfaAffx.30436.1.S1_at	1.368	0.036
GTP binding protein 5 (putative)	Cfa.14720.1.A1_at	1.252	0.036
GTP binding protein 6 (putative)	CfaAffx.17078.1.S1_at	1.171	0.036
Gycogenin 1	Cfa.4710.1.A1_at	2.078	0.036

Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	CfaAffx.7356.1.S1_s_at	-1.429	0.036
Hepcidin antimicrobial peptide	Cfa.14486.1.S1_at	-1.064	0.036
Helicase, lymphoid-specific	CfaAffx.12909.1.S1_at	-1.208	0.036
Hexosaminidase (glycosyl hydrolase family 20, catalytic domain) containing	CfaAffx.20065.1.S1_s_at	-1.137	0.036
3-hydroxyisobutyryl-CoA hydrolase	CfaAffx.14905.1.S1_at	1.192	0.036
MBD2-interacting zinc finger	CfaAffx.19014.1.S1_at	1.292	0.036
3-hydroxymethyl-3-methylglutaryl-CoA lyase	CfaAffx.20284.1.S1_at	1.586	0.036
HNF1 homeobox B	CfaAffx.27789.1.S1_at	1.166	0.036
Heterogeneous nuclear ribonucleoprotein A3	Cfa.4338.1.S1_at	-1.253	0.036
Heterogeneous nuclear ribonucleoprotein R	Cfa.11232.1.A1_at	-1.123	0.036
5-hydroxytryptamine (serotonin) receptor 4	CfaAffx.28081.1.S1_at	1.272	0.036
Isoleucyl-tRNA synthetase	CfaAffx.4356.1.S1_s_at	-1.223	0.036
Indoleamine 2,3-dioxygenase 2	Cfa.14055.1.A1_at	-1.186	0.036
Interferon, alpha 6	Cfa.15825.1.S1_x_at	-1.187	0.036
Intraflagellar transport 27 homolog (Chlamydomonas)	Cfa.9654.1.S1_at	1.197	0.036
Immunoglobulin superfamily, member 10	CfaAffx.13511.1.S1_at	1.168	0.036
Interleukin 1, beta	CfaAffx.11741.1.S1_s_at	1.189	0.036
Interleukin 20	CfaAffx.17816.1.S1_at	1.233	0.036
Interleukin 23, alpha subunit p19	CfaAffx.1156.1.S1_at	4.263	0.036
Interleukin enhancer binding factor 3, 90kDa	CfaAffx.27097.1.S1_s_at	-1.462	0.036
INO80 complex subunit E	CfaAffx.26108.1.S1_s_at	-1.378	0.036
Inositol polyphosphate-4-phosphatase, type II, 105kDa	CfaAffx.6377.1.S1_s_at	1.282	0.036
Insulin	Cfa.18796.1.S1_s_at	-1.053	0.036
IQ motif containing E	Cfa.16498.1.S1_at	1.360	0.036
Interferon regulatory factor 3	Cfa.18322.1.S1_at	1.237	0.036
Interferon stimulated exonuclease gene 20kDa	CfaAffx.17917.1.S1_at	2.777	0.036
Integrin, alpha 7	Cfa.18226.1.S1_s_at	-1.064	0.036

Integrin, alpha D	CfaAffx.22103.1.S1_at	-1.119	0.036
Inter-alpha (globulin) inhibitor H2	Cfa.13306.1.A1_s_at	-1.190	0.036
Katanin p60 subunit A-like 2	CfaAffx.26904.1.S1_s_at	1.183	0.036
Katanin p80 (WD repeat containing) subunit B 1	Cfa.8784.1.S1_at	1.168	0.036
Kazal-type serine peptidase inhibitor domain 1	CfaAffx.15418.1.S1_at	-1.205	0.036
Potassium channel, subfamily T, member 1	CfaAffx.30205.1.S1_s_at	1.148	0.036
Potassium channel tetramerisation domain containing 15	CfaAffx.11895.1.S1_at	-1.231	0.036
Potassium channel tetramerisation domain containing 9	CfaAffx.14165.1.S1_s_at	-2.251	0.036
Kinesin family member 26B	CfaAffx.24281.1.S1_s_at	-1.154	0.036
Kinesin family member 6	CfaAffx.3233.1.S1_at	1.130	0.036
Kinesin family member C3	CfaAffx.13645.1.S1_at	1.413	0.036
Kin of IRRE like 2 (Drosophila)	CfaAffx.11006.1.S1_at	-1.178	0.036
Kinesin light chain 2	CfaAffx.20017.1.S1_at	-1.120	0.036
Kelch-like 32 (Drosophila)	CfaAffx.6115.1.S1_at	-1.196	0.036
KRI1 homolog (S. cerevisiae)	Cfa.21226.1.S1_s_at	1.223	0.036
Kinectin 1 (kinesin receptor)	CfaAffx.23345.1.S1_s_at	-1.114	0.036
Lysosomal-associated membrane protein 1	CfaAffx.10473.1.S1_s_at	-2.425	0.036
La ribonucleoprotein domain family, member 7	CfaAffx.18216.1.S1_s_at	-1.330	0.036
Lipocalin 2	CfaAffx.30748.1.S1_at	2.561	0.036
Leptin receptor overlapping transcript-like 1	Cfa.9151.1.A1_s_at	1.258	0.036
LETM1 domain containing 1	Cfa.10550.2.S1_at	-1.782	0.036
Lectin, galactoside-binding, soluble, 12	CfaAffx.23223.1.S1_s_at	1.119	0.036
Legumain	CfaAffx.17218.1.S1_at	1.393	0.036
LIM homeobox 2	CfaAffx.30978.1.S1_at	-1.065	0.036
Lens intrinsic membrane protein 2, 19kDa	CfaAffx.5185.1.S1_at	-1.279	0.036
Lethal giant larvae homolog 2 (Drosophila)	CfaAffx.8193.1.S1_at	-1.275	0.036
Leiomodin 3 (fetal)	Cfa.15741.1.A1_at	1.233	0.036
LON peptidase N-terminal domain and ring finger 2	CfaAffx.4233.1.S1_at	1.191	0.036
Low density lipoprotein receptor-related protein 2	Cfa.14034.1.A1_at	1.372	0.036

Leucine rich repeat containing 36	CfaAffx.31181.1.S1_s_at	1.148	0.036
Leucine rich repeat containing 49	CfaAffx.26965.1.S1_s_at	1.316	0.036
Leucine-rich repeats and IQ motif containing 3	CfaAffx.31251.1.S1_at	1.213	0.036
Leucine-rich repeats and IQ motif containing 4	CfaAffx.22698.1.S1_at	1.217	0.036
Leucine rich repeat and sterile alpha motif containing 1	Cfa.17052.1.S1_at	-1.347	0.036
LSM10, U7 small nuclear RNA associated	Cfa.16663.1.A1_at	-1.204	0.036
LUC7-like 2 (S. cerevisiae)	Cfa.17776.1.S1_at	-1.143	0.036
LysM, putative peptidoglycan-binding, domain containing 2	CfaAffx.23800.1.S1_s_at	-1.551	0.036
Leucine zipper transcription factor-like 1	Cfa.17180.1.S1_at	1.289	0.036
Male germ cell-associated kinase	CfaAffx.15292.1.S1_at	1.468	0.036
Mitogen-activated protein kinase kinase kinase 10	CfaAffx.9015.1.S1_at	-1.168	0.036
Mitogen-activated protein kinase kinase kinase kinase 1	CfaAffx.9778.1.S1_s_at	-1.270	0.036
Microtubule-associated protein 6	CfaAffx.9005.1.S1_at	1.319	0.036
Mannan-binding lectin serine peptidase 1 (C4/C2 activating component of Ra-reactive factor)	CfaAffx.21414.1.S1_s_at	-1.190	0.036
Methionine adenosyltransferase I, alpha	Cfa.18757.1.S1_at	1.130	0.036
Matrin 3	Cfa.4360.1.S1_s_at	-1.141	0.036
Minichromosome maintenance complex component 4	Cfa.18077.1.S1_s_at	-1.432	0.036
Microspherule protein 1	Cfa.18343.1.S1_at	1.181	0.036
Multiple C2 domains, transmembrane 1	CfaAffx.12819.1.S1_s_at	-1.233	0.036
Mediator complex subunit 30	Cfa.21628.1.S1_at	-1.789	0.036
Mediator complex subunit 7	CfaAffx.26895.1.S1_s_at	-1.110	0.036
Methyltransferase like 14	Cfa.10848.1.S1_at	-1.177	0.036
Melanoma inhibitory activity 2	CfaAffx.21431.1.S1_at	1.177	0.036
Microtubule associated monoxygenase, calponin and LIM domain containing 2	CfaAffx.12627.1.S1_at	-1.121	0.036
Myo-inositol oxygenase	CfaAffx.1775.1.S1_s_at	-1.192	0.036
Microphthalmia-associated transcription factor	CfaAffx.10709.1.S1_s_at	1.203	0.036
Myeloid leukemia factor 1	Cfa.20466.1.S1_at	1.272	0.036

MTOR associated protein, LST8 homolog (S. cerevisiae)	CfaAffx.29667.1.S1_at	1.095	0.036
Monoacylglycerol O-acyltransferase 3	Cfa.13722.1.A1_s_at	1.146	0.036
Mov10, Moloney leukemia virus 10, homolog (mouse)	CfaAffx.20801.1.S1_at	-1.126	0.036
M-phase phosphoprotein 6	Cfa.1229.2.S1_a_at	-1.283	0.036
Mercaptopyruvate sulfurtransferase	Cfa.21333.1.S1_at	1.143	0.036
Myelin protein zero-like 1	CfaAffx.23659.1.S1_at	-1.488	0.036
Mitochondrial ribosomal protein L14	Cfa.307.9.A1_s_at	1.286	0.036
Mitochondrial ribosomal protein L4	CfaAffx.27357.1.S1_at	-1.316	0.036
Mitochondrial ribosomal protein S28	CfaAffx.13318.1.S1_s_at	-1.240	0.036
MRS2 magnesium homeostasis factor homolog (S. cerevisiae)	CfaAffx.16047.1.S1_at	-1.220	0.036
Membrane-spanning 4-domains, subfamily A, member 4	Cfa.15710.1.A1_at	1.328	0.036
Methionine sulfoxide reductase B3	CfaAffx.1500.1.S1_s_at	2.560	0.036
Serine/threonine protein kinase MST4	CfaAffx.28751.1.S1_at	1.193	0.036
Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	Cfa.4755.1.A1_at	1.119	0.036
Myotubularin related protein 11	CfaAffx.18111.1.S1_s_at	1.237	0.036
Myotubularin related protein 4	CfaAffx.26777.1.S1_s_at	-1.455	0.036
Myotubularin related protein 9	CfaAffx.12888.1.S1_at	-1.203	0.036
Metastasis suppressor 1-like	CfaAffx.30863.1.S1_at	1.182	0.036
Mucin 19, oligomeric	CfaAffx.15555.1.S1_at	1.096	0.036
Mucin 5B, oligomeric mucus/gel-forming	CfaAffx.15591.1.S1_at	-1.070	0.036
Myogenic factor 5	CfaAffx.9675.1.S1_at	1.207	0.036
Myosin, heavy chain 9, non-muscle	CfaAffx.3434.1.S1_at	-1.888	0.036
Myosin IIIB	CfaAffx.19378.1.S1_at	1.108	0.036
Myomesin family, member 3	CfaAffx.20206.1.S1_at	-1.083	0.036
N(alpha)-acetyltransferase 16, NatA auxiliary subunit	CfaAffx.8127.1.S1_s_at	-1.270	0.036
N(alpha)-acetyltransferase 35, NatC auxiliary subunit	CfaAffx.2910.1.S1_at	-1.176	0.036
N-acetylneuraminic acid synthase	Cfa.16909.1.S1_s_at	1.438	0.036

Neurocalcin delta	Cfa.16838.1.S1_at	-1.231	0.036
Neural cell adhesion molecule 2	CfaAffx.13380.1.S1_at	1.213	0.036
Non-specific cytotoxic cell receptor protein 1 homolog (zebrafish)	CfaAffx.9335.1.S1_at	2.758	0.036
Nuclear receptor coactivator 6	Cfa.12454.1.A1_at	-1.209	0.036
NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8kDa	Cfa.10920.1.S1_x_at	-1.090	0.036
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11, 17.3kDa	Cfa.4152.1.A1_s_at	1.111	0.036
Nebulette	CfaAffx.7112.1.S1_s_at	1.107	0.036
Nei endonuclease VIII-like 2 (E. coli)	Cfa.2134.1.S1_at	-1.176	0.036
Nuclear factor, interleukin 3 regulated	Cfa.6371.1.A1_at	1.789	0.036
Ninein (GSK3B interacting protein)	CfaAffx.22466.1.S1_at	-1.178	0.036
NME1-NME2 readthrough	CfaAffx.26483.1.S1_s_at	1.290	0.036
N-myristoyltransferase 1	Cfa.17031.1.S1_at	1.318	0.036
Neuronatin	CfaAffx.13888.1.S1_at	-1.226	0.036
Nucleolar and coiled-body phosphoprotein 1	CfaAffx.15780.1.S1_s_at	-1.937	0.036
Nuclear protein localization 4 homolog (S. cerevisiae)	CfaAffx.9604.1.S1_at	1.251	0.036
Natriuretic peptide precursor C	CfaAffx.17373.1.S1_at	-1.262	0.036
Nik related kinase	CfaAffx.27370.1.S1_at	1.212	0.036
Non-SMC element 1 homolog (S. cerevisiae)	Cfa.6987.1.S1_at	1.120	0.036
5'-nucleotidase, ecto (CD73)	CfaAffx.5438.1.S1_at	1.244	0.036
N-terminal asparagine amidase	CfaAffx.28681.1.S1_at	1.268	0.036
NudC domain containing 3	Cfa.18578.1.S1_at	-1.081	0.036
Nudix (nucleoside diphosphate linked moiety X)-type motif 9	Cfa.1573.1.S1_s_at	-1.175	0.036
Numb homolog (Drosophila)-like	CfaAffx.8624.1.S1_at	1.195	0.036
Nucleoporin 210kDa	CfaAffx.7516.1.S1_at	-1.131	0.036
Nucleoporin 214kDa	CfaAffx.30488.1.S1_s_at	-1.376	0.036
Nucleoporin 54kDa	Cfa.17092.1.S1_at	-1.175	0.036
Nucleoporin 93kDa	CfaAffx.14363.1.S1_at	-1.306	0.036
Nucleoporin like 2	CfaAffx.5029.1.S1_at	-1.203	0.036
Allergen Can f 4	CfaAffx.17457.1.S1_at	1.175	0.036

Obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	CfaAffx.2641.1.S1_at	1.202	0.036
Otoconin 90	CfaAffx.2523.1.S1_s_at	1.179	0.036
OCIA domain containing 1	Cfa.18958.2.S1_at	1.381	0.036
Outer dense fiber of sperm tails 1	CfaAffx.1877.1.S1_s_at	1.216	0.036
Oral-facial-digital syndrome 1	CfaAffx.18295.1.S1_s_at	-1.266	0.036
Opsin 4	CfaAffx.24493.1.S1_at	1.159	0.036
Olfactory receptor	CfaAffx.24879.1.S1_at	-1.083	0.036
Olfactory receptor	CfaAffx.29984.1.S1_at	1.129	0.036
Olfactory receptor	CfaAffx.17625.1.S1_at	1.112	0.036
Olfactory receptor	CfaAffx.16866.1.S1_at	1.113	0.036
Origin recognition complex, subunit 5	Cfa.16807.2.S1_at	-1.257	0.036
OTU domain containing 5	CfaAffx.24037.1.S1_at	-1.357	0.036
Oxidation resistance 1	CfaAffx.1950.1.S1_s_at	-1.288	0.036
Platelet-activating factor acetylhydrolase 2, 40kDa	Cfa.18324.1.S1_s_at	1.489	0.036
Phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	Cfa.1028.1.A1_s_at	-1.154	0.036
Poly (ADP-ribose) polymerase 1	Cfa.21541.1.S1_s_at	-1.436	0.036
Pyruvate carboxylase	Cfa.18692.1.S1_s_at	1.166	0.036
Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	CfaAffx.18467.1.S1_at	-1.631	0.036
Protein-L-isoaspartate (D-aspartate) O-methyltransferase	CfaAffx.1550.1.S1_at	-1.376	0.036
Programmed cell death 1	CfaAffx.20414.1.S1_at	1.165	0.036
Phosphodiesterase 4D interacting protein	Cfa.20329.1.S1_at	1.140	0.036
Peptide deformylase (mitochondrial)	CfaAffx.31057.1.S1_at	-1.317	0.036
PDZ domain containing 1	CfaAffx.17419.1.S1_s_at	-1.119	0.036
Proenkephalin	Cfa.1416.1.A1_at	1.228	0.036
Peptidase D	CfaAffx.11958.1.S1_s_at	1.329	0.036
Peroxisomal biogenesis factor 19	Cfa.11963.1.A1_at	-1.082	0.036
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	Cfa.13232.1.A1_at	-1.274	0.036
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	Cfa.21468.1.S1_at	-1.359	0.036

Peptidoglycan recognition protein 2	CfaAffx.24479.1.S1_s_at	-1.144	0.036
PHD and ring finger domains 1	CfaAffx.10774.1.S1_s_at	-1.246	0.036
Phosphatidylinositol glycan anchor biosynthesis, class M	CfaAffx.18440.1.S1_at	-1.404	0.036
Protein (peptidylprolyl cis/trans isomerase) NIMA-interacting, 4 (parvulin)	CfaAffx.9310.1.S1_x_at	-1.309	0.036
Phosphatidylinositol-4-phosphate 5-kinase, type I, gamma	CfaAffx.29305.1.S1_at	-1.112	0.036
Phosphatidylinositol-4-phosphate 5-kinase-like 1	CfaAffx.30766.1.S1_at	-1.373	0.036
Pirin (iron-binding nuclear protein)	CfaAffx.18617.1.S1_at	1.184	0.036
Phosphatidylinositol transfer protein, cytoplasmic 1	CfaAffx.17853.1.S1_s_at	1.358	0.036
Polycystic kidney disease 2-like 2	CfaAffx.2600.1.S1_s_at	-1.233	0.036
Polycystic kidney and hepatic disease 1 (autosomal recessive)	CfaAffx.4150.1.S1_s_at	-1.170	0.036
Phospholipase A2, group VI (cytosolic, calcium-independent)	CfaAffx.3029.1.S1_s_at	-1.181	0.036
Plasminogen activator, urokinase receptor	CfaAffx.8028.1.S1_s_at	2.816	0.036
Pleckstrin homology domain containing, family M (with RUN domain) member 1	CfaAffx.21270.1.S1_at	1.643	0.036
Polo-like kinase 1 substrate 1	Cfa.9547.1.A1_at	1.097	0.036
Phospholamban	Cfa.3165.2.A1_at	1.207	0.036
Phospholipid scramblase 4	CfaAffx.12996.1.S1_at	-1.694	0.036
Pro-melanin-concentrating hormone	CfaAffx.11803.1.S1_at	1.243	0.036
Phosphomannomutase 2	CfaAffx.29042.1.S1_at	1.377	0.036
Peripheral myelin protein 22	CfaAffx.27421.1.S1_at	1.453	0.036
PMS1 postmeiotic segregation increased 1 (S. cerevisiae)	CfaAffx.14811.1.S1_at	-1.220	0.036
Pancreatic lipase	Cfa.3882.3.S1_at	-1.123	0.036
Patatin-like phospholipase domain containing 5	CfaAffx.2256.1.S1_s_at	1.109	0.036
POC1 centriolar protein homolog A (Chlamydomonas)	CfaAffx.15464.1.S1_s_at	1.324	0.036
POC1 centriolar protein homolog B (Chlamydomonas)	CfaAffx.10156.1.S1_at	-1.566	0.036
Phosphatidic acid phosphatase type 2C	Cfa.18746.1.S1_s_at	1.510	0.036
Peroxisome proliferator-activated receptor alpha	Cfa.3519.1.S1_s_at	-1.129	0.036
Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	CfaAffx.25283.1.S1_at	-1.133	0.036

Protein phosphatase 4, regulatory subunit 1	Cfa.12339.1.A1_at	1.326	0.036
Protein kinase C, zeta	Cfa.19185.2.A1_at	-1.204	0.036
Protein kinase D1	Cfa.12558.1.A1_s_at	-1.612	0.036
PRP40 pre-mRNA processing factor 40 homolog A (S. cerevisiae)	Cfa.3344.1.S1_at	-1.239	0.036
Proline rich Gla (G-carboxyglutamic acid) 2	CfaAffx.6355.1.S1_at	-1.262	0.036
Protease, serine, 42	CfaAffx.21079.1.S1_s_at	-1.604	0.036
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	CfaAffx.15655.1.S1_s_at	-1.197	0.036
Proteasome (prosome, macropain) activator subunit 4	CfaAffx.4992.1.S1_s_at	1.357	0.036
Prostaglandin E receptor 3 (subtype EP3)	Cfa.132.2.S1_a_at	1.134	0.036
Parathyroid hormone 1 receptor	Cfa.3643.1.S1_s_at	-1.449	0.036
PTK2 protein tyrosine kinase 2	Cfa.5377.1.A1_at	1.207	0.036
Protein tyrosine phosphatase, mitochondrial 1	CfaAffx.13364.1.S1_at	1.264	0.036
Protein tyrosine phosphatase, non-receptor type 14	CfaAffx.19519.1.S1_s_at	-1.220	0.036
Protein tyrosine phosphatase, non-receptor type 2	CfaAffx.28811.1.S1_at	1.506	0.036
PWP1 homolog (S. cerevisiae)	Cfa.10480.1.A1_at	-1.257	0.036
Pygopus homolog 2 (Drosophila)	CfaAffx.26250.1.S1_at	-1.220	0.036
Glutamine rich 2	CfaAffx.8523.1.S1_s_at	-1.281	0.036
queuine tRNA-ribosyltransferase domain containing 1	CfaAffx.16818.1.S1_at	-1.456	0.036
RAB4B, member RAS oncogene family	CfaAffx.8550.1.S1_s_at	1.217	0.036
RAB, member RAS oncogene family-like 5	CfaAffx.21318.1.S1_at	1.191	0.036
RalA binding protein 1	Cfa.14547.1.A1_at	2.421	0.036
Receptor (G protein-coupled) activity modifying protein 2	Cfa.11182.1.A1_s_at	-1.934	0.036
RAS association (RalGDS/AF-6) domain family member 6	CfaAffx.5490.1.S1_at	1.205	0.036
RNA binding protein, fox-1 homolog (C. elegans) 1	CfaAffx.29131.1.S1_s_at	1.136	0.036
RNA binding motif protein 34	Cfa.2425.1.S1_s_at	-1.335	0.036
Retinol binding protein 1, cellular	CfaAffx.12273.1.S1_s_at	1.398	0.036
Retinol binding protein 2, cellular	Cfa.6245.1.A1_at	1.288	0.036
Reticulocalbin 2, EF-hand calcium binding domain	Cfa.21641.1.S1_at	-1.335	0.036
REST corepressor 3	CfaAffx.18828.1.S1_s_at	-1.264	0.036

Retinol dehydrogenase 5 (11-cis/9-cis)	CfaAffx.1065.1.S1_at	1.230	0.036
REV1 homolog (S. cerevisiae)	CfaAffx.4279.1.S1_s_at	-1.368	0.036
Regulatory factor X, 2 (influences HLA class II expression)	CfaAffx.28610.1.S1_s_at	1.341	0.036
Regulatory factor X, 4 (influences HLA class II expression)	CfaAffx.3623.1.S1_at	-1.208	0.036
Ral guanine nucleotide dissociation stimulator-like 3	CfaAffx.26633.1.S1_s_at	1.170	0.036
Regulator of G-protein signaling 6	Cfa.1840.1.S1_s_at	1.193	0.036
Rh blood group, D antigen	CfaAffx.19949.1.S1_at	-1.196	0.036
RAS homolog gene family, member T1	CfaAffx.28233.1.S1_s_at	-1.449	0.036
Resistance to inhibitors of cholinesterase 3 homolog (C. elegans)	CfaAffx.11252.1.S1_s_at	-1.280	0.036
Rab interacting lysosomal protein-like 1	CfaAffx.12089.1.S1_s_at	-1.245	0.036
Retinaldehyde binding protein 1	CfaAffx.18016.1.S1_at	1.224	0.036
Ring finger protein 144A	CfaAffx.5868.1.S1_at	-1.106	0.036
Ring finger protein 148	CfaAffx.6272.1.S1_at	1.364	0.036
Ring finger protein 213	CfaAffx.9471.1.S1_s_at	-1.783	0.036
Ring finger protein 217	CfaAffx.2434.1.S1_at	1.228	0.036
Ring finger protein 25	Cfa.11589.1.A1_at	1.192	0.036
RNA guanylyltransferase and 5'-phosphatase	CfaAffx.5573.1.S1_at	-1.383	0.036
Roundabout, axon guidance receptor, homolog 1 (Drosophila)	CfaAffx.12712.1.S1_s_at	-2.098	0.036
Rho-associated, coiled-coil containing protein kinase 1	CfaAffx.27952.1.S1_at	1.240	0.036
Reactive oxygen species modulator 1	Cfa.8706.1.S1_at	1.129	0.036
Ribonuclease P/MRP 21kDa subunit	Cfa.8855.2.S1_at	-1.216	0.036
Regulation of nuclear pre-mRNA domain containing 1B	CfaAffx.13968.1.S1_at	-1.165	0.036
RNA pseudouridylate synthase domain containing 2	Cfa.4769.1.A1_at	1.551	0.036
Ras-related associated with diabetes	Cfa.17583.1.S1_at	1.874	0.036
RRN3 RNA polymerase I transcription factor homolog (S. cerevisiae)	CfaAffx.28690.1.S1_at	-1.555	0.036
Ribosomal RNA processing 15 homolog (S. cerevisiae)	CfaAffx.16920.1.S1_at	-1.579	0.036
Retinoschisin 1	CfaAffx.19864.1.S1_at	-1.189	0.036
Rhotekin 2	CfaAffx.20164.1.S1_at	-1.235	0.036
Shwachman-Bodian-Diamond syndrome	Cfa.17197.1.S1_at	-1.183	0.036

Saccharopine dehydrogenase (putative)	Cfa.5433.1.A1_at	1.514	0.036
Sodium channel, voltage-gated, type IX, alpha subunit	CfaAffx.17961.1.S1_at	1.109	0.036
Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	CfaAffx.24257.1.S1_s_at	1.129	0.036
SEC14-like 2 (S. cerevisiae)	Cfa.10916.1.A1_s_at	-1.224	0.036
SECIS binding protein 2	Cfa.14796.1.S1_at	-1.061	0.036
SEH1-like (S. cerevisiae)	Cfa.18120.1.S1_s_at	-2.035	0.036
SUMO1/sentrin specific peptidase 6	CfaAffx.4947.1.S1_at	-1.244	0.036
Serpin peptidase inhibitor, clade B (ovalbumin), member 6	Cfa.6273.1.S1_at	1.444	0.036
SET translocation	Cfa.73.1.S1_x_at	-1.233	0.036
SET domain containing 6	CfaAffx.13402.1.S1_at	-1.441	0.036
SET domain, bifurcated 2	Cfa.1534.1.A1_at	1.138	0.036
Seizure related 6 homolog (mouse)	CfaAffx.28798.1.S1_at	-1.382	0.036
SH3-domain GRB2-like 3	CfaAffx.20540.1.S1_at	1.159	0.036
Src homology 2 domain containing E	CfaAffx.26278.1.S1_at	-1.147	0.036
Sucrase-isomaltase (alpha-glucosidase)	CfaAffx.22283.1.S1_s_at	1.204	0.036
Sialic acid acetylesterase	CfaAffx.17446.1.S1_at	1.159	0.036
Stem-loop binding protein	Cfa.15304.1.A1_at	-1.187	0.036
Solute carrier family 13 (sodium/sulfate symporters), member 4	CfaAffx.5962.1.S1_at	1.267	0.036
Solute carrier family 16, member 10 (aromatic amino acid transporter)	CfaAffx.6815.1.S1_at	-1.650	0.036
Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6	CfaAffx.15821.1.S1_s_at	1.231	0.036
Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 8	CfaAffx.11134.1.S1_at	-1.123	0.036
Solute carrier family 22 (organic anion/urate transporter), member 11	CfaAffx.22191.1.S1_s_at	1.131	0.036
Solute carrier family 22 (organic anion transporter), member 6	Cfa.14330.1.A1_at	-1.183	0.036
Solute carrier family 24 (sodium/potassium/calcium exchanger), member 2	CfaAffx.3350.1.S1_at	1.189	0.036
Solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	CfaAffx.8076.1.S1_s_at	1.259	0.036

Solute carrier family 25, member 27	Cfa.2665.1.A1_at	1.319	0.036
Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 31	CfaAffx.6830.1.S1_at	-1.129	0.036
Solute carrier family 25, member 34	Cfa.17246.1.S1_s_at	-1.139	0.036
Solute carrier family 26, member 4	CfaAffx.6797.1.S1_at	1.310	0.036
Solute carrier family 30 (zinc transporter), member 3	Cfa.6122.1.A1_at	1.234	0.036
Solute carrier family 33 (acetyl-CoA transporter), member 1	Cfa.593.1.A1_at	-1.224	0.036
Solute carrier family 35, member D3	CfaAffx.1344.1.S1_at	-1.292	0.036
Solute carrier family 36 (proton/amino acid symporter), member 2	Cfa.5442.1.A1_at	1.164	0.036
Solute carrier family 9 (sodium/hydrogen exchanger), member 2	CfaAffx.4098.1.S1_at	-1.155	0.036
Slowmo homolog 1 (Drosophila)	CfaAffx.28774.1.S1_at	1.190	0.036
Structural maintenance of chromosomes 3	CfaAffx.16837.1.S1_at	-1.250	0.036
SMEK homolog 1, suppressor of mek1 (Dictyostelium)	CfaAffx.26905.1.S1_at	1.171	0.036
Smu-1 suppressor of mec-8 and unc-52 homolog (C. elegans)	Cfa.1924.1.A1_at	-1.183	0.036
Small nuclear RNA activating complex, polypeptide 2, 45kDa	Cfa.18618.1.S1_s_at	-1.227	0.036
Small nuclear RNA activating complex, polypeptide 4, 190kDa	Cfa.16205.1.S1_at	-1.151	0.036
SNAP-associated protein	Cfa.6879.1.A1_at	-1.163	0.036
Small nuclear ribonucleoprotein polypeptide N /// SNRPN upstream reading frame	CfaAffx.928.1.S1_at	1.143	0.036
Sorting nexin 14	Cfa.2011.1.S1_s_at	-1.100	0.036
Sorting nexin 5	CfaAffx.9150.1.S1_s_at	-1.231	0.036
SP100 nuclear antigen	CfaAffx.16746.1.S1_at	-1.261	0.036
Spermatogenesis associated 18 homolog (rat)	CfaAffx.3931.1.S1_s_at	1.115	0.036
SPC24, NDC80 kinetochore complex component, homolog (S. cerevisiae)	CfaAffx.26814.1.S1_at	1.346	0.036
Sulfide quinone reductase-like (yeast)	CfaAffx.21303.1.S1_at	1.261	0.036
Steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2)	Cfa.3579.1.S1_s_at	1.162	0.036
SRSF protein kinase 2	Cfa.10743.1.S1_s_at	1.410	0.036

Serine/arginine-rich splicing factor 2	Cfa.10605.1.S1_at	-1.286	0.036
Serine/arginine-rich splicing factor 6	Cfa.277.1.S1_at	-1.252	0.036
Somatostatin receptor 3	CfaAffx.3154.1.S1_at	-1.097	0.036
ST3 beta-galactoside alpha-2,3-sialyltransferase 5	Cfa.15365.1.A1_at	-1.418	0.036
SH3 and cysteine rich domain 2	CfaAffx.25236.1.S1_s_at	1.130	0.036
StAR-related lipid transfer (START) domain containing 3	Cfa.20925.1.S1_s_at	1.314	0.036
StAR-related lipid transfer (START) domain containing 9	CfaAffx.17398.1.S1_s_at	1.107	0.036
Syntaxin 16	CfaAffx.18848.1.S1_s_at	-1.440	0.036
Syntaxin 17	CfaAffx.4679.1.S1_at	1.504	0.036
Syntaxin 6	CfaAffx.19887.1.S1_at	1.258	0.036
Succinate-CoA ligase, GDP-forming, beta subunit	Cfa.1485.1.S1_at	-1.137	0.036
Sulfotransferase family, cytosolic, 1C, member 3	CfaAffx.3994.1.S1_s_at	1.263	0.036
Sulfotransferase family, cytosolic, 6B, member 1	CfaAffx.10100.1.S1_at	1.106	0.036
Sad1 and UNC84 domain containing 3	CfaAffx.21815.1.S1_at	1.255	0.036
Suppressor of variegation 3-9 homolog 1 (Drosophila)	CfaAffx.23834.1.S1_s_at	-1.169	0.036
Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	Cfa.2424.1.S1_at	1.555	0.036
Syncollin	Cfa.16560.1.S1_at	-1.117	0.036
Spectrin repeat containing, nuclear envelope 1	Cfa.14004.1.A1_at	1.276	0.036
Synaptogyrin 1	CfaAffx.2905.1.S1_s_at	2.048	0.036
SYS1 Golgi-localized integral membrane protein homolog (S. cerevisiae)	Cfa.4337.1.A1_at	1.316	0.036
synaptotagmin-like 5	CfaAffx.21552.1.S1_s_at	-1.252	0.036
TAF6 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 80kDa	CfaAffx.22602.1.S1_at	-1.164	0.036
Taste receptor, type 2, member 40	Cfa.12619.1.A1_at	-1.056	0.036
T-box 4	Cfa.11.1.S1_s_at	1.372	0.036
Trichohyalin	CfaAffx.20088.1.S1_x_at	1.425	0.036
Tectonic family member 2	Cfa.5605.1.A1_at	1.678	0.036
Tyrosyl-DNA phosphodiesterase 1	CfaAffx.26760.1.S1_s_at	-1.301	0.036

thyrotrophic embryonic factor	CfaAffx.2512.1.S1_s_at	1.164	0.036
Thrombospondin, type I, domain containing 4	CfaAffx.26988.1.S1_at	-1.187	0.036
Thiamine triphosphatase	CfaAffx.18104.1.S1_at	-1.280	0.036
THUMP domain containing 1	CfaAffx.27547.1.S1_at	-1.225	0.036
Tubulointerstitial nephritis antigen	CfaAffx.4346.1.S1_s_at	-1.103	0.036
TCDD-inducible poly(ADP-ribose) polymerase	CfaAffx.14014.1.S1_at	1.459	0.036
Tousled-like kinase 1	CfaAffx.19441.1.S1_at	-1.338	0.036
Toll-like receptor 3	CfaAffx.11983.1.S1_at	-2.115	0.036
Transmembrane and coiled-coil domains 3	Cfa.9679.1.S1_at	-1.297	0.036
Transmembrane protein 110	CfaAffx.13932.1.S1_at	1.342	0.036
Transmembrane protein 138	CfaAffx.24818.1.S1_at	-1.306	0.036
Transmembrane protein 161B	CfaAffx.13247.1.S1_s_at	-1.523	0.036
Transmembrane protein 165	CfaAffx.4052.1.S1_at	-1.283	0.036
Transmembrane protein 176B	CfaAffx.7828.1.S1_at	-1.398	0.036
Transmembrane protein 195	Cfa.14573.1.A1_s_at	1.138	0.036
Transmembrane protein 51	CfaAffx.25028.1.S1_at	-1.354	0.036
Transmembrane protein 70	CfaAffx.12987.1.S1_s_at	-1.150	0.036
Tumor necrosis factor (ligand) superfamily, member 15	CfaAffx.5977.1.S1_at	1.220	0.036
Two pore segment channel 1	CfaAffx.14287.1.S1_at	1.167	0.036
Tropomyosin 1 (alpha)	CfaAffx.25960.1.S1_at	1.250	0.036
Triadin	CfaAffx.2407.1.S1_s_at	-1.184	0.036
Thyrotropin-releasing hormone	CfaAffx.7825.1.S1_at	-1.881	0.036
Tribbles homolog 1 (Drosophila)	Cfa.12502.1.A1_at	1.542	0.036
Tripartite motif-containing 10	CfaAffx.18982.1.S1_s_at	-1.117	0.036
Tripartite motif-containing 22	CfaAffx.10368.1.S1_s_at	-1.923	0.036
Tripartite motif-containing 45	CfaAffx.15460.1.S1_at	-1.541	0.036
Tripartite motif-containing 68	CfaAffx.10097.1.S1_at	-1.112	0.036
Tripartite motif family-like 1	CfaAffx.11713.1.S1_at	1.138	0.036
TSC22 domain family, member 1	CfaAffx.7831.1.S1_s_at	-1.259	0.036

Tetraspanin 17	Cfa.21314.1.S1_at	1.305	0.036
Translocator protein (18kDa)	Cfa.16771.1.S1_s_at	1.334	0.036
Tetratricopeptide repeat domain 19	Cfa.17411.1.S1_at	-1.472	0.036
Tubulin tyrosine ligase-like family, member 3	CfaAffx.8903.1.S1_s_at	-1.182	0.036
Titin	CfaAffx.21671.1.S1_s_at	-1.064	0.036
Tubulin, gamma complex associated protein 4	CfaAffx.19226.1.S1_at	-1.443	0.036
Tubulin, gamma complex associated protein 6	CfaAffx.1962.1.S1_at	-1.150	0.036
Taxilin gamma	CfaAffx.19577.1.S1_at	-1.176	0.036
Ubiquitin associated protein 2-like	CfaAffx.26406.1.S1_at	1.171	0.036
UBX domain protein 1	Cfa.13688.1.A1_s_at	1.181	0.036
Upper zone of growth plate and cartilage matrix associated	CfaAffx.8054.1.S1_s_at	1.112	0.036
Unc-5 homolog C (C. elegans)	CfaAffx.16004.1.S1_s_at	1.187	0.036
Ubiquinol-cytochrome c reductase core protein I	Cfa.794.1.A1_at	1.107	0.036
URB1 ribosome biogenesis 1 homolog (S. cerevisiae)	Cfa.4795.1.A1_at	-1.342	0.036
USO1 vesicle docking protein homolog (yeast)	CfaAffx.13431.1.S1_at	1.371	0.036
Ubiquitin specific peptidase 1	Cfa.19994.1.S1_s_at	-1.434	0.036
Ubiquitin specific peptidase 20	CfaAffx.30576.1.S1_s_at	1.707	0.036
Ubiquitin specific peptidase 36	CfaAffx.9238.1.S1_at	-1.335	0.036
USP6 N-terminal like	Cfa.19504.1.S1_at	1.392	0.036
Vesicle-associated membrane protein 8 (endobrevin)	Cfa.8500.1.A1_at	1.279	0.036
Ventricular zone expressed PH domain homolog 1 (zebrafish)	CfaAffx.14042.1.S1_at	1.129	0.036
Vezatin, adherens junctions transmembrane protein	CfaAffx.10479.1.S1_s_at	1.537	0.036
visinin-like 1	CfaAffx.5538.1.S1_s_at	1.186	0.036
von Willebrand factor A domain containing 3B	Cfa.16521.1.S1_s_at	1.212	0.036
WAS protein family, member 2	CfaAffx.18830.1.S1_s_at	-1.281	0.036
WAS protein family, member 3	CfaAffx.11185.1.S1_s_at	-1.148	0.036
WW domain binding protein 11	Cfa.8372.1.S1_s_at	-1.446	0.036
WD repeat domain 11	Cfa.8362.1.A1_at	1.226	0.036
WD repeat domain 12	Cfa.10515.1.S1_at	-1.215	0.036

WD repeat domain 48	Cfa.2333.2.S1_s_at	-1.654	0.036
WD repeat domain 62	Cfa.6553.1.S1_s_at	-1.181	0.036
WD repeat domain 82 pseudogene 1	Cfa.19770.1.S1_s_at	-1.204	0.036
WD repeat domain 87	CfaAffx.9944.1.S1_at	1.157	0.036
Wolf-Hirschhorn syndrome candidate 1	CfaAffx.23014.1.S1_s_at	1.291	0.036
Wolf-Hirschhorn syndrome candidate 2	CfaAffx.22972.1.S1_s_at	-1.215	0.036
X-ray repair complementing defective repair in Chinese hamster cells 4	CfaAffx.13644.1.S1_at	-1.538	0.036
5'-3' exoribonuclease 2	Cfa.10494.1.A1_s_at	-1.107	0.036
YLP motif containing 1	CfaAffx.25973.1.S1_s_at	-1.594	0.036
YME1-like 1 (S. cerevisiae)	Cfa.1083.1.S1_at	-1.161	0.036
Zonadhesin	CfaAffx.21670.1.S1_s_at	-1.163	0.036
Zinc finger, BED-type containing 1	CfaAffx.17291.1.S1_s_at	-1.634	0.036
Zinc finger and BTB domain containing 1	Cfa.1992.1.S1_at	-1.160	0.036
Zinc finger CCCH-type containing 13	Cfa.3076.1.S1_at	-1.171	0.036
Zinc finger, CCHC domain containing 6	CfaAffx.2895.1.S1_s_at	-1.290	0.036
Zinc finger, CW type with PWWP domain 1	CfaAffx.22353.1.S1_at	1.254	0.036
Zinc finger, DHHC-type containing 2	Cfa.9227.1.A1_s_at	1.361	0.036
Zinc finger, MYM-type 3	CfaAffx.26083.1.S1_s_at	-1.349	0.036
Zinc finger protein 132	CfaAffx.598.1.S1_at	-1.260	0.036
Zinc finger protein 157	CfaAffx.23137.1.S1_at	1.403	0.036
Zinc finger protein 227	CfaAffx.7983.1.S1_s_at	-1.348	0.036
Zinc finger protein 280D	CfaAffx.24776.1.S1_s_at	-2.201	0.036
Zinc finger protein 283	CfaAffx.8009.1.S1_s_at	-2.294	0.036
Zinc finger protein 295	CfaAffx.16005.1.S1_s_at	-1.418	0.036
Zinc finger protein 362	CfaAffx.16127.1.S1_at	-1.463	0.036
Zinc finger protein 43	CfaAffx.692.1.S1_x_at	-1.453	0.036
Zinc finger protein 470	CfaAffx.4569.1.S1_s_at	-1.207	0.036
Zinc finger protein 471	CfaAffx.4586.1.S1_s_at	-1.368	0.036
Zinc finger protein 644	CfaAffx.30924.1.S1_s_at	-1.320	0.036

Zinc finger protein 684	CfaAffx.5406.1.S1_x_at	-1.356	0.036
Zinc finger protein 691	CfaAffx.4632.1.S1_at	1.275	0.036
Zinc finger protein 705A	CfaAffx.13503.1.S1_s_at	-1.510	0.036
ZInc finger protein 835	CfaAffx.156.1.S1_at	1.138	0.036
Zona pellucida binding protein 2	CfaAffx.24994.1.S1_s_at	-1.121	0.036
Zinc finger and SCAN domain containing 10	CfaAffx.29433.1.S1_s_at	-1.085	0.036
Gene Title			